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(Signature)

Department of Experimental Medicine

The University of British Columbia
Vancouver, Canada

Date Sept 23/95
ABSTRACT

Pim-1 is an oncogene-encoded serine-threonine kinase, expressed primarily in hematopoietic and germ cells. Previously identified only in mammalian systems, pim-1 was cloned and sequenced from Xenopus laevis, the African clawed frog. The coding region of X. laevis Pim-1 encoded a protein of 324 amino acids, which exhibited 64% amino acid sequence identity with the full-length human protein. PCR was also used to demonstrate the existence of Pim-1 in Pisaster ochraceus, the purple sea star. The high sequence conservation observed in the catalytic domain of Pim-1 between divergent species support a conserved and important function for this kinase.

The full-length coding regions of both human and X. laevis Pim-1 were expressed as recombinant bacterial fusion proteins which exhibited phosphotransferase activity towards exogenous substrates as well as serine, threonine and tyrosine autophosphorylation activity. A kinase-inactive mutant was engineered to serve as a negative control. The phosphorylation site consensus sequence for recognition by Pim-1 was defined by stepwise alterations in the amino acid sequences of peptide substrate analogues to determine which of the amino acid residues surrounding the substrate phosphorylation site were critical for kinase recognition. The optimal substrate peptide for Pim-1 was determined to be K/R - K/R - R - K/R - L - S/T - X, where X is an amino acid residue with a small side chain.

Studies were undertaken to determine the autophosphorylation sites of the GST-Pim-1 kinase, using electron spray ionization mass spectroscopy (ESI-MS). The autophosphorylation sites of the GST-Pim-1 were identified as Ser-4, Ser-190 and Thr-205. These sites were conserved amongst all Pim-1 homologues. An additional site was identified on the GST protein, Thr-17. To assess the importance of the Ser-190 site on phosphotransferase activity, the Ser-190 residue was changed to alanine and to glutamic acid using PCR site-directed mutagenesis. These mutants were expressed in bacteria as GST-fusion proteins, and their activities were compared to the wild-type.

Together with Pim-1-specific antibodies, the optimal Pim-1 peptide substrate was used to study endogenous Pim-1 during X. laevis and P. ochraceus oocyte maturation. Pim-1 did not exhibit maturation-induced activation in sea star oocytes and the quantity of Pim-1 remained constant during the oocyte maturation process. This is the first study carried out to investigate this kinase in the oocyte system.
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NOMENCLATURE AND ABBREVIATIONS

1. MEASUREMENTS, REAGENTS AND UNITS

**aa** amino acid  
**ALP** alkaline phosphatase  
**amp** ampicillin  
**amp**^R^ ampicillin resistance  
**amu** atomic mass unit  
**APS** ammonium persulfate  
**ATP** adenosine 5'-triphosphate disodium salt  
**BCIP** 5-bromo-1-chloro-3-indoyl phosphate  
**BSA** bovine serum albumin  
**BSE** bovine spleen extracts  
**bp** base pair  
**°C** degrees celsius  
**C-terminal** carboxyl-terminal  
**CaFASW** calcium free artificial sea water  
**cDNA** copy DNA-product of reverse transcriptase reaction  
**Ci** Currie, 2.22 x 10^{12} disintegrations per minute  
**ConA** Concanavalin A  
**dH2O** distilled water  
**ddH2O** autoclaved millipore water  
**DNA** deoxyribonucleic acid  
**DMF** dimethylformamide  
**DMSO** dimethyl sulfoxide  
**dNTP** 2'-deoxynucleoside 5'-triphosphate  
**DTT** dithiothreitol  
**ECL** enhanced chemiluminescence  
**EDTA** ethylene diamine tetraacetate disodium salt  
**EGTA** ethylene bis (oxyethylenenitrilo) tetraacetic acid  
**Epo** erythropoetin  
**ESI-MS** electrospray ionization mass spectrometry  
**FCS** fetal calf serum  
**FeLV** feline leukemia virus  
**F-MuLV** friend-helper leukemia virus  
**g** gram
<table>
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<tr>
<td>$x, g$</td>
<td>times the force of gravity</td>
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<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>GVBD</td>
<td>germinal vesicle breakdown</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
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<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>hsp</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>IMAC</td>
<td>immobilized metal affinity chromatography</td>
</tr>
<tr>
<td>IP</td>
<td>immunoprecipitation, immunoprecipitate</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton, a measure of molecular mass</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase (1000 bp)</td>
</tr>
<tr>
<td>KOAc</td>
<td>potassium acetate</td>
</tr>
<tr>
<td>l</td>
<td>litre</td>
</tr>
<tr>
<td>LC$_{50}$</td>
<td>lethal concentration for 50% of animals</td>
</tr>
<tr>
<td>LCMS</td>
<td>liquid chromatography/mass spectrometry</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>mA</td>
<td>milli Amps</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>mg</td>
<td>milligram (10$^{-3}$)</td>
</tr>
<tr>
<td>M</td>
<td>moles/litre</td>
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<tr>
<td>mMol</td>
<td>millimol</td>
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<td>millimolar</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>2-ME</td>
<td>2-mercaptoethanol</td>
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<tr>
<td>MES</td>
<td>2-[N-morpholino]ethanesulfonic acid</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-[N-morpholino]propanesulfonic acid</td>
</tr>
<tr>
<td>$M_r$</td>
<td>molecular mass</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MuLV</td>
<td>murine leukemia virus</td>
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<tr>
<td>m/z</td>
<td>mass to charge ratio</td>
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<tr>
<td>N-terminus</td>
<td>amino terminus</td>
</tr>
<tr>
<td>NaOAc</td>
<td>sodium acetate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>NBT</td>
<td>nitro blue tetrazolium</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethyl maleimide</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>nM</td>
<td>nanomoles (10^{-9} moles)</td>
</tr>
<tr>
<td>nm</td>
<td>nanometres (10^{-9} meters)</td>
</tr>
<tr>
<td>NSW</td>
<td>natural sea water</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonidet P-40</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotides</td>
</tr>
<tr>
<td>O/N</td>
<td>overnight</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PAA</td>
<td>phosphoamino acid analysis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming unit</td>
</tr>
<tr>
<td>PKI</td>
<td>cAMP-dependent protein kinase inhibitor peptide</td>
</tr>
<tr>
<td>PLB</td>
<td>phospholysis buffer</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenyl methylsulphonyl fluoride</td>
</tr>
<tr>
<td>PNP</td>
<td>p-nitrophenyl phosphate</td>
</tr>
<tr>
<td>poly(A)+</td>
<td>polyadenylated</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride membrane</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SBTI</td>
<td>soybean trypsin inhibitor</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polymerase gel electrophoresis</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>SLS</td>
<td>sodium lauryl sarcosinate</td>
</tr>
<tr>
<td>T_{1/2}</td>
<td>half-life</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with tween</td>
</tr>
<tr>
<td>tet</td>
<td>tetracycline</td>
</tr>
<tr>
<td>TIC</td>
<td>total ion chromatogram</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>μ</td>
<td>micron (10^{-6} meters)</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μl</td>
<td>microlitre</td>
</tr>
</tbody>
</table>
UTR  untranslated region
UV   ultraviolet
vol  volume
v    volume
w    weight
XOM  *Xenopus* oocyte media
### 2. AMINO ACIDS

<table>
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<th>Name</th>
<th>Three letter abbreviation</th>
<th>One letter symbol</th>
<th>Characteristics</th>
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<td>A</td>
<td>non-polar</td>
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<tr>
<td>Arginine</td>
<td>Arg</td>
<td>R</td>
<td>basic</td>
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<tr>
<td>Asparagine</td>
<td>Asn</td>
<td>N</td>
<td>polar</td>
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<td>Asp</td>
<td>D</td>
<td>acidic</td>
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<td>Leu</td>
<td>L</td>
<td>non-polar</td>
</tr>
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<td>Lysine</td>
<td>Lys</td>
<td>K</td>
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<td>Pro</td>
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<td>S</td>
<td>polar</td>
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<td>Thr</td>
<td>T</td>
<td>polar</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Trp</td>
<td>W</td>
<td>basic (weak)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Tyr</td>
<td>Y</td>
<td>polar (weak)</td>
</tr>
<tr>
<td>Valine</td>
<td>Val</td>
<td>V</td>
<td>non-polar</td>
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### 3. NUCLEOTIDES

<table>
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<tr>
<td>Cytosine</td>
<td>C</td>
</tr>
<tr>
<td>Guanine</td>
<td>G</td>
</tr>
<tr>
<td>Thymine</td>
<td>T</td>
</tr>
<tr>
<td>Deoxyadenosine 5’-triphosphate</td>
<td>dATP</td>
</tr>
<tr>
<td>Deoxycytosine 5’-triphosphate</td>
<td>dACP</td>
</tr>
<tr>
<td>Deoxyguanine 5’-triphosphate</td>
<td>dAGP</td>
</tr>
<tr>
<td>Deoxythymidine 5’-triphosphate</td>
<td>dTTP</td>
</tr>
</tbody>
</table>
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There are many people whom I would like to thank for their assistance in the past 6 years. I would like to thank my supervisor Dr. Steven Pelech for everything, especially for giving me the opportunity to do my graduate studies in his laboratory. Grateful appreciation to my committee, Drs. Gerry Krystal, Michael Gold and Roger Brownsey for all their guidance and support. I would like to thank my "non-official graduate supervisors" Drs. Gabe Kalmar and Jasbinder Sanghera, for all the advice and encouragement during the roughest times. I would like to express my gratitude to Mr. Roman Babicki for the generous financial support during the last two years, which allowed me to continue and complete the most interesting part of my research.

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CHAPTER I.

INTRODUCTION

PIM-1 EXPRESSION AND REGULATION

The transformation from normal to neoplastic cells involves a sequential series of genetic events, often resulting in the activation or deactivation of genes concerned with the regulation of the cell cycle or the control of differentiation. The overexpression of the pim-1 gene is thought to be one of the steps involved in the tumorogenic process. Despite large amounts of research devoted to pim-1, this protein kinase has been poorly characterized and the physiological function of Pim-1 remains unknown. Resolving the many unanswered questions about the regulation, activity and function of Pim-1 could provide insight into lymphomagenesis and could contribute greater understanding of the mysteries of cell cycle regulation. This work attempts to characterize the kinase activity and expression of Pim-1 using bacterially-expressed enzyme, with the aim of achieving a fuller understanding of the physiological function of the kinase, especially as applied to the oocyte maturation system.

1. VIRAL ORIGINS

i. DISCOVERY OF THE PIM-1 ONCOGENE

Many early oncogenes, such as src, abl and erbB, were first identified as altered versions of cellular genes that had been extricated and incorporated into the viral genome, their altered function or expression lending a survival advantage to the virus [Hunter, 1987; Ramakrishnan and Rosenberg, 1989; Graf and Beug, 1983]. In contrast, the pim-1 oncogene was first identified in Murine Leukemia Virus (MuLV) induced T cell and B cell lymphomas not as a pirated viral component but as a common site of proviral integration [Cuypers et al., 1984; Berns et al., 1988a, 1988b]. Hence the nomenclature of pim-1; the Proviral Integration site of MuLV. Other oncogenes activated by proviral insertion include the c-myc, c-myb, int-1, int-2 and bmi-1 [reviewed by van Lohuizen and Berns 1990]. The pim-1 is also a site of integration in the Friend helper leukemia virus (F-MuLV) -induced murine erythroleukemias and feline leukemia virus (FeLV) -induced lymphomas [Dreyfus et al., 1990; Tsatsanis et al., 1994]. As the MuLV contains no transforming genes of its own, it was inferred that the neoplastic manifestations of the integration must be attributable to the gene located at the site of integration. This
assumption proved correct, as proviral integration into the pim-1 gene caused a 10-50% increase of pim-1 mRNA transcripts as compared to lymphomas not having integrations in the pim-1 site [Cuypers et al., 1984; Selton et al., 1985]. As the gene was found to contain little homology to any of the known oncogenes, pim-1 was acknowledged as a new oncogene [Cuypers et al., 1984].

ii. ACTIVATION BY PROVIRAL INSERTION

The exact mechanics of proviral insertion into the pim-1 gene are still unclear. The MuLV prefers to integrate at specific, conserved nucleotide positions in the pim-1 gene; this integration region is not homologous with the integration sites commonly used by other viruses [Cuypers et al., 1984]. Integrations are almost always found outside of the open reading frame (ORF) in the 3' untranslated region (UTR), suggesting that for successful transformation, a full-length pim-1 protein is required [Selton et al., 1985; Haupt et al., 1991]. One study of viral integration into the pim-1 locus found that of thirty-six lymphomas with an activated pim-1 gene, twenty-five had integrations in the 3' non-coding region of the pim-1 gene, eight had integrations downstream of pim-1 and three had integration upstream of the pim-1 transcription unit. In some lymphomas, the levels of pim-1 mRNA were elevated even though no viral integration was detected within the gene, possibly because the virus integrated elsewhere [Selton et al., 1985]. Another study by Mally et al. [1985] failed to detect the expression of pim-1 in murine T-cell lymphomas. However, the level of detection required may not have been achieved.

Proviral integration significantly enhances the expression of pim-1 mRNA, by increasing the stability of the pim-1 mRNA transcripts. This is achieved by removal of the AUUUA motif, a 'destabilization signal' targeting the mRNA transcript for rapid turnover [Berns et al., 1988a]. Indeed, almost all viral integrations located in the 3' untranslated region are located upstream of the AUUUA sequence, leading to a truncated mRNA transcript without this AUUUA sequence. The size of the pim-1 mRNA in the MMLV-induced leukemias varies from 2.0 to 2.6 kB and depends on the site of viral integration [Selton et al., 1985; Nagarajan et al., 1986]. Alternatively, the activation of the gene may be a function of the provirus itself; all inserted proviruses examined from the pim-1 area have duplicate or triplicate enhancer regions in the long terminal repeat (LTR), possibly causing an increase in the rate of pim-1 gene transcription. However, no mRNA was produced and no transforming activity was observed when NIH/3T3 cells and rat embryo fibroblasts were transfected with genomic pim-1 "provirally activated" by
the MuLV LTR, thus disputing the importance of the LTR in pim-1 activation [Berns et al., 1988a].

The resulting Pim-1 protein remains unchanged by the proviral insertion, not only was the size of the Pim-1 consistent with that of uninfected cells, but there were no mutations or amino acid changes observed, even when upstream insertion occurred [Berns et al., 1988a]. Therefore, Pim-1 becomes an oncoprotein by virtue of overexpression alone; proviral insertion contributes to cellular transformation by causing an increase in the amount of active Pim-1 protein in the cell produced as a result of pim-1 mRNA stabilization.

2. CLONING THE PIM-1 GENE

The genomic and cDNA sequences of the murine pim-1 gene were first reported by Selton et al. [1986]. The murine pim-1 gene is located on chromosome 17, close to the MHC complex, localized to the a-crystallin and tw-12 markers [Berns et al., 1988a], and encodes a 2.8 kilobase (kb) mRNA transcript [Domen et al., 1987]. The size of somatic murine and human pim-1 mRNA transcripts have been reported to be similar [Meeker et al., 1987a].

The human genomic pim-1 clone was first obtained from the human B cell leukemia 380 cell line [Nagarajan et al., 1986] and from a human genomic library [Zakut-Houri et al., 1987; Reeves et al., 1990]. The 2.9 kB human pim-1 cDNA was cloned by several groups from the human chronic myelogenous leukemia tumor cell line K562, which expresses high levels of pim-1 [Domen et al., 1987; Meeker et al., 1987a; Padma and Nagarajan, 1991; Zakut-Houri et al., 1987]. A single non-coding polymorphism at site 1003 distinguishes the pim-1 sequence from the K562 cell line from that of the 380 genomic clone [Meeker et al., 1987b].

The sequence of pim-1 is highly conserved and homologues have been identified by Southern analysis in such diverse vertebrate species as cat, hamster, human, chicken and mouse, but not Drosophila hydei [Cuypers et al., 1986; Tsatsanis et al., 1994]. The human genomic clone has an overall nucleotide homology to the murine pim-1 gene of 53%. Both human and mouse cDNA ORF are 939 nucleotides, with a predicted protein product of 313 amino acids [Berns et al., 1988a; Domen et al., 1987; Reeves et al., 1990].
The primary sequence of the coding regions of the human and mouse genes are 88% to 90% homologous, while the amino acid sequences are 94% homologous [Berns et al., 1988a; Reeves et al., 1990]. The amino terminal 250 amino acids are 98% homologous, with most of the amino acid substitutions clustered at the carboxy terminus. Both human and mouse contain a single sequence for N-linked glycosylation, Asn-Gly-Thr, at Asn-82 [Domen et al., 1987; Berns et al., 1988a].

Until recently, there was believed to be only one pim-1 locus in mammalian genomes. A second related gene called pim-2, located on the X chromosome was recently cloned from pim-1-deficient mice using complementation tagging [van der Lught et al., 1995]. This gene is expressed in hematopoietic cells and in the brain, and the protein encoded by this gene has many characteristics similar to murine pim-1 including upstream translation from CTG sites and similar substrate specificity. The pim-1 and pim-2 genes were demonstrated to be functionally redundant. A pim pseudogene was also identified on chromosome 8. With the discovery of this second pim family member, an unrelated M-MuLV proviral integration region in chromosome 17, called pim-2, was renamed Tic-1 [Breuer et al., 1989a; van Lohuizen and Berns, 1990; Haupt et al., 1991; van Lohuizen and Berns, 1990].

3. ONCOGENIC POTENTIAL OF PIM-1

i. STUDIES IN MURINE LEUKEMOGENESIS

Studies of proviral integration in murine lymphomagenesis have been extensive and have implicated the involvement of pim-1 in cellular transformation. Pim-1 is a site of proviral insertion not only in murine T cell lymphomas [Cuypers et al., 1984; Dreyfus et al., 1990], but in erythroleukemias and in B cell lymphomas as well [Muscenski et al., 1988; Dreyfus et al., 1990; Verbeek et al., 1991]. Genetic examination of the sites of F-MuLV integration in erythroid, lymphoid and myeloid leukemias demonstrated that 50% of F-MuLV were integrated in pim-1, c-myc or pvt-1, and that this integration was often associated with rearrangements in p53 in the same tumor [Dreyfus et al., 1990]. The pim-1 site, along with the c-myc gene, is one of the most common MuLV integration sites in murine lymphoid tumors, with at least 75% of early murine T-cell lymphomas showing proviral integration in pim-1 or near c-myc [Cuypers et al., 1984]. When Balb C mice were infected with Moloney Murine leukemia virus (M-MuLV), 31 of the 66 mice that developed lymphomas within a six month period had a provirus integrated in the pim-1
region [Selton et al., 1985]. In another study, changes were observed in the pim-1 region in 23 of 93 murine lymphomas screened and over 50% of the T-cell lymphomas examined demonstrated integration in this region [Cuypers et al., 1984]. In a study involving inbred mouse strains with a high incidence of spontaneous lymphomas, only 24% of the lymphomas examined contained any type of observable genetic rearrangements, and rearrangements of pim-1 (as well as fis-1, mlvi-1, mlvi-2) were observed only in T cell lymphomas [Muscenski et al., 1988]. Finally, studies with transgenic animals overexpressing pim-1 have confirmed that this oncogene is involved in murine lymphomagenesis [van Lohuizen et al., 1989; Möröy et al., 1991].

ii. NO PROOF THAT PIM-1 IS A HUMAN ONCOGENE

Despite the strong link between pim-1 overexpression and mouse lymphomagenesis, there is very little evidence to prove that pim-1 is a human oncogene. Although pim-1 mRNA and protein are overexpressed in many human cell lines and leukemias, there is no evidence that viral integration in humans occurs in or near the pim-1 gene or that chromosomal translocations involve the pim-1 locus [Meeker et al., 1987b; Amson et al., 1989]. It seems that the levels of the pim-1 gene products are elevated in many human leukemias by methods other than translocation or amplification [Amson et al., 1989].

The human pim-1 gene was mapped by somatic cell hybrid analysis and in situ hybridization to site 6p21 [von Lindern et al., 1989], more specifically to the 6pter-q12 segment [Cuypers et al., 1986]. The gene is located quite close to the human HLA (human leukocyte antigen) complex, but it is not known if the two regions are linked [Cuypers et al., 1986]. Some chromosomal translocations associated with lymphomas and leukemia do occur at 6p21, but it is doubtful if any of the observed genetic defects actually involve pim-1 [Amson et al., 1989]. Genetic defects involving 6p21 include 6p deletions associated with T cell lymphoma [Meeker et al., 1987b] as well as a reciprocal translocation in t(6:9)(p21;q33) as the sole chromosomal anomaly in some human chronic myeloid (CML), acute myeloblastic (AML) [Nagarajan et al., 1986] and acute nonlymphocytic leukemias (ANLL) [von Lindern et al., 1989]. A detailed study of the breakpoints in AML found that the translocation did not involve pim-1, but rather introns of the can gene, icb-9, on chromosome 9 and of the dek gene, icb-6, on chromosome 6, which were spliced to produce a transcript yielding a 165 kDa DEK-CAN fusion protein [von Lindern et al., 1992]. ANLL patients were examined specifically for the involvement of the pim-1 (6p21) and c-abl (9q34) genes in the reciprocal (t6;9)(p23;q34) translocation. Although the expression of pim-1 mRNA was elevated in 2/3 of the ANLL...
patients examined, the transcript size (2.7 kB) was unaltered, so the *pim-1* gene remained on chromosome 6 during the translocation and no chromosomal breakpoints were detected within 165 kb of the *pim-1* locus [von Lindern et al., 1989]. In addition, tumors from 51 patients revealed trisomies and diploidies but no gene rearrangements or gene translocations in chromosome 6 [Amson et al., 1989]. Coincidentally, the human erythroleukemia cell line K562, which over-expresses *pim-1*, has a 6p21 rearrangement not involving the *pim-1* gene [Nagarajan et al., 1986].

### iii. EXPRESSION OF PIM-1 IN HUMAN CANCERS AND CELL LINES

**a. *pim-1* mRNA expression in human cancers and cell lines**

Several detailed studies have been conducted to examine the expression patterns of *pim-1* mRNA in various human tumor cells and cell lines. Examination of 38 human cell lines revealed that the *pim-1* mRNA is detected mainly in B cell and myeloid cell lines. Of 19 B-cell lines examined, most were positive for *pim-1* except for the very immature and the very mature [Meeker et al., 1987b]. These results agree with those of Nagarajan et al., [1986] who also examined *pim-1* mRNA expression in various human hematopoietic cell lines and found that the *pim-1* transcripts were expressed at various levels in the different cell types. Myeloid cell lines (K562 and KG-1) expressed the highest amounts of *pim-1*; the high frequency of positive *pim-1* cDNA clones (0.1%-0.01%) in the K562 library confirmed that *pim-1* mRNA is highly expressed in this erythroleukemia cell line [Nagarajan et al., 1986; Meeker et al., 1987b]. Despite the fact that *pim-1* is causal in viral-induced murine T cell leukemia, *pim-1* mRNA was not detected in any of the human T cell lines examined in one study [Meeker et al., 1987b], and detected in only one of seven T cell lines tested in a second study [Nagarajan et al., 1986]. A recent study examining *pim-1* mRNA levels in primary bovine lymphocytes confirmed that *pim-1* is constitutively expressed in primary B cells, and is expressed at only low levels in T cells [Wingette et al., 1995]. In summary, *pim-1* mRNA is preferentially expressed in a some myeloid leukemias and B-cell lymphomas, including Burkitts.

**b. Pim-1 protein expression in human cancers and cell lines**

The expression of Pim-1 protein was examined in 70 malignancies of human origin, ranging from fresh tumor tissues to cell lines representing various stages of differentiation including myeloid, myelomonocyte and cells of B and T cell origin. Pim-1 protein was detected/overexpressed in 30% of the samples tested but expression was not correlated
with any particular cell type or stage of cell differentiation and was not caused by rearrangement of amplification of the gene [Amson et al., 1989]. Pim-1 was overexpressed at various stages in 24 AML patients, in a stage IV B-lineage ALL and a completely immature ALL. In these patients, the protein levels of c-Myc were also examined; although 90% of tumors from 51 patients overexpressed c-Myc and 30% overexpressed Pim-1, the two genes did not display cooperativity with each other [Amson et al., 1989]. This lack of cooperativity observed in the human tumors contrasts sharply to the cooperativity observed in pim-1/myc double transgenic mice [Möröy et al., 1991; Verbeek et al., 1991].

In the human cell lines tested, expression of Pim-1 was variable between the different cell types and differentiation stages [Amson et al., 1989]. The highest expression was in myeloid cells (K562 and KCL cells) and in SUDHL-6 cells of histiocytic origin. Pim-1 protein was detected in most myeloid leukemia cell lines examined including HL60, PLB985, KG1, K562, U937, WEHI3B and M1 cell lines, but not in EM2 [Lilly, 1989]. The lowest expression was found in T cells and myelomonocytic cell lines [Amson et al., 1989], a result in agreement with other studies [Padma and Nagarajan, 1991]. Relative levels of pim-1 mRNA and Pim-1 protein detected in the Daudi, SB, HL60, K562 cell lines were in agreement [Amson et al., 1989; Meeker et al., 1987b], suggesting that mRNA and protein levels of pim-1 may be correlated.

In summary, expression of pim-1 in human neoplasms does not involve proviral insertions, genetic rearrangements, translocations or truncations. Pim-1 protein appears to be overexpressed primarily in myeloid and B-cell tumors and cell lines and expression does not seem cooperative with the expression of c-myc. Although pim-1 unquestionably acts as an oncogene in the mouse, it is uncertain if pim-1 has a similar role in humans.
4. PIM-1 GENE EXPRESSION

i. SELECTIVE mRNA EXPRESSION PATTERNS

Pim-1 mRNA has very selective patterns of expression, with levels varying considerably between stages of development, tissues and cell lines [Sorrentino et al., 1988; Amson et al., 1989; Wingette et al., 1995]. Between some cell lines, the levels of the mRNA transcript vary from 0-0.2% of total mRNA [Meeker et al., 1987a; Zakut-Houri et al., 1987; Nagarajan and Narayana, 1993]. Variation in pim-1 expression levels and stability have been demonstrated between different tissue types in the same animal, possibly reflecting a difference in Pim-1 function between the various tissues [Wingette et al., 1991, 1995]. Although it is unusual for such a highly conserved gene to be expressed differently in various species, the patterns of pim-1 expression vary considerably between mouse and human [Meeker et al., 1987b]. It is unclear if variations in the amounts of pim-1 mRNA detected are the result of different experimental methods, expression levels or a reflection of post-transcriptional regulation.

Murine pim-1 mRNA is detected in both B and T cells as well as in many hematopoietic cell lines [Berns et al., 1988a]. During murine embryonic development, the expression of the gene follows migration of the hematopoietic cells, with pim-1 mRNA expression restricted to liver, thymus and spleen. High levels of pim-1 mRNA are also detected in 10-12 day-old placenta [Selton et al., 1985]. In normal newborn and adult mice, pim-1 mRNA is detected predominantly in the spleen and thymus, with minor amounts in the liver and none in the kidney, lung, heart, ovaries, testes or brain [Selton et al., 1985]. The levels of pim-1 mRNA are higher in the embryonic tissues than in the corresponding adult tissues [Berns et al., 1988a]. Maximum pim-1 mRNA levels were detected in the thymus at birth, in the liver at 16-19 days of gestation, and pim-1 mRNA levels increase up to 14 days after birth in the spleen [Selton et al., 1985].

ii. ALTERNATE mRNA TRANSCRIPTS & DIFFERENT HALF-LIVES

The 2.8 kb pim-1 mRNA transcript detected in somatic cells is inducible and short-lived, and can be upregulated by mitogen and growth factor stimulation [Dautry et al., 1988; Wingette et al., 1991; Lilly et al., 1992; Wingette et al., 1995]. Growth factors increase the stability of the transcripts, contributing in part to increases in the levels of pim-1 mRNA [Wingette et al., 1991; Yip-Schneider et al., 1995].
The stability of *pim-1* mRNA transcripts varies substantially between different cell lines, species and laboratories. The half-life (T1/2) of *pim-1* was measured in several unstimulated human cell lines and ranged from 47 min to over 3 hours [Meeker *et al.*, 1990; Wingette *et al.*, 1995]. Stimulation of bovine lymph node lymphocytes and bovine PBMC lymphocytes with Con A (a T cell mitogen) and phorbol ester (PMA) caused a 3.5-fold increase in *pim-1* mRNA in 4 hours (T1/2 > 80 min), which had decreased to 50% of peak levels by 17 hours post-stimulation (T1/2 ~ 35 min) [Wingette *et al.*, 1991]. The induction of *pim-1* mRNA in primary lymphocytes stimulated with PMA and ionomycin, peaked in 4 hours (T1/2 ~ 40 min) and dropped by 17 hours (T1/2 ~ 120 min), indicating that more than just mRNA stabilization was involved in this increase in *pim-1* mRNA. For unknown reasons, similar cells from different species yield different mRNA stabilities; rat lymphocyte *pim-1* mRNA transcripts have a longer half life (T1/2 ~ 140 min) than those found in activated bovine lymphocytes (T1/2 ~ 80 min) [Wingette *et al.*, 1991].

There are differences in the size and half-life of *pim-1* mRNA transcripts in various tissues from the same animal. Somatic tissues of rats and mice contain a 2.8 kb *pim-1* mRNA transcript, while a shorter 2.4 kb transcript has been observed in the testes [Sorentino *et al.*, 1988; Wingette *et al.*, 1992]. This alternate 2.4 kb fragment is not a product of a related gene or a different isoform of *pim-1* generated by alternative splicing, but results from the removal of the A/U rich destabilizing regulatory region in the 3' untranslated region of the gene and polyadenylation at an alternate site, nucleotide number 1302 [Wingette *et al.*, 1992]. The removal of this same A/U rich region is thought to occur during proviral integration. Consequently, the 2.4 kb mRNA message in germ cells is more stable (T1/2 > 3.5) than the longer 2.8 kb message produced in lymphoid cells (T1/2 ~140 min) [Wingette *et al.*, 1991, 1992].

This 2.4 kB form is specifically expressed in the haploid postmeiotic early spermatids of mature adult mouse testes, and seems to correlate with sexual maturation and the development of spermatids [Sorentino *et al.*, 1988]. This transcript was not observed in the testes of newborn mice, ovaries of mature female mice, nor in spermatogonia, suggesting that the *pim-1* plays a developmental role in male gametes [Sorentino *et al.*, 1988].

One functional justification for the short, stable germ-cell specific transcript could be that the increased stability of the transcript could allow it to survive through the
translational delay that occurs in post-meiotic germ cells as the early spermatids mature into differentiated spermatozoa [Sorrentino et al., 1988]. There are several other genes that have shorter testes-specific transcripts, including the mos [Sorrentino et al., 1988], abl, t-fer, calmodulin, cAMP-dependent protein kinase regulatory and catalytic subunits, possibly due to the existence of a unique germ-cell specific polyadenylation system [Sorrentino et al., 1988; Wingette et al., 1992]. With abl, a more stable mRNA transcript occurs in the mouse spermatids than in somatic cells [Wingette et al., 1992]. The PKA-R1α was examined but unlike pim-I, no difference in stability was found between the short, testes-specific transcript and the longer somatic cell-specific transcript. However, the A/U rich region of PKA-R1α is not as extensive as that of pim-I [Wingette et al., 1992].

iii. REGULATION OF PIM-I GENE EXPRESSION

Pim-1 mRNA expression is tightly regulated and occurs at many different levels, the specifics of which remain hypothetical. The promoter region of pim-1 was found to have many characteristics of a constitutively expressed housekeeping promoter [Meeker et al., 1990; Nagarajan and Narayana, 1993], yet expression is selective and highly variable between different cell types and lines and during stimulation by growth factors [Dautry et al., 1988; Meeker et al., 1990; Wingette et al., 1991; Lilly et al., 1992; Wingette et al., 1995]. Although elements located outside of the promoter region are probably responsible for the differences in mRNA transcription, the pim-1 promoter region has undergone extensive examination for clues about how the gene may be physiologically regulated.

Extensive examination of the 1.7 kb promoter region has revealed that pim-1 has many features of a constitutively expressed housekeeping gene including Sp1 binding sites and a lack of TATA and CAAT boxes [Meeker et al., 1987b; Meeker et al., 1990; Reeves et al., 1990; Nagarajan and Narayana, 1993]. The human promoter region also has AP-1 sites and NF-A2 and NF-kB binding sites that may be involved in transcriptional regulation [Meeker et al., 1987b; Reeves et al., 1990]. The transcription initiation site of human pim-1 was identified by S1 nuclease protection, from which it was determined that only one transcription initiation site is viable [Meeker et al., 1987b]. The human pim-1 promoter region is more than 80% identical with the mouse pim-1 promoter region, and the -1 to -876 region is extremely G+C rich (71%) [Reeves et al., 1990; Meeker et al., 1990; Wingette et al., 1992].
Recently an interferon gamma (IFNγ) responsive element was identified in the 5' region of Pim-1 [Yip-Schneider et al., 1995]. This region, called PMGAS, contained a Stat (signal transducers and activators of transcription) binding site TTCCCAGAA that bound Stat 1α, a 91kDa subunit of the interferon-stimulated gene factor 3 (ISGF3). This Stat binding site was functional and is capable of conferring IFNγ responsiveness onto heterologous promoters. As many growth factors utilize the JAK-Stat pathway, this might be the method by which this gene is stimulated in growth factor-activated cells.

Two major functional regions of the human pim-1 promoter were defined by deletion mutations; a proximal element at -104 to -1 and a distal element at -429 to -336 [Meeker et al., 1990]. DNase I protection assays identified the specific binding sites for SP1 and AP2 proteins within these elements, four of five Sp1 boxes [(G/T) (G/A) G G G C G (G/T) (G/A) (G/A) (C/T)] are conserved between mouse and human [Meeker et al., 1990].

The pim-1 promoter also contains a lymphoid-specific octamer motif beginning at -248, similar to one that is found in the promoter or enhancer regions of IgG genes that binds lymphoid-specific transacting transcriptional regulating proteins [Selton et al., 1986; Meeker et al., 1987b; Berns et al., 1988a]. This region is normally found 70 kb upstream of the RNA cap site, but is located in a slightly different region in pim-1 [Selton et al., 1986]. In the human, this motif (ATGCAGAT) is similar (7/8) to that of the IgG octomer motif, while in the mouse gene, this octomer (ATGCAAAT) is identical. The role of this octamer in pim-1 expression is unclear.

Examination of the pim-1 promoter has revealed little information about the regulation of the oncogene or why there is such a tissue-specific expression of the pim-1. Several theories have emerged as to how pim-1 is regulated including transcriptional attenuation, transcription control by the rate of transcription and tissue-specific transcriptional control elements and selective degradation of RNA by protein factors. All theories propose that unknown control factors, other than the sequence itself, are required in the regulation of pim-1 mRNA, and are discussed below.
a. Transcriptional attenuation by DNA secondary structure

Transcriptional attenuation/repression is one theory to explain the differences in the steady state levels of pim-1 mRNA; formation of stem-loop structures or triple helix binding in genomic DNA blocks transcriptional machinery in the first exon-intron, leading to a "transcriptional pause" [Nagarajan and Narayana, 1993; Svinarchuk et al., 1994]. Dyad symmetry elements which could possibly form stem-loop structures were found within the first 488 base pairs of the genomic coding sequence of pim-1, but neither the location nor the identity of these sequences was specified [Nagarajan and Narayana, 1993]. This block might possibly be prevented or overcome by the presence of a mystery factor that could interact with the DNA directly, overcoming this "transcriptional pause", allowing mRNA transcription and the subsequent protein translation.

A novel potential method of eukaryotic gene regulation involving triple-helix binding has recently been suggested for pim-1 [Svinarchuk et al., 1994]. A polypurine oligonucleotide (GGGAGGGGGAGG) based murine pim-1 promoter residues -182 to -194, binds to a homopyrimidine duplex in the murine pim-1 promoter (-358 to -370 and -425 to -437) forming a stable triple-helix complex. This complex is stable to 65°C, binds irreversibly at 37°C and the interaction is dependent on the sequence in the pim-1 promoter region; a one base pair substitution abrogates oligonucleotide binding and triple-helix formation. A suggested method of gene regulation involves a regulatory molecule interacting with the DNA on the basis of triple-helix recognition [Svinarchuk et al., 1994]. Interestingly, a yet unidentified transcription factor, PPF-348 (Pim-1 promoter factor 348) binds to residues -348 to -374 [Meeker et al., 1990] which overlaps with the homopyrimidine domain. If regions of the pim-1 promoter are able to trimerize into a stable complex, this PPF-348 may be the transcription factor that interacts with this region and regulates gene expression.

Recently another group has reported that elements within the pim-1 5' untranslated region (UTR) may be responsible for translational repression, and that this repression may be relieved in vivo by a factor-dependent mechanism [Hoover, et al., 1995].

b. Pim-1 regulation by mRNA destabilization

Messenger RNA stability is involved in the modulation and regulation of pim-1 mRNA levels in normal lymphocytes and germ cells by protein synthesis-dependent post-transcriptional mRNA degradation [Wingette et al., 1991]. The 1.3 kb 3' untranslated region of both the murine and human pim-1 genes contain conserved UUAUUUAUU
motifs, which are believed to mediate mRNA instability [Lagnado et al., 1994; Zubiaga et al., 1995]. The removal of this region occurs during pim-1 proviral integration and in the production of stable germ-cell specific pim-1 transcripts [Domen et al., 1987; Zakut-Houri et al., 1987; Wingette et al., 1991, 1992].

The selective and rapid degradation of mRNA transcripts containing AU-rich elements (AREs) are mediated by protein factors and is protein synthesis-dependent. Actinomycin D (an inhibitor of de novo mRNA synthesis) inhibits mRNA degradation, and cycloheximide increases the stability of pim-1 transcripts about three-fold [Wingette et al., 1991; Meeker et al., 1990]. ARE-containing mRNA sequences are stabilized by growth factors, phorbol esters, calcium ionophores, mitogenic antibodies, protein synthesis inhibitors and Con A, all of which may modulate the action of mRNA degrading proteins [reviewed in Reeves and Magnuson, 1990].

c. Pim-1 regulation by differing rates of transcription

Differences in the rates of pim-1 transcription may be responsible for differences in the levels of pim-1 mRNA observed. Levels of pim-1 mRNA expression were studied in three cell lines that exhibited levels of expression that were typical for unstimulated cells of the myeloid (K562), B-cell (Daudi) and T-cell (Jurkat) lineages [Meeker et al., 1990]. pim-1 mRNA was most highly expressed in K562 cells, which had 20-fold greater expression than in Daudi and 50-fold greater expression than in Jurkat cells. The variations in the amount of mRNA were primarily due to differences in the rate of transcription of the gene, as the rates of transcription were similar to the amounts of mRNA. As the pim-1 promoters were compared between the different cell lines and were found to be identical, some tissue or cell specific mechanism must control the rate of transcription in these cells [Meeker et al., 1990]. The half-life of the pim-1 mRNA varied between these different cell lines, with 47 minutes in the K562 cells, 71 minutes for the Daudi cells and 35 minutes for Jurkat. In this case, the half-life of the mRNA transcripts were not related to the amounts of pim-1 mRNA detected in the cells [Meeker et al., 1990]. A second study showed that the amount of pim-1 mRNA transcript in a cell is not solely related to the stability of the transcript, and suggested that other processes such as increased transcription, mRNA processing or transport from the nucleus are involved [Wingette et al., 1995]. Indeed, Yip-Schneider et al., [1995] demonstrated that an increase in the rate of transcription of the pim-1 gene was partially responsible for elevations in the pim-1 levels in the cell in response to IFNγ.
The whole question of regulation of \textit{pim-1} has been complicated by the recent claim that the Pim-1 protein and \textit{pim-1} mRNA are not necessarily induced under the same conditions [Hoover, et al., 1995], a finding that is in direct conflict with other groups [Yip-Schneider et al., 1995]. Although different levels of transcriptional and translational control over \textit{pim-1} expression exist, it is not known whether they operate independently.

5. \textbf{PIM-1: THE PROTEIN}

\textbf{i. EXPRESSION IN NORMAL TISSUE}

In consideration of the wide disparity between levels of \textit{pim-1} mRNA expression, it is hardly surprising that Pim-1 protein levels vary between different tissues and cell types. Amson \textit{et al.}, measured Pim-1 protein expression during fetal development and in hematopoietic malignancies [Amson \textit{et al.}, 1989]. Very high protein expression was detected in the liver and spleen during human fetal hematopoiesis, and low expression was detected in the kidney. In the fetal liver, expression was limited to the typical round cells, which are hematopoietic progenitors, but not in surrounding tissue [Amson \textit{et al.}, 1989]. In human adults, the protein was expressed only slightly in circulating granulocytes and in bone marrow [Amson \textit{et al.}, 1989]. These results imply that Pim-1 may play a role during embryonal and fetal hematopoiesis, but does not reveal anything further about the actual function of the kinase.

The protein product of the \textit{pim-1} gene is localized to the cytoplasm in \textit{pim-1} over-expressing (K562, 679thy) and low expressing (NIH3T3) cell lines and in primary lymphocytes of normal and Pim-1 transgenic mice [Telerman \textit{et al.}, 1988; Saris \textit{et al.}, 1991]. The kinase does not have any hydrophobic stretches nor signal sequences characteristic of a membrane directed protein [Domen \textit{et al.}, 1987]. This cytoplasmic localization indicates that the kinase is potentially a component of a signal transduction pathway.

\textbf{ii. THE PRODUCT OF THE PIM-1 GENE IS A KINASE}

When first sequenced, the protein products of both the human and murine \textit{pim-1} genes were found to be structurally related to kinases, possessing all of the conserved residues and domains of protein kinases [Hanks \textit{et al.}, 1989]. All studies examining the activity of endogenous and expressed Pim-1 have confirmed that the protein has auto-kinase activity. The presence of a tyrosine at residue 198 corresponds to Tyr-416 in Src,
which is a conserved autophosphorylation site in all tyrosine kinases [Cooper and MacAuley, 1988; Telerman et al., 1988; Hanks et al., 1989], led to the initial classification of Pim-1 as a tyrosine kinase [Meeker et al., 1987a; Telerman et al., 1988]. An early study with endogenous Pim-1 protein immunoprecipitated from K562 cells and translated in vitro, confirmed this belief [Telerman et al., 1988]. Not only did the immunoprecipitated Pim-1 autophosphorylate on tyrosine residues, but this tyrosine-specific activity was recovered even after the immunoprecipitated Pim-1 was boiled, electroluted and removed from an SDS-PAGE gel, suggesting that this activity was due specifically to the Pim-1 and not a contaminating kinase. Protein phosphotransferase activity towards serine and threonine with the Pim-1 was not detected [Telerman et al., 1988].

After extensive investigation by many different groups, Pim-1 was reclassified as a serine/threonine protein kinase. Both the mouse and human pim-1 cDNA undergo in vitro transcription-coupled translation in the rabbit reticulocyte system producing an active kinase with serine-specific autophosphorylating activity [Saris et al., 1991; Padma and Nagarajan, 1991]. Human and murine Pim-1 expressed in bacteria [Saris et al., 1991; Hoover et al., 1991; Friedmann et al., 1992] and in COS cells, and immunoprecipitated from the 679thy and K562 cell lines [Saris et al., 1991], was found to have autophosphorylating activity only on serine and/or threonine residues and have only serine/threonine phosphotransferase activity towards exogenous substrates. Phosphotyrosine-containing proteins were not detected on Western blots of E.coli cells expressing wild-type or mutated Pim-1 [Saris et al., 1991]. In contrast to the earlier study, Pim-1 was found to be thermolabile [Hoover et al., 1991]. It would seem from these studies that Pim-1 possesses serine/threonine but not tyrosine kinase activity. Among the groups claiming that Pim-1 was not a tyrosine kinase, some conflict still existed; some groups claimed that the Pim-1 was strictly a serine kinase [Saris et al., 1991; Padma and Nagarajan, 1991], while others detected kinase activity towards threonine residues as well [Hoover et al., 1991; Friedmann et al., 1992]. The protein product of the recently discovered pim-2 gene was also found to possess serine autocatalytic activity [van der Lugt et al., 1995].
Expression of the Pim-1 Protein Product

Examination of the protein products of human and murine pim-1 genes has revealed that several forms of Pim-1 protein are produced in various systems as a result of alternate initiation of translation. These multiple protein forms result from a single mRNA transcript [Nagarajan et al., 1986].

Human Pim-1 protein often appears as a tight 33-34 kDa doublet in Western blots and immunoprecipitations of various cell lines [Telerman et al., 1988; Saris et al., 1991; Lilly, et al., 1992]. In vitro translation of the human Pim-1 in the rabbit reticulocyte system yields a doublet of 32 and 33 (34 kDa) [Domen et al., 1987; Padma and Nagarajan, 1991] in contrast to a predicted protein product of 35.6 kDa [Reeves et al., 1990]. In addition, a smear is often detected at about 50 kDa on SDS-PAGE gels [Domen et al., 1987]. Although initially thought to be the results of degradation or phosphorylation, mutant studies with murine Pim-1 have found that the 34-35 doublet is a result of independent initiation from an alternate initiation site, CUG, 4 codons upstream of the normal start site [Saris et al., 1991]. Some groups also detected two minor, 28-29 kDa bands, in-frame products of the translation, which immunoprecipitate with the Pim-1 C-terminal antibodies [Domen et al., 1987]. These proteins are speculated to arise from internal initiation events, possibly at the Met-88 residue conserved in mouse, rat and human Pim-1 [Domen et al., 1987; Wingette et al., 1992].

Expression of murine Pim-1 differs from the human in its production of an additional protein product. Murine Pim-1 was expressed as a 33 to 34 kDa doublet [Domen et al., 1987] and a 41 kDa [van Lohuizen et al., 1989] (or 44 kDa [Saris et al, 1991]) protein in equimolar amounts in all murine tissues and cell lines tested [Saris et al, 1991]. These proteins were also coexpressed in the in vitro rabbit reticulocyte translation system, bacterial and COS expression systems, and were detected by immunoprecipitation from a murine thymoma cell line, 679thy (from a transgenic overexpressing Eμ-pim-1) [Saris et al, 1991]. The larger sized protein was due to an in-frame amino terminal extension, resulting from an alternate, albeit inefficient, translation event at an upstream CUG site [Saris et al, 1991]. This upstream initiation is not conserved between mice and humans, but was observed during translation of the pim-2 gene product [van der Lught et al., 1995]. Overexpression of the Pim-1 did not change the ratio or the cellular localization of the two proteins. However, the in vivo association state of the cell-free synthesized kinase varies, with the p34 existing in a monomeric state and the p44 forming either a dimer or a complex with other proteins [Saris et al, 1991].
iv. PROTEIN HALF-LIFE OF PIM-1

In K562 cells, the Pim-1 protein has a very short half-life implying that it is tightly regulated. The half life of the Pim-1 proteins immunoprecipitated from murine 679thy cells were determined to be 1 hour for 44 kDa protein and 10 minutes for the 34 kDa protein [Saris et al., 1991]. This is in agreement with another group that found the half-life of human Pim-1 to be 10 minutes [Amson et al., 1989].
STUDIES INTO THE FUNCTION OF PIM-1

Pim-1 is highly conserved between all species examined and is tightly regulated pre-transcriptionally, post-transcriptionally, and post-translationally. The fact that pim-1 is a proto-oncogene implies that it serves an important function in the cell. Expression patterns of pim-1 mRNA and protein implicate the kinase in hematopoiesis and male germ cell development. However, differences in expression levels between different subtypes, developmental stages and species have made it difficult to assign a specific role to the kinase based on expression patterns alone. Studies to elucidate the function of pim-1 are divided into three major categories: substrate studies to identify downstream targets of the kinase, studies with growth factors and receptors to understand if Pim-1 is regulated in a specific signal transduction pathway, and studies with transgenic animals to analyze the in vivo effects of pim-1 overexpression or knockout.

6. PIM-1 SUBSTRATE SPECIFICITY

Substrate specificity studies have been performed to a limited extent with endogenous Pim-1 [Saris et al., 1991] and extensively with bacterially [Hoover et al., 1991; Friedmann et al., 1992] and in vitro expressed Pim-1 [Padma and Nagarajan, 1991]. Studies have defined optimal conditions for kinase activity, and have tested a wide variety of proteins as exogenous substrates of Pim-1. As will be described below, preliminary estimations of a substrate consensus sequence for Pim-1 were determined using peptide and protein substrates.

Studies using endogenous Pim-1 immunoprecipitated from K562 cells found that the autophosphorylating activity of the kinase was constant in the range pH 5.5-7.5. The optimal divalent cations for Pim-1 peptide assays, as defined by studies with bacterially expressed Pim-1, were 10 mM MgCl₂ or 5mM MnCl₂ [Friedmann et al., 1992]. Autophosphorylation activity was inhibited by higher cation concentrations as well as by the presence of zinc and sodium in concentrations over 50 mM [Saris et al. 1991; Friedmann et al., 1992]. Thrombin-cleaved Pim-1 and GST-Pim-1 phosphorylated the same spectrum of substrate proteins and peptides in vitro but GST-Pim-1 was more efficient than thrombin-cleaved Pim-1 [Hoover et al., 1991].

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Many proteins were tested and found to be substrates of Pim-1 including histone H1 (on serine and threonine), enolase (on serine and threonine) [Hoover et al., 1991; Friedmann et al., 1992], histone 2B (on serine), salmon protamine [Saris et al., 1991] which contains no tyrosine residues, and lactate dehydrogenase, which was only slightly phosphorylated [Hoover et al., 1991]. Histone H1 was found to be the best substrate of mammalian Pim-1, with a \( K_m \) of 7 \( \mu \)M under optimal conditions [Friedmann et al., 1992]. All exogenous substrates were found to be phosphorylated on serine and/or threonine residues only. Proteins tested but not phosphorylated by Pim-1 were casein, BSA, poly(GLU:TYR 4:1), GST [Hoover et al., 1991], angiotensin I peptide (DRVYIHPFHL), Src peptide (RRLIEDAELYAARG) and c-Myc [Saris et al., 1991].

V8 proteolysis of histone H1 phosphorylated by Pim-1, followed by HPLC and sequencing revealed two sequences in histone H1 that are similar to the Kemptide: TAPAETAAPAP and AKPKA. One corresponded to the N-terminal fragment containing a cAMP-dependent protein kinase phosphorylation site, LRRASGP, which was referred to as "H1PEP" [Friedmann et al., 1992]. This H1PEP sequence is conserved in many histone H1 proteins and may be the site of Pim-1 phosphorylation [Friedmann et al., 1992].

Peptide substrates have been used to define the substrate consensus sequence of Pim-1. Pim-1 exhibited strong preference for peptide "H1PEP" (KRRASGP) six-fold over Kemptide (LRRASLG), the optimal substrate of cAMP dependent protein kinase. H1PEP and Kemptide both exhibited \( K_m \) values of 0.4 mM with Pim-1. Both N-terminal arginine residues (-2,-3) in the peptides were equally important for recognition, as replacement of these residues with alanine reduced the efficiency of the peptides as substrates to the same extent. Basic amino acids, in this case arginine, were essential on the N-terminal side, while basic residues on the carboxy terminus of the phosphoacceptor site were inhibitory, and interfered with substrate recognition [Friedmann et al., 1992]. Saris suggested that in addition to a preference for serine surrounded by basic residues (arginine), the Pim-1 also requires a proline residue [Saris et al., 1991]. Under optimized conditions, the consensus site was deduced to be \((R/K)3-X-S/T-X'\), where X' cannot be arginine, lysine, or a large hydrophobic residue [Friedmann et al., 1992].

Pim-1 showed a higher \( K_m \) value for Kemptide than cAMP-dependent protein kinase, possibly due to the Pim-1 having less hydrophobic residues at the sites that interact directly with the substrate [Friedmann et al., 1992]. Examination of the crystal structure
of the cAMP-dependent protein kinase revealed that the main residues interacting with the substrate were Leu-198, Pro-202 and Leu-205 [Knighton et al., 1991]. In contrast, Pim-1 has Phe-201, Arg-205 and Ser-209 at these same sites, suggesting that the less hydrophobic residues at the +1 site of the peptide are preferred for Pim-1 recognition [Friedmann et al., 1992].

The vague definition of a Pim-1 substrate consensus sequence has had very few functional implications. Although Pim-1 phosphorylates several proteins and peptides with relatively high affinity in vitro, it is uncertain if these phosphorylations reveal any physiological relationship between the kinase and these substrates in vivo. Further work is required before the physiological substrates of Pim-1 can be identified.

7. UPSTREAM REGULATORS: GROWTH FACTORS AND MITOGENS

i. MITOGEN AND GROWTH FACTOR STIMULATION OF PIM-1

pim-1 mRNA expression is increased in response to growth factors and mitogens. Pim-1 mRNA and protein expression is induced by cytokines associated with the hematopoietic (HP) receptor superfamily, such as GM-CSF, G-CSF, IL-2, IL-3, IL-5, IL-6, IL-7 but is not limited to these types, as it is also stimulated with ConA, PMA, IFNγ, Steel factor (SF) when costimulated with other mitogens and in response to TCR cross-linking [Dautry et al., 1988; Wingette et al. 1991; Lilly et al., 1992; Saito et al., 1992; Domen et al., 1993b, 1993c; Wingette et al. 1995; Yip-Schneider et al., 1995]. Pim-1 expression is dependent on the mitogen used with effects being proportional to the proliferative effects of the cytokine [Wingette et al. 1991; Lilly et al., 1992]. Expression of Pim-1 in response to myeloid cytokines is varied, and depends on the nature of the growth factor and the response phenotype of the cell examined. However, it seems to be generally associated with a proliferative response to GM-CSF and similar cytokines [Lilly, et al., 1992; Polotskaya et al., 1993]. Pim-1 does not seem to be induced in response to SF alone [Domen et al., 1993c], TNFα [Saito et al., 1992] or by receptor tyrosine kinases [Polotskaya et al., 1993]. Many of the cytokines, such as IL-3, IL-5 and GM-CSF, have similar functions in common target cells such as eosinophils, implying that Pim-1 may have a similar function in response to each of these cytokines [Kinoshita et al., 1995].
Many growth factors were found to synergistically upregulate the expression of Pim-1 when combined. For example, the induction of Pim-1 mRNA and protein is much stronger when stimulated with both IL-7 and SF, IFNγ and SF, PMA and ConA, or PMA and ionomycin than when each of these growth factors is used separately [Domen et al., 1993b; Yip-Schneider et al., 1995; Wingette et al., 1995]

ii. EFFECTS OF MITOGEN STIMULATION ON PIM-1

In unstimulated, factor-dependent myeloid or lymphoid cells, there is normally a very small amount of pim-1 mRNA present and no protein. Mitogen stimulation causes a rapid accumulation of pim-1 mRNA within an hour, which is followed by an increase in protein levels. For example, stimulation of two IL-2-dependent lymphoid (CTLL-2 and B6.1) and one IL-3 dependent myeloid (FDC-P2) cell line, with IL-2 and IL-3 caused and increased accumulation of pim-1 mRNA. All cell lines showed similar patterns of pim-1 mRNA accumulation, with a small amount of pim-1 mRNA present in serum-starved cells which increased by 40 minutes, peaked between 2-8 hours and declined after 10 hours post-stimulation [Dautry et al., 1988]. Upregulation of pim-1 mRNA in response to IL-2 and IL-3 occurred at the transcriptional level in the absence of protein synthesis [Dautry et al., 1988]. pim-1 mRNA and protein levels are sustained for the duration of growth factor exposure [Lilly, et al., 1992]. This time course of pim-1 mRNA accumulation is similar to that of c-myc [Dautry et al., 1988].

As discussed in a previous section, pim-1 mRNA is regulated post-transcriptionally in mitogen-stimulated lymphoid cells by a protein synthesis-dependent mRNA degradation [Wingette et al., 1991]. Changes in the levels of pim-1 mRNA are controlled in part by changes in pim-1 mRNA stability, with mitogens mediating the stabilization of the mRNA transcript. mRNA stabilization leads to an accumulation of pim-1 transcript in the cell which results in a greater amount of protein product translated. Other methods, such as an increase in the rate of transcription, contribute to an accumulation of mRNA in addition to mRNA stabilization.

iii. PIM-1 MAY PLAY A ROLE IN T CELL RECEPTOR SIGNALING

Pim-1 is likely to be active in T cells, as increased pim-1 expression is associated with murine T cell lymphomas [Meeker et al., 1987b], the kinase is expressed in the thymus and also in ConA-stimulated spleen cells, a model system that is representative of proliferating peripheral T cells [Mally et al., 1985]. Expression of pim-1 mRNA can be induced in both Tα/β (five-fold) and Tγ/δ cells (eight-fold) by PMA and ionomycin.
[Wingette et al., 1995]. *Pim-1* mRNA expression can also be induced by T cell receptor (TCR) cross-linking with anti-CD3 antibodies [Wingette and Magnuson, 1995], confirming that enhanced expression of the *pim-1* gene is an early and transient event in activation of normal lymphocytes [Wingette et al., 1991].

iv. **INDUCTION OF PIM-1 EXPRESSION BY PKA AND PKC**

Although IL-3 can produce an increase in Pim-1 protein levels in 2-4 hours, the PKC activator bryostatin, which can substitute for IL-3 as a mitogen in MAC-II or U937 cells, did not lead to any increase in Pim-1 in these cells [Lilly et al., 1992]. PMA did not cause prolonged changes in *c-myc*, *pim-1* or cyclin D2 mRNA [Polotskaya et al., 1993], and failed to induce protein production in MO7E cells [M. Lilly, personal communication]. TPA treatment to stimulate PKC, and forskolin or dibutyryl-cAMP treatment to stimulate PKA did not have any effect on *pim-1* mRNA levels, indicating that PKC and PKA are not involved in *pim-1* induction [Dautry et al., 1988]. This implies that *pim-1* is not stimulated through the PKC pathway, and even though binding of GM-CSF to the receptor causes PKC translocation, this may not be how most of the signal is transduced.

By contrast, another group found that PMA synergized with ConA and with ionomycin to induce a strong *pim-1* mRNA induction in primary lymphocytes [Wingette et al., 1991, 1995]. In this system, *pim-1* gene expression seemed to be upregulated transcriptionally and post-transcriptionally following activation of PKC. Not only did PMA induce rapid *pim-1* expression, but PKC inhibitors H-7 and staurosporine blocked *pim-1* expression. In stimulated T cells, *pim-1* mRNA was not induced after elevation of intracellular free Ca\(^{2+}\) [Wingette and Magnuson, 1995] and was induced by PMA alone, suggesting that the activation of Pim-1 involved a PKC signalling pathway [Wingette et al., 1995]

v. **PIM-1 IS INDUCED IN RESPONSE TO SIGNALING THROUGH RECEPTORS OF THE GM-CSF FAMILY**

Although *pim-1* expression is upregulated in response to a large number of growth factors and mitogens, studies with the GM-CSF and IL-3 pathways have been most productive [Lilly, 1989; Polotskaya et al., 1993]. Recent studies with mutant GM-CSFβ receptor subunits have delineated specific signalling pathways and have identified potential upstream regulators of Pim-1 [Polotskaya et al., 1993; Sato et al., 1993].
a) Induction of Pim-1 by GM-CSF

Many different cell types have been tested and found to exhibit induction of *pim-1* in response to GM-CSF. The cell growth response to GM-CSF is correlated with prolonged increases in cell levels of *c-myc, pim-1* and cyclin D2 mRNA, but not with changes in either immediate early genes or mitogen-activated protein kinase (MAPK) phosphorylation [Polotskaya et al., 1993]. The expression of Pim-1 protein is selectively induced in the human factor-dependent myeloid leukemia cell line MO7E by IL-3 and GM-CSF [Lilly et al., 1992]. The promyelocytic cell line HL60 does not express *pim-1* with or without GM-CSF (and does not proliferate in response to the cytokine), but when the cell was made GM-CSF-dependent by DMSO treatment, *pim-1* was induced. In the murine cell line MAC-II, *pim-1* is induced by IL-3 and GM-CSF in 2-4 hours, but not by bryostatin or M-CSF [Lilly et al., 1992]. The human line U937 (myeloid leukemia) expressed *pim-1* in response to GM-CSF, G-CSF and IL-6 but not bryostatin, even though the cell line does not proliferate in response to GM-CSF [Lilly et al., 1992]. As additional proof that *pim-1* may mediate GM-CSF signalling, *pim-1* anti-sense oligonucleotides reduced cell growth in GM-CSF cultures by 50-80% [Lilly, 1989].

b) Background information on the GM-CSF/IL-3/IL-5 receptor family

Interleukin 3 (IL-3) and granulocyte-macrophage colony stimulating factor (GM-CSF) are produced by activated T cells and mast cells, and serve as potent growth factors for immature multipotential hematopoietic progenitors. GM-CSF causes a dose-dependent proliferative response in immature myeloid cell lines, but acts on more differentiated cell types to cause activation as opposed to proliferation [Lilly et al., 1992]. The receptors for human IL-3, IL-5 and GM-CSF are heterodimeric and share a common βc subunit, but have distinct cytokine-specific α subunits. Ligand binding to the specific α subunit causes dimerization and activation of β subunits.

The cytoplasmic portion of the 881 amino acid β-subunit has two distinct functional domains. The membrane distal domain between Leu-626 and Ser-763 is necessary for many of the functions of the GM-CSF receptor including the activation of tyrosine phosphorylation of cellular proteins including SHC, the activation of Ras, Raf-1, MAPK, p70S6K and the induction of *c-fos/c-jun*, yet it is dispensable for growth factor-induced proliferation [Sato et al., 1993; Kinoshita et al., 1995]. The membrane proximal domain between Arg-455 and Glu-517 was essential for proliferation, JAK2 activation, and for the induction of *c-myc* and *pim-1* [Sato et al., 1993; Quelle et al., 1994; Kinoshita et al., 1995]. This proximal region is believed to stimulate a tyrosine kinase, as induction of *c-
myc and pim-1 by GM-CSF is sensitive to herbimycin A, a tyrosine kinase inhibitor, and receptor deletion mutants containing only the proximal domain are also sensitive to herbimycin A [Sato et al., 1993; Quelle et al., 1994].

The 400 amino acid cytokine-specific α subunit functions not only in ligand binding but is necessary for signal transduction by protein phosphorylation and entry into the cell cycle [Polotskaya et al., 1993]. A short intracytoplasmic region of the α subunit (aa 346-382) is necessary for cell growth and is involved in translocation of PKC to the cell membrane [Polotskaya et al., 1994]. Phosphorylation and activation of the β subunit requires the entire intracytoplasmic domain of the α subunit. The functions of the α and β subunits are complementary and cooperative; not only is the β subunit necessary for high affinity ligand binding by the α subunit [Ronco et al., 1994] but the 54 amino acid α subunit cytoplasmic tail may mediate the specificity of the cellular response to cytokines, possibly by interacting with secondary signalling proteins [Polotskaya et al., 1993].

c) Pathways induced by the IL-3/GM-CSF receptors

Long term cell proliferation of cultured cells requires two distinct pathways. One pathway leads to the induction of DNA synthesis and is inhibited by both staurosporin (a protein kinase C inhibitor that generally inhibits tyrosine and serine/threonine kinases) and genistein (an inactive ATP analogue that acts as a tyrosine kinase inhibitor). The other route is an anti-apoptotic pathway that is inhibited only by staurosporin [Kinoshita et al., 1995]. The IL-3 and GM-CSF activate the anti-apoptotic pathway even in the presence of genistein by activating a signalling pathway distinct from the pathway that causes induction of DNA synthesis [Kinoshita et al., 1995]. Analysis of mutant IL-3/GM-CSF receptor β subunits lacking one or more of the defined functional domains has allowed definition of the downstream events and has permitted discrimination of the two signal pathways involved in receptor signalling.

Signals transduced through the membrane distal domain (Leu-626 and Ser-763) of the GM-CSF receptor are responsible for the prevention of apoptosis. The membrane distal domain is necessary for phosphorylation and stimulation of SHC, ras, Raf-1 and MAPK and is not sensitive to inhibition by genistein. Removal of this domain causes the cells to undergo apoptosis, even in the presence of IL-3 [Kinoshita et al., 1995]. Expression of v-Ras in cells lacking this domain, rescues the cell from apoptosis but does not allow the cells to proliferate in long term culture [Kinoshita et al., 1995].
The membrane proximal domain (Arg-455 and Glu-517) of the GM-CSF/IL-3 receptor β subunit is necessary for DNA synthesis and cell cycle progression, but not for the prevention of apoptosis [Kinoshita et al., 1995]. This DNA synthetic pathway, repressed by both staurosporin and genistein, is responsible for JAK2 activation and cyclin E, c-myc and pim-1 mRNA induction. This domain does not seem to be necessary for the induction of cyclin D2, D3, CDK4 and bcl-2 [Kinoshita et al., 1995]. This data strongly indicates that pim-1 induction in response to IL-3/GM-CSF is mediated through the membrane proximal domain and that Pim-1 may be involved in a proliferative pathway [Fig. 1]. Pim-1 may occupy a similar position in pathways stimulated by IL-3 and erythropoietin (Epo), a glycoprotein produced in mammalian kidney and liver and is a member of the cytokine receptor superfamily.
Figure 1: Model of GM-CSF receptor signal transduction as mediated by two distinct domains of the βc subunit. Protein tyrosine kinases (PYK) may be involved. Modified from Sato et al., 1993.
d) Potential upstream activators of pim-1 transcription

One candidate for the upstream activator of pim-1 is the JAK2 tyrosine kinase, a member of the family of receptor-associated soluble tyrosine kinases. JAK2 lacks SH2 and SH3 domains and is activated and tyrosine-phosphorylated in response to GM-CSF, Epo, IL-3, IL-5, IL-6, IFNγ, prolactin as well as several other ligands [Schindler, 1995]. JAK2 activation and pim-1 expression are both mediated through the membrane proximal regions of the EpoR and the GM-CSF βc chains, suggesting that JAK2 may be an intermediate factor upstream of Pim-1 [Sato et al., 1993; Quelle et al., 1994; Miura et al., 1994]. JAK2 associates with the proximal domain of the βc chain independently of either GM-CSF or the GM-CSF α chain, implying that the GM-CSF βc receptor subunit and JAK2 may be constitutively associated [Quelle et al., 1994]. JAK2 phosphorylates a Stat protein in response growth factor stimulation, which then dimerizes, translocates to the nucleus and binds DNA, influencing the transcriptional response of cytokine and growth factor-inducible genes [Hill and Treisman, 1995; Schindler, 1995]. Recently, the 5' flanking region of pim-1 was found to contain a functional Stat1α binding site [Yip-Schneider et al., 1995]. That Stat1α is a specific target of JAK2, implies that Pim-1 expression is upregulated through the JAK2-STAT pathway in contrast to acting as a direct substrate for the JAK2 kinase.

Another protein that may function in the same signal transduction pathway as Pim-1 is the proto-oncogene-encoded protein Vav. This 95-kDa protein contains one SH2 and two SH3 domains and has some of the structural features of a transcription factor. Vav is expressed only in hematopoietic cells, and is tyrosine-phosphorylated in response to many of the same growth factors that induce pim-1 expression. Vav is tyrosine phosphorylated in response to stimulation of the T-cell antigen receptor, cross-linking of the IgE or IgM receptors by stimulation with Epo, and by p210BCR/Ab expression in MO7e cells [Matusuguchi et al., 1995; Miura et al., 1994]. In GM-CSF stimulated cells, Vav coprecipitates with JAK2, possibly by interaction of the SH2 domain of Vav with JAK2 [Matusuguchi et al., 1995]. Vav is tyrosine phosphorylated in response to Steel factor (SF), but not GM-CSF or IL-3 [Alai et al., 1992], while pim-1 mRNA is induced in response to GM-CSF and IL-3 but not SF alone. This implies that Pim-1 and Vav may participate in a similar pathways, but in response to different stimuli.

There are not many reports of other cytoplasmic mediators that have been tested as upstream activators of Pim-1. Neither c-Fms tyrosine kinase [Lilly et al., 1992] nor c-Raf-1 [Wingette and Magnuson, 1995] was found to influence pim-1 expression.

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e) Other signal transduction pathways possibly involving Pim-1

Pim-1 may play a role in growth factor signalling from the erythropoietin receptor (EpoR). Epo affects erythroid progenitors in hematopoietic organs and induced Pim-1 expression in various 32D/EpoR cell lines. Stimulation of the EpoR caused receptor association with the JAK2 kinase, led to tyrosine phosphorylation of Vav and induced expression of the Pim-1 protein [Miura et al., 1994]. Pim-1 expression was stimulated in various mutant EpoR-containing cell lines, except for those having an inactivated EpoR mutant; in these cell lines, only IL-3 induced Pim-1 expression [Miura et al., 1994].

Pim-1 expression is also stimulated in response to IFNγ and SF in Mo7e cells [Yip-Schneider et al., 1995]. Stimulation of Mo7e cells with both growth factors has a synergistic effect on Pim-1 levels in the cell. Stimulation with SF alone does not elicit a Pim-1 response, stimulation with IFNγ induces both Pim-1 mRNA and protein accumulation and stimulation with both growth factors causes a 2- to 3-fold increase of both Pim-1 mRNA and protein levels. In this system, the upregulation of Pim-1 occurs both transcriptionally and post-translationally: IFNγ alone causes an increase in the rate of pim-1 gene expression while the IFNγ/SF stimulation increases the stability of the mRNA transcript. STAT1α is thought to mediate the transcriptional effects of IFNγ on pim-1 [Yip-Schneider et al., 1995].

f) Possible involvement in an apoptotic pathway?

Many of the growth factors that stimulate pim-1, including IL-3, IL-5, GM-CSF and Epo, have an anti-apoptotic function [Koury and Bondurant, 1990; Kinoshita et al., 1995]. Apoptosis is a normal part of hematopoietic differentiation, with T cell apoptosis taking part during thymic selection and B cell apoptosis during the development of self-tolerance [Kinoshita et al., 1995]. As tempting as it is to speculate about Pim-1 being an apoptotic inhibitor, studies with mutant GM-CSF receptors have implicated Pim-1 as a component of the cell proliferation pathway as opposed to the anti-apoptotic pathway [Sato et al., 1993; Kinoshita et al., 1995]. Defining the function of Pim-1 in the cell cycle and in lymphopoiesis requires further study.

The induction of pim-1 gene during the primary response to growth factors suggests that Pim-1 functions as a cytoplasmic mediator in myeloid growth factor signal cascades. Although the mRNA and protein levels are upregulated, it is unclear if the activity of the kinase is modified in any way by phosphorylation. Indeed, changes in phosphorylation state of Pim-1 (as evidenced by band shifts on SDS-PAGE gels) in response to growth
factors have not yet been demonstrated. The recent identification of a STAT1α binding site in the \( pim-1 \) gene strongly imply that response of Pim-1 to growth factor stimulation may be a secondary effect, and that Pim-1 is not part of the primary signal transduction pathway from the cell membrane to the nucleus.

8. **PIM-1 TRANSGENIC MICE**

Studies with transgenic mice have contributed the most to our understanding of the role of \( pim-1 \) and have confirmed that it is, in fact, an oncogene. Provirus tagging with slow growing retroviruses and the creation of double transgenics have led to the identification of other oncogenes with which \( pim-1 \) cooperates. The creation of \( pim-1 \) null mice has permitted comparison of the growth factor response of cells from \( pim-1 \) null, wild type and overexpressing animals, allowing the role of \( pim-1 \) to be assessed in specific signal transduction pathways.

i. **TRANSGENIC ANIMALS CONFIRM THAT PIM-1 IS AN ONCOGENE**

Pim-1 transgenic mice were produced with an upstream immunoglobulin enhancer (Eμ) in the promoter and a single MuLV long terminal repeat (LTR) inserted in the 3' UTR to boost expression of the transgene further [van Lohuizen et al., 1989]. The Eμ enhancer was used to achieve a high level of transcription, as fusion genes between \( pim-1 \) and proviral sequences alone were not expressed in earlier studies. The transgene was expressed at similar high levels in in both B and T cells, and high levels of Eμ-\( pim-1 \) mRNA were detected in the thymus, bone marrow and spleen. No expression was detected in the testes and only low levels were detected in most other tissues, possibly as a result of the presence of circulating lymphocytes. Expression of the transgene did not interfere with expression of the endogenous \( pim-1 \) mRNA; levels of endogenous Pim-1 were comparable between tissues from transgenic and control mice [van Lohuizen et al., 1989]. Pim-1 protein levels were increased in thymic and splenocytes of transgenic animals. Histological examination and fluorescence-activated cell sorting (FACS) analysis of various tissues (liver, spleen, thymus, lymph node and bone marrow) in non-diseased Eμ-\( pim-1 \) transgenic mice revealed no abnormalities nor increase proliferation of hematopoietic cell populations, although a slight enlargement of the spleen was observed [van Lohuizen et al., 1989; Verbeek et al., 1991].
Overexpression of the \( \text{E} \mu \text{-} \text{pim-1} \) transgene caused a rise in the incidence of tumor development in transgenic animals. These tumor cells adapted to \textit{in vitro} culture and could cause tumors in syngeneic hosts, confirming the identity of \text{pim-1} as an oncogene [van Lohuizen et al., 1989]. The target cell population for transformation by \( \text{E} \mu \text{-} \text{pim-1} \) is not a reflection of the expression pattern of this oncogene; despite similar expression levels in both B and T cells, the \( \text{E} \mu \text{-} \text{pim-1} \) transgenic animals developed T cell lymphomas exclusively. The tumors had a long, varied latency period, with tumors appearing in 5-10% of the mice after 7 months [van Lohuizen et al., 1989]. This long latency and the fact that lymphomas were monoclonal in nature implies that high \text{pim-1} expression is not sufficient, and that other genetic events are required to induce the fully malignant phenotype [van Lohuizen et al., 1989].

\text{ii. PROVIRAL TAGGING TO IDENTIFY COOPERATING ONCOGENES}

Studies of proviral integration in murine lymphomagenesis indicate that usually more than one gene is activated [Berns 1988b; 1991]. The method of proviral tagging by MuLV neonatal infection has been used to identify new oncogenes and to define sets of cooperating oncogenes; taking advantage of the fact that slow transforming retroviruses contain no oncogenes, genomic oncogenes are activated and 'tagged' by insertion of the provirus. Oncogene-expressing transgenics can be infected with the virus and malignant transformants analysed for the site of viral integration. \( \text{Pim-1} \) transgenic mice are very susceptible to MuLV-infection, and thus form an ideal system to study provirus tagging.

Provir al tagging identified \( \text{N-} \text{myc} \) and \( \text{c-} \text{myc} \) as being oncogenes that cooperate with \( \text{pim-1} \) [van Lohuizen et al., 1989], despite the lack of cooperativity initially demonstrated between \( \text{pim-1} \) and \( \text{myc} \) in tumors from human patients and in FeLV-induced murine leukemias [Amson et al., 1989; Tsatsanis et al., 1994]. Infection of \( \text{E} \mu \text{-} \text{pim-1} \) transgenic mice with MuLV decreased the latency period of tumor formation to 7-8 weeks as compared to 7 months in uninfected mice and in all lymphomas either \( \text{c-} \text{myc} \) (80%) or \( \text{N-} \text{myc} \) (20%) had been activated by MuLV proviral integration [van Lohuizen et al., 1989].

\( \text{Myc/Pim-1} \) cooperativity was confirmed after Mo-MuLV-infected \( \text{E} \mu \text{-} \text{c-} \text{myc} \) transgenic mice also experienced acceleration of pre-B cell leukemia [Haupt et al., 1991; van Lohuizen et al., 1991]. \( \text{E} \mu \text{-} \text{c-} \text{myc} \) is expressed in B cells, but not T cells or other somatic tissues, and \( \text{E} \mu \text{-} \text{c-} \text{myc} \) transgenic animals showed a predisposition to pre-B cell lymphomas characterized by an enlarged pre-B cell compartment with some aberrantly expressed cell surface markers. MuLV infection decreased the tumor latency period in
Eμ-\textit{myc} transgenic mice from 150 to 50 days, and the pre-B lymphomas that developed had high proportions of proviral integration in the \textit{pim-1}, \textit{bmi-1}, \textit{pim-2} and \textit{emi-1} loci [Haupt \textit{et al.}, 1991; van Lohuizen \textit{et al.}, 1991].

A second transgene cooperating with \textit{pim-1} identified by proviral tagging was \textit{v-abl}. [Haupt \textit{et al.}, 1993]. Eμ-\textit{v-abl} transgenic animals develop plasmacytomas and after infection with MuLV, experience accelerated T cell tumor development. Insertions in \textit{c-myc}, \textit{N-myc} or \textit{pim-1} were observed in 42\% of tumors and of tumors involving \textit{c-myc} activation, 14\% also had \textit{pim-1} insertions, suggesting that all three oncogenes may cooperate in tumorigenesis.

\textbf{iii. STUDIES WITH PIM-1/MYC DOUBLE TRANSGENIC MICE}

The creation of double transgenic animals strongly confirmed the synergistic effects of \textit{pim-1} and members of the \textit{myc} family of oncogenes. All the \textit{myc} family genes can synergize with \textit{pim-1} to cause lymphoid tumors \textit{in vivo}. The relative transforming efficiency (c-\textit{myc}>N-\textit{myc}>L-\textit{myc} ) was maintained when transgenics were crossed with Eμ-\textit{pim-1} mice, but the latency period was accelerated and the disease was more extensive [Möröy \textit{et al.}, 1991].

Overexpression of \textit{pim-1} and \textit{c-myc} caused severe synergistic effects, as Eμ-\textit{pim-1}/Eμ-\textit{c-myc} double transgenic mice developed pre B cell leukemia \textit{in utero} [Verbeek \textit{et al.}, 1991]. Analysis of 17-19 day fetuses revealed that although the gross morphology of double transgenics was normal, the spleen was enlarged and there was a dramatic expansion of pre-B lymphoid cells in the peripheral blood. Despite this strong cooperativity, additional genetic events were still needed for the development of a fully malignant phenotype [Möröy \textit{et al.}, 1991; Verbeek \textit{et al.}, 1991].

Transgenic mice were examined to determine if the N- and L-\textit{myc} family members maintain lineage specific neoplasia when co-expressed with Eμ-\textit{pim-1}, and to assess the relative transforming activities of the \textit{myc} genes when collaborating with other oncogenes [Möröy \textit{et al.}, 1991]. The Eμ-N-\textit{myc} mice expressed the transgene preferentially in B cells and were predisposed to B-cell neoplasia. The Eμ-N-\textit{myc}/Eμ-\textit{pim-1} double transgenic mice were smaller and more sickly than single transgene littermates and experienced accelerated lymphomagenesis, with pre-B lymphoma developing in 36 days in contrast to 13-16 weeks with N-\textit{myc} alone [Möröy \textit{et al.}, 1991].
The Eu-L-myc transgene had the longest latency and the lowest tumor incidence when compared to the other two myc transgenics, and was expressed preferentially in T cells, leading to the development of thymic hyperplasia. Alone, Eu-L-myc and Eu-pim-1 transgenics each had a transforming efficiency of about 5-10%. When expressed together, L-myc/pim-1 double transgenics developed tumors in the thymus with some involvement of spleen and lymph nodes; efficiency was increased to 82% with a shortened latency period of 94 days [Möröy et al., 1991].

To summarize, all three myc genes cooperate with pim-1 in vivo to generate lymphoid tumors but N- and L-myc synergized less efficiently than with c-myc. When expressed alone or with other oncogenes, the myc family members maintained descending efficiency and lineage specificity but had more rapid development of tumors when co-expressed with pim-1. The myc/pim tumors were monoclonal, indicating that other events are required for full tumorigenesis [Möröy et al., 1991]. Previous studies have indicated two additional steps are required for tumorigenesis in pim-1/myc mice [Berns, 1991].

iv. OTHER STUDIES WITH DOUBLE TRANSGENIC MICE

The bcl-2 gene was expressed in transgenic mice and was found to also cooperate with pim-1 [Acton et al., 1992]. The bcl-2 oncogene is an important inhibitor of apoptosis and cooperates with myc [Marin et al., 1995]. Bcl-2-Ig transgenic mice had a low tumor incidence and the expression of the transgene caused B cell malignancies by cellular immortalization. Coexpression of pim-1 and bcl-2 affected several different cell types and accelerated tumorigenesis with a long and variable latency. MuLV infection of bcl-2/pim-1 transgenic mice led to increased tumorigenesis, with proviral integrations in sites including N-myc, c-myc and pal-1. Surprisingly, when bcl-2 transgenics were infected with MuLV, there was no increase in the incidence of tumor formation than with MuLV-infected non-transgenics; bcl-2 is not important for MuLV-induced expression [Acton et al., 1992].

Studies with Eu-pim-1 lpr/lpr double transgenic mice suggest a role for pim-1 in apoptotic pathways [Möröy et al., 1993]. The lpr mutation in mice causes a structural rearrangement of the FAS gene product, a 35 kDa surface receptor molecule expressed in lymphoid cells, structurally homologous to the TNF receptor and to CD40. The FAS gene product is involved in the thymic selection process by transducing an apoptotic signal upon antibody binding at the extracellular domain. C57BL/6 mice homozygous
for the lpr mutation, developed a well described lymphoproliferative syndrome at 26-30 weeks, characterized by the accumulation of abnormal T cells. Expression of the pim-1 transgene rescued lpr lymph node cells from programmed cell death in vitro and prevented steroid-induced apoptosis in vivo. Expression of the pim-1 transgene in the lpr/lpr mice led to a strong acceleration of lymphoproliferation and an increased accumulation of the non-malignant abnormal lpr T cells leading to a dramatic enlargement of lymph nodes in all areas of the body. Eu-pim-1/lpr/lpr thymocytes were protected from steroid-induced apoptotic signals, as dexamethasone was unable to induce apoptosis in these cells.

Cross breeding bcl-2, lpr/lpr and myc transgenic mice with the Eu-pim-1 transgenics caused them to maintain the lymphocytic lineage specificity of the original transgene, but resulted in rapid development and a high incidence of the lymphoid malignancies [Möröy et al., 1991; Verbeek et al., 1991; Acton et al., 1992; Möröy et al., 1993]. Pim-1 overexpression seems to enhance the effects of oncogenes promoting cell proliferation and cell survival by acting as an apoptotic inhibitor rather than a stimulator of cell proliferation [Möröy et al., 1993].

Recently Eu-myc/Pim-1(-/-) double transgenic mice were used to identify the pim-2 gene, a second pim family member [van der Lugt et al., 1995]. By complementation tagging, they managed to identify the pim-2 gene which became activated in the absence of pim-1 expression, implying that the proteins encoded by the pim-1 and pim-2 genes are functionally redundant.

v. PIM-1 NULL MUTANTS - EMBRYONIC STEM CELLS

In vitro studies of effects of pim-1 in early embryonic development were done by consecutive inactivation of pim-1 by homologous recombination in embryonic stem (ES) cells [te Riele et al., 1990]. A knockout was achieved in two steps by homologous recombination, using G418 and hygromycin B for selection. The null allele was created by deletion of the promoter, transcription and translation initiation sites and by removing a large segment of the coding region containing the conserved lysine residue of the ATP binding site in subdomain II of the protein kinase catalytic domain. As the pim-1 gene was highly expressed in ES cells, it was thought that effects of pim-1 gene knockout may be manifested during differentiation and during in vitro propagation. However, no differences in ES cell morphology were observed, with characteristic embryoid bodies developing in all cases, and with no selection against the double pim-1 knockout occuring
[te Riele et al., 1990]. This suggests that pim-1 is not required for normal ES cell proliferation and differentiation.

vi. THE LACK OF PHYSIOLOGICAL EFFECTS IN PIM-1 NULL MICE

Pim-1 deficient or null transgenic mice were constructed and found to have a surprisingly lack of phenotypic abnormalities [Laird et al., 1993; Domen et al., 1993c]. The pim-1 null mice displayed normal behavior, normal body weights, no morphological and histological abnormalities, no differences in tissue distributions were observed, and both male and female were fertile [Laird et al., 1993]. The immature lymphoid compartments were analysed by flow cytometry to reveal no difference between the pim-1 null and normal mice. Splenocytes had stimulatory responses to ConA and lipopolysaccharide (LPS) similar to those of controls [Laird et al., 1993].

The only phenotypic abnormality observed in pim-1 null mice was erythrocyte microcytosis; the MCV (Mean Cell Volume) of erythrocytes was smaller in pim-1 null mice compared to wild-type littermates [Laird et al., 1993]. The concentration of red blood cells was not elevated to compensate for the microcytosis, so hemoglobin levels were reduced. Conversely, the erythrocytes in Eu-pim-1 transgenics overexpressing the protein were larger than normal, and there was a compensatory decrease in erythrocyte concentrations resulting in normal hematocrit and hemoglobin levels. The pim-1 gene was responsible for this abnormality, as introducing a pim-1 transgene with its own promoter into the pim-1 null transgenics restored the low erythrocyte MCV to wild-type levels [Laird et al., 1993]. It has not been determined if the lack of physiological effects in pim-1 null mice is due to a compensatory effects of the pim-2 gene product [van der Lugt et al., 1995].

vii. GROWTH FACTOR STIMULATION OF CELLS FROM TRANSGENIC ANIMALS

A significant functional anomaly observed in cells from the pim-1 null mice was an impaired, but not absent, proliferative response to IL-3 induction in bone marrow-derived mast cells (BMMC) [Domen et al., 1993a, 1993c]. Conversely, expression of high amounts of pim-1 did not lead to growth factor independence, as withdrawal of IL-3 led to apoptosis in BMMC cells from Eu-pim-1 mice [Domen et al., 1993c]. Cells heterozygous for pim-1 expression (pim(+/-)) showed dosage effects with IL-3 [Domen et al., 1993a]. No difference in cell viability was observed between the wild-type, pim-1 overexpressing and pim-1 null cells. Pim-1 null mice had a normal mast cell response to
nematode infection, despite impaired response to IL-3, possibly because of the multiple growth factors (i.e. IL-4) orchestrating an immune response in vivo.

The effects of pim-1 levels on the size of the early B lymphoid compartments in bone marrow were studied using cells from wild-type (WT) and from pim-1 null and pim-1 overexpressing transgenic mice. The pim-1 levels determined the number of SF and IL-7 responsive early B-lymphoid colony forming cells (CFC) in the bone marrow [Domen et al., 1993b]. IL-7 induced pre-B colonies, while the combination of IL-7 and SF induced more primitive cells to differentiate into pre-B cells. The pim-1 levels affected the size of the earliest B cell progenitor compartments in the bone marrow most profoundly; in the pim(+) CFCs there was an increased response to IL-7 and SF, as evidenced by an increased number of pre-B colonies responsive to IL-7 and SF and a reduction in the size in the mature B cell compartment by half as compared to the WT. In contrast, the pim(-) colonies had a reduced growth rates in response to IL-7 and SF, as reflected in the reduced numbers of early IL-7 and SF-responsive B lymphocytes. The lack of IL-7 response in the pim(-) cells was rescued by a transgene that restored pim-1 expression to WT levels [Domen et al., 1993b]. Pim-1 affects IL-7 response; pim-1 functioned in a dose-dependent manner but while pim-1 overexpression did not cause IL-7 growth factor independence, the pim(-) cells were still partly responsive to IL-7 and SF.

The results indicate that Pim-1 seems to function in B-lymphopoiesis and is involved in the response to growth factors acting on the early B-lymphocyte compartment by transducing signals or halting differentiation. These response differences were not observed when complex cell-cell interactions occurred between stomal and lymphoid cells in Whitlock-Witte cultures [Domen et al., 1993b]. These studies have shown that pim-1 is somehow involved in the IL-3, IL-7 signal transduction pathways. However, the function of pim-1 may be redundant as effects of overexpression or knockout are masked by complex cellular interactions.

viii. SUSCEPTIBILITY OF PIM-1 TRANSGENIC ANIMALS TO CHEMICAL CARCINOGENS

Eμ-pim-1 transgenic mice are tumor-prone and display a high incidence of tumor formation when exposed to chemical carcinogens or viral infection [Breuer et al., 1989b, 1991; Armstrong and Galloway, 1993]. Eμ-pim-1 transgenics are approximately 25-fold more susceptible to ENU (N-ethyl-N-nitrosourea)-induced lymphomagenesis [Breuer et al., 1989b, 1991]. H2K-pim-1 transgenics were also tested, but with less impressive
results [Breuer et al., 1989b]. When a simple low dose of ENU was administered, almost all Eμ-pim-1 transgenic mice but only 15% of control mice developed T cell lymphomas, and c-myc levels were strongly elevated in most tumors supporting the concept of pim-1/c-myc cooperativity [Breuer et al., 1989b, 1991]. Approximately 10% of the tumors also had a ras mutation, but this was thought to be a later event, independent of the ENU induction [Breuer et al., 1991]. The levels of pim-1 in ENU-induced lymphomas of both Eu-pim-1 and normal mice were highly variable, so tumor formation could not be correlated to the levels of pim-1 expression [Breuer et al., 1989b, 1991].

Several groups have exploited this susceptibility of Eμ-pim-1 transgenic mice to test the effects of various carcinogens. Armstrong and Galloway [1993] found that 2-acetylaminofluorene (2-AAF) and benzene led to accelerated lymphogenesis in pim-1 transgenics, but 1,2-diethyl nitrosamine (DEN) and 1,2-dichloroethane (1,2-DCE) did not. They used the formation of blood micronuclei (micronucleated erythrocytes) as a measure of bone marrow genotoxicity, with the intention of developing the Eμ-pim-1 transgenic mice as a model for testing other oncogenic agents. A second study also demonstrated that both 2-AAF and N-nitrosodiethyamine (NDEA) led to a significant increase in lymphomas in Eμ-pim-1 transgenic mice [Storer et al., 1995]. In contrast to the previous study, 1,2-DCE was also shown to cause an increase in murine lymphomagenesis, while benzene did not produce any significant increases in lymphoma induction [Storer et al., 1995]. The conflicting results from these two studies may result from differing methods of chemical administration (oral versus injection) and from different methods of assessing toxicity (formation of micronuclei versus lymphoma induction). These studies demonstrate that while the Eμ-pim-1 transgenic mice do have an increased sensitivity to chemical carcinogens, their sensitivity may not be sufficient to justify their use as a tool to screen chemical carcinogens. Further work needs to be done to standardize methods of chemical administration and assessment of effects before this transgenic model can be used in carcinogenesis screening assays.
9. THE ROLE OF OTHER ONCOGENE-ENCODED SERINE/THREONINE KINASES IN OOCYTE MATURATION

Pim-1 belongs to a small family of oncogenic serine/threonine kinases that includes Tpl-2/Cot, Mos, Raf-1, and Akt/Rac. Although the sequences and structures of these kinases are not related, the fact that these are the only known serine/threonine kinases encoded by oncogenes is indeed significant and implies that these kinase may have similar functions in the cells. Indeed, studies have indicated that some interesting functional similarities exist between these kinases.

Tpl-2 (tumor progression locus 2) is the murine homologue of the human Cot (cancer osaka thyroid) protein [Miyoshi et al., 1991; Aoki et al., 1991; Makris et al., 1993]. The gene was first identified as being involved in the progression of Moloney murine leukemia virus-induced leukemia in rats, with proviral insertion in the 3' end of the gene leading to the production of truncated stabilized mRNA transcripts in a similar manner to pim-1 [Patriotis et al., 1994]. The protein is a close relative of MEK-1 and MEKK, acts downstream of Ras and Raf-1, and contributes to the activation of the MAPK cascade [Patriotis et al., 1994].

Mos was one of the first kinases from this family to be studied. It first received recognition as an active component of cytostatic factor (CSF) necessary for both the stabilization of maturation promoting factor (MPF) consisting of cyclin B and p34^cdc2, and for activating MPF to promote the maturation of *X. laevis* oocytes [Sagata et al., 1989]. Mos is also necessary for the insulin and progesterone-induced maturation of *X. laevis* oocytes. Recently Mos has been demonstrated to activate maturation-activated protein kinase (MAPK) in *X. laevis* oocytes and to maintain the activity of MAPK during meiosis [Posada et al., 1993; Nebreda and Hunt, 1993]. Mos phosphorylates MAPKKK in vitro, suggesting a method by which it may activate MAPK in vivo [Posada et al., 1993].

Raf-1 has also been shown to be a member of the MAPK signal transduction pathway, and is located upstream of MEK-1 and downstream of Mos [Muslin et al., 1993]. Importantly, Raf-1 plays a significant role in the regulation of progesterone-induced *X. laevis* oocyte maturation as well as in the early development of the *X. laevis* embryo [MacNicol et al., 1993]. Raf-1 is a key signalling molecule in the development of the posterior structure of the *X. laevis* embryo, mediating the cell differentiating response to FGF in vertebrates [MacNicol et al., 1993].
The product of the AKT8 retrovirus, Akt/Rac, was first isolated form a rodent T cell lymphoma. The cellular homologue is expressed in most tissues including testes, and expression is especially high in the thymus [Bellacosa et al., 1991]. Unlike the other oncogene encoded serine/threonine kinases, akt contains a protein interaction motif, a SH2-like domain called the Pleckstrin homology box in the N-terminal regulatory region.

Although Mos, TPL-2 and Raf function in the MAPK cascade, there is no evidence that Pim-1 has a role in this pathway. While Pim-1 has been demonstrated to play a part in mammalian male germ cell development with the production of an alternative, 2.4 kb Pim-1 transcript in testicular tissue, no studies have examined the expression or regulation of Pim-1 in oocyte maturation [Sorrentino et al., 1988; Wingette et al., 1992]. Although pim-1 mRNA transcripts were not initially detected in ovaries, this early study also did not detect pim-1 transcripts in testes, indicating that the sensitivity of detection may have been very low [Selton et al., 1985]. Because other oncogene-encoded serine/threonine kinase family members, Mos and Raf are important for *X. laevis* oocyte maturation and because pim-1 has been shown to play a role during male germ cell development, we hypothesize that Pim-1 may function during oocyte maturation or development.

10. SUMMARY OF PIM-1

The *pim-1* oncogene is highly conserved between species, suggesting that it plays an important function in the cell. Studies with transgenic mice have confirmed that *pim-1* is an oncogene, producing a low spontaneous rate of tumor incidence. The susceptibility of Pim-1 overexpressing mice to chemical carcinogens and to MuLV infection has made the identification of cooperating oncogenes possible. Pim-1 cooperates strongly with *c-myc*, less strongly with *N-myc, L-myc, bcl-2, lpr* and possibly *v-abl* to contribute to transformation. In all cases, the coexpression of these oncogenes alone is not sufficient to cause malignant transformation; additional genetic events are required, supporting the model of multistep tumorigenesis.

Very little is known about the function of Pim-1, but expression patterns indicate that it is involved in hematopoietic signal transduction and in the development of male germ cells. Expression of *pim-1* mRNA and protein is very tightly regulated at different levels,
implying that is functionally potent. *Pim-1* is induced by mitogens and growth factors and may take part as a cytoplasmic mediator in a signal transduction pathway. Upregulation of Pim-1 by signalling through the GM-CSF receptor family is dependent on the presence of the membrane proximal domain, and may involve the JAK2 tyrosine kinase. During GM-CSF stimulation, *pim-1* is involved in the DNA synthetic as opposed to the apoptotic inhibition pathway.

The only phenotypic anomaly detected in *pim-1* transgenic mice is an alteration in the size of erythrocytes. The lack of physiological effects in transgenic mice both overexpressing and deficient in *pim-1* implies that the oncogene is functionally redundant and any effects of the expression (or lack of) are masked by the effects of other growth factors or pathways *in vivo*. The *pim-1* null mice have an impaired response to several growth factors, implying that the kinase functions as a signal transduction modulator, possibly in the pathways stimulated by IL-3 and IL-7. Pim-1 may act to cause an inhibition of differentiation of early progenitors (especially B cell progenitors), or may act to inhibit apoptosis.

Although extensively studied in the hematopoietic system, the expression and regulation of *pim-1* has never been studied in the oocyte system. Recent studies of other oncogene-encoded serine/threonine kinases (Mos, Raf) has implicated these kinases as having a vital role during oocyte maturation. It was the aim of this study to examine the role of this kinase in the maturing oocyte system.
CHAPTER II.

HYPOTHESIS

1. *pim-1* is expressed in germ cells and is thought to play a role in male germ cell development [Sorrentino *et al.*, 1988, Wingette *et al.*, 1992]. *pim-1* may possibly play a similar developmental role during oocyte maturation.

2. As *pim-1* is present and is highly conserved among all mammalian species examined, we expect the enzyme to also be expressed in *Xenopus laevis*. It is expected that Pim-1 from *Xenopus laevis* will behave similarly to mammalian Pim-1.

3. As a kinase with a high degree of autophosphorylation activity, autophosphorylation of Pim-1 is likely to serve a functional purpose. Autophosphorylation may modify the kinase activity of the enzyme or may allow the protein to participate in interactions with other proteins. Identification and modification of the autophosphorylation site(s) will allow examination of changes to the autokinase activity and the kinase activity towards exogenous substrates resulting from mutations introduced at these sites.

4. As a highly conserved kinase, Pim-1 may phosphorylate important physiological substrates at specific sites that conform to distinct sequence motifs. Characterization of this consensus phosphorylation site sequence will aid in the identification of targets of Pim-1.
RATIONALE

1. *X. laevis* and *P. ochraceus* were selected as model systems, because these systems provide abundant sources of oocytes for biological characterization. The oocytes are arrested at the same stage of maturation and can be induced to mature by progesterone stimulation allowing the activity of the enzyme of interest to be studied at discrete stages of maturation.

2. The role of Pim-1 in oocyte maturation and early development has not been explored.

3. The *X. laevis* oocyte maturation system is well characterized with respect to other oncogene-encoded serine/threonine protein kinases (Mos and Raf). As well, this system offers the opportunity to perform microinjection experiments to assess the activity of the kinase *in vivo*.

4. Sequences important for the function and regulation of Pim-1 are likely to be conserved between divergent species. Comparing the non-mammalian Pim-1 sequences with the human sequence will help determine regions of the protein that are important for catalytic function, substrate binding or regulation of the protein.

5. Pim-1 has never been purified to homogeneity from an endogenous source, so very little enzymological characterization of Pim-1 has been performed. Expression of Pim-1 as a recombinant protein will allow large amounts of the enzyme to be produced for analysis.

6. Many protein kinases autophosphorylate then become active towards other substrates. Despite the strong autophosphorylation activity of Pim-1, there is no proof that Pim-1 activity is modulated by autophosphorylation. Novel regulatory sites may potentially be identified in the Pim-1 protein, which may then act as a model for regulation by autophosphorylation for other kinases containing homologous residues.

7. There are no known or suspected physiological substrates of Pim-1. Understanding the requirements for substrate recognition by Pim-1 will help to identify potential physiological substrates and confirm the exact location of phosphorylation in a suspected substrate.
OBJECTIVES

1. To clone \textit{pim-1} from a non-mammalian species, in this case, from \textit{Xenopus laevis}.

2. To develop specific antibodies that can be used to detect Pim-1 in the \textit{X. laevis} oocyte system.

3. To express the cloned Pim-1 from frog and human as bacterial fusion proteins and to use these expressed proteins to determine the substrate recognition sequence using a series of peptide substrates.

4. To use the Pim-1-specific antibody and peptide reagents to examine changes in expression and activity of this kinase during oocyte maturation.

5. To identify the sites of autophosphorylation in Pim-1, to mutate these autophosphorylation sites using site-directed mutagenesis and to determine their potential roles in the regulation of Pim-1 activities.
### METHODS

#### 1. SUPPLIES AND SOURCES

**Boehringer Mannheim = BM, Fisher Scientific = FS, New England Biolabs = NEB**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic Acid (CH₃COOH)</td>
<td>FS</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>Applied Biosystems</td>
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<td>Acid phosphatase</td>
<td>Sigma</td>
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<tr>
<td>Acrylamide</td>
<td>FS/ICN</td>
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<td>Sigma</td>
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<tr>
<td>Agarose</td>
<td>Gibco BRL</td>
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<tr>
<td>Agarose (low melting point)</td>
<td>BRL</td>
</tr>
<tr>
<td>Amido black 10B</td>
<td>ICN</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>BM</td>
</tr>
<tr>
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<tr>
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<td>Sigma</td>
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<tr>
<td>Bind-Silane</td>
<td>LKB</td>
</tr>
<tr>
<td>Bis-acrylamide</td>
<td>FS</td>
</tr>
<tr>
<td>N,N'- Methylene bis-acrylamide</td>
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<tr>
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<tr>
<td>Brilliant Blue G</td>
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<td>5-Bromo-4-chloro-3 indoyl phosphate (BCIP)</td>
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<tr>
<td>1-Butanol</td>
<td>FS</td>
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<tr>
<td>iso-Butanol</td>
<td>FS</td>
</tr>
<tr>
<td>Benzamidine</td>
<td>ICN</td>
</tr>
<tr>
<td>β-Glycerophosphate</td>
<td>ICN</td>
</tr>
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β-Methyl aspartic acid  
Calcium chloride \((\text{CaCl}_2)\)  
Centricon tubes (10 and 30)  
Citric acid  
Chloroform  
Collagenase  
Concanavalin A  
Coomassie Brilliant Blue R  
Counting scintillant  
α-Chymotrypsin  
α-casein, dephosphorylated  
Denatured alcohol  
2′-Deoxynucleoside 5′-Triphosphate (dNTP kit)  
Diethyl pyrocarbonate  
N,N-dimethyl formamide \((\text{DMF})\)  
Dimethyl sulfoxide \((\text{DMSO})\)  
Disodium pyrophosphate  
Dispase  
Deoxyribonuclease  
Dithiothreitol \((\text{DTT})\)  
DNA 1 kb ladder  
DNA -herring sperm  
Dynabeads oligo \((\text{dT})_{25}\)  
Enhanced chemiluminescence kit  
Enolase  
Ethanolamine  
Ethylene bis (oxyethylenenitrilo)] tetraacetic acid \((\text{EGTA})\)  
Ethylene diamine tetraacetate disodium salt \((\text{EDTA})\)  
Ethidium bromide  
Finquel (methyl trisulphonate)  
Formaldehyde solution \((\text{HCOC})\)  
Formalin  
Gelatin  
Geneclean kit  
Glacial acetic acid
Glutathione
Glutathione cross-linked 4% beaded agarose
Glycerol
Glycine
Guanidine thiocyanate
Histone IIA
Histone IIS
Histone III-S
Histone VII-S
Hybond-N hybridization membrane
Hydrochloric Acid
Isopropyl β-D-thiogalactopyranoside (IPTG)
Kemptide
N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid) (HEPES)
T4 DNA ligase
N-Lauryl sarcosine
Lauryl sulfate (dodecyl lithium sulphate)
Leupeptin
Liquid paraffin
Lithium chloride anhydrous
Lysozyme
Magnesium acetate tetrahydrate
Magnesium sulphate (MgSO4·7H2O)
Magnesium chloride (MgCl2·6H2O)
Maltose
Manganous chloride (MnCl2·4H2O)
2-Mercaptoethanol
Methanol
1-Methyladenine
DL-Threo-β-methylaspartic acid
MES (2-[N-Morpholino]ethanesulfonic acid)
MonoS column
MonoQ column
MOPS 3-[N-Morpholino]propanesulphonic acid

Sigma
Sigma
Anachemia
ICN/Sigma/FS
ICN
Sigma
Sigma
Sigma
Sigma
Amersham
FS
Fisher
Biotech/Promega
Sigma
Sigma
Sigma
Sigma
BDH
Sigma/ICN
BDH
Sigma/BM
BDH
FS
FS
BDH
BDH
BioRad
FS/BDH
Sigma
Sigma
Sigma
Pharmacia
Pharmaica
Sigma/ICN
Myelin basic protein
N-ethyl maleimide
Ninhydrin
Nitric acid (HNO₃)
Nitro blue tetrazolium (NBT)
Nitrophenyl phosphate disodium salt
Nonidet P-40
Oligo(dT)-cellulose type 7
Petroleum ether (50-110°C)
Petroleum ether (60-80°C)
Phenyl phosphate disodium salt (P'ase inhibitor)
Phenol
Phenolsulfonphthalein (Phenol red dye)
Phenyl methylsulphonyl fluoride (PMSF)
p-nitrophenyl phosphate
p81 phosphocellulose filter paper
ortho-Phosphoric acid (H₃PO₄)
Phosphatase, alkaline (high conc)
Phosphate-buffered Saline (PBS)
O-Phospho-L-serine
O-Phospho-DL-threonine
O-Phospho-L-tyrosine
Phosphorylase B
Phosvitin
Plasmid kit
Potassium acetate (C₂H₃O₂K)
Potassium chloride (KCl)
Potassium dichromate (K₂Cr₂O₇)
Potassium dihydrogen orthophosphate monobasic
Potassium hydroxide (KOH)
Potassium phosphate (dibasic)
di-Potassium hydrogen orthophosphate 3-hydrate
Potassium dihydrogen orthophosphate (KH₂PO₄)
Ponceau S concentrate
T7 polymerase
DNA polymerase I large fragment (Klenow)

Kinetek/Sigma
Sigma
BDH
FS
Sigma
Sigma
BDH
Pharmacia
J.T. Baker
BDH
ICN
ICN
Sigma
Sigma
Sigma
Whatmann
FS
BM
Gibco
Sigma
Sigma
Sigma
Sigma
Sigma
Quiagen
BDH
FS
BDH
BDH
BDH
Sigma
BDH
Sigma
NEB/Pharmacia
NEB/Gibco-BRL/BM
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<td>Silver nitrate</td>
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<td>Sodium acetate (dibasic)</td>
<td>BDH</td>
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<td>Sodium azide</td>
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<td>Sodium borate</td>
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<td>Sodium dodecyl sulphate (SDS)</td>
<td>FS</td>
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<td>Sodium fluoride (NaF)</td>
<td>BDH/FS</td>
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</table>
2. PHOTOGRAPHY SUPPLIES

Developer and replenisher
Fixer and replenisher
ISO 3000 Polaroid film 667
ISO 100 Polaroid film
Reflection NEF- autoradiography film
X-OMAT AR imaging film
3. PLASMIDS AND BACTERIAL STRAINS

Bluescript II KS M13(+) plasmid
pGEX-2T vector
R408 Interference resistant helper phage
XL1-Blues bacteria
JM110 bacteria
DH5α bacteria
DH5α high competence cells
UT5600 protease-deficient bacteria

4. ANTIBODY REAGENTS

i. Primary Antibody Reagents
Anti-glutathione S-transferase (GST) antibody
PY20 anti-phosphotyrosine antibody
4G10 anti-phosphotyrosine antibody
CRB anti-Pim-1 antibody
Pim-CT (C2)
Pim-CT (Daniel/A2)
Tel, anti-Pim-1 antibody

ii. Secondary Antibody Reagents
Blotting grade affinity purified goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate
EIA grade affinity purified goat anti-mouse IgG (H+L) alkaline phosphatase conjugate
Affinity purified rabbit anti-sheep IgG (H+L) alkaline phosphatase conjugate
Goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate

Molecular probes
Santa Cruz
UBI
Cambridge Research Biochemicals
Dr. M. Lilly (Seattle VA Hospital)
Drs. A. Telerman and R. Amson, (CEPH, Paris, France)
Bio-Rad
Calbiochem
Bio-Rad
Amersham
iii. **Unique Antibody Reagents**

Unique antibodies created in this laboratory are listed below, and described in extensive detail in Appendixes I and II.

- **Pim1-111** Anti-human and murine Pim-1 (Available from UBI)
- **Pim1-NT** Anti-human and murine Pim-1 (Available from UBI)
- **Pim1-X1** Anti-\textit{X. laevis} Pim-1 peptide (Available from UBI)
- **GXP** Against \textit{X. laevis} GST-Pim-1 fusion protein

### 5. SOURCES OF OLIGONUCLEOTIDES

A detailed description of all oligonucleotides is located in Appendix III. Oligonucleotides K1 and K2 were synthesized on an Applied Biosystems 392 DNA synthesizer by Georgia Tai at the Biomedical Research Centre, UBC. Oligonucleotides 11A, 12A, 13B, 14B, 9205, 9204, were synthesized by core technicians at the Biomedical Research Centre. Oligonucleotides were obtained bound to a column and required cleavage and deprotection.

Oligonucleotides Pim-3', Pim-5', PM1, PM2, PM3 and PM4 were synthesized in the laboratory of Dr. Gabe Kalmar (Simon Fraser University, Burnaby) on an Applied Biosystems 392 DNA synthesizer. These oligonucleotides were obtained in a deprotected and lyophilized state, ready to hydrate and use.

### 6. PEPTIDE PRODUCTION/SOURCES

The sequence of peptides Pim1-111, Pim1-NT and Pim-X1 are provided in Appendix I. The sequences of the substrate analog peptides are detailed in Appendix IV.

Peptides Pim1-111 and Pim1-NT, used for antibody production and well as those used for kinase assays were synthesized on an A.B.I. 430A peptide synthesizer in the laboratory of Ian Clark-Lewis (Biomedical Research Centre), and cleaved from the resin by hydrofluoric acid. Purity of the peptides was demonstrated by reverse phase HPLC, and identity was confirmed by ion spray mass spectrophotometry analysis (model API-III). Due to the small size of the peptides and the high purity as confirmed by reverse-phase HPLC, the kinase substrate peptides did not require further purification after
lyophilization. Peptides Pim1-111 and Pim1-NT were further purified by HPLC and lyophilized. Peptides were carefully dried before weighing on an analytical balance. All peptides were readily soluble in assay dilution buffer or water.

Peptide Pim-X1 was synthesized by contract through UBI.

7. SOURCES OF CELL LINES/CELL LYSATES

Primary human lymphocytes in culture were purified by Dr. Bill Sahl in our laboratory. K562 cells were kindly grown by Ms. Helen Merkins (Biomedical Research Centre).

8. ADDITIONAL REAGENTS

Phosphatase HPTPβ was a kind gift from Mr. Ken Harder (Biomedical Research Centre). The 40S ribosomes were a gift from Dr. J. McNeil's Lab (Dept. of Pharmacy, UBC). Lck was a generous gift from Dr. Julian Watts (Biomedical Research Centre). S6 kinase was a kind gift from Ms. Lorin Charlton (Pelech Lab), and GST-Raf-1 was a kind gift from Mr. Dan Leung (Pelech Lab).
2. EXPERIMENTAL PROCEDURES - MOLECULAR BIOLOGY

1. GENERAL MOLECULAR BIOLOGY TECHNIQUES

   i. Isolation of PCR bands from an agarose gel

   PCR products were separated on 0.75-1.5% agarose gels in TAE buffer (10 mM Tris base, 200 mM EDTA, glacial acetic acid (1.142 ml per litre)), were visualized by ethidium bromide staining and photographed on a shortwave UV light. Size determination was by comparison to 1 kb DNA ladder. DNA bands of interest were excised from the gel with a scalpel blade and the DNA was recovered from the agarose by electrolution or by Sephglas or Geneclean systems.

   For electrolution, the DNA-containing agarose band was inserted into a piece of dialysis tube with several ml of TAE buffer and clamped. The dialysis tube was electroluted in the gel box at 100 mA for 30 min. The current was reversed for 30 s, the TAE was carefully removed from the dialysis bag and the DNA precipitated with 3 vol of ice-cold ethanol.

   The Sephglas and Geneclean kits both relied on the affinity of DNA for a novel glass matrix. The procedure was followed as recommended by the manufacturer, with the reagents supplied.

   ii. Restriction digests

   For restriction digests, 0.5 - 1 µg of DNA were digested in a 20 µl reaction with 2 µl of the appropriate buffer and 1-2 µl of enzyme. Each µg of DNA should ideally be digested in a 10 µl volume. Digests were performed for 1- 2 h (BamH1) or O/N at 37°C except for Sma1 digestions which were performed at 16°C. After the digests were complete, 2 µl of agarose gel loading buffer [Maniatis et al., 1989, Section 6.12. Type 2: 0.25% bromophenol blue (w/v), 0.25% Xylene cyanol FF (w/v) and 30% glycerol (v/v)] were added to each reaction, the DNA was subjected to electrophoresis in an agarose gel, visualized by ethidium bromide staining and was photographed on a shortwave UV light. PCR reactions were assessed for the presence of DNA bands of the expected size and for the amount of background amplification. The PCR band of interest was excised and purified by electrolution, Geneclean or Sephglas system. The DNA fragment was then used for ligation, probe construction or for further restriction digests.
The P. ochraceus and X. laevis pim-1 PCR products were digested with SmaI to create blunt ends. Bluescript plasmid was likewise digested to create compatible cohesive ends and treated with alkaline phosphatase.

### iii. Alkaline phosphatase treatment of linearized plasmids

Linearized plasmids were treated with alkaline phosphatase to remove the 5'-phosphate group, preventing the plasmid from ligating to itself with the exclusion of the PCR fragment. Approximately 30 μl of plasmid were incubated with 1.0 μl diluted alkaline phosphatase (0.024 units/μl, 1/1000 dilution) in a total volume of 50 μl of 1X alkaline phosphatase buffer (provided by manufacturer) for 30 min at 37°C. One additional μl of alkaline phosphatase was added and the reaction continued for an additional 30 min. The reaction was terminated by the addition of 0.5 mM EDTA (pH 8.0) and incubated at 70°C for 20 min. The volume was increased to 200 μl and the plasmid extracted sequentially with phenol:chloroform (1:1; v/v) and chloroform.

### iv. Ligations

Ligation reactions were performed in a final volume of 10 μl, containing 1 μl of ligation buffer, 1 μl of T4 DNA ligase, and varying concentrations of insert, plasmid (cleaved) and ddH₂O water. For blunt-ended ligations, the amount of T4 DNA ligase was increased to 2-4 μl of enzyme per reaction. The ligations were performed for 16-48 h at 16°C. Various controls were performed including insert alone (control for ligated or undigested plasmid contamination), plasmid alone with no ligase (control for spontaneous antibiotic-resistant bacterial revertants) and supercoiled plasmid (control for bacterial competence).

### v. Transformations

Bacteria were made competent using a modified CaCl₂ method of Maniatis et al. [1989, Section 1.82]. E. coli bacterial strains DH5α and UT5600 (protease deficient) were grown until an optical density of 0.4-0.6 (600 nm) was achieved. Aliquots (50 ml) were centrifuged and the pellets resuspended in 20 ml of ice cold 50 mM CaCl₂, and incubated on ice for 20 min. The cells were pelleted and resuspended in 2.5 ml of ice cold 50 mM CaCl₂ containing 20% glycerol, aliquoted and frozen immediately at -70°C.

Transformations were carried out as described in Maniatis et al. [1989, Section 1.83]. In brief, 0.5 μl of supercoiled plasmid or diluted ligation mixture were added to the competent bacterial solution and incubated on ice for 15 min. For ligations, 90 μl of
freshly prepared 10-10-10 buffer (10 mM Tris-Cl, pH 7.5, 10 mM MgCl₂, 10 mM CaCl₂) were added. The solution was then gently agitated at 45°C for 90 s, then incubated on ice for 5 min. Antibiotic-free media (2xYT) was added and the bacteria was incubated at 37°C for 20 min to induce amp<sup>R</sup> gene expression. The transformed bacteria was then plated onto 2xYT(amp+) plates and grown at 37°C O/N.

Isolated colonies were selected and restreaked or used to inoculate an O/N culture. For construction of the PCR probes, XL1-blue E. coli was used for all the transformations and positive colonies were selected by blue/white selection on LB-amp plates. A white colony indicated that the ß-galactosidase gene was interrupted by the insertion of a fragment of DNA, and was selected as potentially positive.

vi. Small scale plasmid preparation

The small scale plasmid preparation protocol was that developed by He et al., [1989]. In brief, O/N cultures of bacteria were grown in media with ampicillin (100 µg/ml). Bacteria were pelleted by centrifugation at maximum speed for 2 min in an Eppendorf centrifuge and resuspended in 200 µl of TELT (2.5 M LiCl, 50 mM Tris-HCl (pH 8.0), 4% Triton X-100 (v/v), 62.5 mM EDTA). The plasmid was extracted with phenol:chloroform (1:1; v/v) and precipitated with 500 µl of 100% ethanol. The pellet was washed with 70% ethanol, air dried for 10 min and resuspended in 15-20 µl of TE buffer (10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0)). Restriction digests were then performed using 5 µl of the DNA solution per digest.

vii. Large scale plasmid preparation

Large scale preparations were initially done using a protocol obtained from Dr. F. Jirik (Biomedical Research Centre). Later (1994 and beyond), plasmid preparations were performed using the Quiagen Midi prep kit, using the protocol and reagents supplied. The procedure recommended with the kit is based on a modified alkaline lysis procedure.

The alkaline lysis protocol obtained from Dr. F. Jirik is briefly detailed. A 500 ml culture of transformed bacteria was grown O/N in LB media containing ampicillin (0.05 mg/ml). The bacteria was pelleted by centrifugation at 4420 x g for 10 min. The supernatant was discarded, the pellet was resuspended by intense vortexing and was kept on ice for the remainder of the procedure. Seven ml of glucose solution (25 mM Tris-HCl, 10 mM EDTA, 50 mM glucose) at 4°C were added to the pellets and mixed thoroughly. In a 50 ml conical centrifuge tube, 14.0 ml of 0.2 N NaOH/1% SDS solution
were added, mixed by gentle inversion and incubated on ice for 10 min. Fourteen mls of KOAc solution (pH 5.7, 60 ml of 5M KOAc, 11.5 ml glacial acetic acid, 28.5 ml dH2O) were then added, mixed by gentle inversion and incubated on ice for 10 min. The plasmid solution was centrifuged twice at 7250 x g for 10 min, 4°C. The supernatant was then extracted with an equal volume of phenol/chloroform (1:1; v/v), vortexed and centrifuged for 10 min at 5000 x g, at RT. The aqueous layer was extracted with an equal volume of isopropyl alcohol and incubated on ice for at least 15 min with occasional inversions. The plasmid was pelleted by centrifugation at 12000 x g for 15 min at 4°C and air-dried for 20 min. The plasmid pellet was resuspended in 200 µl of TE buffer and 5 µl of RNase A (10 mg/ml) were added. After a 20 min incubation at 37°C, the solution was extracted three times with phenol/chloroform (1:1; v/v) and centrifuged at 16000 x g for 5 min. The plasmid was precipitated for 20 min at -20°C by the addition of 1 ml of 100% ethanol and centrifuged for 30 min at 16000 x g. After briefly drying in a dessicator, the plasmid DNA was resuspended in 500-1000 µl of 1x TE buffer and quantitated by measuring the OD260 (OD260 x dilution x 42/50 = µg / ml).


Amplified pim-1 PCR fragments were excised from pBluescript with SmaI and labeled with γ-32P, by a method obtained from Ms. Nicole Janzen (BRC). The radiolabeled DNA fragment used as a probe to screen Northern blots, Southern blots and the P. ochraceus and X. laevis cDNA libraries.

For each probe, 100 ng of double stranded DNA in a volume of 9 µl were denatured by boiling for 5 min then cooled immediately on ice. The labeling reaction was carried out for 30 min at 37°C in a final volume of 20 µl with 2.0 µl of 10X Klenow buffer (supplied by Gibco-BRL (400 mM Tris-Cl, pH 7.5, 66 mM MgCl2, 10 mM 2-ME), 250 µM each of dATP, dTTP, and dGTP, 5.0 µM of random hexamer, 1.0 µl of DNA polymerase large fragment (Klenow) and 5.0 µl of [γ-32P]dCTP. Addition of 60 µl of buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% SDS), 80 µl of 5M NH4OAc, 4 µl tRNA (10 mg/ml), and 400 µl of 95% ethanol was followed by centrifugation for 15 min at RT. The probe was dissolved by boiling in 100 µl of dH2O for 5 min, briefly chilled on ice, then added to hybridization solution.

ix. Prehybridization and hybridization of membranes with radiolabeled probe

Membranes were prehybridized for 30 min at 55°C in hybridization buffer (350 mM NaPO4, 30% deionized formamide (v/v), 7% SDS (w/v) and 1% BSA (w/v)). The
labeled probe was hybridized to the membranes in a volume of 10 ml of hybridization buffer for 16 h at 55°C. The membranes were subjected to multiple washes with 150 mM NaPO₄, 0.1% SDS (w/v) for 10 min at 55°C, until the radioactive counts emitted from the membranes was significantly reduced when measured with a hand-held Geiger counter. For more stringent washes, 50 mM NaP, 0.1% SDS (w/v) was used. When counts were sufficiently reduced, the membrane was air dried and autoradiographed.

2. OOCYTE MATURATION

i. Isolation of *Xenopus laevis* oocytes

Mature female *Xenopus laevis* (African clawed frog) were immersed in a 3% Finquel (tricaine methanesulfonate in tap water) solution for approximately 10 min, until no response was elicited by gentle pinching of feet and claws. As the LC₅₀ of Finquel is 30 min in a 6.2% solution (w/v) [Argent Chemical Laboratories' specification sheet], frogs were monitored carefully during the procedure to ensure that they were not over-anaesthetized. Ovaries were surgically removed, and cardial puncture was performed. Ovaries were washed by gentle swirling in tissue culture plates with 1X *X. laevis* oocyte media (XOM) containing 5.4 mM Tris-base, 86 mM NaCl, 0.8 mM KCl, 0.5 mM CaCl₂, 0.6 mM MgSO₄ and 4.0 mM sulphadiazine (pH 7.6) [Zhang and Masui, 1992]. The sack-like covering of the ovary was gently pulled apart to expose eggs and ovary material to the media and to wash away crushed eggs.

The oocytes were isolated from the ovaries using a modified method [Belle *et al.*, 1986] by digestion with dispase (0.04% (w/v) in XOM) for 4 h at RT with occasional gentle swirling. The oocytes were washed with buffer, then digested in collagenase (0.1% (w/v) in XOM)) for 2 h at RT. Media was replaced when cloudy.

Stage VI oocytes displaying definite bipolar pigmentation were selected by visual inspection, and placed in fresh media. Oocytes that were mottled or had an irregular shape were discarded. Isolated oocytes were stored O/N in sulphadiazine-containing media at room temperature.

ii. Progesterone maturation of *X. laevis* oocytes

Oocytes were matured at ambient temperature with 100 μM of progesterone. Fresh media was added to oocytes, and a 1/1000 dilution of progesterone in ethanol (100 mM
stock) was added. Germinal vesicle breakdown (GVBD) became visible between 4-9 h, as evidenced by the appearance of a symmetrical white spot in the middle of the dark hemisphere. Mature oocytes were selected and set aside for homogenization. Oocytes that became cloudy or mottled were discarded.

iii. *P. ochraceus* oocyte maturation

Ovaries were surgically removed from the arms of *P. ochraceus* (purple sea stars) and incubated in calcium free artificial sea water, CaFASW (475 mM NaCl, 10 mM KCl, 31 mM MgCl₂.6H₂O, 18 mM MgSO₄, 10 mM Tris) on ice. Ovaries were gently teased apart with forceps to release the oocytes, and were strained through a large mesh to remove connective tissue and residue. Oocytes were washed three times in cold CaFASW by pelleting the oocytes by centrifugation at 400 x g for 5 min in the Beckman centrifuge. After the third wash, the oocytes were resuspended in natural sea water (NSW) containing 4 μM 1-methyladenine at 14°C. Oocytes were allowed to mature by gentle stirring at 14°C for 70-100 min.

Maturation was achieved by the onset of GVBD, as evidenced by the disappearance of the nucleus within the oocyte when viewed under high magnification. Mature oocytes were harvested when GVBD occurred in over 80% of the oocytes, or 2 h after the initiation of maturation. For the maturation time course, measured volumes of oocyte suspension were removed at discrete time points after the addition of 1-methyladenine.

3. ISOLATION OF TOTAL RNA FROM OOCYTES

i. Homogenization of oocytes for total RNA

Total RNA was isolated from immature and mature stage *V. laevis* and from *P. ochraceus* oocytes by the method of Maniatis *et al.* [1989, Section 7.16]. All glassware including the homogenizer was washed with 0.1 M NaOH and rinsed several times with ddH₂O to remove RNAses. Approximately 1 ml of oocytes were homogenized with about 10 volumes of homogenization buffer (50mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 8.0), 0.5% SDS (w/v) and 200 μg/ml proteinase K). The homogenate was incubated at 37°C for one h and then extracted with phenol:chloroform (1:1; v/v). Phases were separated by centrifugation at 1480 x g for 10 min in the Beckman centrifuge at RT, the upper phase was removed and re-extracted with phenol: chloroform (1:1; v/v) and a third extraction was performed with chloroform to remove any traces of
phenol. The aqueous phase was transferred to a fresh tube and 0.1 vol (1.1 ml) of 3 M NaOAc (pH 5.2) and 25 ml of ice cold ethanol (95%) were added, then the solution was incubated on ice for 2 h.

After centrifugation at 5000 x g for 15 min at 4°C, the supernatant was discarded and the pellet was briefly air dried. Pellets were resuspended in 5 ml of ddH2O, 5 ml of 7.44 M LiCl were added and the pellets were stored at -20°C O/N. The RNA was pelleted by centrifugation in 2059 tubes at 9800 x g for 30 min at 4°C, and washed with cold 70% ethanol. The pellet was briefly air dried and resuspended in 2 ml of ddH2O and 3 vol of 100% ethanol, with the addition of 20 µl of RNAsin. The RNA was stored at -70°C until use.

To recover the RNA, a volume of the RNA/ethanol solution was removed, 0.1 vol of 3 M NaOAc (pH 5.2) was added and mixed and the solution was centrifuged at 4°C for 5 min in an Eppendorf centrifuge. The supernatant was discarded, the RNA pellet washed once with 70% ethanol, dried very briefly and resuspended in the buffer of choice.

ii. Quantitation and purity assessment of RNA

To quantitate the amount of RNA in a purified sample, an aliquot of the ethanol solution was withdrawn and recovered as described above. The pellet was briefly air dried and dissolved in ddH2O. The absorbance of the solution at 260 nm was measured, one optical density unit contained 40 µg of RNA per ml. The value 44.19 corresponds to the extinction coefficient for RNA.

\[ \text{[RNA]} \mu\text{g/ml} = A_{260} \times 44.19 \times \text{dilution factor} \]

The ratio of the optical density of 260/280 was obtained to determine the purity of the RNA, with a value of 2.0 being optimal.

iii. Selection of poly(A)+ RNA

Poly(A)+ RNA for reverse transcription reactions was selected using affinity chromatography on oligo(dT)-cellulose as described by Maniatis et al. [1989, Section 7.26] with minor modifications. In brief, the RNA was dissolved in ddH2O and an equal amount of 2X column loading buffer (40 mM Tris-HCl (pH 7.6), 1.0 M NaCl, 2 mM EDTA (pH 8.0), 0.2% SLS (w/v)) was added to the RNA. The RNA was not always heated at 70°C to dissociate.
Oligo(dT)-cellulose (0.1 g) was hydrated with 0.1 N NaOH, and poured into a plastic column that was prewashed with 0.1 M NaOH. The volume of the column varied from 250 - 500 µl (each ml of resin bound 10 mg of RNA). The column was first washed with 5 column volumes of ddH₂O, then washed with 1X column loading buffer (20 mM Tris-HCl (pH 7.6), 0.5 M NaCl, 1 mM EDTA (pH 8.0), 0.1% sodium lauryl sulphate) until the pH of the effluent was less than 8.0. An alternate recipe for column loading buffer that was used was 20 mM Tris-HCl (pH 7.5), 1.0 M LiCl and 2 mM EDTA.

The RNA containing solution was applied to the column, followed by one vol of 1X column loading buffer. The column flow-through was collected and reapplied to the column. The column was washed with 10 vol of 1X column loading buffer and the removal of nonpolyadenylated RNA was monitored by reading the absorbance of the collected fractions at 260 nm.

The poly(A)+ RNA was eluted from the column with 2-3 volumes of sterile RNase-free elution buffer (10 mM Tris-HCl (pH 7.6), 1 mM EDTA (pH 8.0) or alternately, 2 mM EDTA (pH 7.5)). SDS was not added to the elution buffer as recommended by Maniatis et al. [1989]. Fractions (0.3-0.5 vol) were collected, assessed by reading the absorbance of the RNA at 260 nm, and the peak fractions were pooled. NaOAc (pH 5.2) was added to the poly(A)+ RNA to a concentration of 0.3 M, 2.5 vol ice-cold ethanol were added, and then the RNA was stored at -70°C until use.

To recover the mRNA, the RNA was centrifuged at 10 000 x g for 15 min at 4°C, and the pellet was washed with 70% ethanol. The pellet was air-dried, and the RNA resuspended in a small volume of ddH₂O and quantitated.

Poly(A)+ RNA for Northern blots was isolated using Dynal Oligo(dT) beads with the recommended protocol. The protocol is similar to this except that the oligo(dT) is adsorbed to metal beads instead of cellulose, allowing very rapid separation of the poly(A)+ RNA in a very small volume. All buffers used were provided in the kit. Approximately 75 µg of total RNA were purified with 200 µl (1.0 mg) of beads.

iv. Northern blot analysis of oocyte RNA

X. laevis and P. ochraceus RNA was analyzed by Northern blotting for the presence of pim-1 mRNA. Before commencing, all equipment including the gel box and comb were rinsed with 0.1 M NaOH and ddH₂O to minimize RNase contamination. Total
RNA (20 μg) and poly(A)+ RNA (0.5-3 μg) were recovered from ethanol and resuspended in 4.7 μl of ddH2O. To each sample of RNA, 3.3 μl of 37% formaldehyde (2.2 M final), 10 μl of formamide and 2.0 μl of 10X MOPS buffer (0.2 M MOPS (pH 7.0), 50 mM NaOAc, 10 mM EDTA (pH 8.0)) were added and the sample was heated at 55°C for 15 min. RNA standards were treated the same as samples, with 3 μl used in the control lanes. Agarose gel loading buffer was added to the samples directly before application onto the gel. The RNA samples were applied onto a dry gel (1.2% agarose, 1X MOPS, 0.66 M formaldehyde) and the gel was electroluted for 10 min at 90V. The gel was then flooded with 1X MOPS buffer and electroluted at 100V. After electrolution, the gel was soaked in 1X MOPS buffer to remove formaldehyde. The RNA was nicked by exposure to 320 nm UV light for 2 min.

The Northern blot was assembled as described in Maniatis et al. [1989, Section 7.46]. Briefly, the gel was placed on a wick made from three pieces of Whatmann 3MM paper, and soaked in 20X SSC (333 mM NaCl, 930 mM NaCitrate, pH 7.0). After a brief hydration in ddH2O, a piece of nylon Hybond membrane was soaked in 10X SSC for 5 to 10 min and placed on the gel without trapping air bubbles. Six sheets of Whatmann paper soaked in 20X SSC were placed on top of the nitrocellulose, followed by a 6 cm stack of paper towels. A glass tray and a 500 g weight were placed on top and the gel was left to blot O/N in the fumehood. The RNA was crosslinked to the air-dried membrane in the Stratalinker. The standards and the 18s and 28s bands were visualized using the hand-held crosslinker and the standards marked with a pencil.

The Northern blot was prehybridized and hybridized with the same probes as detailed in the library screening section. The blots were stripped and reprobed several times, using 15 mM NaP and 1% SDS (w/v), for 30 min at 70°C.

4. AMPLIFYING PIM-1 USING POLYMERASE CHAIN REACTION (PCR)

i. Reverse transcriptase reaction

The cDNA synthesis reaction was carried out using approximately 1-2 μg of X. laevis or P. ochraceus mRNA as a template with 0.5 μl RNAsin (20 units). For each reaction, RNA in a volume of 10 μl (ddH2O) was denatured at 45-50°C for 2-3 min, then incubated on ice as the remaining reagents were added. The reverse transcriptase reaction with 1.0 μl Superscript reverse transcriptase, 100 μM of each nucleotide triphosphate, 1 μg of
random primers, was carried out in a total volume of 20 μl of polymerase chain reaction (PCR) buffer (50 mM KCl, 10 mM Tris-Cl (pH 8.3), 1.5 MgCl₂, 0.01% gelatin (w/v)). The reaction was performed in a Perkin-Elmer Cetus thermal cycler at 23°C for 10 min, 42°C for 45 min, 94°C for 3 min and 4°C for 5 min.

For large scale PCR, the reverse transcriptase reaction was scaled up. For the scaled up reaction, 20 μg of mRNA in 100 μl of dH₂O were denatured for 3 min at 45°C then briefly incubated on ice. The reverse transcriptase reaction was carried out with 10 μl of Superscript reverse transcriptase, 100 μM of each nucleotide triphosphate, 10 μg of random primers, in a total volume of 200 μl of PCR buffer (50 mM KCl, 10 mM Tris-Cl (pH 8.3), 1.5 MgCl₂, 0.01% gelatin (w/v)), at 23°C for 10 min, 42°C for 45 min, then at 94°C for 3 min and 4°C for 5 min.

In place of the random primers, oligo dTTT (1μg/μl) as well as oligo 14B (1.756 μg/μl) were substituted as primers for the reverse transcription reaction. PCR using cDNA prepared in this manner yielded a high background to product ratio, so this method of cDNA preparation was discontinued.

ii. Cleaning the cDNA

Products of large scale reverse transcription reactions were cleaned on a Sephadryl-HR 300 column to remove the random primers from the cDNA. Sephadryl-HR 300 was used to fill a 5 ml syringe plugged with glass wool, and packed by centrifugation in a Beckman at 200 x g for 1 min. The column was equilibrated with TE buffer under the same conditions. The cDNA was loaded on the column and the column centrifuged in a Beckman at 200 x g for 1 min. The eluate was collected and used as a template for preparative PCR reactions.

iii. Deprotection, cleavage and working up oligonucleotides

This protocol was used only on oligonucleotides that were obtained bound to a column. The column was attached to a 1 ml syringe and to a G18 needle using a male-male luer connector. A small amount of fresh ammonia was drawn through the column such that the level of the fluid barely entered the bottom of the syringe. The needle was jabbed into rubber bungie and left to deprotect for 30 min at RT, in the fume hood. After 30 min the crude oligonucleotide solution was expelled into a 1.5 ml screw top, O-ring tube. Ammonia was again drawn through the column and left to deprotect once for 30 min, then twice for 15 min. The oligonucleotide-containing tube was then capped, sealed
with parafilm and incubated in a 45-55°C water bath O/N to cleave the oligonucleotide. The solution was briefly cooled at -20°C, for 15 min, then the fluid dried under vacuum. The dried oligonucleotides were resuspended in 120 ul of 1X STE buffer (100 mM NaCl, 20 mM Tris-Cl (pH 7.5), 10 mM EDTA (pH 7.5)).

Oligonucleotides were purified on a G-50 spin column. G-50 beads were carefully loaded into a 1 ml syringe plugged with glass wool and packed by centrifugation in a 2059 tube in a bench top Clinical Centrifuge (International Equipment Company) at setting "3" for 3 min. The G-50 was topped up to a volume of 1 ml and the centrifugation repeated. The fluid volume of the column was equilibrated by adding 120 µl of 1X STE to top of column and centrifuging as before. The STE was retrieved from the 2059 tube and quantitated with a pipette man to ensure that the entire volume was recovered. If necessary, an additional 120-300 µl of STE were added to top of column and centrifuged as before.

The entire 120 µl of oligonucleotide solution were applied to the top of the column, and the column was centrifuged at setting "3" for 3 min. The purified oligonucleotide was collected at the bottom of the syringe column in a screw-top tube. A second centrifugation with an additional 120 µl of STE buffer was done to remove the remainder of the oligonucleotide from the column and was collected in a second screw-top tube.

iv. Quantitation of oligonucleotides

The concentrations of oligonucleotides in the primary and secondary tubes were determined by measurement at 260 nm in a spectrophotometer. A small amount of the oligonucleotide solution was diluted in dH2O and measured at 260 nm. Each optical density unit equaled a concentration of 1 µg/ml of oligonucleotide.

\[
[\text{oligo}] \mu g/ml = OD_{260} \times 20 \times \text{dilution factor (1000)}
\]

The expected yield was 1-2 µg/µl for the primary tube and less for the secondary tube.

Conversion of µg/µl to mol of oligonucleotides: [oligonucleotides (g/µl)] × 1/325 (number of bases)= mol. \((10^{-11} \text{ mol} = \text{pMol/µl})\)

v. Specific PCR reaction conditions for amplifying pim-1

Pim-1 PCR reactions were designated as A (oligos 11A to 13B), B (oligos 12A-13B), C (oligos 11A-14B) and D (oligos 12A-14B). Oligonucleotide sequences are listed in Appendix III and are based on the human and murine pim-1 sequences [Berns et al.,]
The expected size of the PCR products were 669 bp for reaction A, 423 bp for reaction B, 798 bp for reaction C and 552 bp for reaction D.

Approximately 50-100 pmols of each oligonucleotide were added to each reverse transcriptase reaction (20 μl of single stranded X. laevis or P. ochraceus cDNA template) in a total volume of 49 μl in 1X PCR buffer. The reaction was overlaid with 1-2 drops of PCR oil, heated to 96°C for 1 min, then cooled immediately on ice. Taq polymerase (1 μl) was added to the reaction and the tube was centrifuged briefly and the reaction initiated. For negative controls, 20 μl of dH2O were used in the place of the template.

Initial PCR reactions were performed at low stringency conditions, with initiation at 96°C for 1 min, annealing at 37°C for 90 s and elongation at 73°C for 3 min. The reaction was repeated through 29 cycles. To optimize the reactions, five different PCR reactions were carried out at annealing temperatures of 37°C, 41°C, 46°C, 50°C and 55°C. The reaction times and the rest of the conditions were as above.

Large scale preparative reactions for both P. ochraceus and X. laevis were performed for reaction A only, using 20 μl of reverse transcriptase template, 3 μl of 10X PCR buffer, 1.1 μl of oligo 11A, 1.1 μl of oligo 13B, 23.8 μl of dH2O and 1 μl of Taq polymerase. The reactions were carried out for 29 cycles with an initiation temperature of 94°C for 35 s, annealing at 50°C for 90 s and elongation for 73°C for 90 s.

vi. Confirmation of identity of PCR clones

The PCR clones in pBluescript were first examined by restriction digest analysis. In total, five clones yielded bands of the expected size, and orientation was determined by the restriction analysis. The two X. laevis pim-1 clones (in opposite orientation) were designated as 3A and 3B, and the three P. ochraceus pim-1 clones were designated as 5A, 5B, and 5C. The identity of the PCR clones was confirmed by sequencing, using a double template protocol as dictated by the Pharmacia kit. Details are listed in the section following library screening. The identity of the clones was confirmed as corresponding to pim-1.
5. SCREENING A λZAP cDNA LIBRARY

i. Screening the X. laevis cDNA library

A X. laevis oocyte cDNA library in λZAP (EcoR1, Xho1) was kindly obtained from Dr. Leonard Zon (Children's Hospital, Boston), at a titre of 4 x 10^9 pfu/μl. The library was retired at 1.13 x 10^7 pfu/μl. The library was screened using a protocol modified from Stratagene.

Competent cells were prepared by inoculating a 50 ml culture of LB media containing 0.2% maltose (w/v) and 10 mM MgSO₄ with a single colony of E. coli XL1-blues and grown at 37°C O/N. The cells were pelleted by centrifugation at 2000 rpm for 10 min, and resuspended in 15 ml of 10 mM cold MgSO₄. The cells were stored at 4°C for up to one week.

The competent XL1-blue cells were transformed with an appropriate dilution of phage library by incubation at 37°C for 20 min with agitation and plated onto dried 2xYT plates at approximately 2.2500 - 2.5 x 10⁴ plaque forming units (pfu) per plate. After growing O/N, duplicate plaque lifts were performed using Hybond membranes, which were oriented with respect to the plate by puncturing with an ink-soaked needle. Membranes were denatured in 1.5 M NaCl, 0.5 M NaOH for 2 min. Membranes were neutralized for 5 min in 1.5 M NaCl, 0.5 M Tris-Cl (pH 8.0) and washed with 2X SSC (pH 7.0, 33.3 mM NaCl, 333 mM sodium citrate). After drying briefly, the DNA was cross-linked to the membrane with a Stratalinker UV linker (Stratagene).

Membranes were prehybridized and hybridized with radiolabelled X. laevis pim-1 nucleic acid probe by the method detailed in Section 2.1.ix. Membranes were washed under increasingly stringent conditions, then exposed to X-ray film O/N to identify labeled clones. Potential positives were selected by alignment of the autoradiographs of duplicate membranes. The potential positives were picked and stored in 500 μl of SM buffer (100 mM NaCl, 17 mM MgSO₄, 50 mM Tris-HCl (pH 7.5) and 0.01% gelatin (w/v)) with 20 μl of chloroform. As each plaque contained 10⁶-10⁷ phages, the titre of the phage solution was determined to be between 2 x 10³ - 2 x 10⁵ pfu/μl. Potential positive clones were amplified as per Stratagene protocol and screened several times with increasingly stringent washes (10 - 60 min at 65°C). For secondary and tertiary screens, the phage solution was diluted to yield approximately one hundred pfu per plate.
The protocol was followed for in vivo excision from the Stratagene Instructional Manual (#236211) for the Predigested λZap II/EcoR1 Cloning Kit. This procedure yielded the pBluescript double stranded phagemid with the cloned insert. The pBluescript was amplified and the size of the inserts were examined by digestion with EcoR1.

Potential positives were first analyzed by Southern blotting to confirm their identity, then sequenced by Dr. G. Kalmar (Simon Fraser University) to obtain the sequence of the entire coding region.

**ii. Screening the P. ochraceus cDNA library**

Two P. ochraceus cDNA libraries were obtained from Dr. Michael Smith (Simon Fraser University). Both libraries were in λgt10; one was a cDNA library from P. ochraceus oocytes, the other from P. ochraceus testes. Both libraries were plated and screened with the sea star pim-1 probe, but did not yield any positive clones. Due to the questionable quality of this library, the screening was discontinued.

**iii. Southern blotting**

Three positive pim-1 clones obtained from the X. laevis oocyte cDNA library were quantitated and 60 ng of each were digested by restriction enzymes. The X. laevis clones were digested with AccI/NcoI, Hind III/PvuII and an undigested sample was also prepared as a control. The digests were separated on a 1% agarose gel in TAE buffer, the gel was stained with ethidium bromide and photographed to visualize the DNA bands. The gel was soaked in denaturation solution (0.4 M NaOH) for 2 x 10 min at RT, until the top dye (Xylene) turned green.

The Southern blot was assembled as recommended by Maniatis et al. [1989, Section 9.34]. Normally, Southern blots are left O/N to blot; as we blotted digested plasmid DNA, 4 h was judged to be sufficient. After disassembly, the membrane was washed in 2X SSC for 5 min to rinse away debris, then air dried and crosslinked to the filter under UV light for 3 min manually. The Southern blot was hybridized with a X. laevis pim-1 nucleic acid probe as detailed in Sections 2.1.viii-ix.

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iv. **Sequencing positive clones to confirm identity**

The nucleotide sequence of *X. laevis* and *P. ochraceus pim-1* PCR fragments were determined using the standard dideoxy chain termination method [Sanger *et al.*, 1980]. The protocol detailed in the Pharmacia sequencing kit manual, incorporating 35S-dCTP was utilized. The sequence was visualized by autoradiography and read manually.

Initially, oligonucleotides 11A and 13B were tried as sequencing primers, but they did not yield interpretable sequence. Universal primer (provided with the Pharmacia kit) and oligonucleotides T3 and T7 (see Appendix III) were successfully used for sequencing.

The entire coding region of the *X. laevis pim-1* cDNA clone was sequenced by Dr. G. Kalmar (Simon Fraser University) by automated fluorescent DNA sequencing using an ABI 373A sequencing machine. Sequencing of *pim-1* mutants was performed using the standard dideoxy chain termination method [Sanger *et al.*, 1980] with custom primers. The sequence of *X. laevis pim-1* was entered into the Genome Sequence Database, accession number L29495. A computer search for sequence similarity was performed at the National Center for Biotechnology Information (NCBI) using the BLAST network service.

6. **CONSTRUCTION OF PIM-1 EXPRESSION VECTORS**

i. **PCR of *X. laevis* cDNA clone**

The coding region of the *X. laevis pim-1* was amplified by PCR using oligonucleotides modeled on the amino and carboxyl terminal regions of the open reading frame of the *X. laevis pim-1* sequence. The oligonucleotides, pim5', 5'-CGATGGATACATGCTTCTCTAAATTCGG-3', and pim3', 5'-GATCGAATTCCAGACTCTCGTTGCTTGA-3', were designed to incorporate appropriate restriction sites to allow insertion of the *pim-1* PCR fragment in the correct reading frame into the EcoR1 and BamH1 restriction sites of the pGEX-2T vector. The polymerase chain reaction was used to amplify an approximately 1000 base pair fragment using the *X. laevis pim-1* cDNA clone 12.35 in the pBluescript plasmid as a template. Approximately 50 ng of BamH1-linearized *X. laevis pim-1* cDNA clone were used as a template in a PCR reaction, with 75 pmol of each oligonucleotide, 0.2 μl of non-acylated BSA [10 mg/ml], 50 μM of each nucleotide triphosphate and 1 μl of Vent polymerase in a total volume of 20 μl in PCR
buffer provided by NEB (10 mM KCl, 10 mM (NH₄)₂SO₄, 10 mM Tris-HCl (pH 8.8), 2 mM MgSO₄, 0.1% Triton X-100). The initial PCR reaction was carried out for 25 cycles with denaturation at 96°C for 45 s, annealing at 50°C for 2 min and elongation at 73°C for 2 min. Reaction conditions were optimized with respect to time and temperature and the reaction was scaled up. Preparative PCR reactions were done using approximately 50 ng of template, 75 pmol of each oligonucleotide, 0.5 µl of non-acylated BSA [10 mg/ml], 100 µM of nucleotide triphosphates and 2 µl of Vent polymerase in a total volume of 50 µl in NEB PCR buffer. The PCR reaction was carried out in a Perkin-Elmer Cetus thermal cycler for 25 cycles with denaturation of 96°C for 45 s, annealing at 55°C for 60 s and elongation at 73°C for 90 s. The PCR product was visualized on a 1% agarose gel, purified by Sephglas band prep kit. Subsequent restriction digests were done with both BamH1 and EcoR1, with a Sephglas purification between digestion reactions. pGEX-2T was likewise digested to create compatible cohesive ends, and subjected to alkaline phosphatase treatment.

The *X. laevis* **pim-1** PCR product and the pGEX-2T were ligated together, transformed into UT5600 *E.coli*, and plated onto 2xYT amp plates. Colonies were amplified and digested with restriction enzymes Nco1/Ava1 to determine if the correctly sized fragment was inserted, and with BamH1 to ensure that the 5' restriction site had been maintained. This was designated as "Clone 1" and is shown in Figure 2.

The pGEX-2T vector containing *X. laevis* **pim-1** was used to transform competent UT5600 bacteria as described in Section 2.1.v. The fusion protein was expressed and purified as detailed in the protein biochemistry Section 3.2.i.

### ii. Construction of **pim-1** mutants using PCR site-directed mutagenesis

Non-degenerate oligonucleotides were constructed to incorporate specific changes in the coding region of the *X. laevis* **pim-1** using PCR site directed mutagenesis. A description of oligonucleotides used and mutants constructed is detailed below.

**K69>A**

A kinase-inactive Pim-1 mutant was constructed by changing Lys-69, essential for ATP binding, to an alanine residue. Anti-sense primer K1, 5'- CTC CTT AGC TAC GTG **GCG** CAC AGC GAC CGG CTG -3', corresponding to aa 64-74, contained the lysine codon, TCC (nt 205-207), changed to an alanine codon, GCG (underlined). Sense primer K2 (aa 75-80) was constructed to allow amplification in the opposite direction.
Figure 2  Restriction map of *X. laevis* pim-1 in the pGEX-2T vector
The map shows the 969 base pair coding region of *X. laevis* pim-1 inserted into the BamH1 and EcoR1 cloning sites (shown in bold type) of the pGEX-2T plasmid. The Cla1 site used for construction of the ser-190 mutant is italicized. The unfilled arrow shows the location of the Amp\(^r\) gene, the grey shaded arrow shows the location of the lac \(R\) gene, the hatched box represents the glutathione S-transferase coding region and the black box is the human pim-1 coding region. The line arrow shows the direction of transcription of the fusion construct.
These oligonucleotides were intended to be used with the existing 5' and 3' X. laevis oligonucleotides. Unfortunately, the oligos K1 and K2 were not phosphorylated so the PCR products would not ligate together. Instead, primer K1 was used with the 5' sense primer to first amplify a 232 base pair fragment from BamH1-linearized X. laevis WT pim-1 in a PCR reaction containing approximately 50 ng of template, 100 pmol of oligonucleotides, 0.5 μl of non-acylated BSA [10 mg/ml], 100 μM of nucleotide triphosphates and 2 μl of VENT polymerase in a total volume of 50 μl in PCR buffer provided by NEB (10 mM KCl, 10 mM (NH₄)₂SO₄, 10 mM Tris-HCl (pH 8.8), 2 mM MgSO₄, 0.1% Triton X-100). The PCR reaction was optimized with respect to time and temperature and was carried out in a Perkin-Elmer Cetus thermal cycler for 20 cycles; 96°C for 45 s, 50°C for 60 s and 73°C for 60 s. The PCR fragment containing the K69>A mutation was prepared in large amounts by PCR, visualized on an agarose gel and purified by Sephglas. This PCR product was then used as an oligonucleotide to amplify the full-length coding region of pim-1 with the 3' anti-sense oligonucleotide. The PCR reaction was carried out in a total volume of 100 μl with approximately 100 ng of template, 100 pmol of 3' anti-sense primer, 5 μl of unquantitated 232 base-pair PCR product, 1.0 μl of non-acylated BSA [10 mg/ml], 100 μM of nucleotide triphosphates and 7 μl of Vent polymerase in NEB PCR buffer. The PCR reaction was optimized with respect to time and temperature and was carried out in a Perkin-Elmer Cetus thermal cycler for 25 cycles: 96°C for 45 s, 56°C for 60 s and 73°C for 90 s. After purification, the approximately 1 kb fragment was digested with EcoR1 and BamH1 and ligated into the corresponding sites of pGEX-2T (Pharmacia). The clone was sequenced by Dr. Gabe Kalmar to confirm that the mutation was as expected.

**S190>A**

Sense primer PM1 (1.125 μl), 5' - CTG ATC GAT TTT GGC GCC GGG GCG CTA CTC -3' was used with pim3, the 3' anti-sense primer (4.5 μl) to amplify a 415 base pair fragment that was used to replace the Cla1/EcoR1 fragment of WT X. laevis pim-1. Mutated base pairs are underlined. Conditions are listed below.

**S190>E**

Sense primer PM2 (0.875 μl), 5' - CTG ATC GAT TTT GGC GAA GGG GCG CTA CTC -3' was used with pim3, the 3' anti-sense primer (4.5 μl) to amplify a 415 base pair fragment that was used to replace the Cla1/EcoR1 fragment of WT X. laevis pim-1. Mutated base pairs are underlined. Conditions are listed below.
**Y198>A**

Sense primer PM3 (3.6 µl) 5' - GGA TAC GGT GGA AAC GGA TTT TGA TGG -3' was used with pim3, the 3' anti-sense primer (4.5 µl), to amplify an approximately 400 base pair fragment containing the desired mutation (underlined). A second primer, PM4 (2.7 µl), 5' - TTG AGT AGC GCC CCG GAG CC -3' was used with the 5' sense primer pim5 (4.5 µl), to amplify a 560 base pair fragment. The two PCR products were ligated together and inserted into the BamH1 and EcoR1 sites of pGEX-2T.

The PCR reaction conditions used to create the S190 and Y198 mutants were performed as follows. Approximately 25 µg of BamH1 linearized X. laevis pim-1 cDNA clone were used as a template, with the amounts of oligonucleotides specified, 1 µl of BSA (10 mg/ml), 100 µM dNTPs, and 5 µl VENT polymerase in a total volume of 100 µl of NEB PCR buffer. The PCR reaction was optimized with respect to time and temperature and was carried out for 29 cycles at 96°C for 45 s, 55°C for 120 s and 73°C for 120 s. The reactions continued for one cycle at 96°C for 45 s, 55°C for 120 s and 73°C for 10 min. The reaction was electrophoresed through a 2% agarose gel, visualized by ethidium bromide staining, and purified using Sephglas bandprep kit. PCR products were digested and ligated into appropriate restriction sites of pGEX-2T. The ligations were used to transform DH5α-high competence cells (GIBCO-BRL) and positive clones were selected on the basis of ampicillin resistance. The clones were sequenced by Dr. G. Kalmar to confirm that the mutations were as expected.

**iii. Construction of a H. sapiens Pim-1 expression vector**

The human pim-1 cDNA clone, pCI, was obtained from Dr. T. Meeker via Dr. M. Lilly (Seattle VA hospital). This plasmid was a tetracycline-resistant derivative of pBR322 with the human pim-1 cDNA clone inserted into the PstI site, abolishing the ampR of the plasmid. Restriction digests were done to ensure that the restriction sites in the plasmid were intact. The pCI plasmid was linearized by digestion with SmaI to be used as a template for PCR.

The coding region of the human pim-1 was amplified by PCR, using oligonucleotides based on the amino and carboxyl terminal regions of the open reading frame of the published human pim-1 sequence [Zakut-Houri et al., 1987]. Oligonucleotides 9402 and 9405 (see Appendix III) were designed to incorporate appropriate restriction sites to allow insertion of the pim-1 PCR fragment in the correct reading frame into the EcoR1 and SmaI restriction sites of the pGEX-2T vector.
For each PCR reaction, the amounts of template and oligonucleotides were varied in order to optimize the reaction. Initial reactions were performed in a final volume of 20 μl, and scaled up to 50 μl for preparative purposes. Reactions contained 50 ng of pCI template, 50 pmol of oligo 9204, 50 pmol of oligo 9205, 100 μM of nucleotide triphosphates, 0.5 μl of BSA and 2.5 μl of VENT in a total volume of 50 μl NEB PCR buffer. The PCR reaction was optimized with respect to temperature and time of the various steps, denaturation was at 96°C for 45 s annealing at 55°C for 2 min and elongation at 73°C for 2 min for 25 cycles. The approximately 1 kb PCR product was subjected to electrophoresis on a 1% agarose gel, visualized by ethidium bromide staining, purified by electrolution for 90 min at 50 mA, and then precipitated by the addition of 0.1 volume of NaOAc (pH 4.8) and 2.5 volumes of ethanol.

The pim-1 PCR product and the pGEX-2T plasmid were digested sequentially with EcoR1 and Sma1 to create cohesive ends. In addition, the pGEX-2T fragment was dephosphorylated with alkaline phosphatase. The DNA fragments were ligated for 16 h at 14°C, then for 6 days at RT and then used to transform XL1-blue E. coli. Positive clones were selected with blue-white selection on LB-amp plates containing IPTG and X-gal and were restreaked for mini-preps. Clones were analyzed by restriction digestion with enzymes Xho1, EcoRV, BamH1, EcoR1 and Sma1 to ensure that the insert was the expected size, in the correct orientation and the Sma1 site at the 5' end of the PCR fragment was maintained (Figure 3). A large scale plasmid preparation was performed with one of the positive clones.
Figure 3  Restriction map of *H. sapiens* pim-1 in the pGEX-2T vector
The map shows the the 939 base pair coding region of *H. sapiens* pim-1 inserted into the Smal and EcoRI cloning sites (shown in bold type) of the pGEX-2T plasmid. The unfilled arrow shows the location of the *Amp*<sup>+</sup> gene, the grey shaded arrow shows the location of the *lac Z* gene, the hatched box represents the glutathione S-transferase coding region and the black box is the human pim-1 coding region. The line arrow shows the direction of transcription of the fusion construct.
3. EXPERIMENTAL PROCEDURES - PROTEIN BIOCHEMISTRY

1. GENERAL PROTEIN BIOCHEMISTRY TECHNIQUES

i. Protein quantitation

Proteins were quantitated by the method of Bradford [1976]. A series of protein standards was prepared by adding 0-30 μg of BSA to tubes in 5 μl increments, with 2 ml of Bradford reagent (100 mg Coomassie Blue G, 50 ml ethanol, 100 ml H₃PO₄ made up to 1:1 with dH₂O and filtered) and mixed by gentle vortexing. The protein to be quantitated was diluted with dH₂O so that the absorbance would fall in the linear range of the BSA standards (5-20 μg). The solutions were measured at 595 nm and the concentrations were plotted by linear regression.

For purified bacterially-expressed proteins, the amount of full-length fusion protein was quantitated visually by comparison to BSA protein standards on a SDS-PAGE gel. Serial dilutions of purified protein and BSA standard (0.5, 1, 2, 4, 8 μg) were subjected to electrophoresis on an SDS-PAGE gel, then stained by Coomassie. The amounts of fusion protein were compared to the standards and adjusted by the dilution factor and the volume loaded. The percentage of the fusion protein in the sample was determined by comparison of the fusion protein concentration to the total protein concentration.

ii. Column fractionations of protein extracts

Cell extracts from various sources were fractionated by fast protein liquid chromatography (FPLC) on 2 ml MonoQ, MonoS or ResourceQ columns (Pharmacia). Columns were equilibrated before and after use with 2 ml of 2.0 M NaCl, and washed extensively with buffer A (0 M NaCl). All buffers were filtered (0.22 μ) before using.

Samples were prepared as described and subjected to either high speed centrifugation in the Eppendorf centrifuge at 15000 rpm for 5 min, or by filtration through a 0.22 μ filter. The sample was quantitated if necessary and the appropriate volume was diluted to 2.2 ml with the appropriate buffer A (described below). A maximum of 5.0 mg of total protein were loaded onto the column (numbers of oocytes are indicated where applicable). The sample was loaded onto the 2.0 ml injection loop and applied directly onto the column as part of the program. A standard elution program was followed: the protein was applied onto the column in 2.5 ml of buffer A, eluted in a 10 ml 0-0.8 M
NaCl gradient, and collected in 0.25 ml fractions. Column fractions were either assayed by peptide kinase assays or analyzed by SDS-PAGE.

MonoQ and ResourceQ columns were equilibrated with Buffer A (10 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 2 mM EDTA, 5 mM EGTA, 2 mM Na₃VO₄) and eluted with Buffer B (0.8 M NaCl, 10 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 2 mM EDTA, 5 mM EGTA, 2 mM Na₃VO₄).

MonoS columns were equilibrated with MES buffer A, pH 6.5 (20 mM MES, 25 mM β-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 5 μM β-methyl aspartic acid, 1 mM NaF, 100 μM Na₃VO₄) and eluted with MES buffer B, pH 6.5 (0.8 M NaCl, 20 mM MES, 25 mM β-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 5 μM β-methyl aspartic acid, 1 mM NaF, 100 μM Na₃VO₄).

iii. SDS-PAGE Gels
Proteins were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [Laemmli, 1970]. Proteins were subjected to electrophoresis on 1.5 mm thick polyacrylamide gels, with a stacking gel containing 4% acrylamide and a separating gel containing 10% acrylamide. Before loading, the samples were boiled for 5 min with 1 vol of 2X sample loading buffer (120 mM Tris-Cl (pH 6.8), 4% SDS (w/v), 10% glycerol (v/v), 0.3 M 2-ME, 0.008% bromphenol blue (w/v)), then briefly centrifuged. The gels were subjected to electrophoresis in running buffer (25 mM Tris, 192 mM glycine, 3.5 mM SDS) at 10 mA O/N, until the dye front reached the bottom of the gel.

iv. Staining SDS-PAGE gels

a. Coomassie staining
Gels were immersed in Coomassie stain (0.1% Coomassie Brilliant Blue R/50% methanol/10 % acetic acid (w/v/v)) for 10-30 min, then soaked in 40% methanol/10% acetic acid (v/v) until the gel was sufficiently destained to allow appearance of protein bands. The destaining solution was changed several times, and a sponge was added to soak up the stain.
b. Silver staining

In preparation for silver staining [Merril et al., 1981], gels were first soaked in fixative 1 (40% methanol/10% acetic acid (v/v/v)) for 30 min and fixative 2 (10% ethanol/5% acetic acid (v/v/v)) for 2-15 min periods. The gels were oxidized for 5 min in oxidizer (3.4 mM K$_2$Cr$_2$O$_7$, 3.2 mM nitric acid). The gels were washed three times with dH$_2$O, then stained with 0.204% AgNO$_3$ (w/v) for 20 min. Gels were washed briefly in deionized water and developed with Na$_2$CO$_3$ (0.28M) in a formaldehyde solution (0.166% (v/v)) and stopped with the addition of acetic acid (5% v/v).

c. Amido black staining

Membranes or gels were immersed in 0.1% amido black/45% methanol/10% acetic acid (v/v/v/v) for 15-30 min at RT with rotation. Destaining was carried out in a 5% methanol/10% acetic acid (v/v/v/v) solution until bands were visible and background was reduced.

v. Western blotting of SDS gels

After the dye front reached the bottom of the gel, the electrophoresis apparatus was disassembled and the bottom right corner of the gel cut for orientation. The gel was equilibrated in transfer buffer (pH 8.6, 20 mM Tris, 120 mM glycine, 20% methanol (v/v)) to remove SDS. PVDF membrane was hydrated for 30 s in methanol, then equilibrated for 5 min in transfer buffer. If nitrocellulose membrane was used, it was hydrated in transfer buffer for 60 s. The gel and membrane were placed between 6 pieces of 3 mm filter paper hydrated in transfer buffer and enclosed within the sponges and plastic sandwich apparatus. If more than one gel was transferred at a time, the gels were stacked with 4 pieces of filter paper and a piece of nitrocellulose between them. Care was taken to prevent the trapping of any air bubbles between the gel and the membrane. The transfer sandwich was placed in a Hoefer transfer cell and transferred at 4°C for 3 h at 300 mA, or for 100 mA for 12-16 h.

Membranes were fixed (40% methanol/10% acetic acid (v/v/v)) for 15 min, then washed for 15 min in dH$_2$O. The membranes were then either Ponceau stained to visualize protein or air dried and stored for later use.

The wet membranes were blocked in 5% BSA in TBST (50 mM Tris base, 150 mM NaCl, 0.05% Tween-20, pH 7.5), for 2 h or O/N, then rinsed briefly in TBST. The primary antibody was diluted to the optimum concentration (usually 1/500-1/1000) in
TBST and incubated with the membrane for 4 h at RT, shaking gently. The antibody was then removed and saved (0.1% azide added) and the membrane washed at for three 10 min washes with TBST. The secondary antibody was diluted to the optimum concentration in TBST, and incubated with the membrane for 2 h at RT, with agitation. When using ECL, second antibody was diluted to 1:20000 and discarded after use. Membranes were subjected to at least three 10 min washes with TBST and one wash with TBS.

When using the 4G10 anti-phosphotyrosine antibody, blotting was carried out as above, but with some modifications. Low salt TBS (20 mM Tris-base, 50 mM NaCl, pH 7.5) and 0.05% NP-40 (v/v) instead of Tween-20 was used. The membrane was incubated in the primary antibody for a maximum of 4 h and incubated with an ALP goat anti-mouse secondary antibody for a maximum of 2 h.

a. Alkaline phosphatase (ALP)-conjugated secondary antibody
   The membrane was rinsed briefly with 1X AP buffer (0.1 M Tris-base, 0.1 M NaCl, 5 mM MgCl₂, pH 9.5), then developed in 50 ml AP buffer containing 340 µl of NBT (50 mg/ml in 70% DMF) and 170 µl of BCIP (50 mg/ml in 100% DMF). The color reaction was left to proceed for 30 sec to 1 h (depending on intensity of band) and was terminated by rinsing membrane with dH₂O before air drying.

b. Horseradish peroxidase-conjugated secondary antibody (ECL)
   Membranes were blotted briefly on filter paper to remove excess TBS, then incubated with ECL reagent for exactly 60 s with gentle swirling. Membrane were blotted to remove excess reagent and wrapped carefully in Saran wrap, avoiding bubbles and wrinkles. The membranes were exposed to X-ray film from 10 sec to 30 min, depending on intensity of bands and amount of background.

vi. Stripping and reprobing Western blots
   For ALP blots, membranes were stripped by washing in TBS (pH 2.5) for 10 min, followed by two 5 min washes with TBS (pH 7.5). Membranes were reprobed immediately with primary antibody.

For ECL blots, membrane was stripped with buffer (100 mM 2-ME, 2% SDS (w/v), 62.5 mM Tris-HCl, pH 6.7) at 55°C for 20 min with agitation. The membrane was washed with TBST and then reblocked O/N with 5% BSA in TBST.
vii. Autoradiography and development of film

Radioactive items including SDS-PAGE gels, Western blots, nylon membranes (library screen, Southern and Northern blots), sequencing gels, TLC plates and ECL blots were wrapped in Saran wrap to protect film from moisture. The radioactive item was secured with labeling tape onto cardboard and inserted into cassette. Film was exposed to the radioactive signal for a specified amount of time and developed. Film was developed for 20-30 sec (time dependent on exposure and background) in Kodak Developer with gentle swirling. Development was stopped by immersing the film in 3% acetic acid (v/v) for 60 sec then fixed by soaking in Kodak Fixer for 3-5 min. The film was washed for 10 min in running water, then air-dried at RT.

viii. Immunoprecipitation

The protocol for immunoprecipitation was obtained from Dr. Chris Siow (Kinetek Biotechnology Corp.) and was performed as described below. Protein A-Sepharose CL4B was swollen for 15 min in 3% NEFT (3% NP-40, 100 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, (pH 7.4), 50 mM NaF). The Protein A-beads were pelleted by spinning in a Sorvall RT 6000D lab top centrifuge at 3000 rpm for 1 min, washed twice with 3% NEFT, and resuspended with an equal volume of 3% NEFT to yield a 1:1 suspension.

Antibodies were bound to the Protein A-beads by preincubating the beads (40 µl of the 1:1 suspension) with an appropriate amount of purified antibody or sera. The titre and amount of antibody used is indicated in the results section. The antibody-Protein A solution was mixed by rotation for 45 min at 4°C, then pelleted by centrifugation in an Eppendorf centrifuge at 5000 rpm for 5 min, and washed once with 3% NEFT.

Cell homogenate (approximately 100 µl) or fusion protein (approximately 0.5-2 µg) was clarified by centrifugation in an Eppendorf centrifuge at 4°C for 5 min at 15000 rpm. The solution was aliquoted and the volume increased to 100 µl with 0% NEFT buffer (100 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, (pH 7.4), 50 mM NaF), and SDS was added to a final concentration of 1%. To each sample were added 100 µl of 6% NEFT (6% NP-40, 100 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, (pH 7.4), 50 mM NaF). The samples were precleared with 10 µl of Protein A for 15 min at 4°C, and centrifuged for 5 min at 15000 rpm. This precleared pellet was saved as a control for non-specific protein binding to the protein A-beads. The supernatant was added to the antibody-gel pellet, and rotated for 1 h at 4°C. The immunoprecipitate (IP) was centrifuged at 5000 rpm for 5 min, and the supernatant removed and saved as a control. The pellet was washed with
1.25 ml of 6% NEFT, followed by a wash with 1.25 ml 0% NEFT. If the IP was not to be used for kinase or autophosphorylation assays, the pellet was then resuspended in 2X sample buffer, boiled and loaded on a gel as usual.

Alternatively, when the H-chain interfered with the detection of the immunoprecipitated protein(s) on an SDS page gel, 1 mM N-ethyl maleimide (NEM) and modified sample loading buffer (not containing 2-ME) were added to the IP and the entire sample loaded directly onto a gel without boiling. The remainder of the protocol was followed as usual.

ix. Kinase assays of immunoprecipitations

If peptide assays or autophosphorylation assays were to be performed with IPs, some modifications to the previous protocol were made. If kinase activity was expected to be retained after binding of antibody, the IP was washed several times with ADB. For peptide assays, the peptide cocktail as well as the assay ATP was added to each tube and the assay initiated by gentle vortexing. The peptide assay was terminated by the careful withdrawal (avoiding the protein A-beads) and spotting of the supernatant on filter paper. Autophosphorylations were done in a similar manner, with the appropriate ADB and the ATP added to the IP. The reaction was terminated by the addition of sample buffer and the entire sample, beads and supernatant, was loaded onto gels.

In cases where the kinase activity was immobilized by antibody binding, the kinase was dissociated from the antibody by the addition of 25 μl of 0.1 M glycine, pH 2.5. The IP was mixed and incubated for 5 min at RT, then the beads were quickly pelleted by centrifugation and the supernatant removed to a tube containing 20 μl of 1.0 M Tris-HCl (pH 8.8). Kinase assay constituents were added and the assay carried out as usual. The reaction was terminated by either spotting on filter paper or by the addition of 2X sample buffer.

x. Protein-protein interactions

a. GST-Pim-1 protein affinity columns

Many combinations of cell lysates (immature and mature X. laevis and P. ochraceus oocytes) and affinity matrix (H. sapiens and X. laevis GST-Pim-1) were tested for protein binding. Cell lysates (approximately 4 mg of X. laevis oocyte lysates, 5 mg P. ochraceus oocyte lysates) were filtered with a 45 μ filter. The lysates were precleared to remove
any proteins non-specifically binding to the GST or glutathione by incubating with 250 µl (packed volume) of GST beads in the presence of protease inhibitors (10 µl each of SBTI, aprotinin, leupeptin) for 15 min at 4°C with frequent inversion. The slurry was poured into a column, and the flow-through collected. The column was washed with 8-10 vol of STEC buffer.

The flow through from the GST column was added to 250 µl (packed volume) of GST-Pim-1 beads, and incubated for 10-30 min at 4°C with frequent inversion. The slurry was poured into a column and washed with 8-10 volumes of STEC buffer. The flow-through was applied to the column a second time and the column washed again with 8-10 volumes of STEC buffer.

For in vitro phosphorylation reaction of bound proteins, the column was then washed with ADB. A sample of 250 µl of GST-Pim-1 beads was introduced as a control. The beads were removed from the column and an in vitro phosphorylation reaction was carried out in the presence of 10 mM MgCl₂, 33.3 µM [γ-32P] ATP (10⁴ cpm/pmol), 0.5 µM PKI (cyclic AMP-dependent protein kinase inhibitor) in a final volume of 400 µl ADB. The reaction was initiated with the addition of [γ-32P]ATP and was allowed to proceed for 30 min at 30°C. Beads were resuspended every 10 min by withdrawing and expelling supernatant. The reaction was terminated by the addition of 100 µl of 2X sample buffer and the sample split in two before being subjected to SDS-PAGE. Half of the gel was silver stained to visualize protein and half was blotted onto nitrocellulose and autoradiographed.

b. Far Western blotting procedure

The Far Western blotting protocol was a modified version of that described by Kaelin et al. [1992]. MonoQ fractions of immature and mature X. laevis oocyte extract were separated on an SDS-PAGE gel and Western blotted onto PVDF membrane. Membranes were blocked O/N in 3% BSA in low salt TBS, then briefly washed twice with NBST. Two probe methods were used, [γ-32P] labeling of Pim-1 probe and [γ-32P] labeling of immobilized proteins with X. laevis GST-Pim-1.

1. Labeled Pim-1 probe

Pim-1 protein was prepared as a probe, by radiolabeling X. laevis GST-Pim-1 protein bound to 250 µl of glutathione agarose beads. The beads were washed twice and resuspended in a slurry with 500 µl of ADB (10 mM MgCl₂, 0.5 µM PKI). [γ-32P] ATP
(25 μM, 6 x 10^3 cpm/pmol) was added to the beads and incubated for 1 h at 30°C. The reaction was terminated by the addition of 500 μl of TBST, and the beads were washed 3X with TBST to remove any non-incorporated [γ-32P]ATP. The labeled fusion protein was removed from the beads by washing the beads three times with 500 μl of 10 mM glutathione (15.4 mg/5 ml) in TBST. The eluted GST-Pim-1 was then added to the membrane in 10 ml of 3% BSA in TBST and incubated at RT O/N with constant rotation. The membranes were washed with TBST, air dried and autoradiographed.

2. Labeling of immobilized proteins with *X. laevis* GST-Pim-1

After blocking of membrane O/N with BSA, a negative control kinase assay was performed to ensure that none of the bound proteins would autophosphorylate. Assay dilution buffer (4 ml) was added to the blot, with 0.125 μM PKI and 10 μM [γ-32P]ATP (6 x 10^3 cpm/pmol). The kinase reaction was allowed to proceed O/N at 30°C, with rotation. The blot was washed extensively with NTBS until unincorporated radioisotope was no longer present. The blot was then briefly air-dried and exposed to X-ray film for 16-24 h.

The membrane was rehydrated and blocked for 1 h in 3% BSA (w/v) in NTBS. Assay dilution buffer (4 ml) and 0.125 μM PKI were added to the blot and 200 μl of 1.75 mg/ml of GST-Pim-1 were added, with 10 μM [γ-32P]ATP (6 x 10^3 cpm/pmol). The kinase reaction was allowed to proceed O/N at 30°C, with rotation. The blot was washed extensively with NTBS until unincorporated radioisotope was no longer present. The blot was then briefly air-dried and exposed to X-ray film for 16-24 h.

xi. Antibody Production

a. Pim1-III, Pim1-NT, Pim1-XI

The affinity-purified antibodies Pim1-III, Pim1-NT, and Pim1-XI were prepared as described [Sanghera, et al., 1992] and are commercially available from Upstate Biotechnology Inc. (Lake Placid). The peptides were suspended in PBS and Freund's incomplete adjuvant and injected into rabbits. The rabbits were frequently boosted, and bled for titres. Antibodies were purified from sera using peptide affinity columns and titred by ELISA.
b. Anti-X. laevis GST-Pim 1 serum

Rabbit polyclonal antisera was produced against the full-length X. laevis GST-Pim-1 bacterial fusion protein. The X. laevis GST-Pim-1 bacterial fusion protein was produced, purified and eluted from the glutathione beads as described in Section 3.2.i. The eluted fusion protein was concentrated in a Centricon 30 filter tube by centrifugation in a Beckman centrifuge at 4°C, 3000 rpm for 30-45 min. After several washes with cold PBS, the volume was reduced by continued centrifugation. The protein concentration was quantitated as described in Section 3.1.i, aliquotted and frozen until use. The first injection required 250 μg of protein, 200 μg were used for the second and third injections and 100 μg were used for each successive injection.

The appropriate aliquot of protein was thawed before injection, and mixed with PBS and Freund's incomplete adjuvant (first injection only). Two rabbits (numbers 48, 49) were each injected and boosted according to the regular schedule. The rabbits were bled and the sera purified by protein G purification following the manufacturers protocol [Pharmacia]. The Protein G-purified sera was incubated with GST-beads to remove any antibodies specific for the GST portion of the fusion protein. The sera (1.5 ml) was incubated with 500 μl of GST bound to glutathione beads, rotating for 30 min at 4°C. The sera was centrifuged briefly to pellet the beads and the supernatant was removed. The beads were washed once with 500 μl of PBS (4°C), the supernatants combined and aliquoted for future use. The GST-cleared sera was frozen and used for Western blots and immunoprecipitations.

xii. Phosphatase treatment of proteins

The phosphatase activity of various enzymes was tested using p-nitrophenolphosphate (PNP). The PNP was diluted to 10 mg/ml in the respective phosphatase buffer, and 2 μl of each phosphatase were added to 1000 μl of the PNP solution, and incubated at RT. Phosphatase activity was evidenced by a color change in the solution, from clear to pale yellow. Phosphatases were considered to be active if the color reaction occurred within 30 s of phosphatase addition.

a. Acid phosphatase

Acid phosphatase was used to non-specifically dephosphorylate GST-Pim-1. Acid phosphatase (10 μl) was added to 200 μl of a 1:1 suspension of GST-Pim-1 beads (prewashed in TBS, pH 5.0) and the reaction allowed to proceed for 30 min at RT. To
terminate the phosphatase reaction, the beads were washed three times with isotonic assay dilution buffer.

de.

**Alkaline phosphatase**

Alkaline phosphatase was used to dephosphorylate GST-Pim-1 non-specifically. Alkaline phosphatase (5 μl) was added to 200 μl of a 1:1 suspension of GST-Pim-1 beads (prewashed in the buffer provided, containing MgCl₂, pH 9.0) and the reaction allowed to proceed for 30 min at RT. To terminate that reaction, the beads were washed three times with isotonic assay dilution buffer.

c.

**Phosphatase HPTPB**

The HPTPB, was obtained from Mr. Ken Harder (Biomedical Research Centre) bound to glutathione beads and was thrombin cleaved prior to use. The HPTPB beads were washed in thrombin buffer (0.05 M sodium citrate, 0.15 M NaCl, pH 6.5), and the supernatant was removed. Thrombin was diluted in buffer to a final concentration of 20 ng/μl, which was added in a 1:1 ratio (v/v) with the HPTPB beads. The cleavage reaction was carried out at 22°C for 30 min, with frequent agitation. After centrifugation, the supernatant containing the cleaved HPTPB was carefully recovered and the beads were washed twice with 100 μl of thrombin buffer. PMSF was added to inactivate the thrombin. The final concentration of the cleaved HPTPB was estimated to be 1 μg/μl, which was confirmed by silver staining.

Phosphatase reactions using HTPTB were carried out in TBS, pH 8.0 in the presence of 5 mM 2-ME and SBTI. Ten μl of HPTPB were added to a reaction containing 200 μl of a 1:1 slurry of GST-Pim-1 beads. Reactions proceeded at RT for 30 min.

2. **PRODUCTION AND PURIFICATION OF GST-FUSION PROTEINS IN BACTERIA**

i. **Expression and purification**

The pGEX-2T vectors containing the various Pim-1 inserts were used to transform UT5600 E. coli. Cultures were grown in 2xYT [Maniatis et al., 1989, Section A.3] media with 75 μg/ml amp O/N at 37°C. After 16 h, the cultures were diluted 10-fold with 2xYT media with 75 μg/ml amp and grown for 1 h at 37°C. The expression of the fusion protein was induced with 200 μM IPTG for 3-6 h at RT. The culture was centrifuged in a
Beckman centrifuge at 2000 x g for 15 min, the pellet washed in 25 ml PBS containing 0.2 mM PMSF, frozen and stored at -70°C until use. To extract the fusion protein, the pellet was resuspended in 10 ml of PBS containing 1 mM EDTA, 0.1 mM EGTA, 0.2 mM PMSF, 1% Triton X-100 and protease inhibitors aprotinin, leupeptin and soybean trypsin inhibitor (1 µg/ml each). The pH was adjusted to 7.5 and 200 µg/ml of lysozyme were added to each pellet. After a 20 min incubation on ice, the final concentration of MgCl₂ was adjusted to 2.5 mM and 30 µl of DNase (50 mg/ml) were added. After incubation on ice for 30 min, the lysate was centrifuged (12000 x g for 10 min at 4°C). The supernatant was added to glutathione-agarose beads and gently mixed on a rotator at 4°C for 30 min. The beads were then poured into a column 0.5 cm in diameter and washed with 10 volumes of STE buffer (50 mM Tris-HCl (pH 8.0), 4 mM EDTA, 150 mM NaCl, 0.1% Triton X-100), followed by 10 volumes of STEC buffer (50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 6 mM CaCl₂). The fusion protein was eluted from the beads with 10 mM glutathione in STEC buffer and immediately frozen at -70°C.

ii. Thrombin cleavage of fusion proteins

Thrombin digestion of GST-Pim-1 fusion protein was carried out on both eluted and immobilized fusion protein. In both cases, the immobilized fusion protein was washed with several volumes of thrombin buffer (0.05 M sodium citrate, 0.15 M NaCl, 5 mM EDTA, pH 6.5). Although the Pharmacia manual recommends thrombin treatment for 2-16 h at RT using 10 cleavage units of thrombin per mg of fusion protein, reactions were performed for 1 h at 30°C, for reasons of enzyme thermolability. For thrombinization of eluted protein, the protein was eluted from the beads with 10 mM glutathione in thrombin buffer and the eluate then subjected to thrombinization at 30°C for 1 h. The thrombinization reaction was terminated by the addition of 5 mM EDTA. Alternatively, the thrombin was added directly to the immobilized GST-Pim-1 to form a 1:1 slurry, and incubated at 30°C for 1 h. The supernatant containing the cleaved protein was then removed from the beads.

In general, for 10 µg of protein, 20 ng of thrombin were used, or 10 cleavage units of thrombin per mg of fusion protein. One thrombin cleavage unit was equal to 0.2 NIH units, and could completely digest 100 µg of fusion protein in 16 h.
3. ASSESSMENT OF ENDOGENOUS KINASE ACTIVITY OF EXPRESSED PIM-1

i. Autophosphorylation of GST-Pim-1

Phosphorylations were performed in a final volume of 50 µl, with 5 µl of eluted GST-Pim-1 in STEC buffer, 50 µM [γ-32P] ATP (6 x 10^3 cpm/pmol), 0.5 µM PKI and ADB containing 10 mM MgCl₂ and 1.25 mM MnCl₂. Alternatively, for large scale preparations, GST-Pim-1 immobilized to 50 µl of glutathione beads was autophosphorylated in a total volume of 200 µl ADB containing 10 mM MgCl₂ and 1.25 mM MnCl₂, 0.5 µM PKI and 50 µM [γ-32P] ATP (6 x 10^3 cpm/pmol). Preincubations were performed on ice and the kinase reaction at 30°C for 30 min was started with the inclusion of the ATP and terminated by the addition of 50 µl of 2X SDS-sample buffer. The sample was boiled for 5 min and clarified by centrifugation, the supernatant was subjected to electrophoresis on a 10% SDS-PAGE gel then transferred onto PVDF membrane for Western blotting. X-ray film was exposed to the PVDF membrane O/N.

ii. Determination of specific activity

The specific activity of autophosphorylation of the fusion proteins was determined by measuring the amount of [γ-32P]ATP incorporated per mg of fusion protein at discrete time points. Varying quantities of fusion protein (5, 2.5 and 1 µg) were autophosphorylated in an assays containing 100 µM [γ-32P] ATP (6 x 10^3 cpm/pmol), in 25 µl of ADB. Assays were started by the addition of the ATP, allowed to proceed at 30°C for various amounts of time and were terminated by the addition of 2X sample buffer. Samples were boiled and centrifuged as usual and were subject to electrophoresis on a SDS-PAGE gel. Gels were stained to ensure that the amount of protein loaded per lane was consistent, the gels were dried and radioactive bands were cut from the gel and counted by scintillation counting. Values obtained from the scintillation counting were adjusted for pmol [γ-32P] incorporated per mg fusion protein and were plotted against the time of assay in min. The slope of the graph was calculated as the specific activity or autophosphorylation of the kinase in units of pmol.min⁻¹.mg⁻¹.

iii. Determining the stoichiometry of autophosphorylation of GST-Pim-1

Stoichiometry of autophosphorylation was determined by measuring the moles of ATP incorporated per mol of GST-Pim-1. The stoichiometry was determined by converting the specific activity to pmol [γ-32P]. pmol⁻¹ GST-Pim-1.
iv. Phosphoamino acid analysis of *in vitro* autophosphorylated Pim-1

Phosphorylations were performed in a final volume of 200 µl, with GST-Pim-1 immobilized to 50 µl of glutathione beads, 25 µM [γ-32P] ATP (10⁴ cpm/pmol), 0.5 µM PKI and ADB containing 10 mM MgCl₂ and 1.25 mM MnCl₂. Preincubations were performed on ice and the kinase reaction at 30°C for 30 min was started with the introduction of the ATP. Reactions were terminated by the addition of 100 µl of 2x SDS-sample buffer, boiled for 5 min, clarified by centrifugation, separated on a 10% SDS-PAGE gel and then transferred onto PVDF membrane by Western blotting. Radioactively labeled bands were visualized by autoradiography and excised from the PVDF membrane and chopped into 0.5 mm² pieces. The membrane bound protein was digested in 300 µl of constant boiling HCl at 105°C for 1.5 h. The acid was removed and the membrane was washed briefly with dH₂O to remove residual amino acids. Water and acid were removed by evaporation in a vacuum centrifuge and amino acids were sequentially washed and vacuum-dried. Amino acids were redissolved in water/acetic acid/pyridine buffer (94.5/5/0.5 v/v/v) containing 1 mg/ml each of phospho-serine, phospho-threonine and phospho-tyrosine standards. Phenol red (0.5% in buffer (w/v)) was spotted on the origin as a control. Approximately 2000 cpm were spotted 2 cm from the bottom of a cellulose sheet (Kodak chromagram) and subjected to electrophoresis for 1.5 h at 750 volts, until the phenol red spot had migrated 7-8 cm from the origin. The plate was air dried and sprayed with ninhydrin solution (0.25% in ethanol) and developed to visualize standards either by heating with a hairdryer or by baking in an oven at 90°C for 5 min. X-ray film was exposed to the cellulose sheet for 18 h and subsequently developed.

4. ASSESSMENT OF EXOGENOUS KINASE ACTIVITY OF EXPRESSED PIM-1

i. Phosphorylation of protein substrates

Phosphorylation reactions were carried out in ADB in a total volume of 50 µl containing 25 µg of protein substrates, 0.5 µM of PKI, 50 µM [γ-32P]ATP (5 x 10³ cpm/pmol) and either 5 µg of *X. laevis* or 15 µg of *H. sapiens* GST-Pim-1. For reactions containing 40S ribosome, 60 µg of ribosome was used. Control reactions containing either substrate alone or enzyme alone were performed. Reactions were started with the addition of the ATP, carried out at 30°C for 40 min and terminated by the addition of 100 µl of 2X sample buffer. Samples were subjected to electrophoresis on SDS-PAGE gels, transferred onto PVDF and autoradiographed for 2 h.
ii. Phosphorylation of synthetic peptides

Peptide phosphorylations were performed in a final volume of 25 µl, with 5.0 µl of diluted peptide, 1.25 µg of *H. sapiens* or 0.5 µg of *X. laevis* GST-Pim-1 in STEC buffer, 50 µM [γ-32P] ATP (2000 cpm/pmol), 0.5 µM PKI and ADB. All preincubations were performed on ice and the kinase reactions at 30°C for 10 min were started with the addition of the ATP and terminated by application of 20 µl of the reaction mixture onto a 1.5 cm² piece of Whatmann P81 phosphocellulose paper. Filter papers were washed in phosphoric acid (1%, v/v) and the radioactivity quantitated in an LKB Wallac 1410 scintillation counter.

iii. Determination of kinetic constants

Kinetic constants $K_m$ and $V_{max}$ were estimated from Lineweaver-Burke plots with at least six different concentrations of a given peptide per determination. Assays were performed in triplicate, and the mean values of the $K_m$ and $V_{max}$ determinations are shown in Tables I-VI. The standard errors for all reported $K_m$ and $V_{max}$ were less than 15%. The value of $V_{max}/K_m$ was calculated for all peptides and inserted into the third column of Tables I-VI. The $V_{max}/K_m$ value was used to compare the relative efficiencies of various peptides as substrates of the GST-Pim-1.

iv. Stability of GST-Pim-1 enzyme at 30°C

The stability of GST-Pim-1 enzyme activity after incubation at 30°C was examined. *H. sapiens* and GST-Pim-1 was incubated at 30°C and at discrete time points, aliquots of the enzyme solution were withdrawn and immediately added to either an autophosphorylation reaction or a peptide kinase reaction.

For autophosphorylation reactions, a 1:1 slurry of GST-Pim-1 beads in ADB was incubated at 30°C with frequent resuspension. At intervals from 0-70 min, 10 µl of the slurry were removed and immediately added to an autophosphorylation reaction containing 50 µM [γ-32P] ATP (2000 cpm/pmol), 20 mM MgCl₂, 0.5 µM PKI in 25 µl of ADB. Reactions were allowed to proceed for 5 min at 30°C then terminated by the addition of 25 µl of 2X SDS-PAGE sample buffer and subjected to electrophoresis on an SDS-PAGE gel. After electrophoresis, the gel was silver stained as a control for protein loading and the radioactive bands were excised and quantitated by scintillation counting.
For peptide substrate assays, GST-Pim-1 was incubated at 30°C. At discrete intervals (from 0-70 min), 5 µl of GST-Pim-1 were removed and immediately added to a peptide substrate reaction containing 50 µM [γ-32P] ATP (2000 cpm/pmol), 0.5 µM PKI in 25 µl of ADB. Reactions were allowed to proceed for 5 min at 30°C, then terminated by spotting 20 µl onto p81 filter papers. Filter papers were washed as usual and counted by scintillation counting.

v. Optimization of kinase reactions

a. Metal ion optimizations of GST-Pim-1 kinase reactions

Assays contained 3 µl GST-Pim-1 (eluted without calcium), 0.5 µM PKI, 250 µM P4 peptide (AKRRRLSA), 50 µM [γ-32P] ATP plus ions, in a final volume of 25 µl of ADB containing 1 mM MgCl2. Assay concentrations of CaCl2 ranged from 3.906 mM to 1 M; assay concentrations of NaCl2, ZnCl2, MnCl2, and MgCl2 ranged from 390.6 µM to 200 mM. Assays were performed in triplicate. The incubations were for 7 min at 30°C, and terminated by spotting 20 µl onto p81 filter papers. Filters were washed and quantitated as usual.

A more detailed study was done to examine the effects of CaCl2 on GST-Pim-1 activity. The same conditions were employed as above, except that a higher concentration of ATP was used (90 µM [γ-32P] ATP (1100 cpm/pmol)).

b. ATP optimizations of GST-Pim-1 kinase reactions

Kinase assays were performed to ensure that the amount of ATP used in assays was not limiting. Assays contained 1.5 µg of X. laevis or 3 µg H. sapiens GST-Pim-1, 250 µM P4 peptide, 0.5 µM PKI in 20 µl of ADB (25 mM MgCl2). Preincubations were over ice and reactions started with the addition of [γ-32P] ATP, from 0.5 µl to 10 µl, in amounts varying from 4 µM to 83 µM. Assays were carried out for 10 min at 30°C and were terminated by spotting 20 µl onto p81 filter papers. Filters were washed and quantitated as usual.

c. Time course of Pim-1 activity

A time course assay was performed to ensure that the activity of Pim-1 was linear for the assay times selected. Kinase assays contained 1.5 µg of X. laevis or 3 µg H. sapiens GST-Pim-1, 250 µM P4 peptide, 0.5 µM PKI, 120 µM [γ-32P] ATP (1100 cpm/pmol) in 29 µl of ADB (25 mM MgCl2). Preincubations were over ice and reactions at 30°C were started with the addition of [γ-32P] ATP. Reactions were terminated at discrete time
points by spotting 20 µl of reaction onto p81 filter papers. Time points tested in triplicate, starting at 30 s then one min intervals from 1-20 min, two min intervals from 20-30 min. Filter papers were washed and quantitated as usual.

vi. Antibody inhibition of GST-Pim-1 kinase activity

Kinase reactions were carried out in a total volume of 25 µl of ADB containing 25 mM MgCl₂, 0.5 µM PKI, 50 µM [γ-³²P] ATP (2000 cpm/pmol), 250 µM P4 peptide, and 0.5 µg of either Homo sapiens or Xenopus laevis GST-Pim-1 and serial dilutions (1:2.5 - 1:160) of antibodies (Pim1-III, Pim1-NT, Tel, CRB-Pim-1, Pim1-XI, GXP and a cocktail of A2 and C2). Assays were started with the addition of ATP and were allowed to proceed for 10 min at 30°C. Reactions were terminated by spotting 20 µl of reaction onto p81 filter papers. Filter papers were washed and quantitated as usual.

vii. Peptide inhibition of endogenous GST-Pim-1 activity

Autophosphorylations were carried out in 100 µl of ADB containing 10 mM MgCl₂, 25 µM [γ-³²P] ATP (6 x 10³ cpm/pmol), 25 µg of GST-Pim-1 and peptides P4 (AKRRRLSA), P7 (negative control peptide AKRRRLAA), and P15 (AKRRRLCA, constructed as an inhibitor) in concentrations varying from 0, 0.125 mM (0.5 K_m), 0.25 mM (K_m), 0.50 mM, 0.75 mM, 1.0 mM, 1.5 mM, 2.5 mM and 4.0 mM. For each reaction, constituents were mixed on ice and started at 10 s intervals with the addition of ATP. Reactions were carried out at 30°C for 20 min, and stopped by the addition of 30 µl of 2X sample buffer. Samples were boiled for 5 min and loaded onto gels, then transferred onto PVDF. Radioactive bands corresponding to the full-length fusion protein and smaller byproducts were cut out and quantitated by scintillation counting.

viii. Peptide inhibition of exogenous GST-Pim-1 activity

Reactions were carried out in 25 µl of ADB containing 10 mM MgCl₂, 1.25 mM MnCl₂, 50 µM [γ-³²P] ATP (2000 cpm/pmol), 1.5 µg of GST-Pim-1 and dilutions of substrate peptide P4 (0, 1.953, 3.906, 7.812, 15.625, 31.25, 62.5, 125, 250, 500 and 1000 µM). Inhibitor peptide P7 was used at final concentrations of 100, 200, and 500 µM, and P15 was used at final concentrations of 100 and 200 µM. An additional assay with 200 µM of P15 was carried out under reducing conditions, with 284 mM 2-ME added to prevent dimerization of the P15 peptide during the assay. For each reaction, constituents were mixed over ice and the reactions started at 10 s intervals with the addition of the ATP. Reactions were carried out at 30°C for 10 min, and stopped by spotting on to p81
filter papers. Filter papers were washed extensively and counted by scintillation counting.
5. IDENTIFICATION OF AUTOPHOSPHORYLATION SITES OF EXPRESSED PIM-1

i. In vitro autophosphorylation and tryptic digestion of fusion protein for tryptic phosphopeptide analysis

In vitro autophosphorylation of 25 µg of expressed fusion protein (immobilized on glutathione beads) was carried out in a total volume of 100 µl ADB containing 160 µM [γ-32P]ATP (100 cpm/pmol), 1.25 µM PKI, for 30 min at 30°C. Reactions were terminated by the addition of SDS-PAGE sample buffer, boiled for 5 min and were subjected to SDS-PAGE electrophoresis on a 10% acrylamide gel. The full-length radiolabelled fusion protein was detected by autoradiography and excised from the gel using a scalpel blade. Gel particles were minced, washed with water to remove methanol and SDS and partially dried under vacuum. The gel pieces were resuspended in 50 mM ammonium bicarbonate (pH 8-8.5), containing trypsin (10 µg/ml) and digested at 37°C for 24 h with constant agitation. The gel pieces were extracted several times with dH2O and 20% acetonitrile to recover the peptides, washes were combined and dried down under vacuum. The peptides were resuspended in decreasing volumes of dH2O and redried to ensure removal of all of the ammonium bicarbonate. The radioactivity of the dried peptides was quantified by scintillation counting; from this it was determined that more than 60% of the counts had been removed from the fragmented acrylamide gel.

ii. Two-dimensional phosphopeptide mapping

The dried peptides were rehydrated in a minimal volume of electrophoresis buffer and approximately 5-20 x 10^3 counts (10-100 pmol of protein) were spotted onto the center of a cellulose TLC (20x20) plate, 1.5 cm from the bottom edge. Phenol red (0.4 µl of a 0.5% solution in 5% acetic acid, 0.5% pyridine w/v/v) was spotted as a migration marker. Electrophoresis in the first dimension was performed in water/acetic acid/pyridine (89/10/1; v/v/v) at 750 V at RT, until the phenol red marker migrated 2 cm from the positive electrode. The plates were air dried and the second dimension was developed by ascending chromatography in water/butan-1-ol/pyridine/acetic acid (34/30/30/6; v/v/v/v) for approximately 1.5 h until the solvent front reached the top of the plate. After the plates were dried extensively, phosphopeptides were visualized by autoradiography O/N at -70°C.
iii. Extraction of tryptic phosphopeptides from TLC plate
Phosphopeptides contained within each visualized spot were eluted from the TLC plates by removing the cellulose matrix from the plastic support with a scalpel blade. The cellulose was extracted twice with 200 µl of 20% acetonitrile (0.1% TFA) and once with 200 µl of 60% acetonitrile. The cellulose was vortexed vigorously after the addition of the acetonitrile solutions and the sample was sonicated in a water bath sonicator for 5 min to break up the cellulose. Following centrifugation, the phosphopeptide containing supernatant was recovered and dried to a volume of about 5 µl in a vacuum centrifuge and quantitated in a scintillation counter. More than 90% of the radioactive counts were located in the supernatant. These counts were used to estimate the quantity of peptides in each sample.

iv. Phosphoamino acid analysis of tryptic phosphopeptides

*In vitro* autophosphorylation of expressed fusion protein bound to glutathione beads was carried out exactly as in Section 3.5.i. except that 100 µM of [γ-32P]ATP (10^4 cpm/pmol) was used. The radiolabelled protein was subjected to electrophoresis on an SDS-PAGE gel, isolated, trypsinized, subjected to two dimensional chromatography and excised from the TLC plate as described above.

The phosphopeptides were extracted from the cellulose twice with 200 µl of 20% acetonitrile (0.1% TFA), and once with 200 µl of 60% acetonitrile. After centrifugation, the supernatant was recovered and dried under vacuum. The samples were digested with 200 µl of boiling HCl for 1.5 h at 105°C. The digested amino acids were sequentially washed with water and dried under vacuum, then subjected to phosphoamino acid analysis as described in Section 3.3.iv.

v. HPLC analysis of tryptic phosphopeptides

a. IMAC-HPLC-ESI-MS analysis of tryptic peptides from 2D mapping
Tryptic peptides were subjected to phosphoamino acid analysis or analyzed by immobilized metal affinity chromatography (IMAC) high pressure liquid chromatography (HPLC) electrospray ionization mass spectrometry (ESI-MS). MicroIMAC-HPLC-ESI-MS was performed by Drs. Lawrence Amankwa and Michael Affolter (Biomedical Research Centre) using instrumentation and protocols as detailed elsewhere [Watts *et al.*, 1994]. In brief, the microIMAC column was assembled by manually filling a Teflon tube (6 cm long x 250 µM inner diameter, 1.59 mm outer
diameter) to about 3 cm with a 50% slurry of chelating Sepharose Fast flow (Pharmacia) in 20% ethanol, to yield a final column volume of approximately 1.5 μl. The column was connected to an injector and syringe pump and washed with water for 10 min, at a flow rate of 5 μl/min. The matrix was activated with 30 mM FeCl₃, in 5 - 5 ul injections at 1 min intervals, then washed with H₂O for 10 min, followed by 0.1 M acetic acid for 10 min. Before the initial use, the column was washed with 5 μl of elution buffer (50 mM Na₂HPO₄ in 0.1% ammonium acetate, pH 8). Samples were loaded onto the microIMAC column in volumes of less than 5 μl, and washed for 10 min in 0.1 M acetic acid, pH 3, to remove unbound phosphopeptides. Bound phosphopeptides were eluted with 5 μl of elution buffer, and applied directly to and purified by reversed phase-HPLC on a Hypersil C₁₈ capillary column (0.32 x 50 mm). After an isocratic wash with buffer A (0.05% TFA, 2% acetonitrile in H₂O) the HPLC column was eluted with a 0-50% gradient of buffer B (0.045% TFA, 80% acetonitrile in H₂O) over 15 min at a flow rate of 5 μl/min.

Samples were subjected to ESI-MS analysis by Dr. Lawrence Amankwa on a PE Sciex (Thornhill, Ontario) API III triple quadruple mass spectrometer equipped with a pneumatically assisted ESI source (ion spray). A total ion chromatogram was used to detect charged particles passing into the spectrometer. The mass to charge ratio (m/z) was scanned repetitively over the range of 300-2000.

The output data from the mass spectrometer was analyzed with computer software provided with the machine. Mass spectra were displayed for observed peaks of ion detection events and mass ranges from the data set could be extracted. Peptide masses were calculated based on computer matching of observed signals with the predicted m/z values for the various possible charge states of the same peptide and the theoretical fragmentation of the peptide sequence listing all possible fragments along with predicted charged mass values.

b. LCMS analysis of tryptic digests of X. laevis Pim-1

Two samples of expressed X. laevis GST-Pim-1 were prepared, with an estimated 4.2 mg of fusion protein bound to 5 mg of glutathione beads per sample. Two samples were prepared: one was autophosphorylated in vitro with cold ATP, the other was assumed to be autophosphorylated or phosphorylated in vivo. GST-Pim-1 was partially purified on the glutathione beads and one sample was autophosphorylated in a total volume of 10 ml in ADB buffer with 25 mM MgCl₂ and 120 μM non-radioactive ATP, for 30 min at 30°C. Both samples were washed extensively with thrombin buffer (Section 3.2.ii.) to change
the buffers and to remove unbound ATP. Both samples were digested with 10 µg of thrombin for 1h, RT. After thrombinization, the slurries were poured into columns and the flow through and elute collected. The columns were washed with 2 volumes of thrombin buffer to remove all cleaved fusion protein and the flow-through and elute concentrated in Centricon-10 tubes by sequential centrifugation and washes with 50 mM ammonium bicarbonate buffer. Once the volume of the Pim-1 was reduced to 100 µl, 10 µl of trypsin were added, and the sample allowed to digest for 24 h at 37°C, with constant agitation.

Both samples (in vitro and in vivo phosphorylated) analyzed by Dr. L. Amankwa (BRC) by LCMS. Half of each sample was dephosphorylated with HPTPB prior to LCMS analysis. Peaks that experienced a shift in retention time after phosphatase treatment, indicating the loss of a phosphate group, were identified and analyzed.
6. SOURCES OF ENDOGENOUS PIM-1 PROTEIN

i. Analysis of Pim-1 protein in X. laevis oocytes

X. laevis oocytes were obtained as described in Section 2.2.i. Oocytes were homogenized in a chilled glass homogenizer with one volume of 2x homogenization buffer (150 mM β-glycerophosphate, 40 mM MOPS, 30 mM of EGTA, 4 mM EDTA, 2 mM Na₃VO₄, 0.25 μM DTT, pH 7.2) containing SBTI. Lysates were centrifuged for 10 min at 4°C at 15000 rpm in TL-100, or for 15 min at 15000 rpm in an Eppendorf microfuge. The clear supernatant was withdrawn, avoiding both the greyish pellet and the yellow lipid layer. Additional protease inhibitors were added (aprotinin, leupeptin, SBTI (1 μg/ml each)), and the homogenate was quantitated, aliquoted and frozen until later use.

X. laevis oocyte extracts were subjected to immunoprecipitation, MonoQ column chromatography, affinity chromatography and Western blotting. These procedures were performed as described earlier.

ii. Pim-1 protein in P. ochraceus oocytes

a. Homogenization of oocytes for protein extraction

P. ochraceus oocytes were obtained as described in section 2.2.iii. Oocytes were pelleted by centrifugation at 1500 rpm for 5 min. The volume of the pellet was determined and two volumes of homogenization buffer (50 mM β-glycerophosphate, 20 mM MOPS, 5 mM EGTA, 2 mM EDTA, 1 mM Na₃VO₄ plus 0.25 μM DTT, 5.0 μM 8-methyl aspartic acid, 1.0 mM PMSF, and 1.0 mM benzamidine) were added. The oocytes were homogenized in a blender in 3-4 bursts of 15-20 s, then centrifuged at 9000 rpm for 15 min to remove particulate matter and organelles. The post-mitochondrial supernatant was centrifuged at 42000 rpm in a Sorval ultracentrifuge for 25 min. The supernatant was immediately aliquoted and frozen at -70°C until further use.

b. P. ochraceus oocyte maturation time courses

A time course of oocyte maturation was prepared, using a total of 60 μl of packed oocytes. Oocytes were washed three times with calcium-free artificial sea water (CaFSW) to remove follicle cells and the oocytes were diluted with artificial sea water to a final volume of 400 ml. At this time, the t=0 time point was removed. The maturation stimulant, 4 μM methyladenine, was added and incubated at 15°C. Aliquots of oocytes

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were removed at specific time points and pelleted by centrifugation for 5 min at 177 x g at 4°C in 50 ml tubes. The supernatant was removed and the oocytes resuspended in 1:1 homogenization buffer. The oocyte solution was sonicated for 30 - 60 s, then clarified by centrifugation for 30 min at 42000 rpm in a Sorval ultracentrifuge at 4°C. The supernatant was aliquoted and frozen for future use.

iii. Probing crude bovine spleen extract for activated Pim-1 protein

Crude bovine spleen extracts (BSE) were fractionated and analyzed for the presence of activated, endogenous Pim-1 protein. Crude extracts were prepared by homogenizing chopped bovine spleen in a blender, (1:3, (w/v)) with homogenization buffer (50 mM β-glycerophosphate, 5 mM EGTA, 2 mM EDTA, 1 mM Na₃VO₄ plus 0.1 μM DTT, 5.0 μM β-methyl aspartic acid, 1.0 mM PMSF and 1.0 mM NaF). Homogenate was filtered through cheesecloth to remove particulate matter and clarified by centrifugation at 12 000 x g for 15 min. Supernatants were centrifuged for 40 min at 42 000 rpm in a Sorval ultracentrifuge, then quantitated, aliquoted and frozen at -70°C until use.

iv. Human erythroblast cell line - K562

Human erythroleukemia cells, K562, were cultured in RPMI 1640, 10% FCS and 5x10⁴ M 2-ME by Ms. Helen Merkins (Biomedical Research Centre). Cells were centrifuged at 300 x g in a lab top centrifuge for 5 min then quantitated using a hemocytometer. Cells were resuspended at a concentration of 3x10⁷ cells per ml in ice-cold phospholysis buffer (PLB) [Telerman et al., 1988] (1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.01 M NaH₂PO₄ (pH 7.5), 0.1 M NaCl and 5 mM EDTA) plus protease inhibitors, PMSF, aprotinin and leupeptin each at 10 μg/ml. Cells were lysed on ice for 20 min then sonicated at 40% for three 5 min bursts. Cell homogenates were centrifuged in a TL-100 for 10 min at 10 000 rpm or in an Eppendorf microfuge for 10 min at 15 000 rpm. Supernatants were aliquoted (2.26 - 3.0 x10⁶ cells per aliquot) and frozen at -70°C until use.

v. Primary human lymphocytes

Human lymphocytes were produced by Dr. Bill Sahl (Pelech Laboratory) as a byproduct of platelet purifications using the method of Fotino et al. [1971]. Purified lymphocytes were obtained and quantitated using a hemocytometer and were cultured at 4 x 10⁶ cells per ml in RPMI media. The lymphocytes were divided to provide control and stimulated samples and were stimulated with 0.666 mM Concanavalin A from Canavalia ensiformis which was obtained at 4 M (1.25 g/10 ml). After stimulation for 4
h at 37°C, cells were pelleted by centrifugation at 300 x g for 10 min at RT. Lymphocytes were washed once with PBS, then resuspended in 200 μl of PLB plus protease inhibitors and incubated on ice for 15 min. Cells were sonicated at 40% with two 2 s bursts, then frozen immediately at -70°C.

7. COMPUTER SEARCH ANALYSIS

Computer analysis used a software package developed at the Biomedical Research Centre by Dr. Allen D. Delaney. Evolutionary trees were prepared using the Desoete Tree algorhythms with the PHYLIP program.

Nucleotide and amino acid sequence searches were performed at the National Center for Biotechnology Information (NCBI) using the BLAST network service. These were done by Dr. G. Kalmar.
PART 2 - RESULTS AND CONCLUSIONS: BACTERIALLY EXPRESSED PIM-1

CHAPTER IV.

CLONING AND EXPRESSION OF XENOPUS LAEVIS GST-Pim-1 AND COMPARISON TO HUMAN GST-PIM-1

1. CLONING PIM-1 FROM AN X. LAEVIS OOCYTE cDNA LIBRARY

Due to sequence conservation between pim-1 genes from different species, degenerate oligonucleotides based on the human pim-1 sequence were successfully used to amplify part of the pim-1 coding region from X. laevis cDNA. This pim-1 PCR fragment was used as a probe to screen a X. laevis oocyte cDNA library, and from 6.7 x10^5 plaques screened, three identical clones were obtained. The full-length X. laevis pim-1 cDNA clones were approximately 2.7 kb in length, similar in size to that found in other species [Zakut-Houri et al., 1987; Domen et al., 1987; Meeker et al., 1987a; Padma and Nagarajan, 1991]. The clones were initially analyzed by Southern blotting, using the X. laevis pim-1 PCR product as a probe (Fig. 4). The Southern blot demonstrated that the three clones (1.6/1.1, 6.22, 12.35) were identical and that the probe hybridized even under conditions of high stringency.

The 1348 nucleotides of the X. laevis pim-1 cDNA sequence include a predicted 969 base pair open reading frame (ORF), which specifies a 323 amino acid protein with a molecular mass of 36 970 daltons and a computed isoelectric point of 5.4998 (Fig. 5). The Pim-1 protein sequence contains all the domains and conserved residues common to all eukaryotic protein kinases, including the critical lysine in subdomain II, important for phosphotransferase activity, and the sequence V-G-S-G-F-G-T-V (residues 46-54) conforming to the [L/I/V]-G-x-G-x-[E/Y/M]-G-x-V protein kinase subdomain I region [Barioch and Claverie, 1988]. X. laevis Pim-1 also contains a serine/threonine kinase-specific signature pattern, V-V-H-R-D-I-K-D-E-N-L (residues 164-175) conforming to the [L/I/V]-G-x-G-x-[E/Y/M]-G-x-V protein kinase subdomain I region [Barioch and Claverie, 1988]. X. laevis Pim-1 also contains a serine/threonine kinase-specific signature pattern, V-V-H-R-D-I-K-D-E-N-L (residues 164-175) conforming to the [L/I/V]-G-x-G-x-[E/Y/M]-G-x-V protein kinase subdomain I region [Barioch and Claverie, 1988].
Figure 4. Southern blot of X. laevis cDNA clones probed with Pim-1 PCR fragment. Clones isolated from the X. laevis cDNA library were digested with Acc1 and Nco1 (lanes 2, 5, 8, 11, 14), HindIII and PvuII (lanes 3, 6, 9, 12, 15) or left undigested (lanes 1, 4, 7, 10, 13). Clone 1.6, lanes 1-3; clone 1.1, lanes 4-6; clone 6.22, lanes 7-9; clone 12.35, lanes 10-12; human pim-1 in pGEX-2T (negative control), lanes 13-15. Panel A, an ethidium bromide stained agarose gel of digested plamids. The agarose gel was Southern blot and probed with a radio-labelled X. laevis pim-1 probe. Panel B is an autoradiograph of the Southern blot. Standards are shown on the left, estimated size of radioactive bands on the right. Clones 1.6 and 1.1 are duplicates.
Figure 5. Nucleotide and amino acid sequence of coding region of X. laevis pim-1.
The amino acid sequence of the coding region is shown above nucleotides, numbered
in bold type. The nucleotides sequenced are numbered starting at "1" for the first
residue of the start codon. Oligonucleotides used to amplify pim-1 from X. laevis
cDNA were based on regions underlined, arrows indicate sense (>) and anti-sense
(<) primers.
1988]. Even though the sequence Y-N-C-G-V-V-H (residues 160-167) conforms to the consensus pattern [F/Y]-x-C-x-[V/A]-x-H, it is not clear if Pim-1 dimerizes or forms intramolecular disulfide bonds. *X. laevis* Pim-1 also contains a coiled hydrophobic region near the 3' end of the protein, from residues Leu282-Asp301, that is conserved in Pim-1 from all species. Other interesting regions that were identified in Pim-1 include a potential N-glycosylation site, (N-x-[S/T]-x at residues 311-313) [Grand, 1989], a potential cAMP-dependent protein kinase site ([R/K]-[R/K]-x-[S/T] at residues 258-262) [Kennelly and Krebs, 1991], 4 potential protein kinase C phosphorylation sites ([S/T]-x-[R/K] at residues 135, 144, 273 and 277) [Kennelly and Krebs, 1991], a potential casein kinase II phosphorylation site ([S/T]-x-x-[D/E] starting at residue 200) [Pinna, 1990], a potential tyrosine kinase phosphorylation site ([K/R]-x-x-x-[D/E]-x-x-Y at residues 33-41), and 5 myristoylation sites (G-[E/D/R/K/H/P/F/Y/W]-x-x-[S/T/A/G/C/N]-P) starting at residues 33, 43, 49, 52, 63 and 80). The physiological relevance of these sites is undetermined.

*X. laevis* Pim-1 shares a high degree of sequence similarity to the mammalian Pim-1 counterparts, especially within the catalytic region (Fig. 6). *X. laevis* Pim-1 amino acid sequence has 86% overall similarity and 65% overall identity with human Pim-1. While *X. laevis* Pim-1 N-terminus shares 69% similarity and 49% identity with human Pim-1, *X. laevis* Pim-1 C-terminus shares only 55% similarity and 15% identity with the human cognate. The C-terminus of the amphibian Pim-1 is eight amino acids longer than the mammalian Pim-1 proteins and contains a high number of serine residues. The catalytic domain of *X. laevis pim-1* shares 71% nucleotide sequence similarity with the other *pim-1* sequences.
Figure 6. Protein sequence alignment of Pim-1 from X. laevis, mouse, rat and human. Stars (*) indicate residues identical and dots (.) indicate residues that are conserved between the four species. Roman numerals indicate protein kinase subdomains. Residues that are identical between all protein kinases are shown in bold type.
2. COMPARISON OF THE X. LAEVIS PIM-1 SEQUENCE WITH OTHER PROTEINS

As sequence similarities between proteins may indicate evolutionary relationships and provide clues about domain function or protein-protein interactions, sequence searches were performed to identify other proteins sharing homology with X. laevis Pim-1. The searches may also help to define important regulatory regions in Pim-1 based on strong conservation (e.g. phosphorylation sites). Most proteins that resulted from the search, including the other Pim-1 homologues, were serine/threonine protein kinases and displayed homology to Pim-1 in the catalytic domain. All proteins are listed with their accession number in boxed brackets.

i. Nucleotide sequence searches

Nucleotide sequence comparisons were performed by NCBI using BLAST programs to identify nucleic acid sequences sharing homology with the sequence of X. laevis pim-1. All regions of homology (except with the other pim-1 homologues) were located in the kinase catalytic domain (nt 259-873). As expected, the nucleotide search identified other pim-1 homologues including human pim-1 sequences [M16750, M27903, M54915], Rattus norvegicus (rat) pim-1 [X63675] and Mus musculus (mouse) pim-1 [M13945]. X. laevis pim-1 was 70-72% identical to pim-1 from other species over a 615 nucleotide region in the catalytic domain.

Several sequences for calcium/calmodulin-dependent protein kinases were identified as being similar to pim-1 including rat calcium/calmodulin dependent kinase type II alpha subunit [JO2942], murine calmodulin kinase type II [X14836], murine calcium/calmodulin dependent kinase type II beta subunit [X63615], and Zea mays calcium-dependent protein kinase [L27484]. These sequences were 61-69% identical to X. laevis pim-1 in kinase catalytic subdomain domain VI. The rabbit gamma-subunit of phosphorylase kinase [Y00684] was identified as having 56% nucleotide sequence homology with X. laevis pim-1 in kinase catalytic subdomains V-VI.

ii. Protein sequence searches

Protein sequence comparisons were done by NCBI using BLAST programs to identify protein sequences that had some homology to the X. laevis Pim-1. Most regions of homology (except with the other Pim-1 homologues) were located within in the kinase catalytic domain (aa 46-291). Apart from the other Pim-1 cognates, the sequence search did
not identify any kinases that were strongly related to \textit{X. laevis} Pim-1. The kinases identified as having the highest homology to Pim-1 by the sequence search are summarized in Table 1 along with the domains and identities.

All proteins displaying homology to \textit{X. laevis} Pim-1 are protein-serine/threonine kinases. Most regions of homology were located in domains V-XI, with very few kinases sharing homology to Pim-1 in domains III and IV. Pim-1 has some homology to the calcium/calmodulin-dependent kinases, SNF1-related serine/threonine protein kinases (carbon catabolite derepressing protein kinase) as well as other signal translation kinases such as the β-adrenergic receptor kinases and G protein-coupled receptor kinases. The protein homology search identified many members from these groups from a diverse range of species. As only the proteins with the highest homology were tabulated, the lesser related β-adrenergic receptor kinases and G protein-coupled kinases were not listed.

Novel and interesting protein kinases identified by the search include p78, a human putative serine/threonine protein kinase [P27448], murine tsk-1 serine/threonine protein kinase [S31333] [Bielke et al., 1994], KKIALRE, human cdc-2-related serine/threonine protein kinase [Q00532, S22745] [Meyerson et al., 1992], and mouse ribosomal protein S6 kinase II [P18653] [Alcorta et al., 1989]. Pim-1 also had homology to the 35.1 kDa serine/threonine protein kinase from the African swine fever virus, a relationship reported previously [Baylis et al., 1993].
TABLE 1.  Kinases sharing homology with X. laevis Pim-1.
The name of the kinase (if known) as well as the species is listed. Identity reflects the percentage of identical residues in the domains indicated and similarity reflects the percentage of conserved substitutions plus identical residues in the domains indicated. Note that only a portion of a domain listed may be represented by the homology search.

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<th>Similarity (%)</th>
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### TABLE 1. KinasessharinghomologywithX. laevis Pim-1...continued.

C. Calcium/calmodulin-dependent protein kinases

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| **E. nidulans** [JN0323]    |                | IV   | 36 | 68 |
|                             | V-VI           | 44   | 62 |    |
|                             | IX-X          | 31   | 53 |    |
|                             | XI            | 34   | 56 |    |

|                                | IX-X    | 38 | 61 |
|                                | XI      | 37 | 56 |

| type I                      | **S. cerevisiae** [P27466] | I-II | 35 | 50 |
|                            |                             | IV   | 31 | 57 |
|                            |                             | V-VI | 37 | 62 |
|                            |                             | VII-IX | 40 | 70 |
|                            |                             | XI   | 35 | 59 |

| type II                     | **S. cerevisiae** [P27466] | I-II | 34 | 50 |
|                            |                             | IV   | 31 | 57 |
|                            |                             | V-VI | 37 | 62 |
|                            |                             | IX   | 33 | 59 |
|                            |                             | XI   | 35 | 59 |

| type II                     | **S. cerevisiae** [X65797] | V-VI | 40 | 57 |
|                            |                             | IX   | 41 | 66 |
|                            |                             | XI   | 40 | 62 |

| type II                     | **S. cerevisiae** [P22517] | I-II | 28 | 54 |
|                            |                             | IV   | 36 | 63 |
|                            |                             | V-VI | 42 | 59 |
|                            |                             | IX   | 41 | 66 |
|                            |                             | XI   | 40 | 62 |

| Cam kinase II              | **S. cerevisiae** [B40896] | I-II | 28 | 54 |
|                            |                             | IV   | 36 | 63 |
|                            |                             | V-VI | 42 | 59 |
|                            |                             | IX   | 41 | 66 |
|                            |                             | XI   | 40 | 62 |

<p>| Sch9                       | <strong>S. cerevisiae</strong> [P11792] | V-X  | 32 | 48 |
|                            |                             |      |    |    |</p>
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iii. Pim-1 homology tree

A Pim-1 homology tree was constructed using PHYLIP [Felsenstein, 1993] to demonstrate the relationship of the *X. laevis* Pim-1 to related proteins. A protein sequence search was performed using genetic data environment (GDE), then *X. laevis* Pim-1 was aligned to the 40 closest relatives using clustal. Because of limitations of the program, only short regions of homology could be compared so the less related regions (the divergent N- and C-termini) had to be deleted to allow the alignments to be plotted on the tree. GDE uses the Desoete Tree Fit algorithm to determine the relationship of the proteins to each other. The protein alignment is shown in Figure 7, and displays catalytic domains VI-XI of Pim-1 (amino acids 151-294) aligned with the homologous regions of other proteins.

PHYLIP was used to build a tree from the species alignments; species were assessed in the order at which they appeared in the input file and as each additional species was added, the best tree was selected from all possible tree combinations. Local rearrangements occurred and if a better tree was made, the new rearrangement was accepted, guaranteeing that the best possible tree was constructed. With this unrooted, additive tree model the genetic distances are expected to equal the sums of vertical branch lengths between the species, with the tree "growing" from the left to the right.

The Pim-1 relatedness tree is shown in Figure 8, with a list of the names and accession numbers of the kinases shown in Table 2. The *X. laevis* Pim-1 is located near the far right of the tree. Its closest relatives are, not surprisingly, the human, murine and rat Pim-1 sequences. The mouse and rat Pim-1 are most closely related to each other, then with the human Pim-1. The *X. laevis* Pim-1 is somewhat divergent from these other three species. The Pim-1 proteins do not have many close relatives, except the kinase encoded by cosmid c06E8 of *C. elegans* (celc06e8). At this time the function is of this kinase is unknown.

The next closely related protein to the Pim-1 family is a SNF1 homologue from wheat (a53467) followed by murine tsk-1 testes-specific serine/threonine kinase (u01840), human p78 serine/threonine kinase that is lost during chemically-induced pancreatic tumors (kp78_human), and calcium/calmodulin-dependent protein kinase from *E. nidulans* (jn0323). The murine, human and *E. nidulans* proteins were identified in the protein sequence search in the previous section.

The remainder of the proteins shown in Figure 8 all share a low amount of relatedness to the *X. laevis* Pim-1 but high relatedness to each other. Many of these proteins are
Ca⁺/calmodulin-dependent protein kinases including the rat IV β (S65840) and II α subunits from cerebellum (kcc4_rat), mouse IV catalytic chain (kcc4_mouse), human (a53036) and rat lung Ca⁺/calmodulin-dependent protein kinase (ratcampkaa) as well as the phosphorylase β kinase gamma catalytic chain from human (kbph_human) and rat (kbph_rat). Members of a second group of calcium/calmodulin-dependent protein kinases that are related to each other include the type IV beta chains from mouse (kccb_mouse), rat (kccb_rat) and X. laevis (xlu06636), the type II alpha chain from Drosophila (ju0270), mouse (kcca_mouse) and rat (kcca_rat).

The next group of kinases include uncharacterized protein kinases from barley (S24578, S24579) and homologues to SNF1, carbon catabolite derepressing protein kinase necessary for the release from glucose repression, from rye (rki1_secce), A. thaliana (jc1446), tobacco (tobpki) and rat (rnampapk). Two additional proteins shown are the yeast proteins, Ca⁺/calmodulin-dependent protein kinase (kcc4_yeast) and probable serine/threonine kinase YKL101W (kkkl_yeast), both previously identified in the protein homology search.

One difficulty with this program is that the limits of divergence in the homology regions allow only the catalytic regions with the highest homology to be assessed. The program is dependent on the order that the species are presented into the input file, so to obtain the most accurate tree, a 'jumble' option must be used to randomly input the species. Because the alignment was performed by computer using a mathematical program, the program does not always select the most obvious residues to align. In addition, this program does not know which changes have happened in the past (i.e. bases changing and then changing back) so cannot predict how the sequences evolved or where they diverged from a common ancestor. This gives the results from this analysis limited statistical significance. Only a few proteins were common to both protein sequence searches; both searches limited to the number of proteins to be identified (100 for the previous search, 30 for this search) as there are many proteins which share this low level of homology with Pim-1.

Most of the proteins on the homology tree are more closely related to each other than to the Pim-1 proteins. As all the proteins plotted on the tree are serine/threonine kinases either involved in cellular metabolism or cell cycle regulation, it is likely that Pim-1 is involved in one of these processes.
Figure 7.  **Protein sequence alignments of X. laevis Pim-1 with homologous proteins.**

This alignment was used to construct the homology tree (Fig. 8). Roman numerals indicate protein kinase catalytic subdomains.

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<td>jn0323</td>
<td>KEVDL WSGVFLFAAL LGLRPLPFEQ NNGVÒBLDLK VQKSSQEM-- PNDTISRSAR DQLIGKDLV DSQQKIRKRD LISLHPWMC*</td>
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<td></td>
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</table>
Figure 8. Homology tree of Pim-1 with related kinases.

*X. laevis* Pim-1 is located to the top right of the tree, with the other Pim-1 sequences including mouse, rat and human. The full names and accession numbers of the other kinases shown are listed in Table 2.
Table 2. Names and Accession numbers of kinases listed in the Pim-1 relatedness tree.
 *= proteins also identified in protein sequence search, Section 2.ii.

<table>
<thead>
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<th>Name of kinase</th>
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<tr>
<td>kpim_xenla</td>
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<tr>
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</tr>
<tr>
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</tr>
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<td>rat Pim-1</td>
</tr>
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<td>C. elegans. cosmid c06E8</td>
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<td>a53467</td>
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<td>u01840</td>
<td>mouse tsk-1 testes-specific serine/threonine kinase</td>
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<td>E. nidulans Ca+/calmodulin-dependent protein kinase</td>
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<td>yeast Ca+/calmodulin-dependent protein kinase</td>
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<tr>
<td>kkk1_yeast</td>
<td>yeast probable serine/threonine kinase</td>
</tr>
</tbody>
</table>
3. BACTERIAL EXPRESSION OF PIM-1 AS A GST-FUSION PROTEIN

The pGEX plasmids allow recombinant genes to be expressed at high intracellular levels in bacteria as fusion proteins with the *Schistosoma japonicum* glutathione S-transferase. The expression is chemically inducible; the fusion protein is under the control of the lac promoter which is induced with the lactose analog isopropyl β-D-thiogalactoside (IPTG). The expressed fusion protein is easily purified from bacterial lysates under non-denaturing conditions by an affinity interaction with glutathione covalently attached to Sepharose 4B and can be eluted from the affinity matrix with glutathione. A thrombin digestion site is encoded between the GST and the fusion protein to allow cleavage of the purified protein from the GST.

i. **Expression of human Pim-1 as a bacterial GST-fusion protein**

The human *pim-1* coding region was subcloned into the pGEX-2T expression vector using non-degenerate oligonucleotides based on the published nucleotide sequence of human *pim-1*. The polymerase chain reaction was optimized to discourage the amplification of unwanted byproducts, by employing stringent annealing conditions. VENT polymerase was used in the PCR reaction because its high accuracy and proof-reading function were essential to maintain the sequence integrity of the PCR product. The expression of the predicted 62 kDa recombinant fusion-protein was confirmed by Western blotting with several different Pim-1 antibodies as well as with an anti-GST antibody (Fig. 9). While most of the GST-Pim-1 existed as a 62 kDa fusion protein, lower molecular mass species between 28 and 40 kDa were also detectable on Western blots with several of the antibodies, even in the presence of protease inhibitors. These bands may have arisen from specific proteolytic cleavage or aborted translation of the protein. While all antibodies immunodetected the full-length fusion protein, Pim1-NT detected 30, 33 and 38 kDa bands while Pim1-III detected 38 and 40 kDa bands and the GST antibody detected a smear from 28-32 and 38 kDa bands. The *X. laevis* antibodies did not detect the full-length human fusion protein. Tel, Lilly cocktail and CRB antibodies strongly detected the full-length fusion protein and CRB and 4G10 also detected a 32 kDa band. Neither of the anti-phosphotyrosine antibodies detected the full-length fusion protein. As all three antibodies directed against regions in the N-terminus detect lower Mr proteins, the lower molecular mass forms are possibly the result of aborted translation.

The human GST-Pim-1 preparation was catalytically active and autophosphorylated in *vitro*. In an autophosphorylation assay, the two lower molecular mass forms (32 and 38
kDa) were phosphorylated in addition to the 62 kDa band predicted to be the full-length GST-Pim-1 (Fig. 9, lane 12). These bands correspond with the three main immunoreactive bands detected by the Pim-1 antibodies. It is uncertain if all the species are catalytically active, or if the observed phosphorylation is a result of cross-phosphorylation by one active protein. Attempts to resolve these Pim-1 species by various forms of column chromatography (e.g. MonoQ and Superose) were unsuccessful.

The expression of GST-Pim-1 was compared between two different E. coli strains, DH5α and UT5600, to determine if a higher proportion of the fusion protein would be produced in the UT5600 protease deficient strain (Fig. 10). Although protein expression was significantly higher in the protease-deficient UT5600 strain, the amounts of the full-length fusion protein relative to the smaller mass bands remained the same. The UT5600 E.coli strain was used for the remainder of the expression experiments.

Phosphoamino acid analysis of human GST-Pim-1 was performed in order to determine the nature of the residues that were phosphorylated in an in vitro autophosphorylation reaction (Fig. 11, Panel A). Although the majority of phosphorylation was on threonine residues, there was also phosphorylation on serine and tyrosine residues as well. The phosphorylation on tyrosine was unexpected, as antiphosphotyrosine antibodies did not detect the full-length human GST-Pim-1 by Western blotting (Fig. 9, lanes 9, 10) and recent studies have reported that the Pim-1 autophosphorylated strictly on serine and threonine residues [Saris et al., 1991; Hoover et al. 1991; Padma and Nagarajan, 1991; Friedmann et al., 1992]. This tyrosine autophosphorylation activity of GST-Pim-1 will be examined further in Chapter VI.

By comparing the relative amounts of the full-length fusion protein with BSA standards on an SDS-PAGE gel, the percentage of full-length human fusion protein in the glutathione affinity-purified Pim-1 preparation relative to the total protein concentration was estimated to be 1.8%. As this quantity was only an estimate, and because we were unable to determine if the lower species were catalytically active, the protein concentration of the entire sample was used for all experiments unless otherwise indicated.
Western blot, silver stain and autoradiograph of *H. sapiens* GST-Pim-1. Western blots (lanes 1-10) of 0.75 ug of GST-Pim-1 expressed in DH5α probed with various antibodies: lane 1, anti-GST; lane 2, Pim1-NT; lane 3, Pim1-III; lane 4, GXP; lane 5, Pim1-XI; lane 6, Tel; lane 7, Lilly; lane 8, CRB; lane 9, 4G10; lane 10, PY20. Lane 11, silver stain of 0.75 ug of *H. sapiens* GST-Pim-1. Lane 12, autoradiogram of 0.75 ug of autophosphorylated GST-Pim-1. Migrations of Mr standards are shown at right, and arrow indicates the mobility of GST-Pim-1.
Figure 10. Expression of human GST-Pim-1 in DH5α and UT5600 _E. coli_ strains.

Approximately 5 µl of affinity purified GST-Pim-1 expressed in DH5α (lanes 1 and 3) and UT5600 (lanes 2 and 4) strains of _E. coli_ were electroluted and silver stained (lanes 1 and 2) or Western blotted with Pim1-NT antibody (lanes 3 and 4).
Figure 11. Phosphoamino acid analysis of bacterially-expressed GST-Pim-1. 

*H. sapiens* and *X. laevis* GST-Pim-1 were auto-phosphorylated *in vitro* and electroluted on an SDS-PAGE gel. The radiolabelled band corresponding to the full-length fusion protein was excised and subjected to phosphoamino acid analysis. Standards were visualized by ninhydrin. The autoradiogram is shown at right with the location of the standard phosphoamino acids shown.
a. Attempted separation of different Pim-1 fragments by column chromatography

To distinguish the activity of the full-length fusion protein from the smaller products, attempts were made to separate the different species by MonoQ column chromatography and by Superose gel exclusion. Uncleaved GST-Pim-1 was loaded onto these columns and eluted by the standard protocols, and the fractions analyzed by peptide substrate assays using the P4 peptide (AKRRRLSA), in vitro autophosphorylation assays and by Western blotting. These experiments indicated that the three main bands present in expressed human GST-Pim-1 preparations were not resolvable by the column chromatography methods attempted.

b. Thrombin cleavage of human GST-Pim-1

Thrombin treatment of the GST-Pim-1 fusion protein was done in order to isolate the Pim-1 portion. Although there was a thrombin-cleavage site located at the GST-Pim-1 junction, cleavage with this enzyme reduced the activity of the kinase. This is likely due to thermolability; GST-Pim-1 is stable and retains most of its activity after a 25 min incubation at 30°C, but begins to lose activity after 35 min at 30°C. Reducing the time of incubation from 60 min, reduced the efficiency of thrombinization. A large proportion of the cleaved product remained bound to beads, and the methods used to separate the thrombin from the cleaved product led to a further reduction in activity. Because of the inefficiency of the thrombinization reaction and the low activity of the end product, the uncleaved fusion protein was utilized in subsequent studies to obtain maximal activity.

ii. Expression of X. laevis Pim-1 as a bacterial fusion protein

X. laevis Pim-1 was subcloned into the pGEX-2T vector and expressed in UT5600 protease-deficient bacteria. Analysis by Western blotting with X. laevis-specific Pim-1 antibodies as well as the anti-GST antibody demonstrated that the expected 549 amino acid, 63.3 kDa fusion protein was produced (Fig. 12). Unlike the expression of human GST-Pim-1 fusion protein, expression of X. laevis GST-Pim-1 yielded a relatively pure preparation with few degradation products. GXP antibody detects a plethora of proteins that may have also been present in the original immunizing preparation. Pim1-XI detects only the full-length protein, indicating that the lower mwt. bands observed in lane 2 are unlikely to be due to Pim-1 degradation or production of alternative Pim-1 products. The X. laevis GST-Pim-1 is immunodetected by both antiphosphotyrosine antibodies as well as by some of the human Pim-1 antibodies, most notably the Tel antibody (lane 6).
Figure 12. Bacterially-expressed *X. laevis* GST-Pim-1. Western blot, silver stain and autoradiograph of *X. laevis* GST-Pim-1. Western blots (lanes 1-10) of 0.75 µg of GST-Pim-1 expressed in DH5α cells probed with various antibodies: lane 1, anti-GST; lane 2, GXP; lane 3, Pim1-XI; lane 4, Pim1-NT; lane 5, Pim1-III; lane 6, Tel; lane 7, Lilly; lane 8, CRB; lane 9, 4G10; lane 10, PY20. Lane 11, silver stain of 0.75 µg of *X. laevis* GST-Pim-1. Lane 12, autoradiogram of 0.75 µg of autophosphorylated GST-Pim-1. Migrations of Mr standards are shown at right, arrow indicates the mobility of GST-Pim-1.
The full-length 62 kDa fusion protein was the major species radiolabelled in an autophosphorylation reaction of *X. laevis* GST-Pim-1 (Fig. 12, lane 12). A very faint 45 kDa doublet was also visible, along with an even fainter band of about 15 kDa. Phosphoamino acid analysis revealed that like the human protein, the *X. laevis* GST-Pim-1 autophosphorylates on serine and threonine as well as on tyrosine residues (Fig. 11). Interestingly, the proportion of radiolabel incorporated by each residue differs between the two enzymes.

The *X. laevis* GST-Pim-1 protein was treated with thrombin to cleave the GST portion away from the Pim-1. The kinase activity of the thrombin-treated Pim-1 was reduced to 2.5% of the uncleaved control (data not shown). Staining of the glutathione-agarose immobilized sample revealed that although the thrombinization reaction was quite efficient, again most of the cleaved product adhered to the beads. Because of low activity and low protein recovery, the full-length GST-Pim-1 was used for all further experiments.

### iii. Expression of a kinase-dead mutant of *X. laevis* GST-Pim-1

A kinase-dead (KD) mutant of *X. laevis* GST-Pim-1 was created as a negative control for peptide substrate assays and to ensure that the GST portion of the fusion protein and other contaminating or co-purifying proteins did not contribute to kinase activity. The KD Pim-1 mutant was created by changing Lys-69, a residue required for ATP binding, to an alanine residue (for another example of where this has been done, refer to Taylor, 1989). Although lysine has a significantly larger side chain, the substitution of alanine for lysine is unlikely to cause a major conformational change in the protein, nor should it interfere with the recognition sites for other substrates. The residue was altered by PCR site-directed mutagenesis using the wild-type (WT) *X. laevis* GST-Pim-1 as a template, and was expressed and purified in the same manner as for WT GST-Pim-1. Western blot analysis with *X. laevis* Pim-1 antibodies revealed that an approximately 63 kDa protein was produced (Fig. 13).

The KD mutant was not radiolabelled in an *in vitro* autophosphorylation reaction, even though other radiolabelled proteins were observed in the preparation (Fig. 13, lane 8). A 16 hr exposure is shown in Figure 13. However, no labeling of the KD GST-Pim-1 was observed even after the autoradiogram was exposed for 2 weeks (data not shown). A radiolabelled 70 kDa protein was present in the KD preparation; as this protein was also detected by GXP antibody, it was probably part of the original immunizing preparation. This protein was possibly the product of the bacterial dnaK gene, a close homologue of the
eukaryotic HSP family of proteins [Craig and Gross, 1991]. This protein, sometimes called chaperonin, is a major component of normally growing cells, binds to proteins and is thought to be important for protein motility within the cell [Craig and Gross, 1991; Leustek et al., 1992; Yu-Sherman and Goldberg, 1992]. The expression of the 70 kDa protein increases in response to stress and may bind preferentially to abnormal (expressed) proteins along with other HSP (grpE, La) enhancing susceptibility to cellular proteases [Craig and Gross, 1991; Leustek et al., 1992; Yu-Sherman and Goldberg, 1992]. Interestingly, the dnaK protein binds ATP with high affinity and possesses a weak ATPase activity [Leustek et al., 1992; Yu-Sherman and Goldberg, 1992]. This could account for the radioactive labeling of the band in an autophosphorylating assay. Although the dnaK protein can be disassociated from the bacterial kinase by performing what is basically a kinase reaction [Yu-Sherman and Goldberg, 1992], this procedure has been tested and found to be essentially ineffective [Dr. U. Dekkart, BRC]. Although this protein possessed autokinase activity, it did not phosphorylate the KD GST-Pim-1.

In summary, the KD GST-Pim-1 had no exogenous or auto kinase activity and although the preparation was contaminated by a 70 kDa protein with autokinase activity, there were no kinases present that phosphorylated the KD mutant. Therefore, it can be concluded that the radio-labeling of the WT GST-Pim-1 was due solely to autophosphorylation. It is likely that the tyrosine phosphorylation of X. laevis GST-Pim-1 that was observed is the result of an autophosphorylation event. This suggests that Pim-1 possesses tyrosine autophosphotransferase activity in addition to serine and threonine autophosphotransferase activity.
Figure 13. Comparison of WT and KD X. laevis GST-Pim-1. 
Bacterially-expressed, purified and autophosphorylated GST-Pim-1 WT (lanes 1, 3, 5, 7) and KD (lanes 2, 4, 6, 8) Western blotted with GXP (lanes 1 and 2) and 4G10 antibody (lanes 3 and 4). Amido black stain of 2 ug of protein shown in lanes 5 and 6. Lanes 7 and 8 show autoradiograph of lanes 5 and 6. Full-length 63 kDa GST-Pim-1 fusion protein indicated by solid arrow (►), 70 kDa DNAK protein indicated by hollow arrow (←). Migration of Mr standards are shown at right.
4. **STABILITY OF GST-PIM-1 ENZYME**

Time courses of autophosphorylation and substrate phosphorylation of both human and *X. laevis* GST-Pim-1 were performed to determine how stable GST-Pim-1 was at 30°C. Preincubation at 30°C only slightly reduced the exogenous kinase activity of human and *X. laevis* GST-Pim-1; the kinase activities were constant for the first 20-25 min and began to drop off slowly at 30 min (data not shown). The autophosphorylation activity of the two Pim-1 enzymes did not seem to vary much after preincubation at 30°C. The specific activity determinations demonstrated that the auto-phosphorylation activity of GST-Pim-1 at 30°C was linear for 25 min, suggesting that preincubation at 30°C for 20 min was unlikely to affect activity.

5. **AUTOPHOSPHORYLATION ACTIVITY OF GST-PIM-1**

Specific activity of autophosphorylation of the bacterially-expressed GST-Pim-1 preparations was quantitated. The specific activity of *X. laevis* GST-Pim-1 autophosphorylation was 122 pmol.mg⁻¹.min⁻¹, and the specific activity for the autophosphorylation of human GST-Pim-1 was calculated to be 10 pmol.mg⁻¹.min⁻¹ (Fig. 14). This calculation takes into account the total concentration of the human GST-Pim-1 preparation as it is unclear which Pim-1 species in the preparation were catalytically-active. Reaction rates were linear for 20 min, but started to drop by 25 min for all concentrations of samples. As this rate reduction was observed even in the samples with the lowest concentrations of protein, this reduction in reaction rate was unlikely due to saturation. Stoichiometry of autophosphorylation after 20 min was determined to be 0.15 mmoles ATP incorporated per mole of *X. laevis* GST-Pim-1 fusion protein, and 0.013 mmoles ATP incorporated per mole of human GST-Pim-1 fusion protein. These findings implied that GST-Pim-1 poorly autophosphorylated *in vitro*, or that it was substantially autophosphorylated in the *E. coli* prior to cell lysis.
Figure 14. Specific activity of autophosphorylation of *H. sapiens* and *X. laevis* GST-Pim-1.

The specific activity of autophosphorylation was determined for both *H. sapiens* and *X. laevis* GST-Pim-1. Panel A - specific activity of *H. sapiens* GST-Pim-1. Panel B - specific activity of *X. laevis* GST-Pim-1. The pmol of $^32$P incorporated per mg of GST-Pim-1 was calculated and plotted against the time of autophosphorylation assay. The slope of the graph equals the specific activity and is shown on the graph.
6. GENERAL CHARACTERIZATION OF BACTERIALLY-EXPRESSED GST-PIM-1

i. Time course of activity

A time course study was performed to ensure that the exogenous activity of GST-Pim-1 towards a synthetic peptide substrate, AKRRRLSA (see later for criteria for selection of this peptide), was linear for the time selected in most standard assays. These experiments demonstrated that the exogenous kinase assay of human GST-Pim-1 was linear between 5 and 10 min, indicating that (a) the enzyme retained full activity during this time and (b) none of the assay constituents were limiting. For this reason, assays with human GST-Pim-1 were performed for 5-10 min. Preliminary results indicated that the X. laevis GST-Pim-1 reaction was linear for only about 3.5 min, then decreased rapidly because of substrate saturation. To optimize the assays the amount of enzyme was reduced from 3.75 ug to 0.5 ug per assay, and when the time course was repeated the reaction was linear with time for at least 10 min (data not shown).

ii. Divalent cation requirement (Mg, Mn, Ca, Zn)

The divalent metal cation requirement of GST-Pim-1 for phosphotransferase activity was examined (Fig. 15a). The phosphotransferase activity of the human GST-Pim-1 towards the peptide substrate AKRRRLSA was optimally stimulated by 1.25 mM MnCl₂ or 10 mM MgCl₂, so these metal ions were included at these concentrations in subsequent experiments. These concentrations agree with the previously reported optimal values of 2 mM MnCl₂ and 10 mM MgCl₂ for GST-Pim-1 [Hoover et al., 1991], but differ slightly with the corresponding value of 5 mM MnCl₂ described by Friedmann et al. [1992]. Zinc and calcium both inhibited GST-Pim-1 kinase activity at concentrations higher than 0.6 mM. All ions were tested to a final concentration of 250 mM, but as concentrations over 100 mM had an effect comparable to 50 mM, values were only shown for up to 50 mM of ions.

The phosphotransferase activity of the X. laevis GST-Pim-1 towards the peptide substrate P4 was optimally stimulated by 1.5 mM MnCl₂ or 25 mM MgCl₂, so these metal ions were included at these concentrations in subsequent experiments (Fig. 15b). These concentrations differ slightly from the values obtained for human GST-Pim-1, possibly because of enzyme quantities in the reaction. There was a slight stimulation of Pim-1 activity with 10 uM calcium, but at higher concentrations zinc and calcium both inhibited GST-Pim-1 kinase activity. Sodium chloride was inhibitory at concentrations over 250 mM.
Figure 15. Cations required for optimal GST-Pim-1 phosphotransferase activity. Peptide assays using the P4 peptide as a substrate were performed in the presence of varying cation concentrations. Cations tested: - - Ca²⁺, - - Mg²⁺, ▲ Na⁺, - - Mn²⁺, - - Zn²⁺. Panel A: results with X. laevis GST-Pim-1, panel B: results with H. sapiens GST-Pim-1.
iii.  $K_m$ of ATP

The amount of ATP used per reaction was assessed using the equation described in Appendix V. The apparent $K_m$ of Pim-1 for ATP was calculated using Lineweaver and Burke plots, as described in Appendix VI. The $K_m$ for ATP of human GST-Pim-1 was determined to be 14 µM ATP in the reaction. As enzyme reactions should optimally contain concentrations of substrate at least three-fold higher than their $K_m$ values, the standard human Pim-1 assays normally contained 50 µM of ATP. The $K_m$ of *X. laevis* GST-Pim-1 for ATP was determined to be 154 µM, so a final concentration of 450 µM was used for all *X. laevis* GST-Pim-1 assays.

iv.  Protein kinase inhibitors

The sensitivity of Pim-1 to a peptide inhibitor (PKI) of cAMP-dependent protein kinase was tested, because of the high amount of sequence homology between Pim-1 and second messenger-dependent protein kinases [Cheng et al., 1985]. The activity of human and frog GST-Pim-1 was independent of the amount of PKI in the reaction, indicating that PKI could be added to assays to eliminate the possibility that the phosphotransferase activity observed was due to cAMP-dependent protein kinase. This was especially important, in retrospect, when the Pim-1 substrate peptides were found to fulfill the consensus sequence requirements of cAMP-dependent protein kinases.
7. SUMMARY OF GST-PIM-1 EXPRESSION

An amphibian pim-1 homologue was cloned from a X. laevis cDNA library. The kinase contained all the conserved residues in catalytic subdomains common to serine/threonine protein kinases, and featured a consensus pattern for a disulfide bond-forming cysteine, and consensus phosphorylation site motifs for cAMP-dependent kinases, PKC, CKII, and a tyrosine kinase. Nucleotide and amino acid searches revealed that Pim-1 had homology to the catalytic domain of serine/threonine kinases, especially Ca²⁺/calmodulin-dependent protein kinases and SNF-1 homologues. The Pim-1 homology tree indicated that the X. laevis kinase was most homologous to the Pim-1 from mammalian species.

The human and X. laevis Pim-1 were subcloned into the pGEX-2T bacterial expression vectors and expressed as GST-Pim-1 fusion proteins. Full-length proteins were produced in both cases, and several smaller human Pim-1 byproducts were produced, possibly a result of alternate or aborted translation. The fusion protein preparations were active and had a low specific activity. They displayed autophosphotransferase activity primarily towards serine, threonine and tyrosine residues. The enzymes were stable at 30°C for about 20 min, and Pim-1 kinase assay conditions were optimized with respect to the nucleotide and ion requirements.
Although the exact function of Pim-1 in the cell has not yet been defined, its nature as a cytoplasmic protein kinase is consistent with a role within a signal transduction pathway. Upstream activators (GM-CSF, Epo, IL-3) [Wingette et al., 1991; Lilly et al., 1992; Sato et al., 1993] and proteins existing in similar signal transduction pathways (i.e. JAK, p95\textsuperscript{vav}) [Matsuguchi et al., 1995; Miura et al., 1994; Quelle et al., 1994] are being intensively investigated by other groups, but little has been done to identify direct targets of Pim-1. To gain insight into the function of the kinase, various proteins and peptides were tested as in vitro substrates of Pim-1. It was intended that the definition of substrate requirements of Pim-1 would lead to the identification of physiological substrates of this kinase.

1. PRELIMINARY SUBSTRATE STUDIES

i. \textit{In vitro} phosphorylation of protein substrates by GST-Pim-1

A large number of proteins were tested and found to be \textit{in vitro} substrates of both \textit{H. sapiens} and \textit{X. laevis} GST-Pim-1. \textit{X. laevis} GST-Pim-1 strongly phosphorylated MBP (21 kDa), and all the histones, including histone IIA (14-17 kDa), histone IIS (14-17 kDa), histone III-S (31 kDa) and histone VII-S (14-17 kDa) (Fig. 16). \textit{X. laevis} GST-Pim-1 moderately phosphorylated the 40 S ribosomal protein (31 kDa), casein (40 kDa), GST-Raf-1 (95 kDa), enolase (40, 42 kDa) and phosphorylase b (94 kDa). This GST-Pim-1 did not phosphorylate GST, phosvitin, protamine sulfate or protamine chloride.

The protein spectrum phosphorylated by human GST-Pim-1 was similar to that phosphorylated by \textit{X. laevis} GST-Pim-1 (Fig. 17). Human GST-Pim-1 strongly phosphorylated MBP (21 kDa) and all the histones, including histone IIA (14-17 kDa), histone IIS (14-17 kDa), histone III-S (31 kDa) and histone VII-S (14-17 kDa). Human GST-Pim-1 moderately phosphorylated the 40 S ribosomal protein (31 kDa), GST-Raf-1 (120 kDa) and phosphorylase B (94 kDa). Human GST-Pim-1 did not phosphorylate GST, phosvitin, protamine sulfate and protamine chloride and it was difficult to determine if it phosphorylated casein (40 kDa) or enolase (40, 42 kDa) as these proteins comigrated with autophosphorylated byproducts in the GST-Pim-1 preparation.
Figure 16. Phosphorylation of various protein substrates by X. laevis GST-Pim-1. Phosphorylation reactions were carried out in the presence (+) or absence (-) of GST-Pim-1. GST-Pim-1 control on left. Reactions contained 5 ug each of protamine sulphate (A), protamine chloride (B), casein (C), phosvitin (D), phosphorylase B (E), enolase (F), GST-Raf-1 (G), GST (H), histone II A (I), histone II S (J), histone VII S (K), histone IIIS (L), 40S ribosome (M) and myelin basic protein (N). Migrations of Mr standards are shown on the left. The autophosphorylated GST-Pim-1 is indicated by arrow (→).
Figure 17. Phosphorylation of various protein substrates by *H. sapiens* GST-Pim-1. Phosphorylation reactions were carried out in the presence (+) or absence (-) of GST-Pim-1. GST-Pim-1 control on left. Reactions contained 5 µg each of protamine sulphate (A), protamine chloride (B), casein (C), phosvitin (D), phosphorylase B (E), enolase (F), GST-Raf-1 (G), GST (H), histone II A (I), histone II S (J), histone VII S (K), histone III S (L), 40S ribosome (M) and myelin basic protein (N). Migrations of Mr standards are shown on the left. The autophosphorylated GST-Pim-1 fragments are indicated by arrows (→→).
ii. Phosphoamino acid analysis of phosphorylated substrates

Phosphoamino acid analysis of GST-Pim-1 phosphorylated substrates was performed to establish the nature of phosphorylation catalyzed by GST-Pim-1. Radiolabelled proteins from Figures 16 and 17 were excised and subjected to acid hydrolysis. Both *H. sapiens* and *X. laevis* GST-Pim-1 phosphorylated proteins on serine and threonine, but not tyrosine residues. Both human and *X. laevis* GST-Pim-1 phosphorylated the 40 S ribosomal protein, histone H2A, histone H2B and histone H3 on both serine and threonine residues, while MBP and histone vH1S were phosphorylated on serine only. Because of low stoichiometries of phosphorylation, the phosphoamino acid analysis was not performed on enolase, casein, phosphorlyase b and GST-Raf1.

In agreement with our results, previously published data indicated that Pim-1 phosphorylated histone H1 on both serine and threonine residues, enolase on serine and threonine residues [Hoover et al., 1991; Friedmann et al., 1992], histone 2B on serine [Saris et al., 1991] and did not phosphorylate GST [Hoover et al., 1991]. In contrast to our results, GST-Pim-1 phosphorylated salmon protamine [Saris et al., 1991] but did not phosphorylate casein [Hoover et al., 1991]. The reason for these conflicts is most likely due to the fact that phosphorylation of these substrates by this kinase was very low and different preparations of these substrates are already phosphorylated to variable extents.

The fact that the GST-Pim-1 phosphorylated so many of these proteins *in vitro* is of minimal physiological significance. This *in vitro* situation involves very large amounts of two highly purified proteins: an active kinase and a potential substrate. When presented with such high concentrations of a potential substrate without interference by regulatory proteins or competing substrates, the GST-Pim-1 is likely to seem more promiscuous in its substrate preference. Therefore, the *in vitro* protein phosphorylation data should be used only as a guideline of substrate preference and not an ultimate determination of physiological activity.

iii. Peptide substrate comparisons to published data

In an earlier study by Hoover et al. [1991], histone H1 and Kemptide (LRRRASLG, a peptide modeled after the cAMP-dependent protein kinase phosphorylation site in pyruvate kinase) were used as substrates for GST-Pim-1. These substrates were used in the present study to test the exogenous phosphotransferase activity of bacterially-expressed human and *X. laevis* GST-Pim-1. Both substrates were found to be phosphorylated by GST-Pim-1; histone H1 was phosphorylated by the *X. laevis* GST-Pim-1 with a $V_{\text{max}}$ of 825 pmol.min$^{-1}$.
1.mg\(^{-1}\), with an apparent \(K_m\) of 2 \(\mu\)M, while Kemptide was phosphorylated with a \(V_{\text{max}}\) of less than 7.5 pmol.min\(^{-1}\).mg\(^{-1}\), with an apparent \(K_m\) of greater than 1200 \(\mu\)M (Table 3). A peptide based on the C-terminus of ribosomal S6 protein routinely used in the lab, was phosphorylated by \(X.\) laevis Pim-1 with a \(V_{\text{max}}\) of 178 pmol.min\(^{-1}\).mg\(^{-1}\) with an apparent \(K_m\) of 70 \(\mu\)M. Human GST-Pim-1 phosphorylated Kemptide with a \(V_{\text{max}}\) of 0.6 pmol.min\(^{-1}\).mg\(^{-1}\), and an apparent \(K_m\) of greater than 800 \(\mu\)M. This same preparation of human GST-Pim-1 phosphorylated the S6-CT peptide with a \(V_{\text{max}}\) of 11 pmol.min\(^{-1}\).mg\(^{-1}\) with an apparent \(K_m\) of 16 \(\mu\)M.

Friedmann et al. [1992], found that Pim-1 phosphorylated histone H1 \textit{in vitro} with an apparent \(K_m\) of 7 \(\mu\)M. They found that the histone H1 was a 6-fold better substrate than Kemptide and suggested that histone might actually be a physiological substrate. Histone H1 was used early in our study to initially test and optimize the activity of the expressed fusion proteins and to analyze Pim-1 activity during early column purifications. However, as histone H1 is a substrate for many other kinases, we decided to explore the activity of Pim-1 using a more specific and convenient substrate. As a panel of S6 peptide analogs was available in the lab, substrate studies were initiated using peptides modeled on the S6.
TABLE 3. COMPARISON OF SEVERAL PEPTIDES PHOSPHORYLATED BY GST-PIM-1.
Vmax is expressed as pmol.min⁻¹.mg⁻¹. Km is expressed as μM.

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<td>Vmax</td>
<td>Km</td>
<td>Vmax</td>
<td>Vmax</td>
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<td></td>
<td></td>
<td></td>
<td>Kᵣm</td>
<td>Kᵣm</td>
</tr>
<tr>
<td>Histone H1</td>
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<td>2</td>
<td>412</td>
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<td>Kemptide LRRASLG</td>
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<td>&gt;800</td>
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</tr>
<tr>
<td>S6-CT AKRRRLSSLRASTSKSESSQK</td>
<td>11.0</td>
<td>16</td>
<td>0.69</td>
<td>178</td>
</tr>
</tbody>
</table>
2. SUBSTRATE ANALYSIS USING PEPTIDE ANALOGS

To delineate the consensus phosphorylation site recognition sequence for GST-Pim-1, we intended to test the affinity of GST-Pim-1 for a series of peptide analogs. Since the S6 protein in the 40S ribosome served as a substrate for both human and X. laevis GST-Pim-1, a peptide that encompassed the major phosphorylation sites located at the C-terminus of the human S6 protein (residues 229 to 249) [Heinze et al., 1988] was tested as a substrate (Table 3). This peptide (S6-CT, AKRRRLSSLRASTSKSESSQK) was at least a 20-fold better substrate for GST-Pim-1 than Kemptide (LRRASLG). A panel of shorter peptide analogs of S6-CT was constructed to identify the critical residues surrounding the phosphoacceptor site that were needed for substrate recognition and phosphorylation by GST-Pim-1.

Peptides were synthesized in the laboratory of Dr. Ian Clark-Lewis (Biomedical Research Centre). Peptides were initially purified by HPLC, but because of the small size and relative purity of the peptides, this step was eliminated. Comparison of the GST-Pim-1 phosphorylation of the HPLC-purified with the non-purified peptides (P1, P2, P3), demonstrated that the activity towards the HPLC treated peptides averaged 15% higher that with the non-purified peptides (data not shown). Since the peptide selectivity was maintained despite the differences in the rate of the reaction, non-HPLC treated peptides were used for the subsequent kinetic determinations. Determinations were conducted in triplicate and each experiment repeated at least three times with highly reproducible results. Values were plotted and kinetic constants determined using Michaelis-Menten and Lineweaver and Burke plots as detailed in Appendix VI. There was some difficulty in the estimation of the $V_{\text{max}}$ values, because substrate inhibition occurred with higher concentrations of certain peptides. The mean values of the apparent $K_m$ and $V_{\text{max}}$ determinations as well as the $V_{\text{max}}/K_m$ value for all peptides are shown in Tables 4-9. The $V_{\text{max}}/K_m$ value was used to compare the relative efficiencies of various peptides as substrates of the GST-Pim-1.

To ensure that the phosphorylation was due to the phosphotransferase activity of Pim-1 and not to a contaminating/copurifying kinase, the KD mutant was incubated with the same peptides in control reactions. No phosphotransferase activity was detected towards any peptide which confirmed that activity was specifically due to Pim-1 (data not shown).
i. Location of phosphoacceptor site

To determine which serine was phosphorylated by the kinase, the rates of phosphorylation of S6-CT (AKRRRLSSLRASTSKSESSQK), P1 (AKRRRLSSLRA), P2 (AKRRRLSALRA), and P3 (AKRRRLASLRA) by GST-Pim-1 were compared (Table 4). To identify which of the two serine residues in P1 was targeted for phosphorylation, the serine residues were selectively replaced with alanine residues in P2 and P3. Alanine residues were selected as the closest non-hydroxylated analogs for serine. The P1 and P2 peptides were similarly effective as substrates and were phosphorylated to a much greater extent than P3. These results demonstrated that GST-Pim-1 exhibited strong preference for the first serine residue in P1. Consequently, all other peptides were constructed with only the first serine residue; for reference, the position of the phosphorylatable residue was designated as "0".

ii. Influence of C-terminal residues

To examine the influence of neighboring C-terminal residues on serine phosphorylation by GST-Pim-1, peptides P4 (ALRRRLSA), P5 (ALRRRLS-amide) and P6 (ALRRRLS-acid) were tested as substrates (Table 5). The P4 peptide was more strongly phosphorylated than P2, implying that the C-terminal residues, "ALRA" of P2 exerted an inhibitory effect on phosphorylation. P5 was phosphorylated more efficiently than P4, confirming the inhibitory effect of residues C-terminal to 0. However, it remains possible that different C-terminal residues might contribute to improved substrate recognition in a physiological substrate of Pim-1.

To evaluate the importance of a peptide bond on the C-terminal side of the phosphoacceptor site, P6 was constructed with a C-terminal free acid instead of an amide. P6 was poorly phosphorylated by GST-Pim-1, suggesting that the negative charge at the C-terminus acted as a negative determinant and physiological substrates of Pim-1 are unlikely to be phosphorylated by this kinase at the C-terminal residue. As P4 was more representative of a natural substrate than P5 or P6 in having a peptide bond after the phosphoacceptor residue, P4 was used as the prototype for the design of additional analog peptides for the remainder of this study and a standard for comparison with other peptides.
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<td></td>
<td></td>
</tr>
<tr>
<td>Kemptide</td>
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<td>&gt;800</td>
<td>&lt;0.001</td>
<td>7.5</td>
<td>&gt;1200</td>
<td>&lt;0.006</td>
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<td>13</td>
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TABLE 5. INFLUENCE OF C-TERMINAL RESIDUES ON SUBSTRATE PHOSPHORYLATION BY GST-PIM-1.

Vmax is expressed as pmol.min\(^{-1}\).mg\(^{-1}\). Km is expressed as µM.

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</tr>
<tr>
<td>P2</td>
<td>AKRRRLSALRA</td>
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<td>13</td>
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<td>AKRRRLSA</td>
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<td>15</td>
</tr>
<tr>
<td>P5</td>
<td>AKRRRLS - amide</td>
<td>15.3</td>
<td>6</td>
</tr>
<tr>
<td>P6</td>
<td>AKRRRLS - free acid</td>
<td>not measurable</td>
<td>9</td>
</tr>
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iii. Amino acid specificity of the phosphoacceptor site

The specificity of the phosphoacceptor site for recognition by GST-Pim-1 was explored with P4 (AKRRRLS_A), P7 (AKRRRLT_A) and P8 (AKRRRLY_A) (Table 6). Peptides P4 and P7, which featured serine and threonine respectively, at the phosphoacceptor site (position 0) were recognized comparably as substrates. Phosphorylation of the peptide P12, which contained a tyrosine residue at position 0 was not detected.

iv. Influence of the -1 amino acid residue

To examine the influence of the residue directly before the phosphoacceptor site, phosphorylations of peptides P4, P9, P10, P11, P12, P13, and P14 by GST-Pim-1 were compared (Table 7). There were marked differences in the selectivity of human and X. laevis GST-Pim-1 within this set. P10 (AKRRRRSA) with a basic arginine residue at the -1 position was poorly phosphorylated peptide by both human and X. laevis GST-Pim-1. Although P8 (AKRRRKSA) with a basic lysine residue at the -1 position was not well tolerated by the human enzyme, P18 was phosphorylated to a comparable if not greater extent than the P4 standard by X. laevis GST-Pim-1. An acidic residue at the -1 site, as in P11 (AKRRRRESA), yielded only low phosphorylation by both human and X. laevis GST-Pim-1. Other polar residues such as glutamine in P12 (AKRRRQSA) at the -1 location also produced only low phosphorylation by human GST-Pim-1, but were phosphorylated to the same extent as the P4 standard by X. laevis GST-Pim-1. Non-polar residues such as alanine in P13 (AKRRRASA) and isoleucine in P14 (AKRRRISA) at the -1 position were tolerated by both enzymes, yielding intermediate results. Thus, most amino acids, except for acidic residues, were acceptable at the -1 site. Basic residues in this location were not accommodated by the human GST-Pim-1, while the X. laevis GST-Pim-1 tolerated a lysine but not an arginine residue at this site, an effect that may be sterically related. Although any non-polar residue was tolerated by the X. laevis GST-Pim-1, the optimal amino acid for the human GST-Pim-1 for the -1 position was found to be leucine, as in P4 (AKRRRLS_A). For this reason, leucine was used for the -1 site for the construction of all further peptide analogs. The fact that the human and the amphibian GST-Pim-1 results did not agree for all the peptides tested indicate that either the sites examined were not particularly critical for substrate recognition by Pim-1 or that minor differences in the primary structure of the proteins contribute to substrate recognition. To our knowledge, this is the first example of a change in substrate specificity for a protein kinase from different species.
TABLE 6. AMINO ACID SPECIFICITY OF THE PHOSPHOACCEPTOR SITE.
Vmax is expressed as pmol.min⁻¹.mg⁻¹. Km is expressed as µM.

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<td>Km</td>
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<td>Km</td>
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<td>1.02</td>
<td>124</td>
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<tr>
<td>P7</td>
<td>18.9</td>
<td>19</td>
<td>0.99</td>
<td>80</td>
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<tr>
<td>P8</td>
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<td>not measurable</td>
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**TABLE 7. INFLUENCE OF THE -1 AMINO ACID RESIDUE.**

Vmax is expressed as pmol.min\(^{-1}\).mg\(^{-1}\). Km is expressed as \(\mu\)M.

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<td>K(_{\text{m}})</td>
<td>V(_{\text{max}})</td>
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<tr>
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<td>AKRRRLSA</td>
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<td>1.02</td>
<td>124</td>
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<td>3.5</td>
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<td>AKRRRKSA</td>
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<td>99</td>
<td>31</td>
<td>3.2</td>
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v. Influence of -2, -3 and -4 amino acid residues

The importance of the arginine residues at the -2, -3, and -4 positions for GST-Pim-1 recognition of a substrate was evaluated with peptides P4, P15, P16, P17, P18, P19, P20 and P21 (Table 8). Peptide P15 (AKRRALSA) demonstrated that substitution of an alanine for the arginine at the -2 position dramatically reduced the phosphorylation of the peptide by GST-Pim-1. A lysine residue at the -2 position in P16 (AKRRKLSA) restored phosphorylation by human GST-Pim-1 and partially restored phosphorylation by X. laevis GST-Pim-1 and indicated a strong preference for a basic residue at this location. When an alanine residue was substituted for arginine at the -3 position in P17 (AKRARLSA), phosphorylation by GST-Pim-1 was markedly reduced. Conservative substitution of a lysine for an arginine at the -3 site in peptide P18 (AKRKLRLSA) did not restore phosphorylation to the original levels, which emphasizes the absolute requirement for an arginine residue at this location. Substitution of an alanine for the arginine at the -4 position in peptide P19 (AKARRRLSA) reduced the affinity of the peptide slightly, but it did not affect the rate of phosphorylation by human GST-Pim-1. However, the alanine substitution at the -4 position in peptide 19 reduced the rate of phosphorylation by X. laevis GST-Pim-1. Peptide P20 (AKRRRLSA) demonstrated that a substitution of lysine for the arginine at the -4 position did not affect phosphorylation, and indicated that that the substrate requirement is fulfilled by any basic residue at the -4 position. A double substitution of alanine at the -2 and -4 positions, further decreased the $K_m$ and $V_{max}$ of phosphorylation of peptide P21 (AKARALSA), as compared to -2 and -4 single substituted peptides, P15 and P19, respectively, and emphasized the need for a strong basic environment upstream of the Pim-1 phosphorylation site.

This data indicated that all three arginine residues are optimal for recognition by GST-Pim-1, and that other basic residues like lysine, cannot completely replace the arginines. The most important arginine is located at the -3 position, followed by the -2 and the -4 arginines. The results from both the H. sapiens and the X. laevis Pim-1 were consistent in this regard.

vi. Influence of the -5 and -6 amino acid residues

The influence of the residues at the -5 and -6 positions for GST-Pim-1 phosphorylation was tested with peptides P4, P22, P23, P24, P25, P26 and P27 (Table 9). The results with P22 (KRRRLRSA) demonstrated that the presence of a specific residue at the -6 position was not essential for phosphorylation by GST-Pim-1. The reduction in phosphorylation by human GST-Pim-1 that was observed with P22 could
TABLE 8. IMPORTANCE OF -2, -3 AND -4 ARGinine RESIDUES.
Vmax is expressed as pmol.min\(^{-1}\).mg\(^{-1}\). Km is expressed as µM.

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TABLE 9. INFLUENCE OF THE -5 AND -6 AMINO ACID RESIDUES.
Vmax is expressed as pmol.min\(^{-1}\).mg\(^{-1}\). Km is expressed as \(\mu\)M.

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<td>Km</td>
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</tbody>
</table>
possibly result from the removal of the peptide bond. In contrast, removal of this alanine caused an increase in phosphorylation by the *X. laevis* GST-Pim-1. The influence of this site on substrate phosphorylation was probably minimal, accounting for the conflict in results between the two enzymes. The influence of this residue was not examined further.

Peptide P23 (RRRLSA) demonstrated that a residue at the -5 position was essential for recognition by both *H. sapiens* and *X. laevis* GST-Pim-1. Replacement of the lysine at the -5 location with hydrophobic residues in P24 (AARRRLSA) and P26 (ALRRRLSA) reduced phosphorylation of the peptide. The restoration of phosphorylation that occurred when a basic arginine was inserted at the -5 position as in P25 (ARRRLRLSA) showed that human GST-Pim-1 exhibited strong preference for a basic residue at this site, while *X. laevis* GST-Pim-1 was ambivalent. Substitution of an acidic glutamic acid residue at this position as in P29 (AERRRLSA) caused very low phosphorylation, confirming the preference for a basic residue at this site.

vii. Determining the substrate consensus sequence

These results demonstrate that *H. sapiens* and *X. laevis* GST-Pim-1 have very similar substrate specificities. Residues at the +1, 0, -2, -3, -4 and -5 sites had similar selectivity with both the human and the *X. laevis* GST-Pim-1 enzymes, indicating that these residues are important determinants in the substrate consensus sequence. Residues at the -1 and -6 sites were not in agreement between the two enzymes, implying that these two sites are of minimal importance for substrate recognition by GST-Pim-1. Although the *K*_m and the *V*_max differ, a similar selectivity was maintained for most peptides.

The deduced consensus sequence for substrate recognition by GST-Pim-1 based on the substrate peptide studies was [K/R] - [K/R] - R - [K/R] - L - [S/T] - X, where X is optimally a residue with a shorter side chain. It is possible that additional residues located N- and C-terminal to this sequence also influence substrate recognition. This sequence is not present in Pim-1 from human, mouse [Breuer *et al.*, 1989] or rat [Wingette *et al.*, 1992]. However, a sequence similar to this, F-R-R-R-I-S-T (amino acid residues 256-263) is located in the primary structure of the *X. laevis* Pim-1 near catalytic subdomain XI. Perhaps this might be an auto- or cross-phosphorylation site in *X. laevis* Pim-1, with phosphorylation at this site leading to altered phosphotransferase activity towards exogenous substrates.
3. DATA BANK SEARCH FOR POTENTIAL SUBSTRATES OF PIM-1

Using deduced consensus phosphorylation site motifs to scan protein sequence data bases is potentially a useful approach to identify putative kinase substrates. However, in some cases, secondary or tertiary structure may deny access to a potential phosphoacceptor site through steric hindrance [Kemp and Pearson, 1990; Kennelly and Krebs, 1991]. A computer search with the consensus sequence, [K/R]-[K/R]-R-[K/R]-L-[S/T] identified many proteins that featured this sequence. In addition to S6, other potential substrates for Pim-1 include: human heterogeneous ribonucleoprotein [KKRRLS]; human cAMP-dependent protein kinase type I-β regulatory chain [RRRRLS]; *Schizosaccharomyces cerevisiae* DNA polymerase delta large chain [KRRRLS]; *Saccharomyces cerevisiae* CTP:cholinephosphate cytidylyltransferase [KRRRLT]; human epidermal growth factor precursor [KRRRLT]; human Ski oncoprotein [RKRKLT]; the mouse CDC-25 protein homologue [RRRKLS]; and the rat guanine-nucleotide releasing protein [RRRKLS].
4. INHIBITION OF PIM-1 ACTIVITY BY INHIBITOR PEPTIDES

i. Inhibition of phosphotransfer activity by pseudo-substrate peptides

Peptides P28 (AKRRRLAA) and P29 (AKRRRLCA) were constructed as analogs of P4 that lacked phosphorylatable amino acid residues. Neither peptide was detectably phosphorylated by GST-Pim-1, which confirmed that all other peptides were radiolabelled with [γ^32P]-ATP from direct phosphorylation and not due to of radioactive ATP being trapped onto the phosphocellulose filters indirectly via the basic residues of the peptide (data not shown).

When P28 and P29 were tested as inhibitors of human GST-Pim-1 phosphorylation of P4 substrate, P28 was found to inhibit the phosphorylation in a competitive fashion (Fig. 18); increasing amounts of P4 competed with P28 for the substrate binding domain of the kinase. This is not surprising as the sequences of the peptides differed only between the serine and alanine residue and these two amino acids are both very small. Peptide P29 inhibited phosphorylation of P4 more potently, acting competitively at low concentrations and non-competitively at higher concentrations (Fig. 19), indicating that a more complex interaction existed between P29 and GST-Pim-1. The inhibition of GST-Pim-1 phosphorylation by P29 was repeated in the presence of 100 mM 2-mercaptoethanol with similar results. This reducing agent was added to inhibit dimerization of the cysteine-containing P29 peptide and disulfide bond formation with GST-Pim-1. The GST-Pim-1 did not rely on intact disulfide bonds to be functional and a disulfide bond did not appear to form between GST-Pim-1 and peptide P29.

Pseudosubstrate sequences are located in regulatory domains of certain protein kinases and regulate phosphotransferase activity [Kennelly and Krebs, 1991]. They correspond to sequences resembling protein kinase phosphorylation site motifs, except that they usually contain an alanine residue substitution for a serine or threonine residue [Kennelly and Krebs, 1991]. Peptide P28, which contained an alanine residue in place of the phosphorylatable serine and P29, which contained a cysteine instead of the serine, were modeled after the consensus phosphorylation site sequence of Pim-1 and tested as inhibitors of Pim-1 phosphotransferase activity. The results with these peptides indicated that the activity of Pim-1 is not likely to be regulated by the presence of a pseudosubstrate site within the kinase.
Figure 18. Competitive inhibition of exogenous phosphotransferase activity of *H. sapiens* GST-Pim-1 by P28 peptide.

The concentrations of P28 peptide (AKRRRLAA) indicated in the legend were used to inhibit the phosphorylation of P4 substrate peptide (AKRRRLSA) by *H. sapiens* GST-Pim-1. The inverse incorporation of $^{32}$P is indicated on the vertical axis, the inverse concentration of P4 peptide is indicated on the horizontal axis. Origin is indicated by vertical dotted line.
Figure 19. Inhibition of exogenous phosphotransferase activity of *H. sapiens* GST-Pim-1 by P29 peptide.
Concentrations of P29 peptide (AKRRRLCA) were used to inhibit the phosphorylation of P4 substrate peptide (AKRRRLSA) by *H. sapiens* GST-Pim-1. The inverse incorporation of $^{32}$P is indicated on the vertical axis, the inverse concentration of P4 peptide is indicated on the horizontal axis. Panel A shows a close-up of panel B. The origin is indicated by the vertical dotted line.
ii. Inhibition of autophosphorylation activity by substrate and pseudo-substrate peptides

Peptides P4, P28 and P29 were tested as a potential substrate and pseudosubstrates of human GST-Pim-1 that might specifically block the active site of the kinase and sterically inhibit autophosphorylation. Peptides Pim1-III and Pim1-NT, used to immunize rabbits for Pim-1 antibodies, were used as controls with peptide concentrations ranging from 0.125 - 4.0 mM per assay.

The autophosphorylation of GST-Pim-1 was inhibited strongly by substrate P4 at concentrations as low as 0.125 mM (data not shown). Peptide P29 slightly inhibited the autophosphorylation at concentrations above 0.5 mM and moderately inhibited autophosphorylation at concentrations greater than 1.0 mM, while P28 caused only very minor inhibition of autophosphorylation at concentrations that exceeded 0.75 mM. These results indicated that GST-Pim-1 preferentially phosphorylated exogenous substrate rather than itself. Peptides used to inject rabbits for antibody production (Pim1-III and Pim1-NT) did not inhibit the autophosphorylation activity whatsoever, indicating that the inhibition of autophosphorylation was selective and did not result from high amounts of non-specific peptide in the reaction.
5. CONCLUSIONS OF SUBSTRATE STUDIES

The substrate consensus recognition sequence for Pim-1 was deduced to be [K/R] - [K/R] - R- [K/R] - L - [S/T] - X, where X cannot be a residue with a large side chain. There were slight differences in peptide substrate selectivity between *H. sapiens* and *X. laevis* GST-Pim-1; the *H. sapiens* enzyme displayed a clear preference for a leucine residue at the -1 site, while the *X. laevis* enzyme tolerated most residues tested at this site. Friedmann *et al.* [1992] published a similar consensus phosphorylation site motif for Pim-1 while this work was in progress, i.e. (R/K)3-X-S/T-X', where X' cannot be arginine, lysine or a large hydrophobic residue. They defined this sequence using a series of only 6-8 peptides, some of which did not resemble this consensus sequence, and the influence of each of the sites near the phosphorylatable residue was not tested.

Potential physiological substrates containing this motif are not yet obvious. Many of the proteins that featured the Pim-1 phosphorylation site consensus sequence are enzymes or structural proteins, many of which are functional in the cytoplasm. This is in agreement with several recent publications implicating Pim-1 upregulation as a cytoplasmic event in response to growth factor receptor stimulation.
CHAPTER VI.

ANALYSIS OF AUTOPHOSPHORYLATION

As demonstrated in Chapter IV, expressed GST-Pim-1 was able to autophosphorylate in vitro on serine, threonine and tyrosine residues. Whether Pim-1 autophosphorylates on tyrosine and/or serine/threonine has been a matter of controversy in the literature [Meeker et al., 1987a; Telerman et al., 1988; Saris et al., 1991; Padma and Nagarajan, 1991; Friedmann et al., 1992]. Although the nature of this autophosphorylation has been extensively investigated, the sites of autophosphorylation have not yet been identified. The goal of this study was to identify the major autophosphorylation site of X. laevis GST-Pim-1, to change this site by PCR mutagenesis, and to assess the functional consequence of this perturbation in the expressed mutant in comparison to WT GST-Pim-1. The X. laevis GST-Pim-1 was used for all experiments as the full-length fusion protein strongly autophosphorylated and the results were easier to assess because of the lack of degradation products as associated with human GST-Pim-1.

1. DETECTION OF GST-PIM-1 WITH ANTI-PHOSPHOTYROSINE ANTIBODIES

In Chapter IV we demonstrated immunodetection of bacterially-expressed X. laevis GST-Pim-1 protein by anti-phosphotyrosine antibodies (Fig. 12). We wanted to determine if this immunodetection was specific for phosphotyrosine or if it was due to cross reactivity with phosphoserine or phosphothreonine residues. We also wanted to assess if in vitro autophosphorylation would cause an increase in the amount of detectable phosphotyrosine associated with Pim-1, and we wanted to confirm that the tyrosine phosphorylation of Pim-1 was due to auto-kinase activity and not due to contamination by bacterial kinases in the GST-Pim-1 preparation.

Western blots of KD, WT Pim-1 and WT Pim-1 autophosphorylated with [γ32P]-ATP were probed with a X. laevis-specific antibody, Pim1-XI, and an anti-phosphotyrosine antibody, PY20 (Fig. 20, panel A). Probing with Pim1-XI antibody demonstrated that the amount of protein loaded in each lane was similar (lanes 1-3). Although WT GST-Pim-1 (lanes 2) did not experience a size shift with in vitro autophosphorylation (lane 3), both unphosphorylated and in vitro autophosphorylated WT GST-Pim-1 samples appeared to be slightly retarded on a Western blot, compared to the KD- GST-Pim-1. The difference in
size between the KD and WT GST-Pim-1 may be due to the incorporation of phosphate residues, as a result of \textit{in vivo} autophosphorylation of the WT GST-Pim-1.

The anti-phosphotyrosine antibody detected GST-Pim-1 both before and after \textit{in vitro} autophosphorylation and indicated that the GST-Pim-1 was already substantially phosphorylated on tyrosine prior to lysis of the bacteria. The KD mutant was not detected by the anti-phosphotyrosine antibody, indicating that the tyrosine phosphorylation was not the result of a contaminating kinase (lanes 4-6). To ensure that this effect was not due to cross-reactivity with phosphoserine and phosphothreonine residues, free phosphoamino acids were used to compete with the PY20 anti-phosphotyrosine antibody. Phosphoserine (lanes 7-9) and phosphothreonine (lanes 10-12) did not compete with the antibody, but phosphotyrosine competed and eliminated detection with the PY20 antibody (lanes 13-15). This experiment was repeated using a second anti-phosphotyrosine antibody (4G10) with similar results (data not shown).

From this experiment we concluded that the \textit{X. laevis} GST-Pim-1 can autophosphorylate on tyrosine residues in addition to serine and threonine. While some tyrosine phosphorylation occurred during an \textit{in vitro} autophosphorylation reaction, it seems that the majority of tyrosine phosphorylation happened during expression in the bacteria.

Experiments were performed to assess if the kinase activity of GST-Pim-1 could be changed by autophosphorylation or by dephosphorylation. The results of these experiments were inconclusive and are described with the results in Appendix VIII.
Figure 20. Tyrosine phosphorylation of expressed X. laevis GST-Pim-1.
Panel A - Western blots of expressed X. laevis GST-Pim-1 probed with anti-phosphotyrosine antibodies. Lanes 1, 4, 7, 10, 13 each contain 0.75 ug of K69-A kinase-inactive GST-Pim-1. Lanes 2, 5, 8, 11, 14 each contain 0.75 ug of wild-type GST-Pim-1. Lanes 3, 6, 9, 12, 15 each contain 0.75 ug if wild-type GST-Pim-1 that has undergone an in vitro kinase reaction with $^{32}$P-ATP. The antibodies used to probe the Western blots are indicated. Antibody binding was inhibited by the addition of phosphoamino acids; lanes 7-9 with 100 ug/ml of phospho-serine, lanes 10-12 with 100 ug/ml of phospho-threonine, and lanes 13-15 with 100 ug/ml of phospho-tyrosine. Panel B - Autoradiograms of panel A.
2. ANALYSIS AND IDENTIFICATION OF GST-PIM-1 AUTOPHOSPHORYLATION SITES

Identification of the autophosphorylation sites of GST-Pim-1 was attempted. The intention was to alter the identified autophosphorylation sites by site-directed mutagenesis and to assess the effect on Pim-1 catalytic activity. As well, a comparison of the Pim-1 site(s) to autophosphorylation sites of other kinases could potentially help determine a mode of activation of Pim-1.

i. Two dimensional phosphopeptide analysis of GST-Pim-1

Two dimensional phosphopeptide analysis of in vitro autophosphorylated X. laevis GST-Pim-1 yielded 2 strongly phosphorylated peptides (spots 1 & 2), as well as a number of moderate and weakly phosphorylated peptides (Fig. 21a). Each spot was numbered (Fig. 21b) for future reference. Addition of increasing amounts of trypsin caused the relative strength of some spots to change, indicating that some of these peptides were the result of incomplete tryptic cleavage. None of the minor spots disappeared completely even when large amounts of trypsin were added. Dephosphorylation of GST-Pim-1 before in vitro autophosphorylation did not result in any significant changes in the relative intensities of the spots.
Figure 21. Two-dimensional phosphopeptide map of *X. laevis* GST-Pim-1. *Panel A*: Autoradiogram of two-dimensional phosphopeptide map of auto-phosphorylated and trypsonized *X. laevis* GST-Pim-1. *Panel B*: labeling of the spots from the 2D map. Origin is indicated by small circle, arrow indicates direction of electrophoresis in first dimension.
ii. Phosphoamino acid analysis of spots from 2D phosphopeptide mapping

After extraction from the TLC plate, peptides identified by 2D phosphopeptide analysis were subject to phosphoamino acid analysis. The results of this experiment are summarized in Table 10. Sample numbers correspond to the spot numbers in Figure 21b. Some samples did not yield enough radioactivity to warrant further analysis by IMAC-HPLC-ESI-MS.

<table>
<thead>
<tr>
<th>Samples #</th>
<th>Phosphoamino acid residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>S</td>
</tr>
<tr>
<td>2.</td>
<td>T</td>
</tr>
<tr>
<td>3.</td>
<td>S</td>
</tr>
<tr>
<td>4.</td>
<td>S (T)</td>
</tr>
<tr>
<td>5.</td>
<td>S (Y)</td>
</tr>
<tr>
<td>6.</td>
<td>S (Y)</td>
</tr>
<tr>
<td>7.</td>
<td>T</td>
</tr>
<tr>
<td>8.</td>
<td>S</td>
</tr>
<tr>
<td>9.</td>
<td>T, S (Y)</td>
</tr>
<tr>
<td>10.</td>
<td>S</td>
</tr>
<tr>
<td>11.</td>
<td>S</td>
</tr>
<tr>
<td>12.</td>
<td>T</td>
</tr>
<tr>
<td>13.</td>
<td>S</td>
</tr>
</tbody>
</table>

Table 10. Phosphoamino acid analysis of peptides isolated from the 2D phosphopeptide map.
Radiolabelled peptides appearing as spots on the 2D phosphopeptide map (Fig. 21) were extracted from the cellulose matrix and subjected to phosphoamino acid analysis. Residues in brackets indicate that the radioactive labelling of that amino acid was difficult to discern. Samples 6 and 8 did not have enough radioactivity to allow analysis by this method.
iii. Identification of Tryptic Phosphopeptides

A collaboration was undertaken with Drs. Lawrence Amankwa and Michael Affolter of Ruedi Aebersold's laboratory at the Biomedical Research Centre to analyze and identify the peptides isolated by 2D tryptic phosphopeptide mapping, utilizing new methods developed in their laboratory.

a. Analysis of isolated tryptic phosphopeptides by IMAC-HPLC-ESI-MS

Immobilized metal affinity chromatography (IMAC) - high pressure liquid chromatography (HPLC) - electron spray ionization mass spectroscopy (ESI-MS) is a sequential series of methods that allow isolation and characterization of tryptic phosphopeptides. IMAC exploits the fact that phosphate adheres to charged ferric cations non-covalently, so FeCl₃ was used to charge a chelating Sepharose Fast Flow matrix. Phosphorylated peptides were selectively retained by the IMAC column and eluted from the matrix with sodium phosphate buffer and were subjected to subsequent analysis by HPLC-ESI-MS.

Charged particles entering the spectrometer were detected by total ion chromatography. A total ion chromatogram (TIC) for sample 7 is shown in Figure 22, which demonstrates the presence of two major charged species. As the amount of information from a TIC is very limited, the chromatograms for the rest of the samples analyzed are not shown.

The peaks observed in the TIC from each sample were subjected to mass spectroscopy. Peaks that corresponded to tryptic peptides of GST-Pim-1 were sequenced by the sequential removal of residues during the partial fragmentation by mass spectrometry. Peptide bonds were broken by electron bombardment during the ionization process (Fig. 23) and species generated by this process are indicated in the mass spectra. Samples not yielding interpretable results were not described.

Figure 23. Fragmentation of peptide bonds by ionization.
N-terminal fragments are represented by A, B and C species, C-terminal fragments are represented by X, Y and Z.
Figure 22. Total Ion Chromatogram of Spot 7. Main peaks corresponding to peptides are shown.
Using IMAC-HPLC-ESI-MS, two definite sites of Pim-1 autophosphorylation were identified, and one additional autophosphorylation site was suggested. A tryptic peptide corresponding to amino acids 185-195 (LIDFGSGALLK), containing the phosphorylated residue Ser-190, was identified in sample 1 (Fig. 24). The mass spectrum shows the singly charged species [M+H]+, the sodium adduct [M+Na]+, the doubly charged species [M+2H]2+, as well as the fragmentation products y7, y8, y9 and y10. A tryptic peptide corresponding to amino acids 196-206 (DTVYTDFDGTR) was identified in sample 7 (Fig. 25). Although this peptide contained four phosphorylatable residues, sequencing of C-terminal fragments indicated that the only residue phosphorylated was the Thr-205.

A third site of Pim-1 autophosphorylation was suggested, Ser-4, in a tryptic peptide corresponding to the last two residues of GST, and the first 5 residues of Pim-1 (GSMLLSK) (Fig. 26). This sample also contained a peptide and peptide fragments corresponding to amino acids 56-60, of GST, PYYID. Although this second peptide was not phosphorylated, it may have been retained on the IMAC column by interaction with the other bound peptides.

An additional autophosphorylation site was identified in the GST portion of the fusion protein, Thr-17, corresponding to GST residues 10-20 (IKGLVQPTRLL) (Fig. 27). The GST phosphorylation site was unexpected, as earlier experiments (Figures 16 and 17) demonstrated that GST was not phosphorylated by either H. sapiens or X. laevis GST-Pim-1. It is possible that this phosphorylation resulted either from the in vivo activity of a bacterial kinase or results from a strictly intramolecular reaction.

\[
\begin{align*}
\text{An} & \quad \text{Bn} & \quad \text{Cn} \\
\text{CH}^{-} & \quad -\text{CO}^{-} & \quad -\text{NH}^{-} & \quad --\text{CH}^{-} \\
\text{Pr} & \quad \text{Xn} & \quad \text{Yn} & \quad \text{Zn} & \quad \text{Pr}^{-1}
\end{align*}
\]
Figure 24. Mass spectrum of an 185-195, LIDFGSGALLK.

- $\text{[M+2H]}^2+$
- $\text{[M+H]}^+$
- $\text{[M+Na]}^+$
- Y7, Y8, Y9, Y10

Sequence: LIDFGSGALLK

(M+PO3H2)
Figure 25. Mass spectrum of aa 196-206, DTVYTDGDGTR.
Figure 26. Mass spectrum of GST-Pim-1 peptide, GSMLLSK.
Figure 27. Mass spectrum of peptide GST aa 10-20, IKGLVQPTRLL.
b. Analysis of trypsinized GST-Pim-1 by LCMS

To determine if the autophosphorylation of GST-Pim-1 detected was the result of an in vivo or an in vitro event, Pim-1 was analyzed after extraction and purification from the bacteria and prior to autophosphorylation. GST-Pim-1 was thrombin-treated to remove the GST, trypsinized, then the entire sample loaded onto the IMAC column and analyzed by liquid phase mass spectrometry (LCMS). Phosphopeptides containing the phosphorylated Ser-190 site (m/z 1213) as well as the dephosphorylated form (m/z 1133) were identified. This implies that the Pim-1 is autophosphorylated in vivo, in the bacteria. The exact amount of phosphorylation by the time of purification was not established as the LCMS treatment causes removal of the phosphate group, but over 50% of GST-Pim-1 was estimated phosphorylated on Ser-190 (data not shown).

A modification of this method was attempted to specifically identify tyrosine phosphorylation sites, using HPTPB to selectively dephosphorylate phosphotyrosine-containing peptides which were then analysed by LCMS. This exploited the fact that dephosphorylation would cause a reduction in the atomic mass of a peptide, and thus would appear shifted in an LCMS chromatogram as compared to non-dephosphorylated peptide. Although several peaks that underwent a shift with HPTPB treatment were identified in both Pim-1 samples (those that had undergone in vitro autophosphorylation as well as those which had not) the peaks did not relate to tryptic peptides of Pim-1. However, all peptides contained a common Tyr-133 site, which may be a potential autophosphorylation site. No other data were obtained to confirm this result.

All the identified autophosphorylation sites of Pim-1 are shown in Figure 28.
Figure 28. Autophosphorylation sites of *X. laevis* Pim-1.
Protein sequence alignments of Pim-1 from *X. laevis*, mouse, rat, and human showing autophosphorylation sites identified by IMAC-HPLC-ESI-MS. Autophosphorylation sites Ser-4, Ser-190 and Thr-205 are boxed, the number of the residue is indicated above the site. The suggested Tyr-133 site is also shown. Roman numerals indicate protein kinase subdomains. Residues that are common to all protein kinases are shown in bold type.
3. IDENTIFYING OTHER KINASES WITH SIMILAR PHOSPHORYLATABLE RESIDUES

Protein sequences were examined to identify kinases with phosphorylatable residues in the same locations as the Pim-1 autophosphorylation sites, as these enzymes may also autophosphorylate at these sites and may possess similar modes of regulation as the Pim-1 [Hanks, 1993]. Kinase catalytic subdomains containing homologous phosphorylatable residues are shown with surrounding residues. The names of the kinases are shown in the text below the figures.

The Ser-190 site was very easy to identify in other kinases, as it immediately follows the conserved DFG region in domain VII (Fig. 29). Sequence comparisons of catalytic domain VII indicate that most other kinases contain hydrophobic methionine or leucine residues at this site. The Thr-205 site was also easy to examine, as it is located seven residues upstream of the conserved glutamic acid residue in domain VIII. This site is conserved as either a serine or a threonine residue in most kinases, so only a few kinases are listed in Figure 30. This site undergoes autophosphorylation in other protein kinases such as Rsk. The suspected Tyr-133 autophosphorylation site in domain V of GST-Pim-1 is shown with several other kinases having similar tyrosine residues in Figure 31.

Many kinases containing homologous phosphorylatable residues to the Pim-1 are homologues of glycogen synthase 3 including the rat GSK-3α/β, S. cerevisiae MCK-1, Drosophila sgg/zw3, Arabidopsis ASK-α/γ, and the product of the S. cerevisiae MDS1 gene. Kinases homologous to cdc/CDC28 family include the KNS1 gene product and the human Clk protein kinase. Of interest is the fact that many of these kinases, like Pim-1, have been reported to possess both tyrosine and serine/threonine autophosphotransferase activity including the ASKα/γ, Clk, and MCK1/YPK1 gene products. Several of the kinases identified as having homologous phosphorylatable residues were also previously identified as having a high overall sequence homology to the Pim-1 including tsk-1, ASFV and p78.
<table>
<thead>
<tr>
<th>Kinase</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pim-1 (Xen)</td>
<td>E L K L I D F G S G A L L K</td>
</tr>
<tr>
<td>ASFV</td>
<td>I I K V I D F G S A V R L N</td>
</tr>
<tr>
<td>DM</td>
<td>H I R L A D F G S C L K L R</td>
</tr>
<tr>
<td>GSK-3α</td>
<td>V L K L C D F G S A K Q L V</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>V L K L C D F G S A K Q L V</td>
</tr>
<tr>
<td>sgg/zw3</td>
<td>V L K L C D F G S A K Q L L</td>
</tr>
<tr>
<td>MCK1/YPK1</td>
<td>V L K I C D F G S A K K L E</td>
</tr>
<tr>
<td>MDS1</td>
<td>S L K L C D F G S A K Q L K</td>
</tr>
<tr>
<td>ASK-α</td>
<td>Q V K L C D F G S A K V L V</td>
</tr>
<tr>
<td>ASK-γ</td>
<td>Q V K L C D F G S A K V L V</td>
</tr>
<tr>
<td>PSK-G1</td>
<td>A A R V V D F G S A T F D H</td>
</tr>
<tr>
<td>PSK-H2</td>
<td>G I K V I D F G S S C Y E H</td>
</tr>
<tr>
<td>Doa</td>
<td>D V R L I D F G S A T F D H</td>
</tr>
<tr>
<td>KNS1</td>
<td>E I K I I D F G S A I F H Y</td>
</tr>
<tr>
<td>YAK1</td>
<td>E L K I I D F G S S C E E A</td>
</tr>
<tr>
<td>ELM-1</td>
<td>V A K L S D F G S C I F T P</td>
</tr>
<tr>
<td>Pstki</td>
<td>N A K L T D F G S A R N I N</td>
</tr>
<tr>
<td>Clk</td>
<td>D I K V V D F G S A T Y D D</td>
</tr>
</tbody>
</table>

Figure 29. Kinases with sites homologous to the Ser-190 in kinase catalytic subdomain VII.

Alignment of amino acids 182-195 of X. laevis Pim-1 with corresponding residues of other kinases. The phosphorylation site is in bold, the conserved kinase motif is underlined. Homologous kinases include: ASFV, kinase from African Swine fever virus [Baylis et al., 1993]; DM, myotonic dystrophy kinase [Brook et al., 1992]; GSK-3α/β, rat glycogen synthase kinase-3 [Woodgett, 1990]; sgg/zw3, kinase encoded by Drosophila segment polarity genes [Siegfried et al., 1990]; MCK1, a S. cerevisiae kinase encoded by a meiotic induction gene [Shero and Hieter, 1991]; MDS1 a S. cerevisiae GSK homologue and suppressor of mck1 mutants [Puziss et al., 1994]; A. thaliana ASK-α/γ, GSK-3 homologues, [Bianchi et al., 1994], PSK-G1 and PSK-H1, putative kinases from HeLa cells [Hanks, S.K.]; Doa, the darkener of apricot locus [Yun et al., submitted]; KNS1, a non essential protein kinase homologue [Padmanabha et al., 1991]; YAK1, S. cerevisiae a kinase downstream/parallel to the Ras/cAMP pathway [Garret and Broach, 1989]; PST-K1, a distant relative of c-mos [Lohia and Samuelson, accession L05668], Elm-1, a S. cerevisiae kinase involved in a differentiation pathway induced by nitrogen starvation [Blacketer et al., 1993]; Clk, a human kinase with homology to cdc2-CDC28 [Johnson and Smith, 1991].
Figure 30. Alignments of subdomain VIII of Pim-1 with several other kinases.

Alignment of amino acids 198-214 of X. laevis Pim-1 with corresponding residues of other kinases. The phosphorylation site is in bold, the conserved glutamic acid residue is underlined. Homologous kinases include: ASFV, kinase from African Swine fever virus [Baylis et al., 1993]; the p78 kinase lost in chemically induced human pancreatic tumors [Maheshwar et al., P27448]; tsk-1, murine testes-specific kinase [Bielke et al., 1994]; GSK-3α, rat glycogen synthase kinase-3 [Woodgett, 1990]; KIN1, a S. cerevisiae kinase involved in cell polarity [Levin et al., 1987]; Clk, a human kinase with homology to cdc2-CDC28 [Johnson and Smith, 1991]; YPK1, a S. cerevisiae putative dual specificity kinase [Dailey et al., 1990]; MCK1, a S. cerevisiae kinase encoded by a meiotic induction gene [Shero and Hieter, 1991].

Figure 31. Kinases having a tyrosine residue homologous to Tyr-133 site of Pim-1 in subdomain V.

Alignment of amino acids 127-137 of X. laevis Pim-1 with corresponding residues of other kinases. The phosphorylation site is in bold. Homologous kinases include: ASFV, kinase from African Swine fever virus [Baylis et al., 1993]; the p78 kinase lost in chemically induced human pancreatic tumors [Maheshwar et al., P27448]; ran+, a gene required for normal meiotic division [Mcleod and Beach, 1986]; AKIN10, an A. thaliana kinase related to SNF1 [Le Guen et al., 1992]; SNF1, a S. cerevisiae glycogen repression release protein [Celenza and Carlson, 1986]; KIN1/KIN2, S. cerevisiae genes involved in cell polarity [Levin et al., 1987]; c-Abl tyrosine kinase [Reddy et al., 1983]; ARG, an abl related gene [Kruh et al., 1990] and the PDGF-R [Gronwald et al., 1988].
4. CONSTRUCTION AND BACTERIAL EXPRESSION OF S190 MUTANTS

The S190 site was selected for further study, as its identity was most definitive, and rather unusual in its placement as compared to the Thr-205. PCR-mutagenesis was used to construct two Pim-1 mutants by changing the Ser-190 residue to alanine (S190>A) and to glutamic acid (S190>E). Alanine, a non-phosphorylatable residue, mimicked the unphosphorylated state of the Pim-1, and glutamic acid, a charged acidic residue, was used to partially mimic phosphorylation at this site. The mutant Pim-1 species were expressed as bacterial fusion proteins. The the sites of mutation are shown in Figure 32.

i. Phosphoamino acid analysis of mutants

The mutants were expressed as bacterial fusion proteins, and products of the expected size were immunodetected with X. laevis Pim-1 antibodies by Western blotting analysis (Fig. 33). The autophosphorylation of both mutants was significantly reduced as compared to the WT Pim-1, which confirmed that the S190 site is an autophosphorylation site of Pim-1. Although phosphoamino acid analysis was repeatedly attempted to determine if the proportion of phosphoserine was reduced, autophosphorylation of the mutants did not allow enough isotope to be incorporated into the protein to allow analysis by this method.

ii. Specific activity determinations

The specific activity of autophosphorylation of the mutants was analyzed and found to be reduced in comparison to that of the wild-type X. laevis Pim-1 (107 pmol.min⁻¹mg⁻¹). The specific activity of S190>A was determined to be 4.9 pmol.min⁻¹mg⁻¹ (Fig. 34a) and the specific activity of S190>E was determined to be 5.2 pmol.min⁻¹mg⁻¹ (Fig. 34b).

In summary, the specific activities of autophosphorylation of the S190 mutants were reduced to about 5% of the activity of the WT GST-Pim-1. The differences in the specific activity determinations between the S190>A and S190>E mutants were not significant. These findings confirm that S190 was indeed one of the major sites of autophosphorylation, since autophosphorylation was reduced in these mutants.
Figure 32. **Nucleotide sequence of coding region of X. laevis pim-1 showing expected location of mutations.** Amino acid sequence is shown above nucleotides, residues changed by site directed mutagenesis shown in bold type. The * indicates the ClaI site where the two PCR fragments were ligated together.

```
MLLSKFGSGLAHICNPSNMEHLPVK
ATGCTTCTCTTCAAATCGGATTGGCTGCTATATCTGACACACAGGACGGCATCATCCGGGTGAG
72
ILQPVKDKEPEFKKYQVCGSVVGS
ATCTTACAGGCAGATGGAAGCGCGATCCCTCCAGAGGTAGTATCAGGGTGGGCTGCTTGGGCA
144
GGFGTVYSGSRIDAGQQPVAVKHVA
GGTGGTTTCCGCACGGGTATCTGGGCAATGGGACAGGCCTGCGCTGATGGAAGCAGCTAG
216
KERVTEWCTLNGVMVPLEIVLLKK
AAGGAGAGAGCTACAGAATGGGCACCTTGGAGATCTATCAGGGGATATCTGAACGGGAC
288
VPTAFRGGVNLDDLWDYERPDFAFLIV
GTGCCACCGCTCTCCAGGGAAGTAATCAACACTTGGGTATGGAACAGCTATCCATAGAAG
360
MERPEPVKDLFDYITEKGPPLDEDT
ATGGAAGAGACCAGCGCTGAGATCTATCAGGGGAAGTATCTTCCATGGAAGCAGGACAC
432
ARGFFRQVLEAVRHCYNCGVHVRD
GCCGCACCCCTTCCGACCCGCTGAGATCTATCAGGGGAAGTATCTTCCATGGAAGCAGGACAC
504
IKDENLVDTRNGELKLDFGSGA
ATCAAGGATGAAACAATTGGGCGCTGAGACGCCGGAAGGAACTGAAACTGAT*CGATTTTGGCTCCGGGC
576
S to A mutant: GCC
S to E mutant: GAA

LLKDTVYTDFFDGTAVYSSPEWVRY
CTACTCAAGGATACGTTTATCAGGATGGAAGCGAGATCTATCAGGGCCTCGGTCAC
648
Y to E mutant: GAA

HRYHGRSATVWSLGLGVLLYDMVYGDCACAGATCACGAGATCAGCAACCGTTGCTTGGGCTTATCAGATACGGGAT
720
IPFEQDEEIVRVRDLCFRRICSTEC
ATTCCTTGGACAGAAGAGATTTGCTGTCGCTTCTTTCTAGCAGTGTGCTTACGGGATCT
792
QQLIKWCLSRLPSPDRPTLEQIFDHCAGCAACTTCATCAAATGAGTCCCGCTTCTCTGTAGACCCACACTTTGACG
864
PWMCKCDLVRKSEDCLDLRLLRTIDNDCCCTGGAGTAGCTACAGGGCGACCTCTTGAGAATTAGCTAAGCAGCAATTGCAAG
936
GCSCTCSSNELL
TCATCAGACGACAGCTACAGGAGGAGCTCG
969
```
Figure 33. Comparison of mutant and wild-type GST-Pim-1. X. laevis GST-Pim-1 mutants S190A (lane 1), S190E (lane 2) and wild-type (lane 3) were subjected to an in vitro autophosphorylation reaction then analysed by SDS-PAGE. Panel A is a Western blot probed with Pim1-XI antibody, panel B shows an autoradiogram of Panel A, exposed for 64 h. Panel C shows a silver stain of the mutants. Approximately 0.33 ug of protein were used for each reaction. Migrations of Mr standards are shown on the left.
Figure 34. Specific activity of autophosphorylation of S190>A and S190>E mutants of Pim-1.

The pmol of [$\gamma^{32}$P] incorporated per mg of GST-Pim-1 was plotted against the time of the assay. The slope, as calculated by the equation, is the specific activity. Panel A is the specific activity determination of the S190>A mutant, panel B is the specific activity determination of the S190>E mutant.
iii. Exogenous kinase activity of mutants

To assess whether the exogenous phosphotransferase activities of the Ser-190 mutants were different from the WT, peptide substrate assays were done using P4 peptide as a substrate. The $K_m$ and $V_{max}$ values of the mutants for the P4 peptide are shown in Table 11. The apparent $K_m$ values were similar between S190>E and the WT, indicating that the replacement of a serine to a glutamic acid did not change the affinity of the enzyme for the substrate peptide. The $K_m$ value for the S190>A mutant was much lower than with the WT, indicating that the mutant has a higher affinity for the substrate peptide. The $V_{max}$ value of both S190>A and S190>E are similar and much lower than the $V_{max}$ value of the WT, indicating that mutations of the Ser-190 site caused a reduction in activity as compared to the WT.

5. CONSTRUCTION OF ADDITIONAL MUTANTS

The Tyr-199 site has been suggested as an autophosphorylation site of Pim-1, based on its homology to the Src autophosphorylation site, Tyr-416 [Cooper and MacAuley, 1988]. A Y199>E mutant was attempted as described in the Methods. Sequencing of the completed mutant revealed that gene duplication had occurred during the PCR mutagenesis. As advanced analysis of Pim-1 by Dr. Lawrence Amankwa failed to identify the Tyr-199 site as being phosphorylated, we elected not to continue construction of this mutant.
<table>
<thead>
<tr>
<th></th>
<th>Vmax (pmol.min⁻¹.mg⁻¹)</th>
<th>Kₘ (µM)</th>
<th>Vmax Kₘ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>124</td>
<td>35</td>
<td>3.5</td>
</tr>
<tr>
<td>S190&gt;A</td>
<td>17</td>
<td>5</td>
<td>3.4</td>
</tr>
<tr>
<td>S190&gt;E</td>
<td>16</td>
<td>42</td>
<td>0.4</td>
</tr>
</tbody>
</table>

**TABLE 11.** ACTIVITY OF SER-190 SITE MUTANTS TOWARD P4 PEPTIDE.
6. CONCLUSIONS OF PIM-1 AUTOPHOSPHORYLATION

Phosphoamino acid analysis of the human and amphibian bacterially-expressed GST-Pim-1 (Chapter IV) revealed that the proteins autophosphorylated on serine, threonine and tyrosine residues. Construction of a kinase dead mutant confirmed that this activity was due to autophosphorylation and not due to the activity of contaminating kinases. Neither autophosphorylation nor dephosphorylation seemed to affect the exogenous phosphotransferase activity of GST-Pim-1. Although there did appear to be a slight reduction in autophosphorylation activity after phosphatase treatment, this could have been due to residual phosphatases in the preparation.

The majority of samples analyzed by IMAC-HPLC-ESI-MS did not contain peptides that corresponded to expected tryptic peptides of GST-Pim-1. This was not surprising, as phosphopeptides may have been purified in amounts too small to be detected or else underwent massive degradation during the analysis procedure. Although the resolution of phosphopeptides by 2D mapping was quite effective, TIC revealed that most samples analyzed contained several peptides that were retained on the IMAC column. Non-phosphorylated peptides might have been non-selectively retained on the IMAC due to the presence of highly acidic residues or by interactions with the selectively retained phosphopeptides. Partial fragmentation bombardment of the peptide during the ionization process in the ion source during mass spectrometry allowed sequencing through the peptide, confirmed the identity of the peptide and allowed identification of the exact site of phosphorylation.

Many of the samples analyzed contained peptides with the Ser-190 site, which indicated that the large numbers of spots on the 2D map resulted from incomplete tryptic digestion and did not represent a large number of different autophosphorylation sites. Peptides containing the S190 site were also identified by LCMS analysis serving to confirm that the main site of phosphorylation of Pim-1 was S190. This site is conserved in all other Pim-1 homologues as well as in several other kinases and may be a physiological site of autophosphorylation.

The importance of the Ser-190 site was investigated using mutants generated by PCR-site directed mutagenesis and expressed in bacteria. Reduced autophosphorylation of the mutants indicate that the Ser-190 was a major autophosphorylation site. It was not determined in the reduction of autophosphorylation was due to the removal of the
phosphorylatable residue or due to a reduction in the activity caused by a conformational change. The S190>A mutant had a higher affinity (apparent Km) for the P4 peptide substrate that the wild-type or the S190>E mutant, indicating that replacement of the serine for an alanine induced some kind of change in the peptide binding site. Despite the increased affinity for the peptide substrate, the S190>A mutant had a 7-fold reduction in catalytic activity compared to wild type Pim-1. This implies that autophosphorylation at this site may be activating. The S190>E mutant displayed a similar affinity for the substrate peptide as with the WT mutant, but also had an 8-fold reduction in activity. Presumably the glutamic acid residue was only able to partially mimic a phosphorylation event.

The other autophosphorylation sites of Pim-1 identified using IMAC-HPLC-ESI-MS analysis of tryptic phosphopeptides were Ser-4 and Thr-205. The Ser-4 phosphorylation site is also conserved in all other Pim-1 homologues, but as it is outside of the catalytic domain, it was difficult to compare to other kinases. Phosphorylation sites are often located near the N- and C- termini of proteins as these regions are generally located at the surface of proteins and are often more accessible. It is not known if phosphorylation of this site has any physiological importance. The functional or physiological importance of the Thr-205 site was not assessed by mutational analysis. In contrast to X. laevis GST-Pim-1 the majority of autophosphorylation of H. sapiens GST-Pim-1 is located on threonine residues (Fig. 20), so it is possible that this Thr-205 site may represent the physiologically most important autophosphorylation site, at least in the human enzyme.

Although the PAA indicates that GST-Pim-1 autophosphorylated on tyrosine residues, we were unable to unequivocally identify a tyrosine phosphorylation site by IMAC-HPLC-ESI-MS. The presence of tyrosine autophosphorylation sites were examined using the novel approach of identifying peaks that were shifted in mass and retention time after treatment with the tyrosine-specific phosphatase HPTPB. Although we were able to observe peptides that shifted in LCMS retention time after HPTPB treatment, these peptides were difficult to relate to the Pim-1. A tentative site, Tyr-133, was suggested by this method. However, we were unable to confirm this result. This site was not conserved in the other pim-1 cognates and therefore was unlikely to be a physiologically important site for the modulation of Pim-1 activity. As well, we have shown that human GST-Pim-1 is not immunodetected by anti-phosphotyrosine residues, which confirms that this tyrosine autophosphorylation may be species-specific, unlikely in such a conserved kinase, or may even be an artifact of bacterial expression. For this reason, this site was not explored further by mutational analysis.
Results of the mutant studies combined with the fact that over 50% of GST-Pim-1 is phosphorylated prior to purification from bacteria would suggest that autophosphorylation of the Ser-190 site is an activating event. This does not preclude the requirement of other protein subunits for the regulation or localization of Pim-1 activity. This will be considered in greater detail in the discussion.
PART 3 - RESULTS AND CONCLUSIONS: PIM-1 IN BIOLOGICAL SYSTEMS

CHAPTER VIII.

EXAMINING THE ACTIVATION OF ENDOGENOUS PIM-1 DURING XENOPUS LAEVIS OOCYTE MATURATION

One of the original aims of this research project was to study the changes in Pim-1 during *Xenopus laevis* oocyte maturation. The regulation and role of Pim-1 in cells of the germ line has been relatively neglected in the scientific literature, despite the high level of Pim-1 expression in spermatozoa. The work described in Chapters IV-VI was initiated in part to develop reagents to assist in the assessment of Pim-1 in the oocyte system. Not only were new antibodies developed to specifically recognize *X. laevis* Pim-1, but peptides were also constructed as substrates to measure the activity of this kinase.

The studies involving bacterially-expressed GST-Pim-1 were very productive. However, it was unclear how these findings applied to the endogenous kinase. It was our intention to exploit the *X. laevis* oocyte system not only to study the Pim-1 during maturation, but to hopefully confirm the earlier results obtained with the bacterially-expressed kinase.

1. DETECTION OF ENDOGENOUS PIM-1 IN XENOPUS LAEVIS OOCYTE EXTRACTS

As described in Appendix II, *X. laevis*-specific Pim-1 antibodies, GXP and Pim1-XI, were developed. Both of these antibodies recognized the bacterially expressed *X. laevis* GST-Pim-1 and detected proteins in crude *X. laevis* oocyte homogenates on Western blots. Unfortunately, not only did these antibodies fail to successfully immunoprecipitate the endogenous Pim-1, but they recognized different proteins on Western blots.

Homogenates from a *X. laevis* oocyte maturation time course were screened with both GXP and Pim1-XI antibodies (Fig. 35). Although the gels were obviously overloaded, a very strong 42 kDa band was detected in all samples with the GXP antibody. Because of the large amount of protein present, it was difficult to assess if the quantity or size of the protein changed during the maturation. The immunoblot probed with the Pim1-XI
antibody fared slightly worse, with a high degree of non-specific binding of the antibody to the blot. In this case it is impossible to assess which band corresponded to Pim-1.

Inhibition of antibody binding with expressed GST-Pim-1 was attempted to confirm the specificity of binding of the antibodies to Pim-1. Inhibition by GST-Pim-1 profoundly changed the pattern of immunoreactivity of the blot, so it was difficult to determine which of the proteins was specifically Pim-1. The most notable changes involved the competitive inhibition of a 42 kDa Pim1-XI immunoreactive band and a 44-46 kDa GXP immunoreactive doublet (data not shown).
Figure 35. Immunodetection of Pim-1 in homogenates of a X. laevis oocyte maturation time course. X. laevis oocyte time course homogenates were Western blotted and probed with Pim-1-XI and GXP antibodies. The numbers below the blots show the time course of maturation in hours from 0 (immature oocytes) to 7 hours post-progesterone. GVBD occurred at 3.5 hours. The antibodies used are indicated above blots, and the migrations of Mr standards are shown on the left. The arrow indicates an approximately 42 kDa protein that may correspond to Pim-1.
2. ANALYZING ACTIVATION OF PIM-1 DURING OOCYTE MATURATION

i. Peptide substrates

The activation profile of Pim-1 during oocyte maturation was examined using peptide substrates. Immature and mature *X. laevis* oocytes were subjected to by MonoQ column chromatography and the fractions were analyzed by kinase assays. Initially, histone H1 was used as a substrate, until a series of Pim-1-specific peptide substrates were developed. The S6, P3 and P5 peptides were used to distinguish between the activities of S6 kinase and Pim-1. No difference was observed in the activation patterns as detected using these peptides, so for later experiments only P4, the optimal Pim-1 peptide substrate peptide, was used.

Preliminary studies detected a large peak of peptide P4 (AKRRRLSA) phosphorylating activity that eluted with 0.3 M NaCl (fractions 24-25) and a second minor peak was eluted with 0.35 M NaCl (fraction 29) (Fig. 36). Maturation induced-activation of the first peak was 1.3 X that of the control, and maturation induced-activation of the second peak was 4.3 X over the control.

Due to the promising results obtained between immature and mature *X. laevis* oocytes, a time course study of P4 phosphorylating activity during *X. laevis* oocyte maturation was undertaken (Fig. 37). A significant increase in total P4 phosphotransferase activity did not occur with oocyte maturation, the peak of P4 phosphorylating activity shifted radically between time points and there were not trends in activation discernable. For this reason, this approach was discontinued.

ii. Immunoreactivity of oocyte maturation time course

Western blots to analyze the Pim-1 protein at each time point of the time course were probed with the Pim1-XI antibodies (Fig. 38). ECL was used to visualize the blots, as we hoped that a more sensitive detection system would allow distinction of the antibody band from the background. In the blots for the time points shown, there was a strong 45 kDa immunoreactive band in fractions 24-26, and a slightly higher band in fractions 26 to 30. The bands were shifted slightly between one fraction and another in the different time points; this is consistent with the elution of the peak fractions as observed in Figure 37. Reprobing the blots with GXP antibody detected a single 40 kDa band in lanes 22-24. The proteins detected by the two antibodies eluted in different fractions (data not shown).
To try to better assess changes between the different time points, fractions 25, 27 and 30 from each time point were analysed by Western blotting with various Pim-1 antibodies (Fig. 39). The Pim1-XI antibody detected a series of proteins with Mr from 42-48 kDa in all fractions. The differences in these proteins between time points were too profound to establish actual changes in protein quantity and size occurring during maturation and were probably a consequence of the peak shifts in the Mono Q elution. Reprobing the Western blots with the GXP demonstrated that some proteins immunoblotted with both antibodies. However, it was not possible to clearly establish whether changes occurred in these proteins during the maturation process.
Figure 36. P4 peptide phosphorylation by fractionated immature and mature *X. laevis* oocyte extracts.

Immature and mature (5 hours) *X. laevis* oocyte extracts were fractionated by MonoQ column chromatography and used to phosphorylate peptide P4 in the presence of [γ-32P]ATP. The enzyme activity of the mature extracts in fractions 22-32, as measured by the activity under the graph, was 1.8X that of the immature extracts. GVBD occurred at 3.5 hours.
Figure 37. P4 peptide phosphorylation by fractionated X. laevis oocyte maturation time course. X. laevis oocyte extracts were fractionated by MonoQ column chromatography and used to phosphorylate peptide P4 in the presence of [γ-32P]ATP. The time points used are shown in the legend. GVDB occurred at 3.5 hours.
Figure 38. Western blots of fractionated *X. laevis* oocyte extracts. *X. laevis* oocyte extracts from various time points were fractionated by MonoQ column chromatography. Western blots of selected fractions (fraction numbers shown below blots) were probed with Pim1-XI antibody. GST-Pim-1 was used as a positive control (on right). Migration of Mr standards are shown on the left. Although the time course was performed from 0-6 hours, only selected time points are shown. Fractions highlighted by stars (25, 27, 30) were used for a comparison of time points (Fig. 39). GVBD occurred at 3.5 h.
Figure 39. Western blots of selected MonoQ fractions of X. laevis oocyte time course.
Time points from a X. laevis oocyte maturation time course were fractionated by MonoQ column chromatography. Selected fractions were Western blotted and probed with the Pim1-XI and the GXP antibodies (labelled above panels). The time points are shown below blots and are expressed in hours. Note: Time point 3 contained almost no protein. GVBD occurred at 3.5 hours.
iii. Summary of *X. laevis* maturation time course results

It was hoped that *X. laevis* oocyte maturation would be an ideal system in which to study the activation of Pim-1 protein, but we experienced several problems with this system and the methods of analysis. First of all, there was ambiguity about which of the immunodetected proteins actually corresponded to Pim-1, as both antibodies detected different bands in the *X. laevis* oocyte extracts. By fractionating the extracts, we hoped to establish which band was Pim-1. Unfortunately, our antibodies detected proteins of the appropriate size range in slightly different fractions. Some of the same proteins were detected by the different antibodies, but it was still a "pick and choose" situation and the uncertainty as to the assignment of which of these was Pim-1 was not resolved.

Secondly, the assessment of Pim-1 activation was difficult because the P4 peptide used to analyze the different fractions was also a substrate for other protein kinases beside Pim-1. As we did not know if the P4 peptide phosphotransferase activity observed arose from Pim-1 or from an other kinase, we hoped that immunodetection by Pim-1-specific antibodies would facilitate identification of which fraction contained Pim-1. Unfortunately, there were immunoreactive bands in the expected size range present in most of the fractions analyzed. Shifting of the peak P4 phosphorylating fractions from one MonoQ elution to another also made analysis of results difficult. Minor shifts (1-3 fractions) in protein retention might be expected to sometimes occur between immature and mature extracts, as proteins that become phosphorylated during the course of activation sometimes experience slight differences in adhesion to the MonoQ matrix. Consistently cleaning and washing the column between uses did not prevent the minor shifting from occurring. As varying characteristics of different Mono Q columns could profoundly influence the outcome of the experiment, all samples in a set were analyzed on the same day with the same Mono Q column.

The *X. laevis* oocyte extracts displayed some biological inconsistencies, possibly due to the frogs themselves (age, health, diet, seasonal). The number of oocytes from a given frog varied immensely, as did the proportion of eggs in the different developmental stages, which was reflected by the differences in time for GVBD between the eggs from different frogs. In some sets of oocytes, GVBD would first begin to appear after 3 hours of progesterone treatment and by 6 hours, a large percentage (65%) of stage VI oocytes would mature. Sometimes only 10% of oocytes would experience GVBD. In other sets of oocytes, GVBD would first occur at 8 hours and by 16 hours, 50% of the oocytes underwent GVBD. Yet in other experiments, the oocytes would just mottle and GVBD
would not occur at all. As the treatment of the oocytes was consistent as possible for more than 20 different frogs was studied, these differences may be due to the genetic or seasonal differences of the *X. laevis* population.
3. CONCLUSIONS

The determination of the regulation of endogenous Pim-1 during *X. laevis* oocyte maturation was not very successful due to inconsistencies of immunodetection with the two antibodies and to difficulties with the *X. laevis* oocyte system. The two different *X. laevis*-based Pim-1 antibodies immunodetected different proteins in fractionated extracts causing some concern about antibody specificity. Although there seemed to be a slight activation in Pim-1 activity as measured by P4 peptide phosphorylation, it is not entirely certain if this activity was due to Pim-1 or to another kinase.

Preliminary experiments were performed to identify proteins in the *X. laevis* extracts that interact with Pim-1. These experiments, Fusion protein affinity columns and Far Western blotting, are detailed in Appendix VIII. Unfortunately, these methods were not very fruitful in this study. Although proteins of 25 and 40 kDa from *X. laevis* oocyte extracts bound to and were phosphorylated by the human GST-Pim-1 matrix, they were not observed when *X. laevis* oocyte extracts were used to bind to *X. laevis* GST-Pim-1. These techniques might be applied in the future when the identity of a downstream target is suspected.

In conclusion, the *X. laevis* oocyte system was not a good model for the study of Pim-1 activation. The variable results that were consistently obtained with this system are in contrast to the reproducible results obtained with the sea star oocyte system. The peptide substrates and reagents developed for use in this system seem to work well in other species. As the results from the different batches of *X. laevis* oocytes varied considerably, and because intensive amounts of work was required to obtain small numbers of oocytes, this system was impractical and not recommended for further study.
CHAPTER IX.

EXAMINING PIM-1 ACTIVITY IN MATURING PISASTER OCHRACEUS OOCYTES

The Pisaster ochraceus (the purple sea star) was selected as a second model system in which to study the role of Pim-1 in oocyte maturation. An advantage of this system is that the immature oocytes are uniformly arrested at the beginning of prophase of the cell cycle, and can be induced to resume meiotic maturation by the addition of 1-methyladenine. Germinal vesicle breakdown (GVBD) typically occurs about 70 minutes following the addition of 1-methyladenine to the P. ochraceus oocytes. Unlike the X. laevis oocyte system, ample quantities of sea star oocyte homogenates can be obtained for purification purposes. Additionally, the echinoderm oocytes can easily be fertilized in vitro, and the relatively large size of the oocyte is ideal for microinjection.

1. PARTIAL CLONING OF SEA STAR PIM-1 BY PCR, AND COMPARISON TO PUBLISHED SEQUENCES

To validate the study of Pim-1 in sea star, we initially exploited PCR techniques to confirm the presence of this kinase in this primitive organism. Oligonucleotides based on the nucleotide sequence of mammalian pim-1 were successfully used to amplify a part of the pim-1 coding region from P. ochraceus cDNA using PCR. The fragment was subcloned into the XL1-blue plasmid and sequenced. The partial nucleotide sequence is shown with the translated amino acid sequence in all three reading frames (Fig. 40). Sequencing of this fragment was carried out until the identity of the fragment was confirmed as being a homologue of pim-1. The alignment of translated regions of the sea star pim-1 with the mammalian and X. laevis amino acid sequences is shown in Figure 41.

This sea star pim-1 PCR fragment was used as a probe to screen three P. ochraceus libraries that were in the lab. One library was labelled simply "P. ochraceus", while the two libraries obtained from Dr. Michael Smith (Simon Fraser University) were P. ochraceus oocyte (2 x 10^8 pfu/ul) and testes (3x10^6 pfu/ul) cDNA libraries in λgt10 (3x10^6 pfu/ul). The oocyte library was retitred at 3.5 x 10^6 pfu/ul and the testes library was retitred at 5.23 x 10^4 pfu/ul. The libraries were plated and screened several times, with little success; the fact that both libraries had been reduced to less than 2% of the original titre indicates that the libraries had probably both undergone degradation due to improper
storage or DNAse contamination. Screening was discontinued due to the questionable quality of the libraries.

The success in amplifying a fragment of pim-1 from sea star cDNA was due to a high level of pim-1 conservation that extends to species as diverse as sea star and X. laevis. In contrast, we were unsuccessful at amplifying raf-1, another oncogene-encoded serine/threonine kinase, from sea star cDNA, also using oligonucleotides based on the mammalian raf-1 sequence. Apparently, pim-1 is more highly conserved between sea star and mammals than raf-1 is.
Figure 40. Partial nucleotide and amino acid sequence of sea star Pim-1.
Partial nucleotide sequence of sea star Pim-1 showing translated regions in three reading frames. Nucleotides in brackets represent uncertain residues. The symbol "@" represents a stop codon.

```
CCCCCCTGACCCTGCTCGAGGCAGCCTGGGAAAGGAGAGAA (A/G) GAGCCXTTC (C) (G) GAAAA
PP @ PGLEAPGKKEK EFFFFRRK
PPDPGRMRGRKRSGS?SGK
PLTRARGAGEGERGRA?PEK

GACGTATGCAACCTGCTCCTGCTCGAGCAGGAGCTTGGACCGCCACCTACCTCGGGGACTCCG
RRMQRVSCHLGAEVLPFTRALDVCNRFATAWEARFRWDRLGLGHS
TYAIGSLLGSGGFVGTYGS

AATCAGAGACATTTGCGTGTTTACATCAAGCTCCTTGAC (G/C) AAGAAAAAGTGAACGATT
ESETICRLLSLSL@PRKKK@TI
NQRQRPAGCYYACDE/QRKSERLFRDNLPVAJKLVTEKEKVND.

GGAACATGATTAATGGACAGAAAGTCTCTCTAGA (A) GT (T) CATCTCTGAGAAGGTGAC
GGTQLMDRFKFL@KFISQ@$RRST
EHY@WTESSSRSRSSPEEGRWNMINOGKVPLEVHLLKKKVND

CACATAACGATGTATAAAGATGCTGATATCATGATGGGGCAGACAATTATATCATCG
TYRCLKMLDPYDPRADNFIIT
PHTDVMRRCWISMIGQTILSS
HIPMYKDAGYLO@GGRQFFHHR

(A/T) CCATGGAACGCCCACCGGACCTGCAAAGGACTTATTTGATTTTCATCCGGAGAAGTGCGG
(D/V) HGTAPNLQRIMITISSPRVGG
S/TEMERPRTKGLIFHHRERWAPWNCPEPAKDLDFFIDESG
```
Fig. 40. (Continued).

CCGC (A/T)GGGAGGAGACAGAGCAGAAATTTTCCACCAAGTTGTAGAGACGAAGATTCGCTGCT
P Q/L G E E T D E S F S T K L @ R R L R L R L
R R/W E R R Q T K V F P P S C R D D F G
P A G R G D R R K F F H O V V E T T S A

ACGGTGCCACGAGGCTGGTGTTCCTCCACAGAGACCTCAAAGACGAGAACATTCCGGTCGATCT
R C H E A C V L H R D L K D E N I P V D L
Y G A T R L V F S T E T S K T R T F R S M
T V P R G W C S P Q R P Q R R E H S G R S

CTCAAAAACCTGAGACCT (T/C)AAACTCATCGACTTTTGATCGGCCTATTCTCAAAGATAC
S K L E T L/S N S S T L D Q A L F S K I
S Q N W R P Q/@ T H R L W I R R Y S Q R Y
L K T G D L K L I D F G S G A I L K D T

CGTCTACAAAGATTTTCGATGGTACCTCGTGTGTAACGCACCAAGAGATGGAGATTCGATCCATCG
P S T K I S M V L V C T A H Q S G F D P I
R L Q R T R W Y S C V Q P T R V D S I P S
V Y K D F D G T R V Y S P P E W I R S H R

TTACCACGGTCGTCCTGCACAGTCAGGTCGTCCGCGCATCCTGCCTGTATGACCATGAGGCTGATG
V T T V V L P Q S G R W A S S C M T W P V
L P R S S C H S L V A G H P P V @ H G L W
Y H G R P A T V W S L G I L Y D M A C G

AGATATACCCCTCAGAACAGACGGAGAAATCTCAGAGCCGGTGTCAG
E I Y P S N T T R K S R A R V S/R
R Y T L R T R G N L E P G S
D I P F E H D E E I S S P G O.
Figure 41. Comparison of the partial amino acid sequence of sea star Pim-1 with other species. Alignment of the sea star Pim-1 amino acid sequence (amino acids 37-241) with the other known homologues. The sea star open reading frame was taken from Figure 40, and frame shifts have not been indicated. Unknown residues are indicated by question marks (?), stars (*) indicate residues identical between all Pim-1 proteins, and dots (.) indicate residues conserved between all Pim-1 proteins. Roman numerals indicate kinase catalytic subdomains. Residues in bold type correspond to Pim-1 autophosphorylation sites.

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<tr>
<td>PIM1 SEASTAR</td>
<td>EKTYAIGSLSSGSFGTYSGLVRDPRLVAIKLVTKEEVDWNMI-NGQKVPLEVHLLK</td>
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<tr>
<td>PIM1c XENLIA</td>
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<tr>
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<td>PIM1c RAT</td>
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<tr>
<td>PIM1 SEASTAR</td>
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<tr>
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2. NORTHERN BLOTTING OF ENDOGENOUS SEA STAR PIM-1

To determine if pim-1 mRNA was expressed in sea star oocytes, the PCR fragment of the *P. ochraceus* pim-1 coding region was used to probe Northern blots of immature sea star oocyte mRNA. The total RNA (Fig. 42, lane 1) contained 1.7, 2.5 and 2.7 kb fragments. The poly(A)+ sample (lane 2) contained only the 2.7 kb species which indicated that only this species was polyadenylated. This 2.7 kb mRNA species was similar in size to that found in other species [Domen *et al.*, 1987; Meeker *et al.*, 1987a]. Although the 2.7 kb fragment in the poly(A)+ lane seemed to be slightly higher than in the poly(A)- lanes, this was probably due to interference by the 2.8 kb tRNA in the poly(A)- samples. The 2.7 kb band present in lane 2 may be poly(A)+ RNA that was not successfully separated from the rest of the RNA. The presence of the 2.6 kb and 1.7 kb bands in the poly(A)- mRNA other bands was unexpected. These shorter transcripts may have resulted from other genes that were highly homologous to pim-1. However, pim-1 has no close relatives and these bands were visible even after stringent washes of the blot. This indicates that these 1.7 and 2.6 kb bands were probably shorter non-polyadenylated versions of the pim-1 transcript, possibly arising as a result of aborted transcription or alternate mRNA splicing. Although no descriptions of pim-1 oocyte mRNA have been published, several other groups have reported finding a shorter, 2.4 kb pim-1 transcript in murine and rat testes [Sorrentino *et al.*, 1988; Wingette *et al.*, 1991]. These blots were not probed with a glycerol-3-phosphate dehydrogenase (GAP-DH) probe to control for the amount of RNA loaded.
Figure 42. Northern blot of sea star RNA.
Northern blot of total RNA (lane 1) and poly(A)+ RNA (lane 2) purified on an oligo(dT) column. RNA was isolated from immature *P. ochraceus* oocytes. Location of 18 and 28s rRNA fragments are indicated by the arrows, migrations of RNA standards are shown on right.
3. EXAMINING CHANGES IN PIM-1 DURING SEA STAR OOCYTE MATURATION

i. Western blotting of Pim-1 from sea star oocyte extracts

As was demonstrated in Appendix II, Pim1-III and Pim1-NT antibodies immunodetected a 42 kDa protein from crude sea star extracts. The antibodies were used to test Mono Q-fractioned sea star extracts, at various time points during oocyte maturation. Initially we probed the Western blots of Mono Q-fractionated immature and mature oocyte extracts with both antibodies, and found a strong, approximately 42 kDa band detected in fractions 25-27 (0.32-0.38 M NaCl) with Pim1-NT, and in fractions 24-26 (0.28-0.32 M NaCl) with Pim1-III (Fig. 43). From this blot, it was difficult to determine if the bands immunodetected were the same size. However, the fact that they were eluted in slightly different fractions strongly implies that the antibodies detected different proteins. The bands were shifted by one fraction from the immature to the mature suggesting that the proteins experience a change during maturation (e.g. phosphorylation) which causes them to be retained on the column. These bands were detected in repeated experiments but there did not seem to be quantitative or qualitative changes in the Pim-1 protein with maturation as evidenced by band shifts.

ii. In vitro peptide studies to examine Pim-1 activation during time courses of oocyte maturation

Changes in endogenous Pim-1 protein during sea star oocyte maturation were assessed by peptide substrate assays of MonoQ-fractionated oocyte extracts. There was some concern that other maturation activated kinases, in particular, S6 kinase, would also phosphorylate the P4 peptide. For this reason, we screened our Mono Q fractions with two different peptides to try to distinguish the activity of Pim-1 from that of S6 kinase. We screened with P3 peptide which was phosphorylated strongly by purified S6 kinase and weakly by GST-Pim-1 and P4 peptide which was phosphorylated efficiently by GST-Pim-1 but poorly by S6 kinase. There was a large maturation-activated P3 activity peak in fractions 27 (0.38 M NaCl), with shoulder peaks at fractions 31 (0.4 M NaCl) and 34 (0.46 M NaCl) (Fig. 44). P4 showed a large maturation-activated peak in fractions 26-27 (0.38 M NaCl) and a shoulder peak in fraction 36 (0.5 M NaCl).

The peak fractions from each time point were Western blotted and probed with both Pim1-III and Pim1-NT antibodies (Fig. 45). The two antibodies detected slightly different bands; Pim1-NT detected a 40 kDa band that eluted in a number of fractions, from 22-26,
Figure 43. Western blot of MonoQ-fractionated immature and mature sea star oocyte extract. Western blots of immature and mature MonoQ-fractionated sea star oocyte cytosolic extracts probed with Pim1-NT antibody, then stripped and reprobed with Pim1-III antibodies. The MonoQ fraction numbers are shown below blots. Immature (I) extracts are on the top, mature (M) extracts on the bottom. Migrations of Mr standards are shown on the left.
Figure 4. Peptide phosphorylation profiles of fractionated sea star oocyte maturation time course. Sea star oocyte maturation time course extracts were fractionated by MonoQ column chromatography and used to phosphorylate substrate peptides P3 (AKRRRASLRA), Panel A or peptide P4 (AKRRRLSA), Panel B, in the presence of [γ-32P]ATP. The bar (-----) indicates fractions containing immuno-reactive protein. Time points tested are indicated in legend in panel A. GVBD occurred at 90 minutes.
Figure 45. Comparison of fractionated sea star oocyte maturation timepoints. Various time points from an oocyte maturation time course were MonoQ-fractionated and electroluted on an SDS-PAGE gel. Western blots were probed with Pim1-NT and Pim1-III antibody. Panel A: immature oocytes; panel B, 15 min; panel C, 30 min; panel D, 45 min; panel E, 60 min; panel F, 90 min; panel G, 120 min. MonoQ fraction numbers are indicated on the bottom of diagram. Migrations of Mr standards are shown on the left.
and Pim1-II seemed to detect a slightly higher molecular mass band of about 42 kDa primarily in fractions 24 and 25. Even though the antibodies detected different proteins, the immunoreactivity of both antibodies corresponded with the minor, non-stimulated P4 kinase activity peak in Mono Q fractions 24-25 (0.28-0.32 M NaCl).

The peak immunoreactive fractions (24-26) from each time point were pooled and Western blotted with Pim-1 antibodies to determine if there are changes in the protein amount or migration during the oocyte maturation (Fig. 46). The Pim-1 antibodies detected slightly different bands; Pim1-NT detected a 40 kDa band strongly and a 42 kDa band weakly, while Pim1-III detected a 40 kDa band weakly and a 42 kDa band strongly. The Lilly antibody visualized only the 42 kDa Pim-1 band. Although there is no easy explanation for why these antibodies have different immunoreactivities, it is possible that there might be two related Pim protein products expressed in the oocyte. Importantly, there was no increase in the amount or size of the immunoreactive proteins in the different time points of maturation.

These results indicate that sea star Pim-1 is present and apparently active in oocyte extracts, but the amount and the size of the protein does not change during the maturation process. There was no significant increase in kinase activity as indicated by the P4 peptide phosphorylation assay. Therefore, Pim-1 does not appear to be regulated during meiotic maturation.
Figure 46. Sea star oocyte time course: peak MonoQ fractions.
Western blots of pooled fractions (24-26) of MonoQ-fractionated sea star oocyte maturation time course. Western blots probed with Pim-1 antibodies indicated below blots in bold type and developed with ECL. Maturation time points (min) are shown below blots. Migrations of Mr standards are shown on right.
4. **SUMMARY**

The sea star oocyte system has potential for future studies with Pim-1, because of high reproducibility of results and large quantities of biological starting materials. Although not enough of the sea star *pim-1* sequence was obtained to warrant a thorough comparison to the mammalian and amphibian sequences, it was enough to confirm that *pim-1* mRNA was expressed in this system. A transcript of the expected size was detected on Northern blots along with two smaller, poly(A)- transcripts of 1.7 and 2.6 kb, possibly resulting from aborted translation or alternative splicing of the mRNA transcript.

Analysis of the sea star oocyte maturation time course with Pim-1 antibodies and peptides indicated that Pim-1 eluted in fractions 24-25 (0.28-0.3 M NaCl), before the main peak of P4 kinase activity. The P4 phosphorylating activity of Pim-1 did not change during maturation. The Pim-1 antibodies immunodetected slightly different proteins from 40-42 kDa, however none of these proteins underwent a change in quantity or size during maturation. The Pim-1 protein seemed to disappear at 6 and 24 hours post-fertilization, reappear at 2 days and disappear again at 4 days post-fertilization. The fertilization studies need to be repeated to confirm these results.

Preliminary work to identify Pim-1 interacting proteins was promising, and has opened some new areas for future study (Appendix VIII). GST-Pim-1 binding proteins in the sea star extracts were not identified by GST-Pim-1 affinity columns and Far Western blotting. Pim-1 kinase (PIK) activity was detected in MonoQ fractions 28-32 (0.35-0.45 M NaCl). This PIK is completely uncharacterized, except for the fact that it undergoes an activation during maturation, unlike Pim-1.
PART 4 - DISCUSSION AND FUTURE DIRECTIONS

DISCUSSION

This study utilized a wide variety of recombinant DNA and biochemistry techniques to examine and characterize the activity of the mammalian, amphibian and echinoderm Pim-1 serine/threonine protein kinases in vitro. Although the findings of this study were obtained from work with expressed enzyme in vitro, these results can be extrapolated to endogenous Pim-1 and can assist in constructing a model of Pim-1 activity in vivo.

The Pim-1 cDNA was cloned from X. laevis, and is the first non-mammalian pim-1 homologue characterized. The cDNA open reading frame encoded a 35-kDa protein containing all the conserved serine/threonine kinase subdomains. The X. laevis Pim-1 has strong sequence homology to the mammalian Pim-1 cognates, implying that the functions of these kinases are highly conserved in all species. The catalytic domain of the sea star Pim-1 was also found to show high homology to Pim-1 of other species, in keeping with this hypothesis.

Although the Pim-1 family members are closely related to each other, they have few immediate relatives. Homology searches identified several proteins sharing some similarity to Pim-1 in the catalytic domain, all of which were serine/threonine kinases. The pim-1 nucleotide sequence is homologous to calcium/calmodulin-dependent protein kinases and phosphorylase b in kinase subdomains V and VI. Pim-1 protein displays homology in the catalytic subdomains with cAMP-dependent protein kinases, calcium/calmodulin-dependent protein kinases, phosphorylase kinases, yeast kinases involved in metabolic control as well as many yet uncharacterized kinases including human p78, the ASFV and tsk-1 proteins.

The structural homology between Pim-1 and these other kinases was high in the catalytic domain, implying that all these kinases may have functional homology. Indeed, the consensus phosphorylation site sequence that we deduced for Pim-1 (K/R-K/R-R-K/R-X-S*/T*-X') resembles the phosphorylation site motifs found for many of the second messenger-dependent protein kinases that have been analyzed [Kennelly and Krebs, 1991]. These include the consensus sequences of calcium/calmodulin dependent protein kinase II (CaMII) (R-X-X-S*/T*), cAMP-dependent protein kinase (R-R/K-X-S*/T*) and cGMP dependent protein kinase (R/K2,3-X-S*/T*). Similarly, the phosphorylation sites recognized by protein kinase C, phosphorylase kinase and the S6
kinase Rsk also feature basic residues on the N-terminal side of the phosphoacceptor residue [Kennelly and Krebs, 1991]. Nonetheless, while Kemptide (LRRASLG) is an excellent substrate for cAMP-dependent protein kinase and Rsk, but it was a very poor substrate for GST-Pim-1. Thus, while there is partial overlap in the substrate requirements of these kinases, they are also distinct.

To study the activity of Pim-1, a concerted effort was made to immunoprecipitate the enzyme from various cell types. However, this did not yield protein with sufficient activity, purity or quantity for kinetic studies. As well, the native Pim-1 has not yet been purified to homogeneity. Both Xenopus laevis and human pim-1 were expressed as bacterial fusion proteins with glutathione S-transferase. Both species were constitutively active and both proteins had auto-phosphotransferase activity and phosphorylated a wide selection of exogenous substrates. Because the substrate specificity of eukaryotic serine/threonine-specific protein kinases is largely determined by the ~250 amino acid catalytic domain [Hanks et al., 1988], the GST protein fused to the amino terminus of the Pim-1 should not have influenced the substrate specificity of the kinase. There is no published evidence for the substrate specificity of a kinase being determined by regions outside of this catalytic domain. However, the catalytic efficiency of protein kinases is often regulated by regions outside of the catalytic domain.

The phosphorylation site consensus sequence for Pim-1 varied slightly between the mammalian and amphibian species. The substrate binding domain is thought to be located in the 60 carboxy-terminal amino acids of the catalytic domain, in kinase subdomains (IV-VI) [Kemp and Pearson, 1990]. As single residues can influence the substrate specificities [Knighton et al., 1991], differences of one or more amino acids within these domains between species could account for minor changes in the substrate consensus sequence for a kinase. This variation in the substrate specificity between species may allow a difference in function of Pim-1 between the two species. Alternatively, the differences observed between X. laevis and H. sapiens Pim-1 may merely indicate that some of the residues surrounding the phosphoacceptor site are not critical for substrate specificity.

Potential physiological substrates containing the K/R - K/R - R - K/R - X - S*/T* - X' motif for phosphorylation by Pim-1 were not immediately obvious. Many of the proteins that featured the Pim-1 phosphorylation site consensus sequence are enzymes or structural proteins, some of which are functional in the cytoplasm. Nuclear localization signals, (NLS) used as targeting signals for nuclear proteins, consist of stretches of basic residues
As well, stretches of basic residues form part of DNA binding domains [Hill and Treisman, 1995]. Regulation of transcription factors is achieved by phosphorylation, which seems to encourage dimerization, nuclear localization and modulate DNA binding activity [Schindler, 1995]. Some of these NLS or DNA binding domains are followed by serine residues, and conform to the Pim-1 phosphorylation sequence. For example, the Calmodulin kinase II δ subunit from rat heart, contains an 11 amino acid insert between the catalytic/regulatory and association domains (VKKRKSSSSS), which conforms to the Pim-1 phosphorylation consensus site [Srinivasan et al., 1994]. Phosphorylation of this sequence is believed to modify nuclear targeting. Similarly, the SV40 T antigen is phosphorylated near the NLS in order to enter the nucleus [Rihs et al., 1991]. In addition, it has been shown that serine phosphorylation of Stat3 by a yet uncharacterized kinase, is necessary for Stat3-Stat3 dimerization and optimal DNA binding [Zhang et al., 1995]. Perhaps Pim-1 functions "late" in signal transduction, regulating transcription factors or other nuclear proteins by the serine phosphorylation of NLS, dimerization or DNA binding domains, to mediate the entry into the nucleus, to promote dimerization or to prevent DNA binding.

Substrate mixing experiments identified many proteins that either phosphorylated Pim-1 or were phosphorylated by Pim-1. Although it is intriguing to speculate that these interactions may have some physiological significance, these reactions involving with mM amounts of proteins may not resemble the in vivo situation.

The main site of autophosphorylation of Pim-1 was identified using IMAC-HPLC-ESI-MS, in collaboration with Drs. Lawrence Amanakwa and Michael Affolter, as Ser-190. The Ser-190 as well as the Ser-4 and Thr-205 residues are conserved in all Pim-1 homologues, suggesting that they may represent physiological sites of autophosphorylation. Many other kinases were identified that contained conserved residues in these locations, including many of yeast kinases involved in cellular metabolism. Most of the cyclin-dependent kinases (CDKs) and cdc homologues contain a threonine residue in catalytic subdomain VIII, which is in the T loop, phosphorylation of which is essential for kinase activity [Morgan, 1995; Pines 1995]. Pim-1 Thr-205 is located in domain VIII, and although it does not seem to align exactly with the Thr-160/Thr-161 residues of CDK2/cdc-2, it may also be have a similar function, to stabilize the structure of the enzyme. Other kinases having activating phosphorylation sites in this domain include MAP kinase (Thr-183, Tyr-185), src (Tyr-416) and cAMP-dependent protein kinase (Thr-197) [Veron et al., 1994].
The Mos serine/threonine protein kinase also contains a major phosphorylation site roughly homologous to the Pim-1 Ser-4 site in the N-terminus, at Ser-3 [Nishizawa et al., 1992; Freeman et al., 1992]. The importance of this site is disputed; one group has determined that phosphorylation of this site is necessary for metabolic stability and for full physiological activity during the cell cycle [Nishizawa et al., 1992]. The phosphorylation is the result of an autokinase event and stabilizes the kinase by preventing ubiquitination of the kinase. In contrast, a second group has determined that the Ser-3 site is phosphorylated by an unidentified protein kinase, and that phosphorylation of this site is not essential for CSF activity or to induce oocyte maturation [Freeman et al., 1992]. The reasons for these discrepancies are not known, but when resolved may provide a model for Pim-1 regulation by phosphorylation/autophosphorylation.

In the search for tyrosine phosphorylation sites, the site corresponding to the Src Tyr-416 autophosphorylation site, i.e. Tyr-199 [Cooper and MacAuley, 1988], was not identified as a site of tyrosine phosphorylation. That we did not detect autophosphorylation of this site does not preclude that it may be an important location of phosphorylation by other kinases. It just means that by our methods, we could not identify it as a phosphorylated residue.

Although the phosphoamino acid analysis indicated that GST-Pim-1 autophosphorylated on tyrosine residues, we were unable to unequivocally identify a tyrosine phosphorylation site by IMAC-HPLC-ESI-MS. The presence of tyrosine autophosphorylation sites were examined using the novel approach of identifying peaks that were shifted in mass and retention time after treatment with the tyrosine-specific phosphatase HPTPB. Although we were able to observe peptides that shifted in LCMS retention time after HPTPB treatment, these peptides were difficult to relate to the Pim-1. A tentative site, Tyr-133, was suggested by this method, but we were unable to confirm this result. This site was conserved only between X. laevis Pim-1 and murine Pim-2 proteins, and therefore was unlikely to be a physiologically important site for the modulation of Pim-1 activity. For this reason, this site was not explored further by mutational analysis. Interestingly, some of the kinases with homologous residues to the Ser-190 site were found to also exhibit dual kinase activity including Clk, ASK-α/γ, and MSDI [Johnson and Smith, 1991; Bianchi et al., 1994; Puziss et al., 1994]. It is not known if the apparent dual specificity of these kinases is physiological or is an effect of the particular expression systems used.
The importance of the Ser-190 site was investigated using mutants generated by PCR-site directed mutagenesis and expressed in bacteria. Reduced autophosphorylation of the mutants confirm that the Ser-190 was the main autophosphorylation site. Both the S190>A and S190>E mutants had a lower phosphotransferase activity (V\textsubscript{max}) towards exogenous substrates than the WT GST-Pim-1, suggesting that a change in the structure of the protein has occurred, inhibiting the activity of the kinase. The S190>A mutant has a much higher affinity (K\textsubscript{m}) for the peptide substrate than the WT GST-Pim-1 implying that a non-phosphorylated residue at this site allows tighter binding of the peptide to the enzyme. The charged glutamic acid of the S190>E mutant partially mimics a phosphorylation event, and the affinity of the S190>E mutant and the WT GST-Pim-1 are identical. This suggests that autophosphorylation of the GST-Pim-1 on Ser-190 is activating. That the Ser-190 site was 50-100% phosphorylated by the time that active GST-Pim-1 was purified from the bacteria, supports this hypothesis. Additional methods are probably also used to control the activity of the kinase posttranslationally.

Despite the changes in kinase activity obtained with the S190 mutants, the activity of the expressed WT GST-Pim-1 did not appear to be modulated by \textit{in vitro} autophosphorylation, dephosphorylation or by phosphorylation by other purified kinases. It is very likely that autophosphorylation is not the sole method of posttranscriptional regulation of Pim-1 activity. Examination of the regulation of other small serine/threonine kinases may provide some insight into the methods by which the activity of Pim-1 is modulated. Many other kinases in the cell are part of dynamic, multiprotein complexes, the constituents changing as the regulatory or localization requirements of the kinase changes. For example, the activity of CDKs are controlled by four highly conserved biochemical mechanisms: activation by cyclin binding, activation by phosphorylation of the Thr-160 residue, deactivation by phosphorylation of residues Thr-14 and Tyr-15 within kinase subdomain I, and deactivation by the binding of cyclin kinase inhibitory domains [Pines, 1995; Morgan, 1995]. The cAMP-dependent protein kinases have a cAMP-binding regulatory domain that binds and represses the catalytic domain. Liberation of the active catalytic domain occurs when two cAMP molecules bind and induce a steric change, releasing the regulatory domain. In addition to regulatory domains, catalytic domains also have targeting domains which direct and localize the activity of the kinase catalytic domain [Hubbard and Cohen, 1993]. An example of a protein employing the target signal hypothesis method of regulation is CaMII, where each 54-60 kDa isoform contains a N-terminal catalytic domain, a regulatory domain containing a kinase autoinhibitory segment.
and an association domain [Srinivasan, 1994]. The subunits, associate to from large 500-600 kDa multimeric complexes composed of 6-12 subunits.

It is possible that Pim-1 is also a component of a larger protein complex. Unlike the CaMII isoforms, Pim-1 consists of a catalytic domain without characterized autoregulatory or association domains to allow interaction with other kinases. Our results with inhibitor peptides indicate that Pim-1 was not inhibited by sequences within the kinase. However, the activity of Pim-1 might be inhibited by pseudosubstrates located on associated regulatory proteins. Pim-1 must associate with other kinases by virtue of still uncharacterized binding domains in the N- or C-terminus. Although Pim-1 does not have any of the known protein modules essential for protein-protein interaction such as the Src-homology domains (SH2 and SH3), the Pleckstrin (PH) domain, PTB, LIM Armadillo and the Notch/ankyrin repeat, this does not preclude the possibility that other proteins containing these domains may bind to Pim-1 [Pawson, 1995; Cohen et al. 1995; van der Geer and Pawson, 1995]. An imperfect amphipathic α-helix motif in the C-terminus of Pim-1, similar to that of cAMP-dependent protein kinase [Veron et al., 1994], as of yet has no defined function, and may be involved in an interaction with associated proteins. Pim-1 activity may be regulated in a manner similar to that of second-messenger kinases, with binding to regulatory proteins causing steric alteration or blocking of active sites.

Although there is little to suggest that Pim-1 shares any structural or functional similarity with other oncogene-encoded serine/threonine kinases such as Mos and Raf, Pim-1 shares homology and may be part of the same family as some of the newly identified protein kinases. Some of the more interesting serine/threonine kinases with homology to Pim-1 include the human p78 protein kinase that is lost during chemically-induced pancreatic tumors, the product of the recently characterized African swine fever virus (ASFV) and tsk-1, a testes-specific murine kinase. The product of the ASFV is similar in size to the Pim-1 (299 residues) and contains residues homologous to the autophosphorylation sites of Pim-1 [Baylis et al., 1993]. The ASFV kinase phosphorylated histones but not BSA, casein, phosvitin or protamine, similar to the Pim-1. The murine testes-specific kinase tsk-1 is also similar in size to Pim-1 (364 residues) and has a conserved serine residue at the location homologous to T205 [Bielke et al., 1994]. Both of these kinases are similar to Pim-1 in that they are comprised of a catalytic domain without a known targeting domain or an obvious autoregulatory domain. It is likely that these kinases, along with Pim-1 may form a new family of protein kinases that interact with
other regulatory or targeting proteins. Many of the yeast kinases identified in homology searches are also of similar sizes and may be part of a homologous protein complexes.

Pim-1 is thought to participate in signal transduction pathways induced by IFN-γ, Epo, IL-3, IL-5 and GM-CSF, and requires an intact receptor membrane proximal domain for upregulation [Polotskaya et al., 1993]. Also stimulated by the same growth factors are JAK2 and the STAT proteins [Hill and Treisman, 1995; Schindler, 1995]. The STAT proteins are dimeric DNA binding proteins that respond to cytokines and growth factors to induce cytokine and growth factor inducible genes. They exist in the cytoplasm as unphosphorylated monomers, but after phosphorylation, dimerize and translocate to the nucleus [Cohen et al., 1995; Schindler, 1995]. Although it has been recently been demonstrated that the pim-1 gene contains a STAT binding site, it is possible that Pim-1 functions to in turn phosphorylate STAT or other STAT-like transcription proteins in the cell.

Most work in other laboratories has focused on characterizing the expression of the Pim-1 mRNA transcript or the upregulation of the Pim-1 protein in cytokine-stimulated hemopoietic cells. This study explored the expression and activity of the Pim-1 protein in the maturing oocyte system. Although the meiotic cell cycle of the maturing oocyte does not have an S phase and has shortened G1 and G2 phases, many of the same kinases that are active during the mitotic cell cycle become active during the meiotic cycle. It was hoped that by defining the activity and expression patterns of Pim-1 in the oocyte, that the results could be applied to the activity and expression of Pim-1 during the cell cycle in general.

Despite many promising results, the studies of Pim-1 in the oocyte model systems were somewhat inconclusive. Immunoreactive Pim-1 displayed phosphotransferase activity towards P4 peptide in sea star oocytes, but it did not become activated, and the quantity did not change during oocyte maturation. This method for examining the activity of endogenous Pim-1, involving the analysis of fractionated extracts, is extremely limited when the previous discussion is taken into account. Although Pim-1 is present during oocyte maturation, the enzyme may be sequestered in a non-active state in multi-protein complexes with changes in the tightly regulated activity too insignificant or transient to be detected by these methods. Although there were changes observed in the quantity of Pim-1 protein after oocyte fertilization, these changes were not thoroughly investigated.
FUTURE DIRECTIONS

This study has advanced the knowledge of the in vitro activity of expressed GST-Pim-1 and in the process has developed many tools and reagents for the further investigation of this kinase in endogenous systems. Many unanswered questions were raised, which form a new platform for future study. The most important questions remaining to be answered are: (1) What are the physiological substrates of Pim-1? (2) What are the regulatory/targeting proteins which associate with Pim-1 in vivo? (3) What is the function of Pim-1? and (4) What is the importance of the other autophosphorylation sites? To answer these remaining questions, some of the following directions could be considered.

1. Identification of proteins interacting/regulating/phosphorylated by Pim-1

The small size and the constitutively active state of Pim-1 suggest that other proteins interact with the kinase to regulate and target the activity of the kinase. Several approaches can be utilized to identify these associated proteins:

i. The yeast two hybrid system

As Pim-1 is a relatively small kinase composed of a catalytic domain with very short N- and C-terminal regions, it is highly likely that other proteins interact with Pim-1 to regulate or modulate the activity of the kinase. Preliminary work to screen a HeLa library with Lex-A human pim-1 was initiated by Mr. Michael Kyba (Biotech Lab, UBC). Initial results from his laboratory and others indicate that Pim-1 non-specifically causes indiscriminant activation of transcription, and removal of acidic domains of the Pim-1 did not change this activity. This implies that Pim-1 either acts as or interacts with a transcription factor to short-circuit the 2-hybrid selection system, or that the Pim-1 phosphorylates a protein that may activate transcription of the markers in this system. Screening the library with a LexA-KD pim-1 would determine if this effect is dependent on kinase activity of the Pim-1 or is strictly due to a protein/protein interaction.

ii. Far Western blots and fusion protein affinity columns

Preliminary work to test new methods of identification of protein-protein interactions were promising. Both the Far Western and fusion protein affinity column methods demonstrated some binding of X. laevis proteins to GST-Pim-1 and some phosphorylation of X. laevis proteins by GST-Pim-1. The identities of these associated proteins need to be investigated further. These methods would be best applied for confirmation when an interaction between Pim-1 and another protein is suspected.
iii. Purification of Pim-1 from endogenous sources.

Purification of Pim-1 from endogenous sources would not only provide material with which to confirm the results of the in vitro activity characterizations, but might allow copurification and identification of associated proteins. Sea star oocytes would be a good source of endogenous Pim-1 for purification, using the peptide reagents and antibodies developed in this study. Although it does not experience further stimulation during maturation, Pim-1 appeared to be active in the sea star oocyte, based on P4 peptide phosphorylation.

Initial characterization of bovine spleen extracts indicates that Pim-1 is active and present in ample quantities to justify purification. Unlike many of the other Pim-1 expressing cell lines, large volumes of starting material can be inexpensively and easily obtained. One disadvantage of using BSE is that the extracts contain many different cell types including various different spleen cells and circulating hemopoietic cells. Not all these different cell types will contain the active Pim-1 protein; analysis of column fractions with the P4 peptide should select for activated Pim-1 species.

iv. Identification of upstream activators of Pim-1 using sea star oocyte extracts

The kinase-dead X. laevis GST-Pim-1 could be used to screen fractions for Pim-1 kinase (PIK) activity, allowing potential upstream activators of Pim-1 to be purified and identified. This could be performed as in Appendix VIII, or the expressed KD GST-Pim-1 could be used as a substrate in an in gel assay.

2. Examining in vivo effects of Pim-1 in the oocyte system by microinjection

Microinjection studies using WT and mutant Pim-1 could be performed to determine the function of Pim-1 in vivo. Although the putative Pim-1 activity does not seem to increase during sea star oocyte maturation, the fact that some activity was observed implies that this kinase has some role in the oocyte. Microinjection studies could be performed with both X. laevis and sea star oocytes. However, as sea star oocytes are easier to obtain, are transparent and yield more consistent results, the sea star oocyte system is recommended. Microinjections of many reagents, including expressed protein, expressed mutant proteins, antibodies and oligonucleotides, could be performed to examine effects in the oocyte.
i. Pim-1 ablations

Microinjection of antibodies directed against a specific protein has been shown to inactivate or neutralize the activity of the protein, i.e. Mos. As the GXP antibody recognizes and inhibits the kinase activity of Pim-1, microinjection of this antibody into oocytes at different stages of maturation or fertilization may allow assessment of Pim-1 ablation in immature oocytes, during maturation and at different stages post-fertilization. An advantage of using embryos, is that just one cell in a two cell blastocyst can be injected, and the effects compared to the non-injected cell. As a negative control, some of the serum that had been depleted of anti-Pim-1 antibodies could be microinjected.

Pim-1 ablation could also be achieved through the microinjection of specific pim-1 antisense oligonucleotides. These oligonucleotides could bind the pim-1 mRNA, blocking protein translation. This approach may be more successful over longer term experiments (during embryo development) where new Pim-1 protein would be translated.

ii. KD Pim-1 dominant-negative effects

Microinjection of KD Pim-1 into oocytes in situations identical to that of the GXP antibody will allow determination of whether or not the KD pim-1 can exert a dominant-negative effect on oocyte maturation or embryonic development. The results of these experiments would be expected to be similar to that of the Pim-1 ablation by antibody injection.

iii. Effects of WT and mutant Pim-1 over-expression

Similar to the experiments described above, expressed active or mutated GST-Pim-1 could be microinjected into oocytes to determine the effects of Pim-1 over-expression in immature, maturing and post-fertilized oocytes and developing embryos. Various autophosphorylation site mutants (i.e. S190>A) could also be microinjected to assess the effects of these mutants in the in vivo situation.

3. Mutation of other autophosphorylation sites of X. laevis Pim-1

The Thr-205 and Tyr-133 phosphorylation sites could be altered by PCR-mutagenesis, and the activity of the expressed mutants assessed to determine the importance of these sites for regulation of the kinase. Pim-1 clones could be constructed to have mutations at 2 or more of these autophosphorylation sites (i.e. both Ser-190 and Thr-205) and the combined effects on expressed Pim-1 activity assessed.
To determine if the autophosphorylation sites mediate protein-protein interactions, the aforementioned approaches (yeast 2-hybrid or affinity columns) could be used with the mutant Pim-1. Results could be compared to those obtained with the wild-type Pim-1, to assess the effects of the mutation on protein-protein interaction.

4. Further studies of Pim-1 in the sea star oocyte system

The studies undertaken with Pim-1 from oocytes were productive, but not conclusive. Additional experiments need to be performed in order to more fully examine the activity of the endogenous kinase in the oocyte system.

i. Further examination of the oocyte-specific pim-1 RNA transcripts.

These alternately sized RNA transcripts (2.6 and 1.7 kb) need to be more completely characterized to determine if they are pim-1, and if so, what they represent (i.e. are they a result of alternative mRNA splicing?). What does the presence of these transcripts indicate about the regulation and expression of pim-1 in the oocyte system?

ii. Cloning of the sea star cDNA

The sea star-specific cDNA probe could be used to screen a sea star cDNA library allowing the pim-1 to be cloned from this system. Experiments such as GST-Pim-1 affinity chromatography, Far Western blotting and microinjection could then be performed using the kinase from sea star.

iii. Fertilization studies of sea star oocytes

Initial results indicate that the amount of Pim-1 changes after oocyte fertilization. Further studies could be done in fertilized oocytes to determine if the activity of the kinase changes during oocyte fertilization and embryo development. Of interest, the activity of another oncogene-encoded serine-threonine kinase, Raf, is essential for mesoderm formation in developing embryo. Pim-1 may have a similar role.

iv. Localization of Pim-1 activity

If more specific Pim-1 antibodies were developed, immunofluorescent studies could be undertaken to determine the exact location of the Pim-1 in the oocyte. Although Pim-1 has been localized to the cytoplasm in the cell types examined, it would be of particular interest to examine if the Pim-1 was located at the endoplasmic reticulum to phosphorylate new proteins, or existed as part of a nuclear transport complex, modulating the transport of proteins to the nucleus.
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### APPENDIX I

**DESCRIPTION OF PRIMARY ANTIBODIES USED**

<table>
<thead>
<tr>
<th>Name</th>
<th>Animal</th>
<th>Usual Dilution</th>
<th>Source &amp; Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRBPim-1</td>
<td>Sheep antisera</td>
<td>1/500</td>
<td>Cambridge Research Biochemicals. Directed against the peptide corresponding to human and murine amino acids 26-38, ATKLAPGKEKEPLESQY.</td>
</tr>
<tr>
<td>A2/Daniel</td>
<td>Rabbit antisera</td>
<td>1/500</td>
<td>Dr. Michael Lilly Seattle VA Hospital. Directed against a peptide corresponding to the 18 carboxy-terminal amino acids of human Pim-1, residues 294-313, PQETAEIHLHSLSPGSK.</td>
</tr>
<tr>
<td>C2</td>
<td>Rabbit antisera</td>
<td>1/500</td>
<td>Dr. Michael Lilly Seattle VA Hospital. Directed against the 12 carboxy-terminal amino acids of murine Pim-1, amino acid residues 302-313, IHLHSLSPGSSK.</td>
</tr>
<tr>
<td>Tel</td>
<td>Mouse monoclonal</td>
<td>1/500</td>
<td>Drs. A. Telerman and R. Amson (CEPH, France). Directed against a TrpE-fusion protein with 69 (128-196) residues of human Pim-1.</td>
</tr>
<tr>
<td>Pim1-111</td>
<td>Rabbit antisera</td>
<td>1/1000</td>
<td>Pelech Lab, UBI. Antipeptide antibody directed against the kinase subdomain III region of the human Pim-1, amino acid residues 70-91, VEKDRISDGELPNTRPMEV-GGC.</td>
</tr>
<tr>
<td>Pim1-NT</td>
<td>Rabbit antisera</td>
<td>1/1000</td>
<td>Pelech Lab, UBI. Antipeptide antibody directed against the amino terminus of human Pim-1, amino acid residues 1-37, MLLSINSLAHLRARACNDLHATKLAPGKEKEPLES-GC.</td>
</tr>
<tr>
<td>Pim1-X1</td>
<td>Rabbit antisera</td>
<td>1/1000</td>
<td>Pelech Lab, UBI. Antipeptide antibody against kinase domain XI of <em>Xenopus</em> Pim-1 amino acids 275-292, RPSDRPTEQIFDHPWMC.</td>
</tr>
</tbody>
</table>
GXP Rabbit antiserum 1/500 Pelech Lab

Sera produced against the bacterially expressed full-length GST-\textit{Xenopus} Pim-1 fusion protein. Sera was purified with Protein G and GST-beads.
APPENDIX II.
ANTIBODY CHARACTERIZATIONS

1. PEPTIDE SELECTION AND WESTERN BLOTTING

   i. *H. sapiens* Pim-1 antibodies - Pim1-III and Pim1-NT

   Unique antibodies were created in order to study the expression patterns of Pim-1 protein during the maturation of *Pisaster ochraceus* (purple sea star) and *Xenopus laevis* (African clawed frog) oocytes. Initially, only the Pim-1 sequences of *Homo sapiens* (human) and *Mus musculus* (mouse) Pim-1 were known. As these mammalian Pim-1 protein sequences were very highly conserved (94%) [Domen et al., 1987], we hypothesized that the Pim-1 sequence would be similar in other species as well. Therefore, the first antibodies were produced against sequences of mammalian Pim-1. The rabbit polyclonal antibodies Pim1-NT and Pim1-III were directed against peptides based on sequences in the amino-terminus and catalytic subdomain III region of human Pim-1, respectively. As demonstrated in Figure 47, these regions are highly conserved between mammalian species. These peptide sequences contain several proline residues which introduce 'kinks' into the secondary structure of a protein and are often located at the surface of the macromolecule. By immunizing rabbits with these peptides, we hoped to create stronger epitopes for antibody recognition and potentially, immunoprecipitation.

   The Pim1-NT and Pim1-III antibodies immunodetected Pim-1 protein from various sources on Western blots. Figure 48 demonstrates the specificity of Pim1-NT and Pim1-III towards bacterially-expressed *H. sapiens* and *X. laevis* glutathione S-transferase (GST)-Pim-1 fusion protein and endogenous Pim-1 from the K562 (human erythroid) cell line. Both antibodies strongly detected the 62 kDa full-length human GST-Pim-1 fusion protein. Pim1-NT detected a strong 34 kDa doublet in K562 lysates. This is in close agreement with previous reports of proteins of 33-35 kDa in K562 extracts [Telemman et al., 1988; Saris et al., 1991]. Pim1-III antibody detected a faint doublet in K562 lysates; low immunoreactivity in K562 extracts is a result observed by other groups as well [M. Lilly, personal communication].
**Figure 47.** Regions of Pim-1 proteins against which antibodies are directed. Protein sequence alignments of Pim-1 from *X. laevis*, mouse, rat and human; open boxes designate peptides/regions against which antibodies were made. The names of the antibodies are italicized above or below the sequence. Roman numerals indicate protein kinase subdomains. Specific details are listed in the text and in Appendix I.
Figure 48. Detection of bacterially-expressed and endogenous Pim-1 with *H. sapiens* antibodies. Pim1-NT (left panel) and Pim1-III (right panel) antibodies were used to probe Western blots of cell extracts. Lanes 1 and 2 contained 0.75 ug of bacterially-expressed *H. sapiens* and *X. laevis* GST-Pim-1, respectively. Lane 3 contained K562 cell extracts (7.5 x 10^5 cells per lane). Lanes 4 and 5 contained immature and mature sea star oocyte extracts, respectively (approx. 350 ug per lane) and lanes 6 and 7 contained immature and mature *X. laevis* oocyte extracts, respectively (approx. 300 ug per lane). Solid arrows (→) show location of expressed GST-Pim-1. Hollow arrows (↔) show location of Pim-1 doublet in K562 cell extracts. Migrations of M_r standards are shown on right.
The Pim1-NT and Pim1-III antibodies were also used to screen for Pim-1 expression in *P. ochraceus* and *X. laevis* oocytes. Pim1-NT and Pim1-III both detected a strong series of proteins of about 44 kDa proteins in immature and mature sea star oocytes (Fig. 48, lanes 4 and 5). Both antibodies recognized the 62 kDa *X. laevis* GST-Pim-1 fusion protein very poorly, and while Pim1-NT detected proteins of 31 and 42 kDa in *X. laevis* oocyte extracts, Pim1-III did not detect any proteins of the appropriate size in these extracts. This is hardly surprising, for when the sequence of *X. laevis* Pim-1 is compared with that of the mammalian sequences in the regions against which the antibodies were directed, the sequences are dissimilar in these regions. Additional proteins of inappropriate sizes were also immunodetected on Western blots with these antibodies. As the antibodies were polyclonal, they contained a mixture of antibodies directed against different epitopes in immunizing peptides. Some of these antibodies might have cross-reacted with unrelated proteins sharing epitopes with Pim-1.

**ii. X. laevis Pim-1 antibodies: GXP and Pim1-XI**

The lack of immunoreactivity of the human-directed Pim-1 antibodies to endogenous and bacterially-expressed *X. laevis* Pim-1 necessitated the production of frog-specific antibodies. The Pim1-XI rabbit polyclonal antibody was directed against the kinase subdomain XI of *X. laevis* Pim-1, which showed considerable conservation between all species (Fig. 47). The GXP rabbit polyclonal antibody was directed against the full-length bacterially-expressed *X. laevis* GST-Pim-1 fusion protein. Although it would have been preferable to immunize the rabbits with the Pim-1 portion of the fusion protein alone, only small quantities of the thrombin-cleaved Pim-1 were obtainable, as will be discussed in Chapter IV, Section 3.i.b. Instead, the GXP serum was passed through a GST-bound glutathione agarose column to remove most of the anti-GST antibody. Although there was still some cross-reactivity, immunoreactivity towards expressed GST was significantly reduced.

Both antibodies were tested for immunodetection with recombinant *X. laevis* GST-Pim-1, *H. sapiens* GST-Pim-1 and native Pim-1 in extracts from *X. laevis* and sea star oocytes and from K562 cells (Fig. 49). Both GXP and Pim1-XI strongly detected recombinant *X. laevis* GST-Pim-1, GXP faintly detected the human recombinant and native Pim-1, and Pim1-XI did not detect human Pim-1 at all (Figure 49, lanes 1 - 3). Both antibodies detected strong 44 kDa proteins in the immature and mature sea star extracts and the GXP antibody detected an additional 33 kDa protein in these lanes (Figure 49, lanes 4 and 5). GXP detected a strong 31 and 42 kDa proteins similar to those
observed with Pim1-NT in the *X. laevis* oocyte extracts, but the Pim1-XI antibody only
detected a 38 kDa band.

The frog Pim-1 antibodies were designed to cross-react with Pim-1 from other species;
the Pim1-XI antibody was directed against a region in the catalytic domain of Pim-1 that is
highly homologous between species, and the GXP antibody was directed against the full-
length *X. laevis* Pim-1 which contains many regions very similar to the mammalian Pim-1.
The GXP antibody displayed some cross-reactivity with sea star and human Pim-1, while
Pim1-XI primarily detected sea star and frog Pim-1. That these antibodies cross-reacted
with Pim-1 from several different species was desirable, as they allowed examination of
Pim-1 expression in other systems (i.e. sea star).
Figure 49. Detection of bacterially-expressed and endogenous Pim-1 with *X. laevis* antibodies. GXP (left panel) and Pim1-XI (right panel) antibodies were used to probe Western blots of cell extracts. Lanes 1 and 2 contained 0.75 μg of bacterially-expressed *H. sapiens* and *X. laevis* GST-Pim-1, respectively. Lane 3 contained K562 cell extracts (7.5 x 10⁵ cells per lane). Lanes 4 and 5 contained immature and mature sea star oocyte extracts, respectively (approx. 350 μg per lane) and lanes 6 and 7 contained immature and mature *X. laevis* oocyte extracts, respectively (approx. 300 μg per lane). Solid arrows (→) show location of expressed GST-Pim-1. Hollow arrows (→) show location of Pim-1 doublet in K562 cell extracts. Migrations of Mₖ standards are shown on right.
2. COMPARISON OF PIM-1 ANTIBODIES FROM VARIOUS SOURCES

Figure 47 shows regions of the Pim-1 sequence against which the antibodies from other sources (detailed in Appendix 1) were directed. The Pim-1 antibodies obtained from other researchers were compared with those produced in our laboratory (Fig. 50). Although titres of our antibodies were available, those from other sources were not. We did not attempt to titre these antibodies as only limited quantities for confirmatory work were obtained. Because both of the antibodies kindly donated by Dr. Michael Lilly were directed against C-terminal residues, a 1:1 cocktail of A2 and C2 antibodies was used unless otherwise indicated. This cocktail was designated as "Lilly".

K562 cell lysates were used as a positive control for Pim-1 detection, as they have been reported to express high amounts of endogenous Pim-1 [Telerman et al., 1988; Saris et al., 1991]. Approximately 7.5 x 10^5 cells were electrophoresed on each lane of a Western blot. A 32-35 kDa doublet was observed with most antibodies tested. A 34 kDa doublet/smear was detected strongly with Tel, CRB, Pim1-NT and Lilly, detected faintly with GXP and Pim1-III, but not detected with the Pim1-IX antibody.

Most antibodies detected the 62-kDa bacterially-expressed H. sapiens and X. laevis GST-Pim-1 fusion proteins by Western blotting (Fig. 50, panels B, C). Human GST-Pim-1 protein was detected very strongly by the Pim-NT, Pim1-III, Tel, Lilly, and CRB antibodies (panel B). The protein was detected only faintly by GXP and was not detected at all by Pim1-XI, 4G10 and PY20. Several lower Mr proteins were observed in the Pim1-III, Pim1-NT, GXP and CRB lanes. These bands are likely to be the products of aborted translation, as these antibodies are directed towards regions in the amino-terminus of the protein. The anti-GST, GXP, Pim1-XI, Tel and 4G10 antibodies strongly detected frog GST-Pim-1, Pim1-III and PY20 faintly visualized frog GST-Pim-1, and Pim1-NT, Lilly and CRB negligibly detected frog GST-Pim-1 (panel C).
Figure 50. Pim-1 antibody comparison by immunodetection of Pim-1 on Western blots. Panels A: K562 cell extracts (7.5 x 10^5 cells). Panel B: bacterially-expressed H. sapiens GST-Pim-1 (0.75 ug per lane). Panel C: bacterially-expressed X. laevis GST-Pim-1 (0.75 ug per lane). Antibodies used: lane 1, anti-GST; lane 2, GXP; lane 3, Pim1-XI; lane 4, Pim1-NT; lane 5, Pim1-III; lane 6, Tel; lane 7, Lilly; lane 8, CRB. Open arrow (←) indicates endogenous Pim-1 protein, filled arrow (→) indicates bacterially-expressed GST-Pim-1. Migrations of Mr standards are shown at right.
3. IMMUNOPRECIPITATION

The antibodies were tested for the ability to immunoprecipitate Pim-1 from various sources including preparations of expressed fusion proteins, sea star and *X. laevis* oocytes and from K562 cell lysates. Immunoprecipitation allows rapid and selective resolution of the target protein from other proteins in a cell homogenate, yielding a relatively pure protein preparation for examination on a Western blot or for enzyme activity analysis (i.e. autophosphorylation or kinase assays). For the purposes of this study, it was hoped that active Pim-1 could be immunoprecipitated from oocyte preparations in order to examine both changes in the protein levels, apparent molecular mass or kinase activity of the endogenous enzyme during different stages of oocyte maturation.

Initially, bacterially-expressed *X. laevis* and *H. sapiens* GST-Pim-1 were used as positive controls for immunoprecipitation experiments. Unfortunately, GST-Pim-1 adhered non-specifically to Protein-A Sepharose, so large quantities of the fusion protein were removed during the Protein-A pre-clearing step. Secondly, because of the high concentrations of fusion protein and the high affinity of the antibodies for the fusion protein, we were never certain if the fusion protein that was visible in the immunoprecipitates was immunoprecipitated or was present in trace amounts as a contaminant. Increased washes did not reduce the amount of fusion protein in the immunoprecipitates. Antibody cocktails with a mixture of dissimilar antibodies were often used to improve the prospects of immunoprecipitation.

i. Immunoprecipitation of Pim-1 from the K562 (human erythroid) cell line

Pim-1 was immunoprecipitated from K562 cells by Tel, GXP, Lilly and weakly by Pim1-XI antibody (Fig. 51). The inefficiency of Pim1-XI for immunoprecipitating Pim-1 from K562 cells was not surprising, as this antibody was directed against a *X. laevis*-specific peptide and did not recognize the human GST-Pim-1. The other *X. laevis* antibody, GXP, was able to immunoprecipitate human Pim-1, probably because it recognized epitopes common to both proteins. The human antibody, Pim1-NT did not immunoprecipitate Pim-1, possibly because it was directed against the amino-terminus of the kinase, which may be folded into the interior and may therefore be unaccessible. The fact that the Pim1-III antibody was able to immunoprecipitate Pim-1 from K562 cells was surprising, as it only faintly detected Pim-1 protein in this cell line on Western blots (Fig. 48, lane 3). The anti-phosphotyrosine mouse monoclonal antibodies 4G10 and PY20 did not immunoprecipitate Pim-1 from K562 cells.
Figure 51. Immunoprecipitation of Pim-1 from K562 cells with various antibodies. K562 cell extracts (+) and PBS controls (-) were immunoprecipitated with the various Pim-1 and antiphosphotyrosine antibodies shown on the top of the blot. Migration of Mr standards are shown on the right. The 33 kDa Pim-1 band is indicated by an arrow (→). Each immunoprecipitation was from approx. 3 x 10⁶ cells.
ii. Immunoprecipitation of Pim-1 from *P. ochraceus* oocyte extracts

A cocktail of Pim1-III and Pim1-NT antibodies was used to immunoprecipitate Pim-1 from sea star oocyte time course homogenates (data not shown). A 40 kDa band thought to be Pim-1 was observed in both the supernatants and in the whole oocyte homogenates, but not in the immunoprecipitates (data not shown). Similar results were observed in subsequent experiments and with the Lilly cocktail (data not shown), leading to the conclusion that Pim1-III and Pim1-NT antibodies did not immunoprecipitate Pim-1 from sea star oocytes, even though they detected the presence of Pim-1 in these lysates.

iii. Immunoprecipitation of Pim-1 from *X. laevis* oocyte extracts

GXP, Pim1-XI and the Lilly antibody cocktail were used to immunoprecipitate Pim-1 from immature and mature *X. laevis* oocytes. There were no unique proteins present in the immunoprecipitates that were not already detected in the controls and proteins at the size expected for the Pim-1 were detected in the supernatants with the GXP and Pim1-XI antibodies, indicating that Pim-1 was not immunoprecipitated. Both antibodies detect different bands in the supernatants; it is uncertain which band is actually Pim-1. The Lilly cocktail did not detect Pim-1 protein in the controls or immunoprecipitates (data not shown).

It was hoped that GXP and Pim1-NT would be the most efficient antibodies for immunoprecipitation of endogenous *X. laevis* Pim-1, as both are directed against epitopes that should be exposed on the surface of the native protein. GXP antibody immunoprecipitated Pim-1 from K562 cells, but was unsuccessful at immunoprecipitating the kinase from *X. laevis* oocyte extracts possibly due to differences in homogenization buffers. While *X. laevis* oocyte extracts were homogenized under non-denaturing conditions, K562 cells were lysed in buffer containing 1% Triton X-100, 0.5% deoxycholate and 0.1% SDS. The presence of these detergents may have caused protein unfolding and denaturation allowing antibodies better access to the epitopes. As we ultimately wanted to obtain active enzyme from the oocyte preparations, non-denaturing conditions were required and the use of high detergent buffers was unacceptable.
4. ANTIBODY INHIBITION OF KINASE ACTIVITY

All antibodies were tested for the ability to inhibit the phosphotransferase activity of expressed GST-Pim-1 toward a peptide substrate in order to determine if any of the epitopes to which the antibodies were directed were important for kinase-substrate binding or catalysis. The kinase activity of 8 nmoles of human or X. laevis GST-Pim-1 was assessed in the presence of 0.184 to 32 nmoles of antibody per assay. The activity towards P4 substrate peptide was plotted against the molar ratio of antibody to enzyme in order to assess the ability of the antibodies to inhibit the kinase activity (Fig. 52). Antibodies that reduced the kinase activity more than 25% in a molar ratio of 2 or less were judged to be inhibitory. X. laevis GST-Pim-1 kinase activity was strongly inhibited by GXP and Tel, and weakly inhibited by Pim1-NT and Pim1-III. H. sapiens GST-Pim-1 was also strongly inhibited by GXP and Tel antibodies, and weakly by the Pim1-III antibody.

The results of antibody inhibition study can be interpreted when the relationship between the antibodies and the Pim-1 protein is considered. Antibodies such as Tel and GXP, which are directed against large portions of the protein are most likely to inhibit the activity of the kinase, as they can recognize epitopes that are on the "outside" of the folded protein. Antibody binding may inhibit or induce a conformational change in Pim-1 necessary for phosphotransferase activity or may sterically block sites important for substrate binding. The Pim1-III antibody, directed at similar regions in the catalytic domain caused a very slight inhibition of Pim-1 activity. As the N-terminus of Pim-1 was fused to GST, normal protein folding of the Pim-1 was likely be hindered, so the inhibition by Pim1-III may not accurately reflect the situation with the endogenous, native protein. The results using the Lilly cocktail were not shown as the antibody did not inhibit the GST-Pim-1 activity even at very high amounts. Of note but of unclear significance, is the fact that low concentrations of the antibodies caused a slight increase in the amount of $^{32}$P incorporated into the P4 peptide by Pim-1.

Although it was hoped that immunoprecipitation would be a suitable method for obtaining active, purified endogenous Pim-1 from oocytes, these results demonstrated that the Pim-1 antibodies inhibited the kinase activity of Pim-1 if they were able to immunoprecipitate the kinase. Therefore, the kinase activity of immunoprecipitated endogenous Pim-1 could not be accurately assessed in our study.
Figure 52. Antibody inhibition of kinase activity.
The activity of 8 nmol of *X. laevis* (panel A) and *H. sapiens* (panel B) GST-Pim-1 towards P4 peptide was inhibited with increasing concentrations Pim-1 antibodies. The amount of 32P incorporation by P4 peptide is shown on the vertical axis and the molar ratio of antibody to GST-Pim-1 protein is shown on the horizontal axis. The 1:1 molar ratio is shown by a dotted vertical line.
5. ANTIBODY SUMMARY

We produced four distinct Pim-1 antibody preparations that detected recombinant and native forms of amphibian and mammalian Pim-1. The antibodies displayed some species specificity; Pim1-NT and Pim1-III recognized mammalian Pim-1, while Pim1-XI and GXP recognized *X. laevis* GST-Pim-1. Comparison studies with Pim-1 antibodies from various sources confirmed that the antibodies constructed for this study immunodetected Pim-1 protein from bacterially-expressed and endogenous sources. Although the GXP and to a lesser extent, Pim1-XI immunoprecipitated Pim-1 from K562 cells, Pim1-III and Pim1-NT did not immunoprecipitate Pim-1 from sea star oocyte extracts, and GXP and Pim1-XI did not immunoprecipitate Pim-1 from *X. laevis* oocyte extracts. Antibodies GXP and Tel inhibited the activity of expressed GST-Pim-1 possibly by blocking substrate binding sites or by inducing or inhibiting a conformational change in the protein that interfered with catalytic activity. These antibodies were used to examine the protein expression of Pim-1 in the bacterial expression system (Chapter IV), in maturing *X. laevis* oocytes (Chapter VII) and in maturing *P. ochraceus* oocytes (Chapter VIII).
APPENDIX III.
DESCRIPTION OF OLIGONUCLEOTIDES

11A 5'-CTG ACC CGG GCT CGA GGC ICC IGG IAA (G/A)GA (G/A)AA (G/A)GA (G/A)CC-3'
Based on the human and mouse sequence APGKEKEP, aa 26-33. 39 bp.
Degenerate sense primer for the amplification of a *pim-1* probe from *X. laevis* and *P. ochraceus* cDNA. CTGA clamp, *CCCGGG* Sma1 site, *CTCGAG* Xho1 site. (0.886 ug/ul, 0.56 ul per PCR reaction)

12A 5'-CTG ACC CGG GCT CGA GGA (C/T)TG GTT (C/T)GA (G/A)AG ICC ICA-3'
Based on human and mouse sequence DWFERPD, aa 108-114. 36 bp.
Degenerate sense primer for the amplification of a *pim-1* probe from *X. laevis* and *P. ochraceus* cDNA. CTGA clamp, *CCCGGG* Sma1 site, *CTCGAG* Xho1 site. (0.714 ug/ul, 0.7 ul per PCR reaction)

13B 5'-CTG ACC CGG GCT CGA GAT (C/T)TC (C/T)TC (G/A)TC (G/A)TGTTC (G/A)AA 100-3'
Based on human and mouse sequence PFEHDEEI, aa 241-248. 39 bp.
Degenerate anti-sense primer for the amplification of a *pim-1* probe from *X. laevis* and *P. ochraceus* cDNA. CTGA clamp, *CCCGGG* Sma1 site, *CTCGAG* Xho1 site. (0.938 ug/ul, 0.53 ul per PCR reaction)

14B 5'-CTGACC CGGGCT CGA GC(C/T) TGC ATC CAI GG(G/A) TG(G/A)TT-3'
Based on human and mouse sequence NHPWMQ, aa 286-291. 35 bp.
Degenerate anti-sense primer for the amplification of a *pim-1* probe from *X. laevis* and *P. ochraceus* cDNA. CTGA clamp, *CCCGGG* Sma1 site, *CTCGAG* Xho1 site. (1.756 ug/ul, 0.285 ul per PCR reaction)

9402 5'-CGT ACC CGG GCC ATG CTC TTG TCC AAA ATC-3'
Based on human sequence MLLSKI, aa 1-6. 30 bp.
Sense primer for the amplification of the human *pim-1* coding region for cloning into the pGEX-2T expression vector. CGTAC clamp, *CCCGGG* Sma1 site. (0.244 ug/ul, 25.02 pmol/ul)
5'-GCG GAA TCC TAT TTG CTG GGC CCC GGC GAC AGG-3'
Based on human sequence LSPGPSK, aa 307-313. 30 bp.
Antisense primer for the amplification of the human Pim-1 coding region for cloning into
the pGEX-2T expression vector. GCG clamp, GAATTC EcoRI site. (0.200 ug/ul, 18.64 pmol/ul)

pim5 5' - CGA TGG ATC CAT GCT TCT CTC TAA ATT CGG -3'
Based on X. laevis sequence MLLSKF, aa 1-6. 30 bp.
Sense primer for the amplification of the X. laevis Pim-1 coding region for cloning into the
pGEX-2T expression vector. CGAT clamp, GAATTC BamHI site. (28.2 pmol/ul)

pim3 5' - GAT CGA ATT CCA GAC TCT CGT TGC TTG A -3'
Based on X. laevis sequence SSNESL, aa 318-323. 33 bp.
Antisense primer for the amplification of the X. laevis Pim-1 coding region for cloning into
the pGEX-2T expression vector. GATC clamp, GAATTC EcoRI site. (27.9 pmol/ul)

K1 5'- CTC CTT AGC TAC GTG GCG CAC AGC GAC CGG CTG -3'
Based on X. laevis sequence QPVAVAHVAKE, aa 64-74. 33 bp.
Antisense primer for site directed mutagenesis (K^69-^A) of X. laevis Pim-1. The lysine
condon, TCC, (nt 205-207) is changed to GCG (alanine). This oligonucleotide was used
with pim5 to amplify a 230 base pair PCR fragment which was designated as oligo 5K1.

K2 5'- AGA GTC ACA GAA TGG GGC -3'
Based on X. laevis sequence RVTEWG, aa 75-80. 18 bp.
Sense primer for site directed mutagenesis (K^69-^A) of X. laevis Pim-1. This
oligonucleotide was meant to be used with pim3 to create a PCR fragment to ligate with the
pim5 - K1 fragment (oligo 5K1).

5K1
232 bp, corresponding to the X. laevis amino acid sequence, aa 1-74.
Sense primer containing the K^69-^A mutation at nt residue 207. This oligonucleotide was
amplified by a PCR reaction with pim5 and K1, and used with pim3 to amplify the entire
coding region of the pim-1.
PM1  5' - CTG ATC GAT TTT GGC GGC GGG GCG CTA CTC -3'
Based on *X. laevis* sequence, aa 184-194, LIDFC§GAKK. Sense primer for site directed mutagenesis (S^190^-A) of *X. laevis* Pim-1. This oligonucleotide was meant to be used with pim3 to create a PCR fragment to ligate into the ClaI/EcoR1 sites of the *X. laevis pim-1* in pGEX-2T. Italics indicate ClaI site, bold underline denotes mutation. [10 ug/ul, 102.562 pmol/ul]

PM2  5' - CTG ATC GAT TTT GGC GAA GGG GCG CTA CTC -3'
Based on *X. laevis* sequence, aa 184-194, LIDFC§GAKK. Sense primer for site directed mutagenesis (S^190^-E) of *X. laevis* Pim-1. This oligonucleotide was meant to be used with pim3 to create a PCR fragment to ligate into the ClaI/EcoR1 sites of the *X. laevis pim-1* in pGEX-2T. Italics indicate ClaI site, bold underline denotes mutation. [13.78 ug/ul, 141.3 pmol/ul]

PM3  5' - GGA TAC GGT GGA AAC GGA TTT TGA TGG -3'
Based on *X. laevis* sequence, aa 195-204, DTVYDTDFG. Sense primer for site directed mutagenesis (Y^199^-E) of *X. laevis* Pim-1. This oligonucleotide was meant to be used either with pim3 to create a large oligo to use with pim5 to amplify the full-length coding region of pim-1, or to be used with oligo pim3 to create a PCR product to ligate with the PCR product of PM4 and pim5'. Bold underline denotes mutation. No restriction sites. [1.8 ug/ul, 20.51 pmol/ul]

PM4  5' - TTG AGT AGC GCC CCG GAG CC -3'
Antisense primer based on *X. laevis* sequence, aa 189-195, KLLAGSG. To be used with pim5 to create a fragment to ligate with the PCR product of PM4 and pim5'. No mutation, no restriction sites. [3.0 ug/ul, 46.153 pmol/ul]

T3  5' - AAT TAA CCC TCA CTA AAG GG - 3'
Primer for sequencing reactions. (0.126 ug/ul)

T7  5' - GTA ATA CGA CTC ACT ATA GGG C - 3'
Primer for sequencing reactions. (0.277 ug/ul)
**5H6Pim** 5' - (P) ATG GAA GAG GAA GAG GAA GAG CTT CTC TCT AAA TTC GGA TCG - 3' MHHHHHHLSSKFGS
Primer used to amplify the coding region of WT *X. laevis pim-1* and mutants to put a histidine tag on Pim-1 to insert into Sma I site of PEF1. Used by Dr. Gabe Kalmar at S.F.U.

**pim3’** 5'- CAA AGC TTT ACA GAC TCT CGT TGC TTG AGC
Primer used to amplify the coding region of WT *pim-1* and mutants and to insert into Sma I site of PEF1. Used by Dr. Gabe Kalmar at S.F.U.
### APPENDIX IV.
#### PEPTIDE SUBSTRATES

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APPENDIX V
FORMULAE FOR CALCULATIONS

1. Calculation of ATP concentration and cpm/pmol
Concentration of $[^\gamma^32P]$-ATP stock: 2.2 uM in 100 ul volume (220 pmoles). Diluted in 4-5 ml of 250 uM ATP to a final concentration of 250 uM, designated as 'Assay ATP'.

\[ \frac{x \text{ counts (cpm in 5 ul of assay ATP)}}{1250 \text{ (pmol in 5 ul)}} = \frac{\text{cpm/pmol=D}}{1} \]

2. Calculation of activity of enzyme in filter paper assay

\[ \frac{x \text{ (cpm of assay)}}{\text{total volume of assay}} \times \frac{\text{pmol/min.mg}}{D \times \text{time (min)} \times \text{[enzyme (mg)} \times \text{vol spotted on filter}} \]
For the calculation of kinetic constants, two methods were employed, the Michaelis-Menten and the Lineweaver Burke equations, which can be expressed as graphs. Representatives of both methods are shown below for the measurement of the activity of expressed wild-type *X. laevis* GST-Pim-1 towards the P4 substrate peptide (AKRRRLSA).

The Michaelis-Menten plot is derived from plotting the enzyme activity against peptide concentration. The best-fit curve is plotted, and the maxima is determined as the $V_{\text{max}}$ value. Half of the $V_{\text{max}}$ is plotted and used to determine the $K_m$ value of the enzyme for the peptide.

**Michaelis-Menten Plot for GST-Pim-1 activity toward P4 peptide**

$V_{\text{max}} = 88 \text{ nmol.min}^{-1}.\text{mg}^{-1}$.

$K_m = 42 \mu\text{M}$
A more accurate method of kinetic constant determination is the Lineweaver-Burke plot. The inverse of the kinase activity is plotted against the inverse of the peptide concentration. The $V_{\text{max}}$ and $K_m$ values are derived from the Y and X-intercepts of the slope equation.

**Lineweaver-Burke plot of GST-Pim-1 activity toward P4 peptide**

Slope equation \( y = 1.5092e-2 + 0.70528x \) $\quad R^2 = 0.991$

$V_{\text{max}} = 66.3 \text{ nmol.min}^{-1}.\text{mg}^{-1}$

$K_m = 47 \mu\text{M}$
APPENDIX VII

AUTOPHOSPHORYLATION/DEPHOSPHORYLATION AND EXOGENOUS KINASE ACTIVITY

i. Dephosphorylation of GST-Pim-1

As GST-Pim-1 was already autophosphorylated by the time it was purified from the bacteria, we wanted to determine if dephosphorylation would cause a shift in molecular mass of GST-Pim-1 or would alter the activity of the kinase. The phosphatases that were tested for Pim-1 dephosphorylation included broad specificity acid and alkaline phosphatases and a tyrosine-specific phosphatase corresponding to the intracellular domain of HPTPB. The phosphatases were removed from the glutathione-bound GST-Pim-1 after phosphatase treatment, so that the in vitro autophosphorylation of the dephosphorylated GST-Pim-1 could be subsequently examined.

Dephosphorylation by the various phosphatases did not cause a shift in apparent molecular weight of GST-Pim-1 (Fig. 53, panel A). The slightly undulating band patterns observed in lanes 2, 4, 5 and 7 were due to a slight problem with the stacking gel and are not due to band shifting. Panel B demonstrates that the dephosphorylated Pim-1 samples were active and able to autophosphorylate. The acid phosphatase treated sample retained 58% of activity, the alkaline phosphatase-treated sample retained 55% of activity and the HPTPB-treated sample retained 65% of activity as compared to the control. Although it is tempting to speculate that the reduction in activity observed was due to dephosphorylation, it likely reflected residual phosphatase contamination. The control sample was incubated in parallel with the other samples, so any effects of protease contamination would have been consistent between samples; as degradation of the samples was not observed in panel A, the reduction in activity was unlikely due to proteolytic degradation. Panel C demonstrates that both the acid and alkaline phosphatases caused a very minor reduction in the amount of tyrosine phosphorylation as detected by the anti-phosphotyrosine antibody (lanes 3 and 5) and autophosphorylation did not change this amount (lanes 4 and 6). HPTPB treatment reduced tyrosine phosphorylation by an estimated 80%, as detected by the anti-phosphotyrosine antibody (lane 7), and autophosphorylation seemed to cause a 5-10% recovery of this signal (lane 8).
Figure 53. Dephosphorylation and subsequent phosphorylation of *X. laevis* GST-Pim-1. Control and phosphatase-treated GST-Pim-1 were loaded directly onto an SDS-PAGE gel (lanes 1, 3, 5, 7) or were subjected to autophosphorylation reactions (2, 4, 6, 8). Panel A, silver stain of SDS-PAGE gel; panel B, autoradiograph of Western blot; panel C, Western blot probed with 4G10 antibody. Control (lanes 1&2), acid phosphatase-treated (lanes 3&4), alkaline phos. -treated (lanes 5&6) and HTP3-treated (lanes 7&8) GST-Pim-1. Boundaries of panels are defined by the 97.2 and 50.0 kDa Mr standards.
ii. Dephosphorylation curve of HPTPβ

A dephosphorylation curve was performed using serial dilutions of HPTPβ to determine if complete dephosphorylation of GST-Pim-1 by HPTPβ could be achieved. At even very high concentrations of HPTPβ, the tyrosine phosphorylation of GST-Pim-1 was not completely reduced as detected by antophosphotyrosine antibodies (data not shown). As Figure 20 demonstrated that recognition of GST-Pim-1 by the antiphosphotyrosine antibody was specific, it is possible that Pim-1 has several tyrosine phosphorylation sites and only certain ones are targeted by the HPTPβ. Indeed, kinetic analysis of the intracellular domain of HPTPβ has revealed that the phosphatase displays substrate specificity [Harder et al, 1994].

iii. Activity of GST-Pim-1 after autophosphorylation/dephosphorylation

To determine if differences in phosphotransferase activity toward exogenous substrates existed between the control and phosphatase-treated GST-Pim-1 and to examine if the exogenous phosphotransferase activity of the control and phosphatase-treated GST-Pim-1 changed after autophosphorylation, a large scale *in vitro* autophosphorylation experiment was performed. At discrete time points from 1-20 min, aliquots of the autophosphorylation reaction were removed and added to P4 peptide substrate assays. Results indicated that the activity of all samples towards exogenous substrates was similar and no trends were apparent as a consequence of autophosphorylation (data not shown).
APPENDIX VIII
PRELIMINARY EXPERIMENTS IN OOCYTE SYSTEMS

1. ASSOCIATED PROTEINS AND SUBSTRATES OF X. LAEVIS PIM-1

Many cytoplasmic proteins associate in large multimeric complexes in cells. These complexes may function to bring regulatory subunits into close proximity to catalytic proteins, or may allow enzyme-substrate combinations to interact with greater efficiency. In many cases, these proteins have been discovered by cross-linking or immunoprecipitation experiments.

Pim-1 is a relatively small kinase with very short N- and C- terminal regions outside of the catalytic domain. It is highly likely that other proteins interact with the Pim-1 to modulate its activity. These putative factors might function in a cell-cycle dependent manner, similar to the interaction of other cell cycle modulators with CDKs and cyclins. Attempts were made to identify proteins that associated with or acted as substrates for the kinase. We used two novel approaches for this study, fusion protein affinity columns and Far Western blotting.

i. Recombinant GST-Pim-1 fusion protein affinity columns

Because of the initial lack of availability of a Pim-1-specific immunoprecipitating antibody, a novel approach was used to isolate regulator or substrate proteins that associate with Pim-1 in the cell. Recombinant GST-Pim-1 bound to glutathione beads by the GST-glutathione affinity interaction were mixed with frog oocyte extracts to allow binding to the Pim-1 portion of the fusion protein. The slurry was poured into a syringe column, the beads were washed extensively, removed from the column and subjected to analysis by SDS-PAGE electrophoresis. The affinity column extracts were examined for protein binding by several different methods. Silver staining was used to detect large amounts of bound proteins. Immunodetection with various antibodies was used to screen for the presence of specific proteins. Phosphorylation assays were done to determine if any of the proteins retained by the columns were substrates for GST-Pim-1. As a control, cellular extracts were first applied to GST columns to remove any proteins that would bind nonspecifically to either GST or to the glutathione beads. To ensure that any of the proteins present were not co-purifying bacterial proteins, identical experiments were performed with PBS or STE buffer instead of cell extracts. Many combinations of cell extracts and affinity matrix were examined with inconsistent results.
a. *X. laevis* oocyte extracts bound to human GST-Pim-1

Before the *X. laevis* Pim-1 was cloned and expressed, immobilized human GST-Pim-1 was used as an affinity matrix to bind proteins in the *X. laevis* oocyte extracts. Silver staining detected a number of bands in all GST-Pim-1 lanes including controls (Fig. 54, panel A). Unique bands of 25 kDa, 40 kDa, 55/60 doublet, 80 kDa and a 80/110 kDa doublet were observed in lanes corresponding to the immature and mature *X. laevis* extracts bound to the GST-Pim-1, but not in control lanes.

The blot was probed with various antibodies directed against Raf (Raf1-CT, UBI) and cyclins A, D and E (from Dr. F. Hall, University of California, L.A.). Raf1-CT and cyclin D antibodies did not detect any bands, cyclin A antibody detected a 65-68 kDa singlet and a 80 kDa triplet in all the *X. laevis* lanes, and the cyclin E antibody detected a 27.5 kDa band in all lanes including controls [data not shown]. When this experiment was repeated, similar results with the cyclin A and E antibodies were not obtained.

Phosphorylation assays revealed strongly autophosphorylated bands of 60 kDa, 30 and 35 kDa in each lane corresponding to the full-length and degraded Pim-1 fusion protein, as well as a faint band at 50 kDa (Fig. 54, panel B). In the immature and mature frog oocyte samples there were phosphorylated proteins of 25, 40 and 45 kDa. It is possible that the 24 and 40 kDa phosphorylated proteins were the same ones as visualized by silver staining.
Figure 54. *X. laevis* oocyte extracts bound to *H. sapiens* GST-Pim-1 fusion protein affinity columns.
Control (lanes 1, 4, 7), immature *X. laevis* oocyte extracts (lanes 2, 5, 8) and mature *X. laevis* oocyte extracts (lanes 3, 6, 9) were precleared on GST (lanes 1-3) then bound to human GST-Pim-1 columns (lanes 4-9). Panel A shows a silver stain of the GST control and GST-Pim-1 affinity matrix, and panel B shows an autoradiograph of the autophosphorylated GST-Pim-1 affinity matrix from lanes 4-6. Sharp arrows (—→) indicate *X. laevis* proteins bound to the GST-Pim-1, open arrows (<→) indicate phosphorylated GST-Pim-1-bound *X. laevis* proteins. Migrations of Mr standards are shown on the left.
b. *X. laevis* oocyte extracts bound to *X. laevis* GST-Pim-1

When the *X. laevis* Pim-1 fusion protein became available, it was used to bind proteins in *X. laevis* oocyte extracts, with the rationale that this would be more representative of physiological protein-protein interaction. Silver staining of the extracts revealed that several proteins bound to the GST controls including those of 30, 40 and 45 kDa (Fig. 55). An 85 - 90 kDa protein bound to the *X. laevis* GST-Pim-1 but not to the control. In an *in vitro* phosphorylation assay of proteins bound to beads, only the fusion protein and fusion protein byproducts were phosphorylated; the 85-90 kDa protein was not. As well, no proteins were phosphorylated when the GST-Pim-1 was used to phosphorylate the membrane in a modified Far Western blot.

The affinity column method initially seemed like a very promising approach to identify proteins that associate with Pim-1. Although this method worked well to confirm the interaction of known proteins, it was not very suitable for detecting the interaction with unknown proteins. Screening bound proteins with a selection of antibodies proved not to be very efficient unless a specific protein to which an antibody was available was expected. The phosphorylation assay detected Pim-1 substrates, but may not have detected regulators that associate and modulate the activity of Pim-1 independent of phosphorylation.
Figure 55. *X. laevis* oocyte extracts bound to *X. laevis* GST-Pim-1 fusion protein affinity columns - silver stain.

Control (lanes 1, 4), immature *X. laevis* oocyte extracts (lanes 2, 5) and mature *X. laevis* oocyte extracts (lanes 3, 6) were precleared on GST-glutathione agarose (lanes 1-3) then bound to *X. laevis* GST-Pim-1 columns (lanes 4-6). Arrows (——) show *X. laevis* proteins bound to the GST-Pim-1 glutathione agarose. Migration of Mr standards are shown on the left.
ii. Far Western Blotting

A second method to detect potential physiological substrates of Pim-1 was Far Western blotting. This method has been successfully used by other groups in the past to identify proteins that specifically interact [Kaelin et al., 1992]. This method relied on the ability of expressed kinase to recognize and phosphorylate denatured proteins on a membrane. Extracts of Mono Q-fractionated X. laevis oocytes were subjected to SDS-PAGE and were transferred onto PVDF membrane. The membranes were then subjected to an "in vitro" autophosphorylation assay and autoradiography to detect any proteins that would autophosphorylate while bound to the PVDF. None were detected. The membrane was then incubated in the presence of radiolabelled ATP with large amounts of X. laevis GST-Pim-1, extensively washed and then autoradiography was performed (Fig. 56). The arrow indicates the GST-Pim-1 used as a control.

The strongest protein bands phosphorylated by GST-Pim-1 were in the MonoQ wash through fractions 8-13. These proteins were 33-35, 38, 53, 55, 66, and 90 kDa. In fractions 22/24 and faintly in 26/28 was a phosphorylated 42 kDa band that might be the endogenous Pim-1 protein, implying that Pim-1 can cross-phosphorylate. There was also a strongly phosphorylated group of proteins of 20-25 kDa of an unknown identity in fractions 33-40 (0.46-0.65 M NaCl).

An elegant aspect of the Far Western blotting procedure is that the phosphorylated blot can be subsequently probed with antibodies to identify potential substrates. Unfortunately, as with the affinity column binding approach, this method is most useful if a specific protein is already suspected of being a potential substrate and does not work well for general screening.

A second variation of Far Western blotting was tested with less successful results. Radiolabelled, inactivated fusion protein was used to screen renatured Western blots which were then washed under conditions of low stringency and autoradiographed. Alternatively, antibodies specific for the fusion proteins could be used to detect the bound fusion protein. This method allows identification of protein-protein interactions between proteins that are not necessarily substrates, an approach that could be especially useful for finding upstream regulators of a protein. This method was attempted, but no signal was detected on the blots and further experiments along these lines were not performed.
Figure 56. Far Western blot of fractionated *X. laevis* oocytes phosphorylated by expressed GST-Pim-1. GST-Pim-1 positive control in far left lane, unfractionated *X. laevis* oocyte extract in far right lane. Numbers below indicate MonoQ fractions loaded in each lane, in most cases several fractions were pooled. Arrow indicates location of GST-Pim-1 control. Migrations of Mr standards is shown on the right. The autoradiogram shown was exposed for 21 h.
2. PRELIMINARY DATA FROM THE *P. OCCHRACEUS* SYSTEM

During the course of examination of Pim-1 in sea star extracts, several other approaches were employed. Although these preliminary studies were not completed, these approaches revealed potentially productive areas of future Pim-1 research.

i. GST-Pim-1 affinity columns

GST-fusion protein affinity columns can be utilized to help identify protein-protein interactions. Using human GST-Pim-1, immature and mature sea star oocyte extracts were examined for proteins that bind to recombinant Pim-1. A 48 kDa protein as well as several higher molecular weight species bound to the GST control lanes (Fig. 57). There were no proteins detectable by silver staining that selectively bound to the GST-Pim-1 column, despite the fact that very large amounts of sea star extracts (10 mg protein) were used. Although no GST-Pim-1 binding proteins were detected, it is possible that silver staining was not sensitive enough to detect this interaction if the binding protein was rarely expressed and present in only low quantities.

ii. Far Western blots of sea star oocyte maturation

Far Western blotting was done with sea star oocyte extracts in an attempt to identify potential substrates of Pim-1. Cytosol from immature and mature sea star oocytes were fractionated on a Mono Q and Western-blotted. The blots were first incubated with ADB and [γ^{32}P]ATP, washed and autoradiographed as a control. No bands were observed in the control. Bacterially expressed human GST-Pim-1 was used to radiolabel the blots in the presence of ADB and [γ^{32}P]ATP and -the blots were washed and autoradiographed. The control lane (GST-Pim-1) was phosphorylated, but unlike with the *X. laevis* oocytes, the sea star blots did not feature many radioactively labelled bands.
Figure 57. Sea star oocyte extracts bound to GST-Pim-1 fusion protein affinity column. PBS control (lanes 1 & 4), 10 mg of immature (lanes 2 & 5) and 10 mg mature (lanes 3 & 6) sea star oocyte extract protein was precleared on GST-glutathione beads (lanes 1-3) then applied to GST-Pim-1-glutathione beads (lanes 4-6). After washing, the entire bead-supernatant slurry was electrophoresed on a SDS-PAGE gel and silver stained. Migrations of Mr standards are shown at right.
iii. Upstream activators of Pim-1

To determine whether there were any maturation-activated kinases in the sea star extract that could phosphorylate Pim-1, Mono Q-fractionated sea star extracts were used to phosphorylate deactivated *H. sapiens* GST-Pim-1 bound to glutathione beads. The GST-Pim-1 was incubated with MonoQ-fractionated immature and mature sea star oocyte extracts in the presence of [γ32P]-ATP, washed extensively and Western blotted with Pim-1 antibodies (Fig. 58). Panel A demonstrated that the amount of protein in each lane was fairly constant. Panel B showed that mature sea star oocytes contained Pim-1 phosphorylating kinases in fractions 28-32 (0.35-0.45 M NaCl). Immature oocyte fractions contained no Pim-1 phosphorylating activity (data not shown). As sea star Pim-1 was eluted primarily in fractions 23-26, this phosphotransferase activity was unlikely to result from endogenous sea star Pim-1 phosphorylating the GST-Pim-1, but may be due to the action of a unique maturation-activated Pim-1 kinase (PIK).

iv. In vitro fertilization studies

Preliminary studies were carried out by Dr. Diana Lefebvre in our laboratory to examine Pim-1 protein following *in vitro* fertilization of 1-methyladenine-treated sea star oocytes. In brief, immature and mature oocyte samples as well as those corresponding to 6 and 24 hours post-fertilization, and 2 and 4 day embryos were fractionated by MonoQ column chromatography. Western blots of the fractions were probed with Pim1-NT antibody (Fig. 59). A 45 kDa band was observed in fractions 13/14 - 15/16, and was detected very strongly in fractions 23/24 -25/26 in the immature oocytes. This 45 kDa band was also observed in the mature extract, fractions 23/24 -25/26. The 45 kDa bands were hardly detected in the 6 and 24 hour post-fertilized samples, were strongly evident in the 2 day embryo, but not seen in the 4 day embryo. This implies that the Pim-1 protein was present in the immature and in the unfertilized, mature oocyte, but that the protein was virtually non-existent after fertilization and reappeared again in the 2 day embryo. The Pim-1 was then gone again in the 4 day embryo. These initial studies are quite promising, as the fluctuating Pim-1 protein levels of indicate that the kinase may have a changing role at different stages of early development.
Figure 58. Phosphorylation of GST-Pim-1 by a maturation-activated kinase.

Panel A- Western blot of *X. laevis* GST-Pim-1 phosphorylated by MonoQ-fractionated mature sea star oocyte cytosolic extracts. Membrane was probed with 4G10 anti-phosphotyrosine antibody.

Panel B- Autoradiogram of membrane from panel A. Western blot and autoradiogram of GST-Pim-1 phosphorylated by immature sea star extracts was not shown. Migrations of *M*₅ standards are shown on right.
Figure 59. Western blot of fractionated in vitro fertilized sea star oocytes.
Immature (panel A), mature (panel B) and fertilized oocytes at 6 h (panel C), 24 h (panel D), 48 h (panel E) and 96 h (panel F) post-fertilization were fractionated on a MonoQ column and electroluted on a 10% SDS-PAGE gel. MonoQ fractions are shown on the top of the blot. Blots probed with Pim1-NT antibody. The 46 kDa Mr marker is shown on right. Western blots done by Diana Lefebvre.