I. ABSTRACT

Interferon-γ (IFN-γ) plays an important role in host defense against infection and cancer. Many of its biological effects are mediated through the IFN-γ receptor (IFN-γR)-which is linked to the cytoplasmic tyrosine kinases Jak1 and Jak2 and to the transcription factor Stat1. However, regulation of IFN-γ receptor signalling is not fully understood and not all responses to IFN-γ are Stat1-dependent. Research described in this thesis used two strategies to identify novel components of IFN-γ signalling. First, a biochemical approach was used to enrich for phosphotyrosine containing proteins regulated by IFN-γ. This involved sub-cellular fractionation, Poly (Glu, Tyr) affinity chromatography, preparative SDS-PAGE, followed by immunoprecipitation of eluted proteins based upon reactivity with antiphosphotyrosine antibodies and microsequencing by mass spectrometry. Using this approach, the transcriptional co-activator TIP120a was identified as a p125 IFN-γ-inducible tyrosine phosphorylated protein in IFN-γ-treated U937 cells, a human monocytic cell line. The finding that tyrosine phosphorylation of TIP120a occurs in IFN-γ treated cells suggests that this post-translational modification may influence its function and that TIP120a itself may be involved in regulating gene expression in response to IFN-γ.

A second, genetic approach was used to identify novel proteins that interact with the IFN-γ receptor. The cytoplasmic domains of the human IFN-γR1 and IFN-γR2 chains were used as bait in a yeast two-hybrid screen of a human monocyte cDNA library. This screen identified two novel interactions. S100A9, a calcium-regulated, EF-hand type
protein was found to bind to the IFN-γR1 chain and annexin A5 (AxV), also a calcium regulated and phospholipid binding protein, was identified as a IFN-γR2 binding partner. These interactions were confirmed in pull-down experiments in which lysates of the human monocytic cell line THP-1 were incubated with the R1 and R2 cytoplasmic domains expressed as glutathione-S-transferase (GST)-fusion proteins. Additional verification of these interactions was obtained when the IFN-γR1 and IFN-γR2 subunits were immunoprecipitated from lysates of THP-1 cells. In this analysis, S100A9 and AxV were found to co-immunoprecipitate with their respective binding partners from the two hybrid screen and in both cases, these associations were shown to be ligand-dependent.

To examine the role of AxV in IFN-γ signalling, small interfering RNA (siRNA) was used to reduce AxV expression in human embryonic kidney cells (293T cells), an IFN-γ responsive cell line. In cells where AxV protein levels were reduced to ≤ 20% of control cells (AxV_{LO} cells), activation of Jak2 and Stat1 in response to IFN-γ, as assessed by tyrosine phosphorylation, was markedly enhanced. The impact of enhanced tyrosine phosphorylation of Stat1 in AxV_{LO} cells was assessed by two approaches. First, analysis of gene transcription by RT-PCR showed that IFN-γ treatment of AxV_{LO} cells was associated with increased expression of the IFN-γ-inducible genes Egr-1 and IFN-γR2. Second, when IFN-γ-induced growth inhibition, a hallmark of the IFN-γ response was examined, the anti-proliferative effect of IFN-γ was found to be significantly enhanced in AxV_{LO} cells. Taken together, results suggest that AxV negatively regulates IFN-γ signalling through an inducible association with the IFN-γR2 subunit that controls the levels of activation of Jak2 and Stat1.
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<th>Description</th>
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<tr>
<td>3-AT</td>
<td>3’-amino-1,2,4-triazole</td>
</tr>
<tr>
<td>4G10</td>
<td>anti-phosphotyrosine antibody</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>AxV</td>
<td>Annexin V</td>
</tr>
<tr>
<td>AxV&lt;sub&gt;Lo&lt;/sub&gt;</td>
<td>Cells expressing low levels of AxV</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine 3’5’ monophosphate</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster domain</td>
</tr>
<tr>
<td>DAP</td>
<td>Death associated protein</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>DsRNA</td>
<td>Double stranded RNA</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(-aminoethyl ether)-N,N,N’,N’-tetraacetic acid</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GAS</td>
<td>Gamma activated sequence</td>
</tr>
<tr>
<td>GATE</td>
<td>Gamma activated transcriptional element</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast performance liquid chromatography</td>
</tr>
<tr>
<td>HA</td>
<td>Hemaglutinin</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>HRPO</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>ICE</td>
<td>Interleukin converting enzyme</td>
</tr>
<tr>
<td>ICER</td>
<td>Inducible cyclic adenosine monophosphate repressor</td>
</tr>
<tr>
<td>ICSBP</td>
<td>Interferon consensus binding protein</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IFN-γR</td>
<td>Interferon gamma receptor</td>
</tr>
<tr>
<td>IFN-γR1</td>
<td>Interferon gamma receptor alpha</td>
</tr>
<tr>
<td>IFN-γR2</td>
<td>Interferon gamma receptor beta</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-thiogalactopyranoside</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon-stimulated gene</td>
</tr>
<tr>
<td>Jak</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide, endotoxin</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-([N\text{-morpholino}])propanesulfonic acid</td>
</tr>
<tr>
<td>NAPS</td>
<td>Nucleic acid and protein sequencing facility</td>
</tr>
<tr>
<td>OAS</td>
<td>2'-5'-oligoadenylate synthetase</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI 3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKR</td>
<td>Double stranded RNA-activated protein kinase</td>
</tr>
<tr>
<td>PLA\textsubscript{2}</td>
<td>Phospholipase A\textsubscript{2}</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>Pyk2</td>
<td>Src/Proline rich tyrosine kinase 2</td>
</tr>
<tr>
<td>RKA</td>
<td>Renaturation kinase assay</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmuno protective assay</td>
</tr>
<tr>
<td>RNAse</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SHP</td>
<td>Src homology domain phosphatase</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signaling</td>
</tr>
<tr>
<td>Stat1(\alpha)</td>
<td>Signal transducer and transcriptional activator 1(\alpha)</td>
</tr>
<tr>
<td>TBP</td>
<td>Tata box-binding protein</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TIP120a</td>
<td>TBP interacting protein of 120 kDa</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
I dedicate this thesis to my dear wife Yane and my daughter Lucia, who provided their love, support, patience and encouragement. I also dedicate this work to my parents Eleuterio and Carlota to whom I owe so much.

I am grateful to my supervisor Neil Reiner for his guidance and support. I learned very much about science, but foremost about myself, from him. I am grateful to the members of Neil’s lab: Zakaria Hmama, Linda Ip, Keith Knutsen, Chrystal Lapinski, Jimmy Lee, Raymond Lo, Martin Lopez, Devki Nandan, Sanaa Noubir, Rafat Sobouti for their help, suggestions and friendship.

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I am grateful to all the people that I met during these years in and outside of UBC and from whom in one way or another I have learned so much. A special thanks for my friends and my community who have supported me in all imaginable ways.

Last but in no way least, I want to thank God for providing me the means to complete this work, but specially for showing me through my life in and outside the lab, how helpless I am without his love and guidance.
VII. INTRODUCTION

A. INTERFERON-γ

In addition to modulating the immune response, interferon gamma (IFN-γ), a member of the interferon family of cytokines, mediates both anti-viral and anti-proliferative responses (1). IFN-γ is a homodimeric protein produced by natural killer cells, CD4⁺ T₁ cells, CD8⁺ T cells and macrophages (2). NK cells secrete IFN-γ in response to recognition of certain microbial components or in response to IL-12, as part of the innate immune response. During the adaptive immune response, T cells produce IFN-γ in response to antigen recognition, and this is also enhanced by IL-12. Homodimeric IFN-γ binds to the IFN-γ receptor in a species-specific manner (3).

Amongst the pleiotropic functional properties of IFN-γ, the most thoroughly studied include host defense against infection and neoplasia as well as its contributions to the inflammatory response associated with tissue injury. Selected functions of IFN-γ are shown in Figure 1. In many instances, the activities of IFN-γ are redundant with those of other cytokines (4). A notable exception to this, however, is the apparently singular role of IFN-γ in activating macrophages to kill certain intracellular pathogens such as Mycobacteria, Salmonella spp and Listeria monocytogenes (5-7). Mechanisms involved in IFN-γ-induced antimicrobial activity include both the production of reactive oxygen and reactive nitrogen intermediates (8;9), through the activation respectively of the phagocyte oxidase and nitric oxide synthase. Interferons are essential for conferring upon cells the ability to rapidly resist viral infection, as has been demonstrated by the
increased susceptibility to viral infection of mice lacking the IFN-α/β or IFN-γ receptors (10). In fact, Type II IFN (or IFN-γ) was initially identified in the mid-1960’s based on its antiviral activities (3).

IFN-γ induces anti-viral activity through activation of double-stranded RNA-dependent protein kinase R (PKR) and 2',5'-oligoadenylate synthetase. The anti-viral effect of PKR is due to its phosphorylation of the alpha subunit of the initiation factor eIF2. Phosphorylation of eIF2α leads to the formation of an inactive complex which results in rapid inhibition of translation (11). The essential role of PKR in IFN-γ-induced anti-viral activity is illustrated by the findings that treatment with either IFN-γ or Poly(I):poly(C) induces resistance to encephalomyocarditis virus infection in wild type mice but not in PKR null mice (12).

The IFN-inducible enzyme 2',5'-oligoadenylate synthetase (OAS) is also critical for anti-viral resistance. 2',5'-OAS is activated in the presence of viral dsRNAs to produce short oligoadenylates that activate the oligoadenylate-dependent RNAse L, leading to the degradation of viral RNA (13). IFN-γ can also induce nitric oxide synthase (iNOS) in murine macrophages to inhibit the replication of many viruses including ectromelia, vaccinia and HSV-1 (9).
Figure 1. Selected biological activities of IFN-γ. IFN-γ plays an important role in the immune response and affects most cells of the immune system, such as the B cells, T cells and antigen presenting cells such as macrophages and dendritic cells.

Modified from Abbas, Lichtman and Pober, 2000
IFN-γ also acts on B cells to influence immunoglobulin class switching (14). Ig class switching is significant because different Ig classes and isotypes promote distinct effector functions in the host. For example, IFN-γ is able to block IL-4 induced Ig class switching in murine B cells from IgM to either IgG1 or IgE (14), thus modulating the immune response.

Other mechanisms through which IFN-γ contributes to resistance to infection are by activation of neutrophils and by stimulating the cytolytic activity of NK cells. Ultimately, the vital role of IFN-γ in resistance to infection is best revealed by experiments of nature wherein genetic defects in the IFN-γ response pathway render macrophages unresponsive to IFN-γ (5-7). For example, Roesler et al., (15) described a patient with a mutation in the R1 subunit of the IFN-γ receptor due to a deletion resulting in the absence of exon 3. Flow cytometric analysis showed that monocytes from this individual could not bind a monoclonal antibody specific for the IFN-γR1. This deficiency was associated with recurrent infections with Mycobacterium bovis BCG and Listeria monocytogenes. Newport et al. (16) characterized the genetic basis for deficient immune responses in four children who presented with increased susceptibility to mycobacterial infection. Using microsatellite analysis and immunofluorescence studies, the authors identified region 6q on chromosome 6 containing the locus for IFN-γR1 where all four children where homozygous for eight different markers. DNA sequencing revealed a common point mutation that generated a truncated receptor deficient in the transmembrane and cytoplasmic domains. Immunofluorescense studies showed that the receptor was not expressed in leukocytes.
from the affected children, linking this mutation to increased susceptibility to mycobacterial infections.

IFN-γ also modulates a subset of genes involved in apoptosis (17). In particular, DAP-1 to -5, thioredoxin and cathepsin D play a role in IFN-γ-mediated apoptosis (18). Depending on the state of differentiation or the presence of factors such as serum or IFN-γ receptor levels, IFN-γ may have either pro- or anti-apoptotic activities (17;19). IFN-γ has also been shown to play a role in inducing growth arrest through the induction of Stat1, a transcriptional activator involved in IFN-γ signalling (20;21).

Understanding how IFN-γ conveys a message from the cell membrane to the nucleus increased rapidly following characterization of the IFN-γ receptor. Knowledge about the subunit composition of the IFN-γ receptor came from key genetic experiments conducted by Pestka and co-workers (22) that examined stable murine:human somatic cell hybrids that contained the full complement of murine chromosomes and a random assortment of human chromosomes. These studies revealed that all hybrids containing human chromosome 6 were able to bind human IFN-γ. However, biologic responsiveness to human IFN-γ was only found in hybrids that contained both human chromosomes 6 and 21. This led to the hypothesis that fully functional human IFN-γ receptor consisted of two or more species-specific and species-matched subunits (22) encoded on these two human chromosomes. Purification of the ligand binding subunit (23) followed by identification of the second signal transducing component (24) by a complementation cloning approach, established formal proof for the two subunit model.
The functional IFN-γ receptor (IFN-γR) is a heterotetramer composed of two proteins, R1 and R2, that belong to the class 2 cytokine receptor superfamily (Figure 2). This group of receptors is characterized by intracellular domains devoid of intrinsic tyrosine kinase or phosphatase activities. IFN-γR1 (also known as the alpha chain or CD119w), a 90 kDa polypeptide encoded by a gene located on human chromosome 6, plays important roles in mediating ligand binding, ligand internalization and signal transduction (2;3). IFN-γR2 (also known as the beta chain or accessory factor-1), a 62-kDa polypeptide encoded on human chromosome 21, plays only a minor role in ligand binding but is required for signalling (24).

In parallel with the characterization of the receptor, critical genetic and biochemical experiments were being carried out in the laboratories of James Darnell, Ian Kerr, George Stark and James Ihle that identified novel signalling pathways activated by the IFN-α and IFN-γ. These pathways involved activation of a family of protein tyrosine kinases, known as Janus kinases or Jaks and a family of latent cytoplasmic transcriptional factors, now known as Stats (signal transducers and activators of transcription) (25). Stat1 has been implicated in regulating IFN-γ signalling (25) as will be described below. Transcription of Stat1 is known to be followed by alternative splicing, resulting in both 84- (Stat1β) and 91-kDa (Stat1α) forms of Stat1, where only the larger form of Stat1α is transcriptionally active (25). In this thesis, further discussion will be restricted to Stat1α, hereafter referred to as Stat1, as it is the only form involved in IFN-γ signalling.
At least three lines of evidence implicated Jaks 1 and 2 and Stat1 in mediating IFN-γ-dependent cellular responses. First, isolation and complementation of mutant human cell lines defective in Jak1 and Jak2, unambiguously showed that these kinases were required for activation of IFN-γ-inducible genes (25). Second, Stat1 was identified as a novel latent transcription factor, which underwent rapid tyrosine phosphorylation and activation in IFN-γ-treated cells (25). Direct implication of Stat1 in IFN-γ signalling was made possible by the identification of a family of genes that were induced rapidly (within 15 to 30 min) in response to IFNs (26). Analysis of the promoter sequences of these genes, termed ISGs (interferon-stimulated genes) revealed the presence of two classes of conserved nucleotide sequences that directed their rapid transcriptional activations by interferons. One of these sequences, termed the gamma-interferon activation site (GAS) was shown to be a nine nucleotide site that affected the transcriptional activation of IFN-γ induced genes and was found to bind only Stat1 (26). Third, through structure-function analyses of the intracellular domains of the IFN-γ receptor, specific binding sites on Jak1 and Jak2 were identified (27). This led to the identification of a motif on the R1 chain containing Y\(^{440}\) that became tyrosine phosphorylated in response to IFN-γ and which functioned as a binding site for Stat1. This provided a mechanism for receptor activation linking the receptor to the signal transduction machinery (2). A key experiment showed that an R1 Y_F\(^{440}\) mutant failed to support IFN-γ-dependent cell activation (28), establishing a link between the ligand-induced receptor phosphorylation of Y\(^{440}\) and activation of the Jak-Stat pathway.
The current model of IFN-γ signalling based on these findings is presented in Figure 2. In this model, the R1 (α) subunit binds ligand leading to receptor oligomerization and activation of Jak kinases 1 and 2 which are constitutively associated with the R1 and R2 subunits, respectively (27). As a result, the activated Jaks trans-phosphorylate each other as well as the IFN-γR1 subunit on Y440 which serves as a docking site for Stat1 (signal transducer and activator of transcription) (25). Recruitment of Stat1 to the receptor results in its phosphorylation on Y701 after which it dissociates from the R1 subunit, dimerizes and translocates to the nucleus where it binds to GAS elements present within the promoter regions of many IFN-γ-activated genes. Stat1 also undergoes IFN-γ-induced phosphorylation on serine727 and this is required for maximal transcriptional activity (29).

Abnormalities in components of the IFN-γR-Jak-Stat1 pathway illustrate its critical importance in mediating cellular responses to IFN-γ. For example, specific mutations or deletions in components of the canonical IFN-γ signalling pathway such as IFN-γR1, IFN-γR2 or Stat1, render both cells and individuals highly susceptible to infection with non-tuberculous mycobacteria, Salmonella spp., Listeria monocytogenes and other pathogens of normally limited virulence (5-7).
Figure 2. Jak-Stat pathway of IFN-γ signalling. IFN-γ binds to the R1 extracellular domain. This leads to receptor oligomerization and activation of Janus kinases Jak1 and Jak2, which are constitutively associated with the receptor chains. The activated Jaks phosphorylate each other and the R1 subunit on residue Y440 which generates a docking site for Stat1. When Stat1 is recruited to the receptor it is tyrosine phosphorylated. This promotes its dissociation from the receptor, along with dimerization and translocation to the nucleus. In the cytosol, Stat1 is phosphorylated on serine by a PKC-dependent mechanism. This serine phosphorylation is required for maximal transcriptional activity. In the nucleus, phosphorylated Stat1 homodimers bind to the GAS sequence present in many IFN-γ-inducible genes.
B. REGULATION OF IFN-γ SIGNALLING

The response to IFN-γ is regulated at multiple levels and it is very likely that not all of the control mechanisms have yet been identified. The ability of IFN-γ to inhibit proliferation of type 2 helper T cells (TH2), but not of type 1 (TH1), was associated with the lack of expression of the IFN-γR2 in the TH1 cells (30). Another level of regulation involves dephosphorylation of the Jaks by the tyrosine phosphatase SHP-1. SHP-1 association with Jak2 is enhanced by Leishmania infection, suggesting that it may dephosphorylate Jak2 leading to the attenuation of the IFN-γ response (31). The association and dephosphorylation of Jak2 by SHP-1 was demonstrated in a co-transfection model of COS-7 cells (32). The association of SHP-2, a dual specificity phosphatase, with the Jaks was characterized by Yin et al. (33). They also showed that SHP-2 is a substrate for Jak1 and Jak2-induced tyrosine phosphorylation. SHP-2 has also been shown to be involved in Stat1 dephosphorylation at both tyrosine and serine residues in the nucleus (34). Interestingly, thus far there is no direct evidence for recruitment of these tyrosine phosphatases to the IFN-γ receptor subunits per se.

Recently, it has been established that IFN-γ induces the expression of members of the suppressors of cytokine signalling (SOCS) family of regulatory proteins. These proteins bind to either Jaks or Stat1 and attenuate signalling through the Jak-Stat1 pathway. Binding of SOCS proteins to Stats or Jaks leads to their ubiquitination and subsequent degradation by the proteasome pathway (35-37).
Negative regulation of IFN-γ by other signalling pathways has also been described. For instance, stimulation of cAMP-, MAPK- or glucocorticoid-dependent signalling pathways can lead to inhibition of subsequent IFN-γ-dependent stimulation of Stat1 phosphorylation (38;39). This crosstalk may play a physiological role in homeostasis and in the control of inflammation.

Positive regulation of IFN-γ signalling includes the effects of type I IFN’s on type II IFN signalling. Pretreatment of cells with IFN-γ augments a subsequent IFN-α response through the induction of the expression of the transcriptional factor p48. IFN-α then increases the intracellular levels of Stat1, thereby enhancing the IFN-γ response (40). As will be discussed below, a variety of other signal transduction proteins are also involved in the IFN-γ response, and regulating either the level or the activity of these various proteins is likely to influence IFN-γ action.
C. POTENTIAL BIOLOGICAL SIGNIFICANCE OF AN ACCESSORY PATHWAY OF INTERFERON GAMMA SIGNALLING

Despite the evident importance of the Jak-Stat pathway, several recent lines of evidence indicate that cellular responses to IFN-\(\gamma\) can occur independently of Stat1 (Figure 3). Two independent reports initially focused attention upon the importance and biological significance of IFN-\(\gamma\)-mediated, Stat1-independent signalling pathways. First, IFN-\(\gamma\)-mediated resistance to Sindbis virus and murine cytomegalovirus infection in Stat1-null mice suggested that an IFN-\(\gamma\)-dependent, Stat1-independent pathway(s) regulates at least some antiviral responses (41;42). Second, the most compelling evidence for Stat1-independent IFN-\(\gamma\) responses came from gene expression studies. Using cDNA microarrays and other approaches, IFN-\(\gamma\) was shown to regulate the expression of as many as 500 genes (20). Studies using Stat1-null mice identified both Stat1-dependent and Stat1-independent mechanisms of transcriptional regulation by IFN-\(\gamma\) (41;42).

Evidence for Stat1-independent signalling has generated considerable interest in identifying other proteins involved in IFN-\(\gamma\) receptor signalling. Figure 3 shows some of the signal transduction pathways that may be involved in IFN-\(\gamma\) signalling. One interesting pathway involves recruitment of the adaptor molecule c-Cbl to the IFN-\(\gamma\)R in a human promyelocytic leukemia cell line. C-Cbl is tyrosine phosphorylated in response to IFN-\(\gamma\) and this provides a docking site for CrkL (43). Recruitment of CrkL results in activation of the guanine nucleotide
Figure 3. IFN-γ receptor signalling pathways. Several different molecules implicated in IFN-γ signalling are depicted in this figure and are discussed in the text. Except in the case of the Jaks and Stat1, there is no direct evidence to show that any of these proteins interact directly with the receptor chains, suggesting that adaptor proteins are likely involved in linking these proteins to IFN-γR signalling.
exchange factor C3G and the small G protein Rap-1 and this pathway appears to account for some of the anti-proliferative effects of IFN-γ (44).

The transcription factors CEBP-β and ICSBP have recently been implicated in IFN-γ signalling. IFN-γ has been shown to activate transcription through the interaction of CEBP-β with a novel gamma activated transcriptional element (GATE) that is distinct from the Stat1-binding GAS element (45). IFN consensus binding protein (ICSBP) is a member of the IFN regulatory factor (IRF) family. This IFN-γ-inducible, Stat1-dependent gene (46) has been shown to play a role in establishing IFN-γ-mediated resistance to various pathogens (47). Contursi et al., 2000 (48) showed that ICSBP is capable of stimulating transcription from IFN-γ-inducible promoters in a GAS-dependent manner, even in mutant cell lines lacking components of the Jak-Stat pathway. The significance of this finding is the potential for transcription factors activated by IFN-γ in a Stat1-dependent manner, to take over and drive the IFN-γ-dependent transcriptional activity after Stat1 activation is no longer present.

Other evidence for IFN-γ signalling independent of Stat1 comes from studies of phosphatidylinositol 3-kinase (PI3K). It was recently shown that IFN-γ induces monocyte cell adhesion in a PI3K-dependent, Stat1-independent manner (49). In this study, IFN-γ was shown to activate the serine threonine kinase Akt and this was abrogated by using PI3K inhibitors. Inhibition of Akt activation led to a reduction in IFN-γ-induced cell adhesion, while the induction of the IFN-γ-induced guanylate binding protein, which is Stat1-dependent, was not affected.
Another example of cellular responses to IFN-γ that are independent of Jak kinases is the induction of the inducible cAMP repressor (ICER) in macrophages (50). IFN-γ mediated activation of ICER, a potent inhibitor of gene transcription, is blocked by the casein kinase inhibitor apigenin, but not by inhibitors of either PKC (bisindolmyde), MAPK (SB202190 and PD98059) or Jak (AG490) under conditions where each inhibitors' action was verified. This work provides a potential basis to explain IFN-γ mediated gene suppression and apoptosis, as ICER has been implicated in mediating neuronal apoptosis (50).

A variety of other signal transducing molecules have also been shown to be activated by IFN-γ concomitant with Jak1 and Jak2. In some cases it is known and in others not, whether these elements intersect with the Jak-Stat1 pathway. For example, the MAP kinases ERK 1/2 (51;52), as well as the tyrosine kinases Pyk2 and Fyn are activated in IFN-γ treated cells (52;53). Pyk2 associates constitutively with Jak2 and is tyrosine phosphorylated and activated by IFN-γ. A dominant negative Pyk2 inhibits IFN-γ-dependent activation of Erk1/2 and phosphorylation of Stat1 on serine727 (52) providing evidence for cross-talk. Taken together, these findings clearly show that cellular responses to IFN-γ are not fully accounted for by simple signalling from the receptor to the nucleus through the Jak-Stat1 pathway alone.

Using a genetic approach, research carried out in this thesis identified two novel IFN-γ receptor binding proteins annexin V and S100A9. Below, characteristics and
properties and how these may in they may be involved IFN-γ signal transduction are discussed.

D. ANNEXIN V

Role of Annexin V (AxV) in cell biology and cell signalling.

Annexins represent a highly conserved family of calcium and phospholipid binding proteins [reviewed in (54)]. Characteristically, annexins bind preferentially to anionic phospholipids. Thirteen vertebrate annexins have thus far been identified, all of which contain the signature α-helical core domain consisting of a conserved 70 amino acid sequence that is repeated either four or eight times. The core domain is responsible for calcium and phospholipid binding, whereas the distinct biological properties of individual annexins are determined by a unique N-terminal domain. Annexins may be either cytosolic, associated with membranes or with the cytoskeleton. These associations are dynamically influenced by calcium as are interactions with negatively charged phospholipids. Specificity of binding to phospholipids by different annexins is determined by both the specific core region and by the amino terminal region. Annexins have been shown to have pleiotropic functional properties including regulation of membrane traffic and organization, apoptosis, cell transformation, ion channel formation, maintenance of calcium homeostasis, secretion of neurotransmitters and hormones, signal transduction and DNA replication (54-56).
Figure 4. Annexins: Calcium-dependent phospholipid binding proteins that interact with receptors and signal transduction molecules, as reviewed in (57) [(-) stands for inhibition of enzymatic activity by the annexin family member].
Although calcium dependent binding to phospholipids is a canonical property of annexins, calcium independent lipid binding has also been described. For example, AxV binds to platelet plasma membranes in a calcium independent fashion (58). It appears that these types of interactions may be mediated via binding of annexins to a protein ligand that is membrane associated. Annexins do in fact bind to diverse non-lipid ligands. (Figure 4). For example, in addition to binding to several well characterized transmembrane receptors, annexins have been shown to interact with several members of the S100 family of EF hand calcium binding proteins. These include AxII with S100A10, AxI with S100A11, AxVI with S100A1 and S100B and AxXI with S100A6 (54).

Other non-lipid ligands for annexins include cytoskeletal proteins and a variety of proteins involved in cell signalling. For example, both AxI and AxII have been shown to bind F-actin and AxV has been shown to interact with γ-actin (59-61). Other miscellaneous annexin ligands include p120 Ras GAP (62) and PKCα, both of which interact with AxVI, and PLA2 which interacts with AxI (63). The complex of AxVI with p120 Ras GAP was also shown to include the tyrosine kinases Fyn and Pyk2 (64).

AxV is one of the most abundant members of the annexin family. Its cDNA encodes a protein with 322 residues with a mass of 36 kDa. AxV has been recognized for its anti-inflammatory, growth inhibitory and anti-coagulant properties, as well as a role in inhibiting apoptosis and regulating calcium influx into cells. These features may relate to its ability to inhibit a variety of signalling molecules including PKC (65;66)
and PLA₂ (56;67-70) and its ability to regulate calcium ion channels (54;55). These findings are of particular interest in the context of this thesis research, since PKC, PLA₂ and calcium have been implicated in IFN-γ signalling (50;71) AxV has also been shown to interact with the vascular endothelial growth factor (VEGF) receptor Flk-1 and VEGF signalling is impaired in cells in which AxV expression was reduced by antisense treatment (72). Taken together, these findings suggest that members of the annexin family and AxV in particular may be involved in regulating cell signalling, perhaps including responses to IFN-γ. Undoubtedly, the recent creation of AxV null mice will be informative in elucidating the diverse roles of AxV including how it influences cell regulation (73).

E. S100A9

S100A9 -also known as p14, MRP-14, L1 heavy chain and calgranulin B- is a member of the S100 family of calcium-regulated proteins of the EF-hand type expressed in vertebrates. These proteins play important roles in intracellular and extracellular regulatory activities (57). Amongst various functions of the S100 proteins are included regulation of protein phosphorylation, transcriptional activity, calcium homeostasis, cell proliferation and differentiation (74).

S100 proteins are characterized by the presence of two calcium binding motifs of the EF-hand type that are joined together by an intermediate region. These two motifs bind calcium with different affinities, with the C-terminal domain having greater
affinity than the N-terminal domain (75). Most S100 proteins exist in the cell as homodimers. S100A9 is an exception as it also forms heterodimers with the S100A8 protein in a calcium-dependent manner (76). In fact, heterodimers of S100A8-S100A9 are the preferred form found in the cell (77). During inflammation, cytosolic monomers of S100A8 and S100A9 dimerize and are recruited to the membrane in a calcium-dependent manner. Human S100A9 has been shown to be phosphorylated on C-terminal Thr^{118} in a protein kinase C-independent and calcium-dependent manner (78). Another calcium-dependent change is the induction of arachidonic acid-binding capacity in S100A8/A9 complexes (79-81). Of interest, arachidonic acid has been shown to be released from IFN-γ-treated endothelial cell membranes in a phospholipase D-dependent manner and it has been suggested that this may influence PKC (82). These findings suggest a potential role for S100A8/A9 in IFN-γ receptor signalling.

Translocation of S100A8-A9 complexes to the plasma membrane has been associated with chemotaxis, degranulation, phagocytosis and oxidative burst activity in phagocytic cells (81;83;84). These functions are all properties of activated macrophages. Interestingly, the expression of S100A8 and S100A9 in monocytes takes place at an early stage of differentiation (85), but is abrogated in resident tissue macrophages (86). This, together with the frequent appearance of S100A8/A9-expressing monocytes in inflammatory lesions, as well as high serum levels of S100A8/A9 in patients with chronic inflammation (86), suggests a key role of these cells and proteins in inflammatory processes.
Extracellular S100A8/S100A9 heterodimers also have functional activity. For
instance these complexes have been shown to inhibit proliferation of HL-60 cells,
which represent immature, monomyelocytic cells (85). S100A8/S100A9 has also been
shown to have cytostatic activity towards bacteria and fungi (87). Additionally, studies
of S100A9-deficient mice (88;89) led to the conclusion that loss of S100A9 protein
reduces the responsiveness of neutrophils to chemoattractant stimuli.

Regarding other regulatory properties, complexes of S100A8/S100A9 have also
been shown to modulate the activity of casein kinase I and II, enzymes involved in the
phosphorylation of topoisomerase I and RNA polymerase I and II (90). These findings
have led to the suggestion these heterodimers might play a role in regulating the
maturation of myeloid cells. Casein kinase has also been shown to be involved in the
IFN-γ-inducible expression of ICER (91). These findings suggest the possibility that
ICER might represent a novel mechanism for IFNγ-dependent inhibition of gene
expression.
**Intracellular S100 family members**

- Regulation of enzyme activity
  - Aldolase, membrane-bound guanylate cyclase
  - Phospholipase A2, casein kinase I and II
- Calcium homeostasis
  - Calcium buffering
- Regulation of phosphorylation
  - Interaction with kinases and substrates
- Cytoskeleton
  - Co-localization with microtubules, filaments, Tropomyosin, myosin, F-actin, calponin
- Interaction with transcription factors
  - MyoD, bHLH, p53

**Extracellular S100A8/S100A9**

- Targeting of fatty acids
- Stimulation of CD11b expression
- Inhibition of immunoglobulin synthesis
- Inhibition of monocytic and mitogen
- Stimulated Lymphocyte proliferation

**Other S100 proteins**

- S100B: iNOS stimulation
- S100A2, A7 and A8: chemoattraction
- S100A4: neurite outgrowth
- S100A12: cytokine secretion

Modified from Donato, 2001 (57)

Figure 5. Intracellular and extracellular roles of S100 proteins. The S100 proteins have been involved in the regulation of protein phosphorylation, transcriptional activity, calcium homeostasis, cell proliferation and cell differentiation (74).
Objective 1. To identify and characterize novel IFN-γ-inducible tyrosine kinases and tyrosine phosphorylated proteins in human macrophages

**Rationale.** Our laboratory has had a longstanding interest in understanding how intracellular pathogens such as *Leishmania, Salmonella* and *M. tuberculosis* cause macrophage dysfunction, thereby promoting their own survival (88;92-100). Experiments with *Leishmania donovani* showed that in macrophages infected with this obligate intracellular pathogen, IFN-γ-induced tyrosine phosphorylation of Jak1, Jak2, and Stat1 was markedly impaired (92). Subsequent results indicated that impaired signalling through Jak-Stat1 was likely due to infection-induced activation of macrophage SHP-1 brought about by leishmania EF-1α (93;94). In contrast to this defect in Jak-Stat1 signalling, analysis of whole-cell lysates by anti-phosphotyrosine immunoblotting showed that incubation with IFN-γ brought about tyrosine phosphorylation of several macrophage proteins and these responses were equivalent in control and infected cells (92). These findings suggested the distinct possibility that IFN-γ activated tyrosine kinases in macrophages distinct from Jak1 and 2 and that these "novel" kinases are resistant to the effects of *Leishmania* infection.

Subsequently, direct evidence was obtained to support the existence of IFN-γ-activated, non-Jak tyrosine kinases that were differentially regulated. Using a renaturation kinase assay, we detected IFN-γ-activated protein tyrosine kinases that, in contrast to the Janus kinases, were sensitive to inhibition by TGF-β (101). These results
and others described in the Introduction to this thesis suggested the existence of non-Jak tyrosine kinases that are activated by IFN-γ in human macrophages. Based upon these results, experiments described in this thesis were designed to identify novel tyrosine kinases and tyrosine phosphorylated proteins activated by IFN-γ.

Objective 2. To identify novel IFN-γ receptor interacting proteins.

Rationale. The current paradigm of IFN-γ cell signalling (see Introduction), emphasizes the roles of Jak1, Jak2 (tyrosine kinases) and Stat1 (transcriptional activator). However, there is increasing evidence that other accessory molecules (Pyk2, Cbl, Abl, SH2-β, Vav) (43;52;102;103) are also involved. In particular, the adaptor molecules, Cbl and SH2-β, may interact directly with the receptor chains, in a similar way to their binding to other cytokine receptors (104). Also, there is no direct evidence for the binding of tyrosine phosphatases to the IFN-γ receptor, though there is evidence that they modulate Jak-Stat activities (31;32;34). To identify novel IFN-γR-interacting proteins, we chose the yeast two hybrid system, which is outlined in Figure 6. Based upon previous reports where intracellular domains of cytokine receptors were used to identify novel protein-protein interactions (105;106), we used intracellular domains of the human IFN-γR, R1 and R2 chains as bait in two-hybrid screens of a human monocyte cDNA library.
Two-hybrid system

Structure-function properties of a typical transcription factor:

- DNA-binding domain (DBD)
- Activation domain (AD)
- Reporter gene

Two-hybrid system: two types of hybrids:

- DBD protein (or domain) of interest ("bait")
- AD protein (or domain) ("prey")

By itself, the DBD bait fusion does not stimulate expression. When bait and prey interact, the reporter gene is expressed.

Figure 6. The Yeast two hybrid system. The yeast two hybrid screen is a genetic method to identify protein-protein interactions. It is based on the fact that the DNA-binding domain (DBD) and an activation domain (AD) of the Gal 4 transcriptional activator can be physically separated. The DBD is cloned as a fusion protein with the bait protein of interest (bait). The AD is used to generate a library of fusion proteins that contain this domain. The system is based on the fact that if an interaction between the bait and an AD-fusion protein occurs, this will bring the DBD and AD close enough to reconstitute a functional transcription factor and drive the expression of a series of reporter genes.
Objective 3. To confirm that annexin V (AxV) is an IFN-γR2 interacting protein as suggested by the yeast two hybrid screen and to examine the role of AxV in IFN-γ signalling.

Rationale. Annexins have been shown to interact with cytokine receptors (72), kinases (64;107), adaptor proteins (62) and phosphatases (108), raising the possibility that they can participate in signal transduction networks. Based upon the identification of AxV as an IFN-γR2 binding protein, experiments were carried out to verify this interaction and to examine whether AxV regulates IFN-γ signalling.
VIII. MATERIALS AND METHODS

A. MATERIALS

1. Antibodies

All antibodies recognized the human protein of interest unless otherwise specified. A mouse monoclonal anti-phosphotyrosine antibody (4G10), as well as anti-Jak1 and anti-Jak2 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): anti-SHP-1, anti-SHP-2, anti-IFN-γR1, anti-Erk, anti-Cbl, anti-Abl, anti-Pyk2, anti-Axl, anti-AxlII and anti-Stat1. Anti-IFN-γR2 was purchased from Pestka Biotechnology Laboratories (New Brunswick, NJ). Anti-phosphoJak2 was purchased from Biosource (Montreal, QC). Mouse monoclonal antibodies against human S100A9 and S100A8 were provided by Dr Nancy Hogg (Imperial College London, UK). Rabbit anti-human TIP120a was developed by Genemed Synthesis, Inc (San Francisco, CA), using as immunogen a synthetic peptide sequence that we selected based on the hydrophilic profile of the protein.

2. Reagents

Unless otherwise specified, reagents used in this thesis were from Sigma Chemical Company (St Louis, MO).
3. Cell lines

The human promonocytic cell lines U937 and THP-1 were from the American Type Culture Collection (Rockville, Maryland). The human embryonic kidney cell line 293T was kindly provided by Dr Alice Mui (Department of Medicine, UBC). The human HeLa cell line was kindly provided by Dr Decheng Yang (Department of Pathology, UBC). U937 and THP-1 cells were cultured in RPMI supplemented with 10% heat inactivated fetal calf serum (FCS), streptomycin and penicillin essentially as described in (109). 293T and HeLa cells were cultured in Dulbecco’s Modified medium supplemented with 10% heat inactivated fetal calf serum (FCS) and streptomycin and penicillin. All human and mouse cell lines were incubated in a humidified atmosphere with 5% CO₂, 95% air. Unless otherwise stated U937 and THP-1 cell were differentiated to adherent monocyte like cells using 10 ng/mL the phorbol 12-myristate 13-acetate (PMA). After 14 h in the presence of PMA, cells were washed with Hank’s Balanced Salt Solution (HBSS), and then cultured in complete RPMI for 4 to 5 h, prior to incubation with 100 Units of human recombinant IFN-γ (a gift from Genentech, San Francisco, CA).

B. METHODS

1. Preparation of cell lysates

   a) Extracts for anti-phosphotyrosine immunoblots or immunoprecipitations were prepared by lysing the cell monolayers on ice for 20 min in modified radioimmunoprotection assay (RIPA) buffer (1% Nonidet P-40, 0.25% sodium deoxycholate, 50 mM Tris pH 7.5, 0.15 M NaCl, 1 mM EGTA, 1 mM NaF, 1 mM
sodium orthovanadate) essentially as described in (110) or co-immunoprecipitation buffer (see Co-immunoprecipitation section for recipe), with the addition of protease and phosphatase inhibitors, as specifically indicated. Lysates were centrifuged at 16,000 x g for 15 min to remove insoluble material.

b) Extracts for anion-exchange chromatography were prepared by lysing cells on ice in fast performance liquid chromatography (FPLC) extraction buffer (1% Nonidet P-40, 12.5 mM MOPS, pH 7.5, 12.5 mM β-glycerophosphate, 2 mM EGTA, 1 mM Na3VO4, 1 mM PMSF, 10 µg/mL aprotinin, 10 µg/mL leupeptin and 10 µg/mL pepstatin) as described in (111). Lysates were centrifuged at 16,000 x g for 15 min to remove insoluble material and passed through a 0.2 µM filter prior to chromatography.

2. Subcellular fractionation

Cells were incubated with hypotonic buffer (Hepes 5 mM, 1 mM EGTA, 1 mM Na3VO4, 1 mM PMSF, 10 µg/mL aprotinin, 10 µg/mL leupeptin and 10 µg/mL pepstatin) for 25 min. After this period, 0.15 M final of NaCl was added to the cell lysates and the cells were passaged through a 24 guage-needle using a syringe for 10 times on ice. The unbroken cells were then removed by centrifugation at 800 x g for 5 min. The supernatant was centrifuged at 40,000 RPM (Beckman TLA 100.3 rotor) for 20 min to obtain the cytosolic material (present in the new supernatant). The pellet containing membrane proteins was solubilized by incubation in the hypotonic buffer plus 0.5 M sodium chloride, for 25 min at 4°C in rotation. To obtain the peripheral membrane proteins, the lysates were centrifuged at 40,000 RPM (Beckman TLA 100.3
rotor) for 20 min and the supernatant containing the proteins was recovered. To obtain membrane proteins the pellet was resuspended in hypotonic buffer containing 1% Triton X-100. The resuspended pellet was incubated for 25 min at 4°C in rotation. To obtain the membrane proteins, the lysates were centrifuged at 40,000 RPM (Beckman TLA 100.3 rotor) for 20 min and the supernatant containing the membrane proteins was saved for subsequent experiments. To control for the purity of the subcellular fractions, aliquots of the fractions were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with antibodies against SHP-1 (a cytosolic protein tyrosine phosphatase) or the IFN-γR1 (an integral membrane protein).

3. Anion-exchange chromatography

Detergent extracts were loaded onto a Mono Q FPLC column pre-equilibrated in buffer A (12.5 mM MOPS, pH 7.5, 12.5 mM β-glycerophosphate, 2 mM EGTA, 1 mM Na₃VO₄), essentially as described in (111). Proteins were resolved with a 20 mL linear gradient of 0-0.8 M NaCl in buffer A at a flow rate of 0.5 mL/min. Fractions of 0.25 mL were collected and aliquots were pooled and assayed for protein kinase activity or immunoblotting as described below.

4. Phosphocellulose paper assay to detect tyrosine kinase activity

Aliquots of either high speed supernatants or Mono Q fractions were assayed for Poly (Glu, Tyr) phosphotransferase activity as described (111). Substrate phosphorylation was carried out in a final volume of 25 µL of kinase assay buffer (pH 7.5) containing 12.5 mM MOPS, pH 7.5, 12.5 mM β-glycerophosphate, 2 mM EGTA, 1
mM Na$_2$VO$_4$, 2 mM DTT, 10 mM MgCl$_2$, Poly (Glu, Tyr) (1 μg/ reaction) and [γ-^{32}P] ATP (50 μM). Reactions were allowed to continue for 10 min at 30°C, and were terminated by spotting 22 μL of assay mixture onto Whatman P-81 phosphocellulose filter (Fisher Scientific) squares (1.5 x 1.5 cm$^2$). Filters were washed six times with ice-cold 175 mM phosphoric acid over a period of 60 min with agitation and incorporation of radioactivity was determined by liquid scintillation counting.

Samples were subjected to SDS-PAGE and separated proteins were transferred to nitrocellulose membranes. Membranes were blocked overnight at 4°C or for 2 h at room temperature in either 3% BSA or 5% milk dissolved in TBS-T (20 mM Tris, pH 7.4, 137 mM NaCl, 0.1% Tween 20), as specified. Immunoblotting was carried out by incubating the membranes for two hours at room temperature with antibodies against the proteins of interest diluted in blocking solution as described in figure legends. After incubation with primary antibody, membranes were washed three times with TBS-T and immunoreactive bands were detected by incubation (45 min at room temperature) with horseradish peroxidase (HRPO) conjugates of either goat anti- mouse, goat anti-rabbit or bovine anti-goat (1/5000 dilution in TBS-T) IgG. After incubation with secondary antibody, the membranes were extensively washed and incubated with enhanced chemiluminiscence (ECL) reagent (Amersham-Pharmacia). If required, stripping of antibodies was carried out by incubating membranes in a 0.2 N NaOH for 5 min. Membranes were then extensively washed, blocked and re-probed with antibodies of interest.
5. Immunoprecipitation

To analyze the tyrosine phosphorylation status of specific proteins, cell lysates (500-1000 μg) were incubated with the antibody or irrelevant control antibody overnight at 4°C as described in (110). Briefly, immune complexes were collected by incubation with protein A or G for 1 h and washed three times with lysis buffer. Samples were resuspended in SDS-Laemmli buffer, boiled for 5 min and separated by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting.

6. Co-immunoprecipitation

Five million THP-1 cells grown in RPMI complete medium were differentiated overnight (12-14 h) using 10 ng/mL PMA. After differentiation, cells at approximately 70% confluence were washed with Hank’s balanced salt solution (HBSS) three times and left to rest for 4 h in complete medium. Cells were then either left untreated or treated with IFN-γ (100 U/mL) for 15 min and then washed with HBSS and solubilized in digitonin buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% digitonin, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM PMSF, 10 μg/mL pepstatin, 10 μg/mL aprotinin and 10 μg/mL leupeptin) for 30 min on ice. Lysates were cleared by centrifugation at 10,000 x g at 4°C for 15 min and supernatants were incubated with antibodies specific for either IFN-γR2 or c-Abl (both mouse IgG1 monoclonal antibodies) for two hours. Protein G (Amersham Pharmacia Biotech) was then added and the incubation continued for 45 min, after which immune complexes were recovered by centrifugation at 5000 x g. Immunoprecipitates were washed in digitonin buffer three times and solubilized in Laemmli buffer without β-mercaptoethanol (non-
reducing conditions). After transfer to nitrocellulose membranes, proteins were immunoblotted for AxV, IFN-γR2 and c-abl.

7. In situ renaturation kinase assay

Whole cell lysates were used for detection of protein tyrosine kinase activity by an in situ renaturation kinase assay with a polydispersed tyrosine and glutamic polypeptide (Poly (Glu, Tyr), 30 to 94 kDa), as described (112). Briefly samples were mixed with the tyrosine kinase substrate Poly (Glu, Tyr) and separated by SDS-PAGE. After electrophoresis, SDS was removed from the gels, proteins were denatured in guanidine hydrochloride and renatured by incubating in buffer containing 0.04% Tween 20 and 10% sucrose. Gels were equilibrated in 10 mM HEPES pH 7.4 plus 10 mM 2-mercaptoethanol, 10 mM MgCl₂, 5 mm MnCl₂ and 0.1 mM Na₃VO₄. The kinase assay was carried out using the same buffer with the addition of 100 μCi of γ³²P ATP. Gels were soaked in 1 M KOH for one hour at 56°C to eliminate signals from serine and threonine kinases. After staining and drying, phosphorylated bands were detected by autoradiography.

8. Purification of a p125 kDa IFN-γ-inducible tyrosine phosphorylated protein

The protocol designed for purifying the p125 IFN-γ-inducible tyrosine phosphorylated protein is outlined in Figure 16. Briefly, PMA-differentiated U937 cells untreated or treated with 100 U/mL of IFN-γ for 15 min, were subjected to a subcellular fractionation as described above. The membrane proteins were incubated with Poly
(Glu, Tyr) agarose beads. The bound material was eluted in SDS-sample buffer and then resolved by SDS-PAGE. The band corresponding to the area around 125 kDa was cut and the proteins were eluted with 2% SDS. The eluted samples were diluted in immunoprecipitating buffer and concentrated by Centriprep™, to remove SDS and replace it with TritonX100 present in the immunoprecipitating buffer. The proteins were immunoprecipitated using an anti-phosphotyrosine antibody (4G10) and then the immunoprecipitated proteins were resolved by SDS-PAGE followed by either silver staining or staining with Coomassie blue.

9. Development of a rabbit polyclonal antibody against TIP120a

A peptide for immunization of rabbits was selected based on hydrophilicity. The peptide was synthesized and used to immunize two rabbits (Genemed Synthesis, Inc). Prior to the immunization, pre-immune serum was obtained. The detailed immunization protocol can be found at ... Reactivity of pre-immune and immune sera against the immunizing peptide was tested by ELISA. Antibody titers in the immune sera were > 1:10,000. Immunoreactivity by Western blot was also tested and is shown in Figure 19.

10. RNA extraction

THP-1 cells were grown in suspension as described elsewhere in this thesis ("Cell Lines" section). Cells were differentiated with PMA overnight in 100 mM cell culture plates and then washed with Hank’s balanced salt solution (HBSS) and left to rest for 5 h. Cells were then treated with 100 U/mL of human recombinant IFN-γ for 18 h. The medium was then removed and 3 mL of TRIZOL (Invitrogen) were added for 5
min at room temperature to disrupt adherent cells. Cells were then scraped and placed into three 1.5 mL microcentrifuge tubes (approximately 1 mL per tube). These tubes and all the plastic material used hereafter for the RNA isolation, were RNase and DNase free, as supplied by the manufacturer. Chloroform (0.2 mL per tube) was added, the tubes were shaken for 15 seconds and then were left to rest for 3 min at room temperature. Samples were then microcentrifuged at 12,000 x g for 15 min at 4°C. After centrifugation, the aqueous phases were transferred to three new tubes. Isopropyl alcohol (0.5 mL per tube) was added and samples were incubated at room temperature for 10 min and then microcentrifuged at 12,000 x g for 10 min at 4°C. With care so as not to disturb the pellet, the supernatant was removed and the RNA pellet was washed with 75% ethanol (at least 1 mL per tube). The pellet was mixed with the ethanol by briefly vortexing and then centrifuged at 7,500 x g for 5 min at 4°C. The supernatant was then discarded and after drying, it was dissolved in 40 μL of diethyl pyrocarbonate (DEPC)-treated water. An aliquot was removed for agarose gel electrophoresis under denaturing conditions and for spectrophotometric analysis in order to determine the purity and amount of the extracted material.

11. cDNA synthesis

RNA samples were placed in duplicate PCR tubes on ice. For 5 μL of RNA sample, 1 μL of 0.5 μg/μL of oligo dT solution and 4 μL of DEPC-treated water were added. Samples were mixed well and the tubes were placed in the PCR machine for five minutes at a temperature at 70 °C to denature the RNA and allow binding of the oligo dTs. Tubes were then placed on ice to snap cool and to prevent the RNA from
returning to its original state. A second RNA reaction mixture was prepared in parallel by adding 2 μL of RNase inhibitor, 4 μL of 5X first strand buffer and 2 μL of 10 mM dNTP. This mix was left at 37°C and afterwards, 2 μL (50 Units) of reverse transcriptase was added to one of the tubes. The mix that did not contain reverse transcriptase was to control for the presence of genomic DNA contamination. After mixing, the first and second reaction tubes were placed in the PCR machine for 1 h at 37°C and then at 70°C for 10 min to denature. At the end of this period the samples were stored at -20°C.

12. Primer design and PCR amplification

Specific primers to amplify the intracellular domains of the IFN-γR1 and R2 were designed for cloning PCR products into the EcoRI and Bgl II sites of the pYTH9 vector (113). The software used to do the restriction enzyme analysis and for primer design was Oligo™. In addition to these, two sets of internal primers were used in a nested PCR reaction to confirm the identity of the PCR products to be used for cloning. DNA sequence of the primers that were used for the cloning and confirmation are shown in Table 1.
### Table 1. Primers for cloning into yeast vector pYTH9

<table>
<thead>
<tr>
<th>Primer Type</th>
<th>Primer Sequence</th>
<th>TM at 50 mM Na⁺</th>
<th>Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>nIFN-γ R1H Forward</td>
<td>ggaattccctggtattcatctg</td>
<td></td>
<td>48°C</td>
</tr>
<tr>
<td>IFN-γ R1RH Reverse</td>
<td>gaagatctgctgtatctcagaaatcc</td>
<td></td>
<td>52°C</td>
</tr>
<tr>
<td>nIFN-γ R2H Forward</td>
<td>ggaattccctggtctgaaatatag</td>
<td></td>
<td>51°C</td>
</tr>
<tr>
<td>IIFN-γ R2H Reverse</td>
<td>gaagatctggtcagctttggag</td>
<td></td>
<td>54°C</td>
</tr>
</tbody>
</table>

### Table 2. Primers for Nested PCR (bait confirmation)

<table>
<thead>
<tr>
<th>Primer Type</th>
<th>Primer Sequence</th>
<th>TM at 50 mM Na⁺</th>
<th>Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ R1FI forward</td>
<td>aggaggtggtcgtcagagag</td>
<td></td>
<td>49°C</td>
</tr>
<tr>
<td>IFN-γ R1RI reverse</td>
<td>agcgatgcgcaggcttcagact</td>
<td></td>
<td>54°C</td>
</tr>
<tr>
<td>IFN-γ R2FI forward</td>
<td>ccatcttagaggcctggac</td>
<td></td>
<td>49°C</td>
</tr>
<tr>
<td>IFN-γ R2RI reverse</td>
<td>cggaaaaacgataatggac</td>
<td></td>
<td>47°C</td>
</tr>
</tbody>
</table>
Table 3. Composition of the PCR reaction used (final volume 20 μL)

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X reaction buffer</td>
<td>1X</td>
</tr>
<tr>
<td>dNTPs</td>
<td>200 μM</td>
</tr>
<tr>
<td>Primers</td>
<td>1 μM</td>
</tr>
<tr>
<td>TAQ polymerase</td>
<td>2.5 Units</td>
</tr>
<tr>
<td>Sample</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td></td>
</tr>
</tbody>
</table>

TAQ polymerase, reaction buffer and deoxynucleotides were purchased from Fermentas (Lithuania). The amplification protocol used for the RT-PCR of the intracellular domains of the receptors and for the nested PCR is shown in Table 4.

Table 4. PCR conditions to amplify the intracellular domain of the receptors

<table>
<thead>
<tr>
<th>Segment</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>One cycle</td>
<td>94 Five minutes</td>
</tr>
<tr>
<td>2</td>
<td>Five cycles</td>
<td>94 One minute</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>One minute</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>One minute</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>4</td>
<td>GO TO 2 for five cycles</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Ten cycles 94 One minute</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>55 One minute</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>72 One minute</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>GO TO 5 for ten cycles</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Fifteen cycles 94 One minute</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>60 One minute</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>72 One minute</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>GO TO 8 for fifteen cycles</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>One cycle 72 Ten minutes</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>End at 4°C</td>
<td></td>
</tr>
</tbody>
</table>

The PCR machine used for the amplification was purchased from Rose Scientific Ltd. (Edmonton, AB). The expected relative molecular size of the PCR products was R1=701 bp and R2=226 bp.
13. Restriction enzyme digestion, DNA ligation and plasmid selection

After amplification of the intracellular domains of the IFN-γR1 and R2 from cDNA derived from IFN-γ-treated THP-1 cells, fragments were purified using agarose gel electrophoresis and a Qiagen gel extraction kit. PCR products were digested overnight with 15 Units of Bgl II (Fermentas, Lithuania). After purification using a nucleotide extraction kit from Qiagen, fragments were digested with 10 Units of Eco R I (Fermentas) overnight. Double digested PCR products were purified again with the nucleotide extraction kit. The vector was prepared in a similar way (Bgl II and Eco R I digestions and subsequent purification), but after the second purification it was treated with 1.5 units of alkaline phosphatase (Fermentas, Lithuania) for one hour at 37°C. To prevent recircularization of the vector, which would reduce the efficiency of cloning of the receptor baits, after digestion it was purified by agarose gel electrophoresis with a gel extraction kit once again. Aliquots of the two inserts and the digested vector were analyzed by gel electrophoresis to quantify the amount of DNA prior to ligation.

Ligation of the two PCR products into the pYTH9 vector was carried out at several different insert:vector ratios, always including a ligation mix without insert to test for recircularization as a control. The ligation was carried out in a final volume of 11 μL including 1 unit of DNA ligase (Invitrogen), at 15°C for 15 h, according to the instructions suggested by the manufacturer. Two μL of ligation mix was used to transform One Shot™ TOP10 competent cells (Invitrogen). After transformation, bacteria were plated on LB (Luria broth) ampicillin plates (final concentration of 100 μg/mL). Following overnight incubation at 37°C, bacterial clones were grown in 5 mL
of LB in the presence of ampicillin for fourteen hours and plasmids were extracted using a miniprep extraction kit (Qiagen). Plasmids were digested simultaneously with EcoRI and Bgl II (in buffer O for two and a half hours at 37°C), to identify clones with inserts. Candidate clones were grown-up and plasmids were extracted using a Midi prep kit (Qiagen). Plasmid DNA was sequenced to check for the correct reading frame and the absence of PCR errors, as compared with the gene sequences present in the gene databases. Confirmed clones were linearized using Xba I (10 Units of Xba I per 50 μL reaction volume were used to digest 20 μg of plasmid overnight at 37°C) and quantified by gel electrophoresis to be used to transform yeast host strain SDY191.

14. Yeast growth and DNA transformation

Yeast strain SDY191 was provided by Dr Martin Sims, Immunology Unit, Glaxo SmithKline UK. SDY191 was grown in YPD medium and stored on YPD agar plates. SDY191 is auxotrophic for tryptophan, leucine and histidine. This protocol and all other protocols related to the yeast two hybrid system, except the construction of the cDNA library were basically derived from (113). To test for this phenotype, we prepared a synthetic medium (CM) with a dropout powder (mix of all essential amino acids minus tryptophan, leucine and histidine), and yeast nitrogenated base without amino acids, ammonium sulphate, adenine, uracil or dextrose, as detailed below. SDY191 was only able to grow in the synthetic medium when supplemented with the three aminoacids, whereas in the absence of any one of them, it failed to grow.
For yeast transformation, a 5 mL culture of SDY191 was grown overnight in a shaker at 30°C in YPD medium. The culture was then diluted 1:20 to a final volume of 50 mL. At an optical density of 0.6 (1.2 \times 10^7 \text{ cells/mL}), equivalent to mid-log phase, yeast cells were centrifuged at 2000 RPM for five minutes at room temperature. The pellet was washed twice by resuspending in 20 mL of sterile water and resuspended in 1 mL of sterile water, placed in a microcentrifuge and spun for one minute at top speed. The new pellet was resuspended in 1 mL of lithium acetate/TE buffer and centrifuged for one minute at top speed. Cells were resuspended in 500 \mu L of the same buffer and then aliquoted in microcentrifuge tubes (50 \mu L per tube). Two \mu L of linearized plasmid DNA (2 \mu g/\mu L) was mixed with 5 \mu L of the salmon DNA (10 mg/mL). The DNA mix was added to the cells and then 300 \mu L of the PEG/lithium acetate in TE buffer was added to each tube. The new mix was incubated for thirty minutes at 30°C with shaking at low speed. Samples were then transferred to a 42°C water bath for 20 min after which they were centrifuged at top speed for one minute and resuspended in 100 \mu L of sterile TE. Cells were placed in CM medium agar plates with lysine and histidine, but without tryptophan to select for transformants. Plates were sealed with Parafilm™ to reduce evaporation and left to grow for two to three days at 30°C. Clones were analyzed by genomic DNA extraction and PCR amplification to select those that had integrons of interest.

15. Genomic yeast DNA extraction using the Yeast Smash and Grab DNA miniprep
A 5 mL yeast cell culture was pelleted in a microcentrifuge tube and resuspended in 0.2 mL of lysis buffer (10 mM Tris pH 8.0, 1 mM EDTA, 100 mM NaCl, 1% SDS and 2% Triton X-100) plus 0.2 mL of a 1:1 mix of phenol and chloroform in the presence of 0.3 g (~0.3 mL) of acid-washed glass beads (Sigma). Tubes were vortexed at top speed for two to three minutes followed by the addition of 0.2 mL TE (10 mM Tris, 1 mM EDTA, pH 8.0) and continued vortexing for a few seconds. Tubes were then centrifuged at 12,000 x g for 5 min in a microcentrifuge, the upper aqueous phase was transferred to a new microcentrifuge tube and the glass beads were discarded. Two volumes of 100% ethanol were added with mixing and the tubes were microcentrifuged for 2 to 3 min at room temperature. The supernatant was discarded very gently so the pellet was not disturbed. The pellet was rinsed with 0.5 mL of cold 70% ethanol, adding the ethanol very slowly down the sides of the tube. Tubes were centrifuged at 12,000 x g for 3-5 seconds, the supernatant was removed and the tubes inverted to dry the pellet. Yeast DNA was resuspended in 50 µL of 1 X TE, pH 8.0 and 1 µL of this mix was used to PCR amplify the R1 and R2 intracellular domains to confirm the presence of the desired recombinant yeast clones.

16. **Library construction**

The cDNA library was constructed following the protocol suggested by the manufacturer (Stratagene). The following flow chart (Figure 7) summarizes the steps taken to prepare the cDNA expression library in the Hybrizap vector.
Peripheral Blood mononuclear cells

\[ \downarrow \]

Total RNA

\[ \downarrow \]

cDNA synthesis

\[ \downarrow \]

Ligation of cDNA into vector arms

\[ \downarrow \]

Packaging reaction to generate viruses

\[ \downarrow \]

Amplification of the HybriZAP library

\[ \downarrow \]

In vivo excision of phagemids from HybriZAP library

\[ \downarrow \]

Amplification of excised phagemid library

Figure 7. Yeast two hybrid library construction. The library was constructed as suggested by the instructions provided by the manufacturer (Stratagene). The excised phagemids were examined for percentage and size of the inserts, as outlined in the results section.
17. Library screening using R1 and R2 recombinant yeast clones

Starting from a single recombinant yeast colony containing either the R1 or R2 bait DNA, a 10 mL culture in selective media was grown overnight at 30°C with shaking. After dilution to 100 mL in fresh selective medium and overnight culture at 30°C, this saturated culture was diluted again in fresh selective medium to give a final volume of 400 mL. When the cells reached an OD$_{600}$ of 0.6, they were examined microscopically to assure the absence of contamination and to confirm active growth, reflected by the presence of visible buds on cells. Mid-log phase cells were distributed into sterile tubes and pelleted in a benchtop centrifuge at 2,000 RPM for 2 min at room temperature. Pellets were washed twice with sterile Milli Q water and then twice with the LiAc/TE solution. Cells were pooled during the washes and after the last wash, were resuspended into 4 mL of LiAc/TE solution and left at room temperature whilst DNA for transformation was prepared. To 100 µg of library plasmid DNA (200 µL), was added 400 µL of salmon DNA, 75 µL of 10 X TE stock solution and 75 µl of 1 M LiAc stock solution were added. Yeast cells and the DNA were then mixed by gently swirling. Twenty-four mL of 40% PEG/LiAc/TE solution was added with gentle mixing. The suspension was placed in the 30°C incubator for 30 min with slow shaking. Cells were then transferred to a 42°C water bath for 20 min, microcentrifuged as before and the pellet was resuspended in 10 mL of sterile water. This mix was plated by adding 1 mL per plate (containing the selective medium plus the 15 mM 3-AT) to each of ten plates (plating area= 245 mm x 245 mm per plate) with incubation at 30°C for four to ten days. The plates were wrapped in plastic bags to avoid dehydration during the incubation period.
18. In situ β-galactosidase assay for yeast cells

Yeast colonies were transferred from culture plates to Whatman™ filters which were then immersed twice in liquid nitrogen. Filters were transferred to Z buffer (Na$_2$HPO$_4$, NaH$_2$PO$_4$, KCl and MgSO$_4$, pH 7.0) containing 2-mercaptoethanol (270 µL of 2-mercaptoethanol in 100 mL of Z-buffer) and X-gal (1mg/mL final) by placing them on top of the buffer in the lid of a culture dish (2 mL of buffer was sufficient for a filter with a 9 cm diameter). Filters were left at room temperature to develop (three hours to overnight incubation).

19. Preparation of DH5α competent cells

A single bacterial colony was inoculated in 5 ml of LB medium and grown at 37°C for 3 to 4 h to an OD$_{600}$ of 0.5 to 0.6. Cells were spun at 9000 RPM for three minutes and the pellet was resuspended in 1 ml of 0.1 M cold CaCl$_2$ by gently mixing without vortexing and placed on ice for 30 min. Cells were then spun at 9000 RPM for 3 min and the pellet was resuspended in 500 µL of 0.1 M cold CaCl$_2$ and placed on ice for 30 min. Cells were then spun again as before and resuspended in 300 µL of 0.1 M cold CaCl$_2$. Competent cells were good for 16 h at 4°C.

20. Back extraction of plasmid DNA

Recombinant yeast transformants were grown overnight (10 mL) in selective medium and 0.5 ml was pelleted in a microcentrifuge tube. All centrifugations in this protocol were done at full speed (12,000 x g) and at room temperature unless otherwise specified. Pellets were resuspended in 300 µl of zymolase solution (1.2 M Sorbitol, 100
mM potassium phosphate pH 7.4, 400 μg/mL of zymolase final) and incubated for 3 h at 37°C. Three hundred μl of solution P2 (from Qiagen plasmid extraction kit), was added with incubation for five minutes at room temperature followed by the addition of an equal volume of solution P3 (from the same kit) and the new mix was incubated on ice for 10 min. Samples were microcentrifuged for 15 min and the supernatants were transferred to fresh tubes to which was added 5 μL of Stratclean™ resin (Stratagene) with vortexing for 3 min. Afterwards the samples were microcentrifuged for 2 min and 800 μL of supernatant was transferred to fresh microcentrifuge tubes with the addition of 560 μL of isopropanol. Tubes were microcentrifuged for thirty minutes and pellets were suspended in 560 μL of 70% ice-cold ethanol for 10 min at room temperature. Samples were then microcentrifuged for 15 min, supernatants were discarded and the pellets air-dried for 10 min. Pellets were resuspended in 50 μL of TE, pH 8.0. Samples were microcentrifuged for one minute to spin out insoluble material and 1 μL of the resulting supernatants were used to transform bacterial competent cells.

21. Chemical transformation with One Shot™ TOP10 (Invitrogen) competent cells

The following protocol is essentially the one suggested by the manufacturer (Invitrogen). One hundred μL of commercially available One Shot™ TOP10 competent cells were mixed with 1 μL of plasmid DNA extracted from yeast transformants incubated on ice for thirty minutes. Cells were heat shocked for ninety seconds at 42°C and transferred to ice for one to two minutes. One ml of LB medium
was added and bacteria were grown for 1 h at 37°C and either 50 or 200 μL of cells were plated on LB agar with the appropriate antibiotic selection.

22. DNA sequencing to characterize the putative IFN-γR1 & R2 interacting clones

Plasmids that had been back extracted were used to re-transform yeast clones recombinant for the bait of interest. Once the expected phenotype was confirmed, plasmid DNA was sequenced in the NAPS facility (University of British Columbia) using a primer that recognizes the GAL 4 activation domain (GAD).

23. Cloning, expression and purification of GST fusion proteins

The intracellular domain of the IFN-γR2 was cloned in a pGEX 4T vector (Amersham Pharmacia Biotech) using the EcoR I and Xho I restriction sites, as per the manufacturer instructions. In a similar way, the intracellular domain of the IFN-γR1 was cloned in the same vector using the Bam H I and Sal I restriction sites. Clones containing the inserts were sequenced and expression of the fusion protein induced by isopropyl β thiogalactopyranoside (IPTG) was confirmed by immunoblotting using an anti-GST antibody. E coli were grown overnight and then subcultured (200 μl of overnight culture diluted into 6 ml of YTA 2X plus ampicillin). After bacterial cells reached an OD600 of 0.6, they were treated with 0.1 mM IPTG for 1 h at 28°C. Cells were then pelleted at 3,400 RPM for seven minutes at 4°C. Pellets were resuspended in 1X PBS plus 1 mM EDTA, sonicated 4 times at power setting 5 on ice. PMSF at 1 mM final and Triton X-100 at 0.5 % final concentrations were added and the lysates thus
generated were microcentrifuged at full speed for 10 min at 4°C. Clarified supernatant was incubated with glutathione agarose, previously equilibrated in PBS, for 1 h by rotation at 4°C in microcentrifuge tubes. Beads were centrifuged at 4500 RPM for 3 min and washed with PBS three times. Beads were incubated in solubilization buffer (described above) for 5 min and then pelleted at 4,500 RPM for 3 minutes. Beads were washed with PBS three times and then incubated with 400 µl of SDS sample buffer by boiling for 5 min. Boiled beads were centrifuged at 3500 RPM for 5 min at 4°C and aliquots were separated by 12% SDS-PAGE.

24. GST pull down experiments

PMA-differentiated THP-1 cells treated with IFN-γ were washed with HBSS and solubilized in a buffer containing 20 mM HEPES, 0.15 M NaCl, 0.5% Triton X-100, 5% glycerol plus protease and phosphatase inhibitors for 30 min on ice and then spun at full speed (13,000 RPM) for 20 min at 4°C. Supernatants were added to the purified GST fusion proteins (see above) and incubated for two hours. Beads were then washed with PBS 0.5% Triton X-100 three times and bound material was released by boiling the coated beads in SDS-Laemmli buffer. The electrophoresis was carried out in 12% gels and continued 1 h after the dye left the gel (at a constant current of 35 mA) in order to better resolve the GST-R2 fusion protein from AxV which have similar electrophoretic mobilities. Separated proteins were probed with an anti-GST, AxV or S100A9 antibodies to detect the expression and binding of specific proteins.
25. Modulation of AxV expression in 293T cells using siRNA

To examine the role of AxV in IFN-γ signalling, three siRNA constructs for AxV downregulation were designed using a web based platform (http://katahdin.cshl.org). The regions chosen were specific to the AxV sequence as determined by a BLAST search. The constructs designated AV1, AV2 and AV3 involved, respectively, nucleotides 193-225, 636-666 and 1042-1075 of the AxV cDNA sequence. Constructs were cloned into the Bse R I/Bam H I cloning site of the pSHAG vector that directs the in vivo synthesis of siRNA molecules using a U6 promoter (114). Positive clones were selected based upon the acquisition of both kanamycin resistance and a new Hind III restriction site. Confirmed clones were grown, purified using an endotoxin free Maxiprep kit (Qiagen) and used to transfect 293T cells. The suitability of these cells as an experimental model to study IFN-γ signalling was confirmed by examining their responses to IFN-γ including ligand-inducible tyrosine phosphorylation of Stat1, Jak1, Jak2, and the IFN-γR1.

Transfection of the pSHAG constructs was carried out using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Briefly, 293T cells were transfected when they reached 95% confluency and were incubated with transfection reagent containing plasmid DNA diluted in OptiMEM medium for 18 h. Cells were washed with HBSS and the medium was then replaced by complete DMEM for an additional 48 h of culture to allow for expression of siRNA and downregulation of AxV. Transfection efficiency was >95% as determined by co-transfection with a pCMV β-galactosidase expression construct combined with measurement of β-
galactosidase activity in situ. The extent to which AxV expression was reduced by the siRNA constructs was assessed by immunoblotting with an AxV antibody coupled with densitometric scanning using Un-Scan-it™. Actin was used as a protein loading control. Specificity of siRNA downregulation was tested by immunoblotting with anti-AxII.

26. Effect of reduced AxV expression on cellular responses to IFN-γ

293T cells transfected with either pSHAG (empty vector) or with pSHAG AV2.1 (referred as AV2.1) plasmids as described above, were either left untreated or incubated with IFN-γ (50-1000 U/ml) for 4 h. Cells were then washed with HBSS and solubilized in 2X Laemmlí sample buffer by boiling for 5 min. After separation of lysates by SDS-PAGE and transfer to nitrocellulose membranes, Western blotting was carried out with antibodies against AxV, Stat1, SHP-2 and actin. Stat1 was immunoprecipitated from pSHAG and AV2.1 transfected cells, that had been left untreated or incubated with IFN-γ to analyze the effect of AxV downregulation on Stat1 tyrosine phosphorylation. IFN-γ-induced activation of Jak2 was measured by immunoblotting of cell lysates using anti-Jak2 pYpY1007/1008 (Biosource, Montreal, QC), which recognizes the activated form of this protein (115).

27. RT-PCR of IFN-γ-inducible genes

The effect of reduced AxV levels was also examined by semiquantitative reverse transcriptase PCR using RNA from pSHAG (vector control) and pSHAG AV2.1 transfected 293T cells, that were incubated in the presence or absence of 100
U/mL of IFN-γ for 4 h. Briefly, cDNA was prepared as outlined above. Transcription of Egr-1, ICSBP, IFN-γR2 and GAPDH was analyzed using the following set of primers (5' to 3'): hEGR-1F: cagcagtcccttactcag; hEGR-1R: gactggtagctggtattg; hICSBPF: ctgatcagcagattgacagtagc; hICSBPR: gaatgctgaatggtgcgcgtcga; hGAPDHF: gccaaaagggtcatcatctc hGAPDHR. Gtagaggcagggatgatgttc; human IFN-γR2 primers were the same as those used to amplify the intracellular domain for cloning the bait for the yeast two hybrid screening described previously. PCR conditions were: 94 °C for 1 min; 50 °C for 1 min; 72 °C for 1 min for 30 cycles.

28. Effect of decreased AxV levels on the IFN-γ-mediated growth inhibition in HeLa cells

HeLa cells transfected with pSHAG and pSHAG AV2.1 constructs were seeded in 12-well plates (0.5 X 10⁴ cells/well) and left to rest for 24 h. The cells were then cultured with or without 100 U of IFN-γ for 72 h. Cells were washed with HBSS, dislodged and viable cells were counted in a hemacytometer using Trypan blue dye, essentially as described in (116).
IX. RESULTS

A. IDENTIFICATION AND CHARACTERIZATION OF NOVEL COMPONENTS OF INTERFERON-γ RECEPTOR SIGNALLING

Evidence in the literature indicates the existence of novel components of IFN-γ signalling other than the Jaks and Stat1 (52;102-104). In this laboratory, we have evidence of tyrosine kinases activities that are activated by IFN-γ in monocytic cells that are distinct from the Jak kinases (101). In the research described below a biochemical approach was used to demonstrate, in a monocytic cell line model, the existence of novel kinase activities. These studies involved FLPC Mono Q fractionation coupled to a phosphocellulose paper assay that has been used in the past to purify kinase activities (111). We obtained novel evidence of non-Jak tyrosine kinase activities induced by IFN-γ. In part this conclusion was based upon the finding that, whereas the Jak kinases were inhibited in cells treated with forskolin to elevate cAMP levels, IFN-γ treatment nevertheless led to tyrosine phosphorylation of cell proteins. In particular a prominent 125 kDa protein showed increased tyrosine phosphorylation in response to IFN-γ in spite of forskolin treatment. This protein was purified to near homogeneity. Mass spectrometry analysis identified it as TIP120a, a co-transcriptional activator. To confirm that TIP120a is tyrosine phosphorylated in response to IFN-γ, we designed both immunoprecipitation and transfection approaches. The immunoprecipitation approach was not informative, since the antibody that we produced did not
immunoprecipitate the protein. The transfection of a his tagged TIP120a was more informative and suggested that TIP120a is indeed tyrosine phosphorylated after IFN-γ treatment.

1. IFN-γ-induced tyrosine phosphorylation of macrophage proteins

An initial objective was to establish that activation of IFN-γ signalling could be detected in a model system of human mononuclear phagocytes. This was done by examining IFN-γ-inducible tyrosine phosphorylation of proteins in whole cell lysates of phorbol ester-differentiated U937 cells. In parallel, immunoprecipitates of the Jak1, Jak2, IFN-γR1 and the transcriptional activator Stat1 were analyzed by anti-phosphotyrosine immunoblotting with the 4G10 antibody (Figure 8A, B). In Figure 8A, lanes 1 and 2 show IFN-γ-induced tyrosine phosphorylation of two proteins with apparent molecular masses of 125 and 85 kDa bands (identified by arrows) after 15 min of incubation with 100 units/mL of IFN-γ (n=8). Also in Figure 8A, lanes 3 and 4 contain immunoprecipitates of IFN-γR1 from control and IFN-γ-treated cell lysates, respectively and demonstrate that the highly glycosylated receptor undergoes tyrosine phosphorylation in response to IFN-γ (n=3). Results shown in Figure 8B demonstrate that IFN-γ-inducible tyrosine phosphorylation of Jak1 also occurs in these cells. The induction of Jak2 and Stat1 tyrosine phosphorylation under similar experimental conditions was also confirmed for phorbol ester-differentiated U937 cells as shown below in Figure 10.
Figure 8. Tyrosine phosphorylation of IFN-γR1 and Jak1 induced by IFN-γ.
PMA differentiated U937 cells were treated with 100 U of IFN-γ for 15 min. Cells were washed and lysed in modified RIPA buffer with protease and phosphatase inhibitors as described in the Materials and Methods section. Cleared whole cell lysates were used as such (WCL) or were used for immunoprecipitation of IFN-γR1 or Jak1. WCL and immunoprecipitates (IP) were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with an anti-phosphotyrosine antibody (4G10). The levels of the immunoprecipitated Jak1 and IFN-γR1 were similar between the control and the IFN-γ-treated samples (data not shown).
2. IFN-γ-induced tyrosine kinase activity in macrophages

Enhanced protein tyrosine phosphorylation in response to IFN-γ as observed above could be explained by activation of tyrosine kinases, by inhibition of phosphotyrosine phosphatases or both. Evidence for tyrosine phosphorylation of Jak1 was consistent with its activation, although it was not clear whether this, with or without the concomitant activation of Jak2, would have accounted for all of the IFN-γ-induced changes in tyrosine phosphorylation that were observed. To seek evidence for activation of other tyrosine kinases, whole cell lysates from phorbol ester differentiated U937 cells, control and IFN-γ-treated cells, were examined using a renaturation kinase assay. This system is based on the property that some protein tyrosine kinases are able to regain their enzymatic activity after SDS-PAGE separation, denaturation, and in gel renaturation. IFN-γ-inducible activation of two tyrosine kinases with apparent molecular masses of 210 and 125 kDa is shown in Figure 9 (see arrows). Induction of these kinases is most evident in Panel B, where the samples were separated by SDS-PAGE in the presence of exogenous Poly (Glu, Tyr), a specific substrate for tyrosine kinases, in the polymerized gel. The activities of these renaturable tyrosine kinases were detected after 15 min of incubation with 100 U/mL of IFN-γ (n=3).
Figure 9. Renaturation kinase assay of extracts from control and IFN-γ-treated PMA-differentiated U937 cells in the absence (A) and presence (B) of Poly (Glu, Tyr), demonstrates IFN-γ-inducible, renaturable tyrosine kinases. Cells were untreated (lane 1) or treated with IFN-γ 100 U/mL for 15 min (lane 2) and lysates were prepared as described for IFN-γR1 and Jak1 immunoprecipitations (see legend to Figure 8). Renaturation kinase assay (RKA) was carried out as described elsewhere (112). Panel A shows the results of the RKA in the absence of Poly (Glu, Tyr), while Panel B shows the results when Poly (Glu, Tyr) was added to the gel solution prior to polymerization. The left arrows refer to the molecular weight markers (205, 116, 95, 66 and 45 kDa). The results shown are from one of three independent experiments that yielded similar results.
cAMP selectively attenuates IFN-γ-induced tyrosine phosphorylation of Jak1, Jak2 and Stat1, without affecting global IFN-γ-induced tyrosine phosphorylation

cAMP is a second messenger that influences cell signalling by activating protein kinase A (PKA). Moreover, there is evidence that PKA is involved in crosstalk with IFN-γ cell regulation with important biological consequences (117). For example, it has been shown that IFN-γ signalling is attenuated in cells with increased levels of cAMP (39). To address potential mechanisms involved, U937 cells were treated with forskolin which acts to increase cAMP levels by directly activating adenylate cyclase. Upon binding cAMP, the regulatory subunits of PKA dissociate from the catalytic subunits. The latter are then available to phosphorylate target proteins and this can be detected by increased PKA activity. As shown in Figure 10A (n=3), treatment with forskolin prior to addition of IFN-γ led to a significant decrease in the IFN-γ-inducible activation of Jak1 and Jak2 without affecting the levels of expression of these kinases. Consistent with this, IFN-γ-inducible tyrosine phosphorylation of Stat1 was significantly diminished by forskolin treatment (Figure 10C, n=2). In contrast and of particular interest, was the finding that IFN-γ-induced tyrosine phosphorylation of phosphotyrosine containing proteins p125 and p85 (initially seen in Figure 8A) was not affected by forskolin (Figure 10B, n=3). Taken together, these findings suggest the existence of other, non Jak, tyrosine kinases involved in IFN-γ signalling.
Figure 10. cAMP selectively attenuates IFN-γ-induced activation of Jak1, Jak2 and Stat1 without affecting global IFN-γ-induced tyrosine phosphorylation.
Figure 10. cAMP selectively attenuates IFN-γ-induced activation of Jak1, Jak2 and Stat1 without affecting global IFN-γ-induced tyrosine phosphorylation. PMA differentiated U937 cells were treated with 30 µM forskolin for 30 min prior to incubation with 100 U/mL of IFN-γ for 15 min. Cells were lysed and immunoprecipitated with the antibodies indicated. Immune complexes were separated on 10% SDS-PAGE, transferred to nitrocellulose and probed with anti-phosphotyrosine (PY) antibodies. The same membrane was stripped and re-probed with anti-Jak1 and anti-Jak2 antibodies. (B) In parallel, total cell lysates from control and treated cells were separated on 10% SDS-PAGE gels, followed by blotting with anti-PY antibodies. (C) Stat1 was immunoprecipitated from control, IFN-γ, forskolin and forskolin + IFN-γ treated cells. Tyrosine phosphorylation of Stat1 was examined by 4G10 immunoblotting. The membrane was then stripped and reprobed with anti-Stat1 antibody. The data are from one of three independent experiments that yielded similar results.
4. Detection of novel (non-Jak) IFN-γ-inducible tyrosine kinase activities by FPLC Mono Q fractionation coupled to a phosphocellulose paper assay

The findings described above suggesting the presence of novel, IFN-γ-inducible tyrosine kinases that are resistant to the effects of increased levels of cAMP, prompted studies aimed at the purification and identification of these novel kinases. FPLC Mono Q fractionation was selected as an initial approach because it had been used extensively to purify and characterize numerous kinase activities (118). Following exposure of cells to IFN-γ (100 U/mL for 15 min), one distinct peak of IFN-γ-activated Poly (Glu, Tyr) phosphotransferase activity was consistently identified in the 0.32-0.33 M fractions of the NaCl gradient (Figure 11, n=3). Compared to lysates prepared from untreated cells (control), the mean fold increase in activity of this major peak of Poly (Glu, Tyr) phosphotransferase activity was 3±0.3 (mean ± SEM, n=3).

Mono Q fractions from control and IFN-γ-treated cells were then subjected to SDS-PAGE and transferred to nitrocellulose for immunoblotting. Using an anti-phosphotyrosine antibody, two tyrosine phosphorylated proteins (with approximate subunit sizes of 100 and 125 kDa) were observed to be enriched in the 0.32-0.33 M fractions of the NaCl gradient derived from the IFN-γ-treated cells. These proteins were not present in the corresponding NaCl gradient fraction from control cells (Figure 12, n=2). Although the identities of the phosphoproteins identified in the Mono Q fractions have not been determined, their presence is consistent with the observation that IFN-γ treatment of PMA-differentiated U937, leads to increased tyrosine phospho-
Figure 11. Assay of IFN-γ-inducible protein tyrosine kinase activity using Poly (Glu,Tyr) as substrate. Detergent lysates of PMA differentiated U937 cells (control and IFN-γ-treated) were applied to a Mono Q anion exchange column, and the column was developed with a linear gradient of NaCl from 0 to 0.8 M. Aliquots of column fractions were assayed for protein tyrosine kinase activity using Poly (Glu, Tyr) as substrate. Separate aliquots of column fractions were subjected to SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting using a mixture of anti-Jak1 and anti-Jak2 antibodies. The localization of Jak1 and Jak2 is shown by an arrow. The data shown are from one of three independent experiments that yielded similar results.
rylation of cell proteins (Figure 8) and that this can occur in the apparent absence of Jak1 and Jak2 activation (Figure 10).

Using rabbit polyclonal antibodies that recognize Jak1 and Jak2, we ruled out the possibility that the kinase activity detected in the 0.32-0.33 M NaCl fraction could be due to the presence of these kinases. The Jaks were identified as eluting in the 0.54 M NaCl fraction (Figures 11 and 13). To establish that the elution gradient was resulting in the detection of kinase activities in their predicted position, we followed the elution of a known protein resolved under identical conditions previously (111). As shown in Figure 13, Erk was detected in Mono Q eluates in the 0.40 M NaCl fraction (Figures 13A and 13B), in agreement with previous findings from this laboratory.

To characterize further the kinase activity in the 0.32-0.33 M NaCl fractions, after incubation in the presence or absence of IFN-γ, cells were subjected to sub-cellular fractionation to obtain, cytosolic, peripheral membrane and membrane fractions, which were separated further by FPLC on Mono Q. Figure 13C shows that the peak of activity identified in the initial Mono Q experiments using total cell lysates, was localized to the peripheral membrane and membrane compartments. When the cytosolic subcellular fraction was analyzed, no peak of activity in this region of the sodium chloride gradient was detected (data not shown).
Figure 12. Anti-phosphotyrosine Western blot analysis of Mono Q fractions. Aliquots of Mono Q fractions from control and IFN-γ-treated cells were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine antibody. The data shown are from one of two independent experiments that yielded similar results.
Figure 13. IFN-γ-inducible tyrosine kinase activities detected by the phosphocellulose paper assay of Mono Q fractions are not related to the Jaks or Erk and are localized to the membrane. Aliquots of the Mono Q fractions were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-Jak1/Jak2 mix and anti-Erk antibodies (panels A & B). Lysates from control (C) and IFN-γ-treated (I) cells were fractionated to obtain cytosol, peripheral membrane (PM) and membrane (ME) fractions, as described in the Materials and Methods section. Whole cell lysates (WCL) and Subcellular fractions were separated further by Mono Q and kinase activity was determined by phosphocellulose paper assay using Poly (Glu, Tyr) as substrate. The cytosolic-derived Mono Q fractions did not show differences between control and IFN-γ samples (data not shown). The data shown are from one of two independent experiments that yielded similar results.
5. Purification and characterization of the IFN-γ-inducible p125 kDa phosphoprotein suggests that it is the transcriptional co-activator, TIP120a

Three findings prompted efforts to identify the IFN-γ-inducible p125 kDa protein (or proteins). First, the renaturation kinase assay identified an apparent 125 kDa IFN-γ-inducible tyrosine kinase (Figure 9). Second, the anti-phosphotyrosine analysis consistently showed the induction of a 125 kDa tyrosine phosphorylated protein in the IFN-γ-treated cell lysates (Figure 8) from multiple cell lines including U937, THP-1, RAW and 293T. Third, the induction of tyrosine phosphorylation of a p125 protein in the 0.32-0.33 M NaCl gradient fraction from the Mono Q fractionation (Figure 12), Initial experiments excluded that p125 corresponded to Pyk2 which was a potential candidate since it has a reported size in the range of 125 kDa and because it has been implicated in IFN-γ signalling (52). Figure 14 shows the results of a Pyk2 immunoprecipitation coupled with anti-phosphotyrosine analysis from PMA-differentiated THP-1 cells. Two findings suggest that Pyk2 is not the p125 tyrosine phosphorylated protein induced by IFN-γ. First, Pyk2 showed a different relative mobility than p125, which is in agreement with its reported relative molecular weight (110 kDa). Second, Pyk2 immunoprecipitates showed similar levels of tyrosine phosphorylation both in control and IFN-γ-treated cells, in contrast to tyrosine phosphorylation of p125 which was IFN-γ-induced.
Figure 14. Pyk2 immunoprecipitation and tyrosine phosphorylation analysis suggest that it is not the IFN-γ-inducible tyrosine phosphorylated p125. PMA-differentiated THP-1 cells were treated with or without 100 U/mL of IFN-γ for 15 min. Cells were washed and lysed in modified RIPA buffer with protease and phosphatase inhibitors. Cleared lysates were used to immunoprecipitate Pyk2. Whole cell lysates (WCL) and immunoprecipitates (Pyk2 IP) were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine antibody. The levels of immunoprecipitated Pyk2 were similar between the control and IFN-γ-treated cell samples (data not shown).
In further attempts to identify the p125 protein, a strategy was designed based upon localization of this phosphoprotein to the membrane compartment, as it was only detected in the 1% Triton-X100 soluble fraction (detergent lysates) but not in the corresponding cytosolic fraction (Figure 15A, lane 2 n=3). Taking into consideration that we had also detected a p125 protein with apparent tyrosine kinase activity induced by IFN-γ which recognized Poly (Glu, Tyr) as a substrate (Fig. 9B), and previous successful efforts to purify kinase activities using this substrate as an affinity reagent (119), a Poly (Glu, Tyr) agarose column was used to further purify this band. Despite the apparent enrichment of this tyrosine phosphorylated band by Poly (Glu, Tyr) affinity binding (Figure 15A, lane 6, n=3), there was no corresponding band detected by silver staining (data not shown) suggesting that additional purification steps would be required.
Figure 15. An IFN-γ-inducible p125 tyrosine phosphorylated protein is membrane associated and binds to a Poly (Glu, Tyr) affinity column. PMA-differentiated U937 cells, control and IFN-γ-treated (100 U/mL for 15 min), were solubilized in MOPS buffer with or without detergent (1% NP-40). Cleared cell lysates were incubated with a pre-equilibrated Poly (Glu, Tyr) column for an hour, bound proteins were washed with buffer, eluted by boiling in SDS sample buffer ["Poly (Glu, Tyr) bound"], separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with 4G10.
Based on the properties of the p125 protein which included membrane localization, binding to Poly (Glu, Tyr) and reactivity with anti-phosphotyrosine antibody (Figure 15A), a revised purification protocol was designed that is shown schematically in Figure 16. Use of this strategy resulted in purification of a band of the appropriate size detected by silver staining (Figure 17) that was modestly enriched in samples from IFN-γ-treated cells, consistent with its undergoing tyrosine phosphorylation in response to ligand. Given these results, the protocol was scaled-up to allow purification of a band detectable by Coomassie blue staining and this was sent for peptide sequencing by mass spectrometry (Cleveland Clinic Research Foundation, Cleveland, Ohio, Dr. Michael Kinter). Mass spectrometry results showed the presence of nineteen peptides corresponding to 20.1% of the amino acid sequence of TIP120a (120), a co-transcriptional activator (Figure 18, see underlined peptides).

Two approaches were used in an attempt to verify that TIP120a actually corresponded to the p125 protein of interest, that underwent increased tyrosine phosphorylation in response to IFN-γ. The first approach required generating an antibody against TIP120a and this was done by designing, synthesizing and immunizing rabbits with an immunogenic peptide. The polyclonal antibody raised detected TIP120a by immunoblotting (Figure 19A). However, it was not able to immunoprecipitate the protein (data not shown) and was therefore not useful for direct examination of the phosphorylation status of TIP120a upon IFN-γ treatment. A second, alternative approach, involved transfection of human embryonic kidney cells.
Figure 16. Protocol for purification of the p125 IFN-γ-inducible phosphoprotein from a human monocytic cell line model.

The protocol outlined above was used to purify a tyrosine phosphorylated protein induced by IFN-γ in PMA-differentiated control or IFN-γ-treated (100 U/mL for 15 min) U937 cells. After SDS-PAGE and silver staining of the purified material, the protocol was scaled-up and the new purified material was resolved by SDS-PAGE, Coomassie blue stained and a band in the area of 125 kDa was cut and sent for mass spectrometry analysis.
Figure 17. Silver staining of purified material obtained using the protocol outlined in Figure 16. Briefly PMA-differentiated U937 cells from control (CTL) and IFN-γ-treated (IFN-γ) cells were washed and subjected to subcellular fractionation. The membrane fraction was incubated with Poly (Glu, Tyr) agarose beads. The material bound to these beads was eluted in SDS-sample buffer and then separated by SDS-PAGE. A piece of gel corresponding to the area of interest (125 kDa) was cut and the proteins eluted with an SDS solution. Eluted proteins were diluted in immunoprecipitating (IP) buffer and concentrated with Centriprep filters. The proteins diluted in IP buffer were incubated with an anti-phosphotyrosine antibody to purify the tyrosine phosphorylated proteins by IP. The samples obtained by the IP were resolved by SDS-PAGE and silver stained (left arrows correspond to the molecular weight markers of 116, 97 and 66 kDa from top to bottom).
(293T) with a construct containing a histidine tagged rat TIP120a, kindly provided by Dr T. Tamura (Chiba University, Japan). His-tagged TIP120 expressed in 293T cells migrated with an apparent size of 140 kDa. As shown in Figures 19B and 19C, in response to IFN-γ treatment of transfected cells, a 140 kDa band became tyrosine phosphorylated and this comigrated with His-TIP120a (panel C, n=2). We have some preliminary data where we show that the his tagged TIP120a can be purified using nickel beads (which bind to the histidine tag present in the recombinant protein) and it is tyrosine phosphorylated in response to IFN-γ (data not shown). These findings suggest that TIP120a undergoes tyrosine phosphorylation in response to IFN-γ treatment and this may be of significance to regulation of transcriptional responses to IFN-γ.
Figure 18 Identification of the IFN-γ-inducible p125 as TIP120a. A. The p125 protein purified according to the protocol described in Figure 16 was cut from the gel, digested with trypsin and subjected to LC/MS analysis. Upper panel. The band was found to contain 26 peptides, the majority of which (19, underlined) matched the sequence of human TIP120a (accession number 8924268). These peptides encompassed 247 amino acids that covered 20.1% of the protein sequence. Lower panel. Schematic diagram of TIP120a. Analysis of putative tyrosine phosphorylation sites in TIP120a (NetPhos 2.0) identified five potential sites of tyrosine phosphorylation (tyrosines 6, 291, 311, 313 and 339, denoted as Y).
Figure 19. (A) TIP120a immunoblot. Whole cell lysates from PMA-differentiated THP-1 cells were separated by preparative SDS-PAGE, transferred to nitrocellulose and immunoblotted with either rabbit pre-immune serum or with serum from a TIP120a peptide immunized rabbit. Serum from a rabbit immunized with a peptide corresponding to...
TIP120a (KQEFEKQDELKRSAMC) recognizes this protein specifically (lanes 7-9). No reactive band in the size range of TIP120a was detected with the preimmune serum (lanes 1-6). BSA = 3% bovine serum albumin, M = 5% milk. (B) His-tagged TIP120a transfection of 293T cells. 293T cells were transfected with His-tagged TIP120a (lane 1) or His-IFN-γR2 (intracellular domain, lane 2). Following overnight transfection, cells were washed, rested for 4 h and then treated or not with 1000 U/mL IFN-γ for 15 min. Panel B shows a 4G10 immunoblot of cell lysates from the transfections and Panel C shows stripping and reprobing of the same membrane with anti TIP120a antibody (n=2).
In summary, the results described in this section show evidence for IFN-\(\gamma\)-induced tyrosine phosphorylation and kinase (non-Jak) activities in human macrophage proteins. IFN-\(\gamma\) induced the tyrosine phosphorylation of Jak1, Jak2 and Stat1, as well as two proteins (molecular masses of 90 and 125 kDa) as detected by an anti-phosphotyrosine antibody. IFN-\(\gamma\) also induced two renaturable tyrosine kinase activities (molecular masses of 210 and 125 kDa) as assessed by an in gel renaturation tyrosine kinase assay. Evidence of the existence of other non-Jak components of IFN-\(\gamma\) signalling was found by using forskolin which induces increased cAMP levels and blocks the Jak-Stat pathway. While forskolin treatment prior to the addition of IFN-\(\gamma\) led to a significant downregulation of the activation of the Jaks, the IFN-\(\gamma\)-inducible tyrosine phosphorylation of proteins with relative molecular masses of 125 and 85 kDa was unaffected. This suggested the existence of non-Jak, IFN-\(\gamma\)-inducible tyrosine kinases. A biochemical approach based on Mono Q fractionation, followed by a phosphocellulose paper assay was designed to detect novel IFN-\(\gamma\)-inducible tyrosine kinases. Using this approach we identified a peak of tyrosine kinase activity in fraction 0.32-0.33 that did not correspond to the Jaks as shown by immunoblotting. An anti-phosphotyrosine immunoblot of the fractions with increased tyrosine kinase activity showed bands of molecular masses of 100 and 125 kDa in the IFN-\(\gamma\)-derived fractions. A subcellular fractionation coupled to the Mono Q fractionation and kinase assay identified that the original peak of IFN-\(\gamma\)-inducible kinase activity was located in the peripheral membrane and membrane compartments, and not in the cytosol.
As we consistently detected a 125 kDa IFN-γ-inducible tyrosine phosphorylated protein, even in the absence of Jak activities as shown by the forskolin experiments, a decision was made to identify this protein. We designed a purification strategy that included a subcellular fractionation to enrich the membrane proteins, a Poly (Glu, Tyr) affinity chromatography, and immunoprecipitation with anti-phosphotyrosine (Figure 16). A band in the p125 region was purified and sent for mass spectrometry, which showed the presence of nineteen peptides corresponding to 20.1% of the amino acid sequence of TIP120a, a co-transcriptional activator. A his-tagged rat TIP120a construct was used to transfect 293T cells. A tyrosine phosphorylated protein co-migrated with his-tagged TIP120a, suggesting that TIP120a undergoes phosphorylation in response to IFN-γ.

TIP120a is a co-transcriptional activator whose expression is induced by vitamin D3 and that may play a role in cell differentiation (121). The potential role of TIP120a in IFN-γ signalling will be described in the Discussion section of this thesis. Experimentally, the role of TIP120a in IFN-γ signalling could be analyzed by determining the tyrosine phosphorylated residues that are affected by IFN-γ, and by downregulating its expression with siRNA (C. Leon, work in progress). During the work that validated the induction of TIP120a tyrosine phosphorylation by IFN-γ, we started a genetic approach using the yeast two hybrid system. This work used the intracellular domains of the IFN-γR1 and R2 and is described in the following Results chapter.
B. IDENTIFICATION OF NOVEL PROTEIN-PROTEIN INTERACTIONS
WITH THE IFN-\(\gamma\)-R1 AND R2 CHAINS BY YEAST TWO HYBRID SCREENING

In the previous chapter we presented evidence of novel kinase activities activated by IFN-\(\gamma\). These kinases or the phosphatases that regulate their activities could be associated directly with the receptor, or indirectly through adaptor proteins that interact with the receptor. To identify either the novel kinases, or phosphatases or adaptor proteins we used a genetic approach with the potential to identify receptor-interacting proteins, irrespective of their nature. The yeast two hybrid system has been successfully used to identify novel receptor interacting proteins in a variety of models (105;106). We used the intracellular domains of the IFN-\(\gamma\)R1 and R2 as bait in a yeast two hybrid screen of a human monocytic cDNA library generated for this purpose. To confirm the putative interactions we used a GST pull down approach and co-immunoprecipitation.

1. Cloning and expression of the intracellular domains of IFN-\(\gamma\)R1 and R2 as bait for yeast two hybrid screening

The intracellular domains of the IFN-\(\gamma\)R1 and R2 were amplified and cloned into the pYTH9 shuttle vector, as described in Materials and Methods. Figures 20A and B show positive clones pYTH9-H1 and pYTH9-H2 selected by plasmid extraction and double restriction enzyme digestion. Figure 20A shows in lane 1 the released R1 insert
band with the predicted size from a positive clone (arrow), lane 2 shows molecular weight markers and lane 3 represents an empty vector digested with the same two enzymes. Similarly, Figure 20B shows in lane 1 the molecular weight markers; lane 2 the purified R2 insert used for the cloning; lane 3 shows a digested empty vector and in lane 4 a positive clone where the R2 insert was released after the double digestion (arrow).

Confirmed positive clones were used to transform yeast host strain SDY191. This resulted in recombinant yeast clones pYTH9-H1 and pYTH9-H2, which were streaked on agar plates with selective medium and then re-grown in the appropriate selective liquid growth medium. Yeast clones were subjected to genomic DNA extraction and PCR to verify chromosomal integration of the Gal 4 Binding Domain (GBD)-insert (data not shown). PCR positive clones were grown, protein was extracted and examined by immunoblotting with an anti-GBD domain antibody (Figures 20C and 20D). As can be seen, immunoblots of extracts from the yeast clones showed that the anti-GBD antibody recognized specific bands corresponding to the GBD-H1 and GBD-H2 fusion proteins (respectively Figures 20C lane 2 and Figure 20D lanes 2-4), as they were absent in the non-transformed yeast host SDY191 (Figure 20C lane 1 and Figure 20D, lane 1). The predicted relative molecular sizes of the GBD-H1 and GBD-H2 fusion proteins were calculated as follows.
Figure 20. Cloning of the intracellular domains of the IFN-γR1 and R2 into the yeast two hybrid bait vector. Upper panels: (A) Restriction enzyme analysis of GBD-R1: lane 1: positive clone; lane 2 1 Kb PLUS™ molecular weight markers (Invitrogen) and lane 3 vector alone. (B) GBD-R2: lane 1 1 Kb PLUS™ molecular weight marker; lane 2 R2 insert; lane 3 vector alone; and lane 4 positive clone. Lower panels: Immunoblot analysis of GBD-H1 (C) and GBD-H2 (D) yeast cells using an anti-GBD antibody. The results are representative of two independent experiments. For both panels (C) and (D), lane 1 contains non-transformed yeast; for panel (C) lane 2 and panel (D) lanes 2-4 contain transformed yeast. Arrows indicate the bands corresponding to GBD-R1, GBD-R2, GBD-H1 and GBD-H2 in panels A, B, C and D, respectively.
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<th>GBD-H1</th>
<th>GBD-H2</th>
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<tr>
<td>Insert</td>
<td>702 bp = 234 aa</td>
<td>226 bp = 75 aa</td>
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<td>Polylinker</td>
<td>10 aa</td>
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<td>HA sequence</td>
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<td>GBD</td>
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<td>Total</td>
<td>400 aa = 44 kDa</td>
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The actual relative molecular sizes of the GBD-H1 and GBD-H2 fusion proteins, as determined by SDS-PAGE and immunoblotting with anti GBD antibody, were respectively 42 and 32 kDa.

In order to rule out false positive interactions in the two hybrid screen, yeast clones containing integrated bait constructs were transformed with the Gal 4 activation domain (GAD) containing vector, pACT2, containing no library inserts, in the presence of 15 mM 3-amino triazole (3-AT), a reagent that reduces histidine leakage. One recombinant yeast clone, H2 Cl 5 (containing an integrated IFN-γR2 cytoplasmic domain GBD fusion construct), transformed with pACT2 and grown in the auxotrophic medium plus 3-AT, showed no growth and was selected to carry out library screening. Similarly, in the case of the R1 cytoplasmic domain construct, yeast clone H1 Cl 1 transformed with the pACT2 plasmid and cultured in the presence of 3-AT showed no growth and was selected for library screening.
2. Library construction in the HybriZap vector for yeast two hybrid screening

The library was constructed according to the manufacturer's specifications as outlined in the Materials and Methods section (Figure 7). The quality of the library was assessed at various steps, as described below.

- First strand and second strand cDNA synthesis were checked by adding \( \alpha^{32}P \) dNTP to an aliquot of the synthesis reaction and the radiolabelled samples were resolved by alkaline agarose gel electrophoresis. The cDNA preparation eluted from the Sepharose column was analyzed by agarose gel electrophoresis. The sizes of the cDNAs ranged between 500 and 3000 bp.

- Titration of the Hybrizap library suggested that it contained \( 1.5 \times 10^6 \) pfu (plaque forming units), which is within the range of the expected number of colonies considering the amount of starting material (4 \( \mu \)g of RNA). According to the manufacturer, a good representational library consists of one million plaque forming units (pfu). Assuming that the number of genes present in a human genome is in the range of 31,000, \( 1.5 \times 10^6 \) pfu represents fifty times this amount.

- The insert percentage and size from the phage library was checked by PCR using a set of primers that recognize the Gal 4 activation domain. We found that we could amplify an insert from at least seven out of ten clones, although based upon PCR alone, it was not possible to positively conclude that the negative clones did not
contain inserts. From the seven positive clones, three had inserts larger than 1000 bp, while the other four clones had inserts between 500-1000 bp (data not shown).

- The library was amplified to ensure its stability. The expected size, considering the amount of starting material (4 μg of RNA) was between $10^8$-$10^{10}$. The titre obtained was $2 \times 10^9$.

- Six colonies grown from the phagemid library were analyzed by plasmid extraction and double restriction enzyme digestion. Five out of six colonies contained inserts; moreover, in each case the size was > 900 bp (Figure 21A).

- The phagemid library was amplified by transformation of *E. coli* and purified plasmids were used as template to amplify a set of IFN-γ-inducible genes IFN-γR1 and R2, GBP (guanylate binding protein) and ICE (interleukin converting enzyme). All of these targets were amplified from the library (Figure 21B) (ICE amplification data is not shown).
Figure 21. Assessment of the quality of the amplified phagemid library. (A) Six plasmids isolated from the phagemid library were digested with EcoRI and Xhol to release inserts. Digestions were separated by 1% agarose gel electrophoresis. Five out of six plasmids released an insert (+). Lane 1: DNA ladder, Lane 2-7 double digestions of phagemid clones (B) Three interferon-responsive genes were PCR amplified from the phagemid library (IFN-γR1, R2 and GBP). Lane 1: DNA ladder; Lanes 2 negative control for R2 amplification; Lane 3, R1 PCR, Lane 4, R2 PCR, and Lane 5, GBP PCR. ICE (interleukin converting enzyme) was also amplified from this phagemid library (data not shown).
3. Library screening, selection, confirmation and identification of IFN-γR1 and R2 interacting clones

The strategy for identifying library clones that interacted with bait constructs included the following criteria:

- Ability to support growth to the recombinant yeast bait clones in auxotrophic medium (absence of leucine, tryptophan and histidine and in the presence of 15 mM 3-AT).
- Positive β-galactosidase activity, a second reporter gene,
- Candidate library plasmid was back extracted from the positive yeast colony, purified and used to retransform the yeast bait to confirm the first two criteria,
- DNA sequencing showed that the insert sequence from the library was in frame with the GAD and corresponded to the coding region of a gene, and
- Absence of premature STOP codons in the protein which would otherwise suggest a truncated protein.

4. Analysis of the sequences of the putative IFN-γR1 interacting clones

Three yeast clones fulfilled the criteria described above for positive interactions in the yeast 2-hybrid screen using yeast clone H1 Cl 1 containing the R1 cytoplasmic domain as bait. The negative control for these interactions was a yeast co-transformed with the plasmid pACT2 (GAD alone) as exemplified with interacting clone B1 (Figure 22A).
(1) S100A9 (also known as MRP-14). One of the R1 interacting clones, B1, contained 553 nucleotides corresponding to the complete sequence of the S100A9 gene in addition to part of the 3' UTR region (Figure 22B). S100A9 is a calcium binding protein of the EF-hand family and has been implicated in the initiation and development of inflammatory processes (57;74) No S100A9 receptor has yet been described thus far.

(2) IP10 [Homo sapiens small inducible cytokine subfamily B (Cys-X-Cys) member 10]. A second clone encompassed 845 nucleotides including the complete coding sequence of IP-10 and part of the 3' UTR region. A functional receptor for IP10, CXCR3, has been previously described (20).

(3) CGI-107. CG107 is a protein of unknown function and analysis of its sequence did not provide evidence for the presence of known functional domains. A BLASTp search showed 97% homology with a mouse gene of unknown function (NCBI accession number BAB28706). The search also found 95% identity with a BCR/ABL regulated protein from the Chinese hamster Crisetulus griseus (direct submission, NCBI accession number AAQ94084) and a 56% homology with MSF-1 a Saccharomyces cerevisiae protein involved in intra-mitocondrial sorting. The amino acid sequence analysis suggested that it is not a transmembrane protein (membrane span probability of 0.05).
Figure 22. Clone B1, encoding the full length sequence of S100A9, interacts with the intracellular domain of the IFN-γR1 in a yeast two hybrid screen. Yeast bearing the R1 bait as a fusion with the GBD (GBD-R1) was transformed with the GAD alone (pACT2). Neither growth nor a positive β-galactosidase assay were obtained. Screening of the cDNA expression library identified a clone B1 which interacted with the GBD-R1 construct as shown by the ability of the yeast to grow in the absence of histidine and the positive β-gal assay. The plasmid from clone B1 was back extracted and used to re-transform the yeast bait. The phenotype to render the yeast bait able to grow in the auxotrophic medium was thus confirmed. After confirmation clone B1 was sent for DNA sequencing, which identified it as the complete amino acid sequence of S100A9, a calcium binding protein.
5. Analysis of the sequence of a putative IFN-γR2 interacting clone

Yeast two hybrid screening revealed an interaction between the IFN-γR2 and annexin V (hereafter referred as AxV). Screening of the library using yeast clone H2 Cl 5 containing the IFN-γR2 cytoplasmic domain as bait led to the identification of three clones able to grow in the auxotrophic medium. One of the clones, D6d, conferred the expected phenotype, including yeast growth in auxotrophic medium and a positive β-galactosidase assay. Moreover, the plasmid from this interacting clone was extracted, amplified in bacteria and used to re-transform yeast clone H2 Cl 5 with the R2 bait sequence. To confirm specificity of the phenotype, a series of control transfections were done as depicted in Figure 23A. Neither yeast growth nor β-galactosidase activity were detected when the R2 bait was transformed with an empty library vector, expressing only GAD. Clone D6d was also used in a cotransfection experiment with yeast expressing the GBD without the R2 sequence and again, no yeast growth was detected. When a yeast clone bearing the GBD fused to the intracellular domain of the IFN-γR1 was transfected with clone D6d, some yeast growth was detected, but the β-galactosidase assay was negative. Together, the results summarized in Figure 23A, suggest that the interaction between D6d and the intracellular domain of the IFNγ-R2, was specific. Clone D6d was sequenced and identified as containing DNA sequence corresponding to the coding region of AxV. Figure 23B depicts the region of AxV present in clone D6d which consisted of 826 nucleotides. These corresponded to aminoacids 157 to 319, including the carboxyl terminal region of AxV and part of the 3' UTR region of the gene. The region of AxV present in clone D6D encompassed...
domains three and four, which are known to be sufficient to form a binding site for calcium and phospholipids.
Figure 23. A. Clone D6d specifically interacts with the cytoplasmic domain of the IFN-γR2 in a yeast two hybrid screen. B. Schematic representation of a clone that interacted with intracellular domain of IFN-γ-R2. Clone D6d, which showed a strong interaction with R2, was sequenced (826 nucleotides) and identified as Annexin V (AxV). This figure shows the region present in clone D6d, (amino acids 157-319), including the carboxyl terminal region of AxV and the 3' UTR region of this gene. The AxV region present in this clone encompasses the domains three and four, which are sufficient to form a binding site for calcium and phospholipids. This truncated AxV chain was apparently also sufficient for the interaction with the intracellular domain of IFN-γR2 receptor.
6. S100A9 (MRP14) as a putative IFN-γR1 interacting protein.

To verify results of the two hybrid screen indicating an interaction between S100A9 and IFN-γR1, co-immunoprecipitations and GST-pull downs were done.

a. Immunoprecipitation of S100A9

An initial approach to validate the S100A9-R1 interaction found by the yeast two hybrid screening involved immunoprecipitation of S100A9 from THP-1 cells. Initially, we immunoprecipitated S100A9 from the control and IFN-γ-treated cells. Interestingly, after a 15 min incubation with IFN-γ, a two-fold increase in the levels of S100A9 protein as detected by Western blot and quantified by densitometry, was observed (n=2) (Figure 24), suggesting a redistribution of the protein. Interestingly, the apparent increase in the abundance of S100A9 that we detected was only apparent in whole cell lysates. The fact that it was not reflected in the S100A9 immunoprecipitates suggested that after IFN-γ treatment, S100A9 may be engaged in specific protein-protein interactions or that it is mobilized to a subcellular fraction in a way such that it is no longer available for immunoprecipitation. For reasons that are not clear, the S100A9 immunoprecipitation did not provide evidence supporting the interaction with the IFN-γR1 (data not shown). Because of this negative result a reciprocal immunoprecipitation was carried out to bring down IFN-γR1 and look for the presence of S100A9.
Figure 24. Immunoprecipitation of S100A9 from control and IFN-γ-treated THP-1 cells and immunoblotting with anti-S100A9 antibody. PMA-differentiated THP-1 cells, control or IFN-γ-treated for 15 min, were solubilized in modified RIPA buffer and cleared by centrifugation (whole cell lysates [WCL], lanes 1,2) to be used to immunoprecipitate S100A9 (lanes 5,6). Lanes 3, 4 immunoprecipitates using an irrelevant antibody to control for specificity.
b. Co-immunoprecipitation of S100A9 with the IFN-γR1

To examine further whether R1 and S100A9 were *bona fide* interacting proteins, IFN-γR1 was immunoprecipitated from lysates of IFN-γ-treated PMA-differentiated THP-1 cells. When the IP was analyzed by Western blotting using an antibody specific for S100A9, a band of 28 kDa was detected (Figure 25B, lane 2, n=1). In light of the fact that S100A9 has an apparent subunit size of 14 kDa, this finding suggested the possibility that S100A9 interacted with R1 as a homodimer or a heterodimer with S100A8. These results are in agreement with previously reported results obtained under similar conditions showing, that S100A9 homodimers and S100A8/A9 heterodimers can be detected by SDS-PAGE even when carried out under denaturing conditions (122) The result shown in Figure 25 also suggested that the interaction of S100A9 with R1 is ligand inducible as it was only seen in lysates from IFN-γ-treated cells.

As an alternate, independent approach to confirm this interaction, IFN-γR1 was immunoprecipitated from neutrophil lysates, as these cells have been reported to express high levels of S100A9 (123). As shown in Figure 25C, a small amount of S100A9 (14 kDa) immunoprecipitated with R1. A third approach to examine the interaction between R1 and S100A9 involved a GST-R1 chain pull down experiment with neutrophil lysates as a source of S100A9. As shown in Figure 26, S100A9 was brought down in the GST-R1 pull down, but not with GST alone or with GST-R2.
Figure 25. Association of IFN-γR1 with S100A9 in vivo. (A) PMA differentiated THP-1 cells were treated with IFN-γ (100 U/ml for 15 min) or left untreated. Cells were then lysed in digitonin containing buffer supplemented with protease and phosphatase inhibitors. Cell lysates were incubated with anti-IFN-γR1 antibody. Immune complexes were recovered using protein-G-sepharose and released into boiling SDS-sample buffer. (A) Solubilized proteins in parallel with an aliquot of total cell lysate were separated on 10% SDS-PAGE and immunoblotted with anti-IFN-γR1 antibody. Lane 1, anti-IFN-γR1 antibody incubated with lysate from control cells, lane 2, anti-IFN-γR1 incubated with lysates from IFN-γ-treated cells and lane 3, irrelevant antibody incubated with lysates from IFN-γ-treated cells (B) The same blot was reprobed with a S100A9 antibody (n=2).
(C) Human neutrophils were purified from whole blood and lysed in co-IP buffer. Cleared lysates were incubated with either IFN-γR1 antibody or irrelevant antibody (anti-Cbl). After the IP, immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose for Western blotting with the anti-S100A9 antibody. Lane 1: Neutrophil lysate; Lane 2: IFN-γR1 IP and Lane 3: Cbl IP. Right arrow indicates the S100A9 protein.
c. *S100A9* interacts with a 90 kDa tyrosine phosphorylated protein in an IFN-γ-dependent manner.

When immunoprecipitates of *S100A9* from PMA-differentiated THP-1 cells were examined by 4G10 analysis, we did not find evidence that it became tyrosine phosphorylated in response to IFN-γ (data not shown). However, a ~ 90 kDa phosphoprotein co-immunoprecipitated with *S100A9* in an IFN-γ-dependent manner and appeared to be tyrosine phosphorylated (Figure 27, n=3). When this blot was stripped and reprobed with the IFN-γR1 antibody, the results indicated that p90 was not the R1 receptor.
Figure 26. GST-R1 pull down suggests that R1 interacts with S100A9 in vitro. IPTG-induced GST, GST-R1 and GST-R2 were expressed and purified using glutathione beads. Equal amounts of beads were mixed with equal aliquots of a neutrophil lysate. Beads were recovered, washed, boiled in SDS-sample buffer, separated by 12% SDS-PAGE followed by transfer to nitrocellulose and immunoblotting for S100A9. S100A9 co-purified with the GST-R1 (lane 3), but not with GST alone (lane 1) or with GST-R2 (lane 2). Lane 4 corresponds to the neutrophil lysate used for the pull down experiment (n=2).
Figure 27. S100A9 co-immunoprecipitates with a 90 kDa protein that becomes tyrosine phosphorylated in response to IFN-γ. PMA-differentiated THP1 cells were untreated (lane 1) or incubated with IFN-γ as shown (lanes 2-4). Lysates were immunoprecipitated with S100A9 antibody and immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose and analyzed by Western blotting for the presence of tyrosine phosphorylated proteins using 4G10.
In summary, a yeast two hybrid approach using the intracellular domains of the IFN-γR1 and R2 was designed to screen a monocytic cDNA library. It identified two novel IFN-γR-interacting proteins (S100A9 with R1 and annexin V with R2). The S100A9-R1 interaction was validated using a GST-pull down approach and by immunoprecipitation using receptor chain antibodies. Preliminary results from the R1 immunoprecipitations suggest that the S100A9-R1 interaction might be ligand-inducible.
C. ANNEXIN V, A NOVEL IFN-γ-R2 INTERACTING PROTEIN THAT REGULATES IFN-γ SIGNALLING

In the previous chapter we identified annexin V as a novel IFN-γR2 interacting protein in a yeast two-hybrid screen. In this chapter, GST pull down and a co-immunoprecipitation approaches were used to validate this interaction. Once the interaction was confirmed, an siRNA approach to downregulate the expression of annexin V in a human embryonic kidney cell model (293T cells) was used to investigate the significance of its association with R2. The results show that in cells with reduced levels of AxV, IFN-γ induced tyrosine phosphorylation of Jak2 and Stat1 was increased and this was associated with enhanced gene transcription and growth arrest in response to IFN-γ.

1. A GST-R2 fusion protein specifically pulls down AxV

To seek independent evidence to support the yeast two-hybrid results, the cytoplasmic domains of IFN-γR2 and R1 were cloned and expressed as GST fusion proteins. After recovery of fusion proteins using glutathione beads, the purity of the preparations was assessed by SDS-PAGE and Coomassie blue staining (Figure 28A, n=3). Equivalent amounts of GST, GST-R1 and GST-R2 fusion proteins were used in pull down experiments. When a lysate of IFN-γ-treated THP-1 cells was incubated with the fusion proteins, AxV bound to the GST-R2 fusion protein, but not to either GST alone or to the GST-R1 fusion (Figure 28B, n=3).
Figure 28. Association of the intracellular domain of the IFN-γ/R2 chain with Annexin V in vitro
Figure 28. Association of the intracellular domain of IFN-γR2 chain with annexin V in vitro. The intracellular domain of R2 (residues 251-316) was amplified using RT-PCR from total RNA of THP-1 cells and cloned into the pGEX-4T expression vector (GST-R2). IPTG-induced GST-R2 fusion protein was purified using glutathione-agarose beads. In addition the intracellular domain of IFN-γR1 chain (residues 252-472) was also expressed and purified as a GST fusion protein (GST-R1) to use as a specificity control. (A) Affinity purified GST-fusion proteins. Lane 1, GST, lane 2, GST-R1, and lane 3, GST-R2. (B) Interaction of GST-R2 with AxV. PMA-differentiated THP-1 cell lysates containing a cocktail of protease and phosphatase inhibitors were incubated with GST-fusion proteins (R1 or R2) or with GST alone immobilized on glutathione-sepharose. After washing, complexes were released by boiling in SDS-sample buffer, electrophoresed on 10% SDS-PAGE and subjected to immunoblotting with anti-AxV antibody. The data shown are from one of three independent experiments that yielded similar results.
2. Confirmation of the interaction between IFN-γR2 and AxV in vivo by co-immunoprecipitation

Based upon the findings from both the yeast two-hybrid screen and the GST pull down experiment, we sought evidence to determine whether AxV and IFN-γR2 associate within the cell. To examine this question, THP-1 cells were either untreated or incubated with IFN-γ and then subjected to immunoprecipitation using an antibody directed at IFN-γR2. As can be seen in Figure 29A, AxV co-immunoprecipitated with IFN-γR2. Of interest, this was only observed when using lysates from cells that had been IFN-γ-treated, suggesting that the interaction was ligand-inducible. When the membrane was stripped and re-probed, the results showed that similar amounts of receptor were brought down from lysates of both IFN-γ-treated and control cells (Figure 29B, lanes 6 & 7). Lysates used for immunoprecipitation for all treatment groups contained equal amounts of AxV and isotype-matched control antibody against c-Abl brought down c-Abl, but not AxV (data not shown).

To examine the specificity of the interaction further, the membrane containing the proteins from the immunoprecipitation was stripped and re-probed with an anti-AxII antibody (Figure 29C, lane 1). This antibody recognized AxII in the whole cell lysates but not in the immunoprecipitates, indicating that the association between AxV and IFN-γR2 was specific. The specificity of the interaction was examined further using other irrelevant antibodies (elongation factor 1α and SHP-1), which did not co-immunoprecipitate AxV (data not shown).
3. Effect of IFN-γ treatment on tyrosine phosphorylation of AxV.

Since tyrosine phosphorylation is a crucial event in IFN-γ signalling, the induction of AxV tyrosine phosphorylation by IFN-γ in 293T cells was investigated as a potential explanation for its ligand-inducible recruitment to the receptor. This question was also of interest as AxV has been previously shown to be tyrosine phosphorylated upon treatment of endothelial cells with vascular endothelial growth factor (VEGF) (72) and other proteins that interact with the IFN-γR are tyrosine phosphorylated, such as Jak1, Jak2 and Stat1. Immunoprecipitation of AxV followed by anti-phosphotyrosine analysis did not provide evidence that AxV is tyrosine phosphorylated after 15 min of IFN-γ treatment (data not shown). Parallel immunoprecipitations of Stat1 and Jak2 showed that these proteins were tyrosine phosphorylated in response to IFN-γ in 293T cells that had been used to immunoprecipitate AxV (Figure 30).
Figure 29. Association of IFN-γR2 with annexin V in vivo. (A) PMA differentiated THP-1 cells were treated with IFN-γ (100 U/ml for 15 min) or left untreated. Cells were then lysed and cleared lysates were incubated with anti-IFN-γR2 antibodies as described in the Methods section. Solubilized proteins in parallel with an aliquot of total cell lysate were separated on SDS-PAGE and immunoblotted with anti AxV antibody. (A) Lane 1, total cell lysate, lanes 2 and 4 are two different irrelevant antibodies to IP control cell lysates, lanes 3 and 5 are the same two irrelevant antibodies incubated with lysates from IFN-γ-stimulated cells, lane 6 anti-IFN-γR2 incubated with lysate from control cells, and lane 7, anti-IFN-γR2 incubated with lysates from IFN-γ-treated cells. (B) The same blot was stripped and reprobed with IFN-γR2 antibodies and (C) AxV antibodies. The data are from one of four independent experiments that yielded similar results.
Figure 30. IFN-γ-dependent induction of the Jak-Stat pathway in 293T cells. (A) Anti-phosphotyrosine analysis of Stat1 immunoprecipitates from control and IFN-γ-treated 293T cells. The upper panel shows levels of tyrosine phosphorylation, while the lower panel shows the levels of immunoprecipitated Stat1 (B). IFN-γ-induction of tyrosine phosphorylation of Jak1 and Jak2. The Jaks were immunoprecipitated from control and IFN-γ-treated 293T cells as described elsewhere in this thesis. Immunoprecipitates were analyzed by immunoblotting using an anti-phosphotyrosine antibody (4G10).
4. Downregulation of AxV expression using siRNA

To address the role of AxV in IFN-γ signalling, siRNA was used to reduce the expression of AxV in 293T cells. As shown in Figures 31A and 31C, transfection of 293T cells with siRNA construct pSHAG AV2.1 brought about a marked reduction in AxV protein levels (70-80% reduction) as determined by immunoblotting. The specificity of the AV2.1 construct was determined by analyzing AxII expression which was unaffected (Figure 31D).

5. Reduced expression of AxV in 293T cells is associated with enhanced tyrosine phosphorylation of Stat1 in response to IFN-γ treatment.

To examine whether AxV regulates cell signalling in response to IFN-γ, either control (pSHAG) or pSHAG AV2.1 (AV2.1) transfected 293T cells were incubated with IFN-γ and whole cell lysates were examined using the anti-phosphotyrosine antibody 4G10. As shown in Figure 32, in cells with reduced AxV levels (AxVLo), increased tyrosine phosphorylation of a band with an approximate subunit size of 90 kDa was observed (n=3). The size of this protein suggested that it might be Stat1. Given the evidence of changes of tyrosine phosphorylation in the AxVLo cells when treated with IFN-γ the same cell lysates were subjected to immunoprecipitation with an antibody to Stat1. Immune complexes were then separated by SDS-PAGE, transferred to nitrocelullose membranes and probed with the anti-phosphotyrosine antibody. As shown in Figure 33, the amount of tyrosine phosphorylated Stat1
(pStat1) recovered from pSHAG AV2.1 transfected 293T cells after 4 h of IFN-γ treatment was increased two-fold in comparison to that recovered from IFN-γ-treated control transfected (pSHAG) cells (n=3). In contrast, levels of pStat1 after 15 min of IFN-γ treatment were equivalent for the two cell types. Together, these findings suggest that AxV may negatively regulate IFN-γ signalling by controlling Stat1 dephosphorylation. In the setting of reduced AxV abundance, Stat1 dephosphorylation may be retarded leading to sustained levels of pStat1.
Western blot:

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Figure 31. Annexin V gene silencing in 293T cells using siRNA. (A) Three AxV specific nucleotide sequences (21 mers) were selected using a web based platform for the design of siRNA constructs. Chemically synthesized oligos were cloned into the pSHAG vector that directs the in vivo synthesis of siRNA using a U6 promoter. Plasmids were purified from positive clones and used for transfecting 293T cells. After 72 h of transfection, cells were solubilized by boiling in SDS-PAGE sample buffer, separated by SDS-PAGE followed by immunoblotting with anti-AxV antibody. (B) To verify equal loading, immunoblotting was also performed with an anti-actin antibody. (C) Densitometric analysis of AxV levels in control and siRNA treated 293T cells. (D) AxII expression was not significantly affected by the AxV siRNA. Values are mean and SD of five independent experiments that yielded similar results.
6. Influence of AxV on activation of Jak2 in response to IFN-γ.

Since Jak2 is known to be involved in the tyrosine phosphorylation of Stat1, experiments were done to examine whether activation of Jak2 is influenced by AxV. To investigate this possibility, an anti-phospho Jak2 antibody that recognizes the activated kinase was used. As shown in Figure 34, when compared to control cells transfected with pSHAG and incubated with IFN-γ, cells with reduced expression of AxV showed an approximate two-fold increase in Jak2 activation in response to IFN-γ. Protein loading between samples was controlled for by immunoblotting for actin.

7. AxV regulates transcriptional responses to IFN-γ.

The results thus far provided evidence that AxV influences activation of Jak2 and Stat1 and potentially signalling through this pathway. To examine whether AxV influences IFN-γ-induced gene transcription, we used semi-quantitative PCR to measure expression of the IFN-γ-inducible genes Egr-1 (124;125), IFN-γR2 (19) and ICSBP (48). As shown in Figure 35, in comparison to IFN-γ-treated pSHAG transfected 293T cells, transcript abundance for EGR-1 and IFN-γR2 was significantly greater in IFN-γ-treated AxVlo cells (n=2).
Figure 32. Downregulation of AxA promotes IFN-γ-induced tyrosine phosphorylation of a 90 kDa protein. 293T cells were transfected with either pSHAG (control) or pSHAG-AV2.1 (siRNA-annexinV) plasmids. After 24h of transfection, cells were washed and left for another 48h in complete medium and then either treated with IFN-γ (100 U/ml) for 4 h or left untreated. Cells were then washed and lysed by boiling in SDS-sample buffer, electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose and probed with anti-phosphotyrosine antibodies. The data shown are from one of three independent experiments with similar results.
Figure 33. AxVLo cells show enhanced tyrosine phosphorylation of Stat1 in response to IFN-γ. 293T cells were transfected, treated with IFN-γ, lysed and immunoprecipitated with anti-Stat1 antibody. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-phosphotyrosine antibodies (A). The same membrane was stripped and probed with anti-Stat1 (B). Lysates were also examined for AxV levels by immunoblotting (C). Panel D shows results of densitometric scanning of the tyrosine phosphorylation of Stat1 from three independent experiments that yielded similar results.
Figure 34. IFN-γ-induced tyrosine phosphorylation of Jak2 in AxVLo cells. (A) 293T cells were transfected with pSHAG or pSHAG AV2.1 plasmids. After 24 h, cells were washed and left for another 48 h in complete medium and subsequently either untreated or incubated with 100 U/ml IFN-γ for 4 h. Cells were then lysed directly in SDS-sample buffer and separated by SDS-PAGE followed by immunoblotting using anti-phospho-Jak2. (B) To verify equal loading, immunoblotting was performed with an anti-actin antibody. Panel C shows level of AxV and panel D shows results of densitometric scanning of the results of Jak2 phosphorylation from three independent experiments that yielded similar results.
Figure 35. 293T cell with reduced levels of AxV show enhanced gene transcription in response to IFN-γ.
293T cells were transfected with pSHAG or pSHAG AV2.1 plasmids. After 24h, cells were washed and left for another 48h in complete medium and subsequently either untreated or incubated with 100 U/ml IFN-γ for 4h. RNA was isolated and RT-PCR was performed for the expression of Egr-1, GAPDH, ICSBP and IFN-γR2. The data are from one of two independent experiments that yielded similar results.
In the case of ICSBP there was no significant difference between pSHAG and pSHAG AV2.1 cells treated with IFN-γ. These results suggest that AxV normally acts to limit signal transmission through the Jak-Stat pathway and that once this constraint is removed, enhanced responses to IFN-γ ensue, at least for some IFN-γ-inducible genes.

8. IFN-γ-mediated growth arrest is potentiated in HeLa cells with reduced levels of AxV expression

To identify an additional phenotype associated with enhanced Jak-Stat activation in AxV<sub>Lo</sub> cells, we examined the anti-proliferative effects of IFN-γ in HeLa cells a well recognized model for IFN-γ growth inhibition response. As shown in Figure 36, in both pSHAG and AV2.1 transfected cells, IFN-γ had an anti-proliferative effect, and this was in agreement with what has been published in the literature (17;21;126). However, this growth inhibitory effect was potentiated in AxV<sub>Lo</sub> HeLa cells (n=3). These results support a model in which AxV has a negative regulatory effect on IFN-γ action. When AxV levels are decreased, in this case through the use of siRNA, IFN-γ action is potentiated.
Figure 36. Reduced levels of AxV potentiate IFN-γ growth arrest in HeLa cells. HeLa cells were transfected with pSHAG, pSHAG Luciferase or pSHAG AV2.1 plasmids. After 24 h, cells were washed and left for another 48 h in complete medium and subsequently were seeded in 12 well plates at 0.5 x 10^5 cells per well. After overnight incubation they were either untreated or incubated with IFN-γ (100 U/ml) for 72 h. Cells were then dislodged and counted in a hemacytometer. Correct function pSHAG siRNA luciferase construct was confirmed by an automated luciferase assay (data not shown). Panel A shows the growth of the cells in the presence of IFN-γ normalized with respect to the growth of corresponding untreated control cells. The effect of the AxV siRNA is shown in panel B (panel C is an actin control for protein loading). The data represent one of three independent experiments that yielded similar results.
In summary, we have identified annexin V as an IFN-γ R2 binding protein by the yeast two hybrid, GST pull down and co-immunoprecipitation studies. The interaction seems to be ligand-inducible, as it its enhanced by the IFN-γ treatment. The cells which present reduced levels of annexin V, show also enhanced Stat1 activation as shown by its tyrosine phosphorylation. The AxV_{L0} cells also presented an enhanced Jak2 activation after IFN-γ treatment. The responses of IFN-γ in these cells, increased transcriptional activity and growth inhibition were enhanced in the AxV_{L0} cells, suggesting that annexin V plays a role of negative modulator of interferon gamma signalling.
Overall strategy to investigate IFN-γ signalling

For most of the past ten to fifteen years, the canonical view of IFN-γ signalling has held that cellular responses are brought about through activation of the Jak-Stat pathway downstream of the IFN-γ receptor (25;27). This view has recently been altered to accommodate new findings indicating that IFN-γ signalling may occur independently of Stat1 (20;41;127;128). Despite these findings, thus far apart from Jak1 and Jak2, there has been no evidence presented showing the direct association of other proteins with either the R1 or the R2 chains of the IFN-γ receptor. In this thesis both biochemical and genetic approaches were used to identify and characterize novel components of IFN-γ receptor signalling. New elements involved in IFN-γ signalling may include phosphatases, kinases, adaptor proteins, transcription factors or combinations of these. The biochemical approach used was originally intended to identify novel IFN-γ-inducible tyrosine phosphorylated proteins, and in particular tyrosine kinases, without a priori invoking any direct association with the IFN-γ receptor. In contrast, by using the cytoplasmic domains of the R1 and R2 chains as bait, the genetic strategy aimed to identify novel proteins that associated directly with receptor subunits.
Biochemical strategy to investigate IFN-γ signalling

Regarding the biochemical approach taken, the initial objective was to determine whether a convenient model to detect activation of IFN-γ signalling in human mononuclear phagocytes could be established. Using either PMA-differentiated U937 or THP-1 human macrophage cell lines, we consistently detected activation of the Jak-Stat1 pathway in addition to the appearance of two IFN-γ-inducible phosphoproteins with subunit sizes of p125 and p85 (Figure 8). Previous work from this laboratory (101), used a renaturation kinase assay to show the activation of high molecular weight kinases by IFN-γ and these findings were reproduced in this thesis (Figure 9). Of interest, when these kinase activities were originally detected they were found to be attenuated by treatment of cells with TGF-β1 whereas the Jak kinases and Stat1 activation were resistant to this effect (101). These findings suggested that there were other tyrosine kinases in addition to Jak1 and Jak2 that may participate in IFN-γ signalling. The exact biological role of these particular renaturable tyrosine kinases remained to be determined. However, TGF-β has been shown to oppose the action of IFN-γ (101;129;130) and thus one attractive possibility is that these kinases may provide a basis for regulation of IFN-γ responses by TGF-β.

We obtained additional evidence to support the existence of novel IFN-γ-inducible tyrosine kinases when investigating a potential regulatory role for cAMP and protein kinase A (PKA). Using forskolin, a compound that increases cAMP levels leading to activation of PKA, selective attenuation of the IFN-γ-induced
activation of Jak1 and Jak2 was observed without affecting global IFN-γ-induced tyrosine phosphorylation (Figure 10). When Stat1 activation was analyzed by immunoprecipitation and antiphosphotyrosine immunoblotting, it was also markedly reduced, indicating a direct biochemical consequence of inhibition of Jak kinases in forskolin-treated cells. The mechanism(s) by which increased cAMP levels and PKA activity attenuate signalling through the Jak-Stat pathway is currently unknown. One possibility is that this may involve the activation of tyrosine phosphatases (131) including the increased association of a tyrosine phosphatase such as SHP-2 with Jak1, as has been suggested (132).

Because of their potential importance, efforts were made in this thesis to purify novel, non-Jak tyrosine kinases activated by IFN-γ. Mono Q chromatography was used because this methodology has been successfully applied in the past to purify tyrosine kinases (118). As shown in Figures 11 and 13, we were able to detect a non-Jak tyrosine kinase activity that was localized to the membrane of IFN-γ-treated cells. The results from the anti-phosphotyrosine analysis of selected Mono Q fractions showed a p125 IFN-γ-inducible tyrosine phosphorylated protein present in the fraction bearing a peak of IFN-γ-inducible tyrosine kinase activity. This p125 protein could have been a substrate of the novel kinase activity or the kinase itself (Figure 12). Although evidence was obtained to shown that the kinase activity in this region of the Mono Q fractionation was neither Jak1 nor Jak2 (Figure 13), based upon the available data, it was not possible to exclude the possibility that its activation occurred downstream of the Jaks. Analysis of Jak kinase negative mutants would
likely be informative with respect to investigating whether this activity was Jak-dependent.

Because of its apparent size, consideration was given to the possibility that the p125 phosphoprotein in the whole cell lysates could have been Pyk2 which is known to be activated by IFN-γ (52). This seemed unlikely, however, in light of the fact that Pyk2 is known to be a cytosolic tyrosine kinase (133). Direct evidence to exclude this possibility was obtained from a Pyk2 immunoprecipitation coupled with anti-phosphotyrosine analysis indicating that Pyk2 was not the IFN-γ-inducible p125 phosphoprotein (Figure 14). This conclusion was based both upon the fact that Pyk2 migrated with a smaller apparent subunit size and that Pyk2 itself did not undergo enhanced tyrosine phosphorylation in response to cell treatment with IFN-γ.

Since the Mono Q fractionation did not provide sufficient material for peptide sequencing by mass spectrometry, an alternative strategy was designed to identify IFN-γ-inducible tyrosine phosphorylated proteins in general and the p125 phosphotyrosine containing protein in particular. The p125 phosphoprotein showed electrophoretic mobility similar to one of the IFN-γ-inducible renaturable tyrosine kinase (Figure 9), suggesting that either they were the same protein or that there were two or more proteins in that area that were regulated by IFN-γ. The initial report indicating the presence of novel IFN-γ-inducible renaturable tyrosine kinases [(101) and Figure 9], indicated that these kinases used Poly Glu Tyr as a substrate. Based on this characteristic, Poly Glu Tyr Sepharose affinity chromatography was identified.
as a strategy to enrich their activity. This approach was also selected based upon prior published successes such as the enrichment and partial purification of protein tyrosine kinases from *S. cerevisiae* based on a single step poly-glutamic acid:tyrosine affinity column (119). In the latter study, a 4000-fold increase in tyrosine kinase activity as determined by a phosphocellulose paper assay was achieved. Using cell lysis conditions that solubilized membrane proteins combined with Poly Glu Tyr chromatography, we obtained substantial enrichment of the p125 protein (Figure 15). To achieve further enrichment, the protocol depicted in Figure 16 was designed and this yielded the desired result as shown by the enriched silver stained band in Figure 17. The purification protocol was scaled up and a Coomassie blue stained band was sent for peptide sequencing by mass spectrometry. This yielded a series of peptide sequences corresponding to the sequence of human TIP120a (Figure 18a). Purification of the phosphoprotein TIP120a using this approach, designed to enrich tyrosine kinase activities using a Poly Glu Tyr column was somewhat surprising. One mechanism to explain this is the possibility that TIP120a could have been copurified with its putative tyrosine kinase. Studies using recombinant TIP120a could be helpful in the identification of this putative tyrosine kinase.

To confirm that TIP120a was in fact the correct identity of the p125 phosphoprotein, an initial attempt was made to determine whether TIP120a became tyrosine phosphorylated in IFN-γ-treated cells. Unfortunately, a rabbit polyclonal antiserum that we raised against an immunogenic peptide selected from the protein sequence was unable to immunoprecipitate TIP120a, notwithstanding the fact that it
was able to detect the peptide by ELISA and the denatured protein by immunoblotting. A second strategy used to determine whether TIP120a became tyrosine phosphorylated in IFN-γ-treated cells, involved transfection of 292T cells with a His-tagged TIP120a construct. The results shown in Figure 19 suggested that a band co-migrating with TIP120a did in fact become tyrosine phosphorylated in response to IFN-γ.

The finding that the phosphorylation state of TIP120a may be regulated by IFN-γ was of significant interest. TIP120a is a transcriptional activator that was identified as a TATA box binding protein (TBP)-interacting protein (120). TBP-interacting proteins such as p53 (134) and c-Myc (135) have been shown to regulate gene expression, and, of interest, c-Myc is a transcriptional factor induced by IFN-γ (136). The primary structure of TIP120a includes two leucine rich regions (Figure 18b). These regions may be involved in interactions with RNA polymerase and TBP (121) through a zipper-like structure. The N-terminal region of TIP120a is rich in acidic amino acids. This region (aa 267-344) contains two consecutive negatively charged regions that are identical to the consensus target site for casein kinase II. This kinase has been implicated in regulating the activities of transcription factors such as CHOP (CAAT/enhancer binding protein homologous transcription factor (137), β-catenin (138) and c-Myc (139). Of interest, casein kinase II has also been implicated in IFN-γ-dependent activation of inducible cAMP repressor (ICER) in macrophages (91), leading to inhibition of gene transcription. Taken together, these findings suggest the possibility that TIP120a could be a target of casein kinase II
during cellular responses to IFN-γ leading to changes in transcriptional responses dependent upon this factor.

The finding that TIP120a appeared to undergo tyrosine phosphorylation in response to IFN-γ raised the question as to the kinase responsible for this event. Although this was not investigated directly, the sequence of the protein was examined to see if this would provide any clues. As shown in Figure 18a, TIP120a contains five potential tyrosine phosphorylation sites (denoted as Y) as determined by a NetPhos 2.0 analysis. A Prosite scan analysis identified an additional site, residue, Y$^{1090}$, as a potential tyrosine kinase phosphorylation site. These sites showed consensus sequences for binding to the SH2 domains of SHP-2 (Y$^{502}$VIL), Abl tyrosine kinase (Y$^{772}$MDL) and Csk (Y$^{980}$ARS) (140). While this analysis did not provide a clear link to the identity of a putative TIP120a tyrosine kinase, it did suggest possible interacting proteins for phospho-TIP120a.

The finding of inducible phosphorylation of TIP120a suggested that this may influence transcriptional responses to IFN-γ. One approach to address this would be to reduce the expression of TIP120a in vivo using either antisense oligonucleotides or siRNA as we did for AxV. One caveat to this approach is that reduced expression of TIP120a could have independent effects on other signalling pathways/cellular processes in addition to affecting IFN-γ signal transduction. An alternative approach, likely to be more specific would involve identifying and targeting the phosphorylation sites in TIP120a specifically modulated by IFN-γ. For example,
once the putative tyrosine phosphorylation site(s) in the protein is known, site directed mutagenesis with a Y_A substitution of the site(s) coupled by transfection and phenotype analysis would likely be informative. Ideally the approach to map the TIP120a phosphorylated sites would consist of state-of-the-art mass spectrometry to detect low levels of phosphotyrosine-containing peptides (141;142).

**Genetic strategy to investigate IFN-γ signalling**

Also with the view to characterizing novel components involved in IFN-γ signalling, a genetic approach based upon the yeast two hybrid screen was used with the intracellular domains of the IFN-γR1 and IFN-γR2 subunits as bait. The yeast two hybrid system has been successfully used to detect novel protein interactions with the intracellular domains of several cytokine and growth factor receptors (105;106;143). Results reported in this dissertation describe two novel protein-IFN-γR interactions: (1) AxV as an interacting partner with the IFN-γR2 (Figure 22) and (2) S100A9 as an IFN-γR1 binding protein (Figures 24 and 25). The discoveries of these novel associations raise at least two important questions including what is the basis for the inducible association of these proteins with the R1 and R2 cytoplasmic domains and what are the biological consequences of these interactions.

In the case of AxV, the finding that the interaction with the R2 subunit is inducible (Figure 29), suggested that AxV may modulate IFN-γ signalling. The
inducibility of the AxV-IFN-γR2 interaction could be due to several factors, such as changes in the phosphorylation status of AxV or to calcium binding. For example, it has been reported that serine phosphorylation of AxV promotes its recruitment to the plasma membrane (58), and this could facilitate its association with various receptors. Alternatively, the induction of AxV tyrosine phosphorylation could promote its interaction with the IFN-γ receptor complex possibly through interactions with other proteins such as Grb2 or other SH2 domain containing proteins such as SHP-2, as has been suggested by the interaction of AxII with SHP-2 (144). AxV is known to undergo inducible phosphorylation, and has been shown to become tyrosine phosphorylated after activation of the VEGF receptor (72). In the latter study, the interaction between AxV and the VEGF receptor was initially identified in a yeast two hybrid screen using the VEGF receptor as bait. The interaction was confirmed by an in vitro transcription and translation system, which presumably did not allow post translational modifications to take place. Moreover, given that the yeast two hybrid system itself does not support this type of post translational modification, it would seem unlikely that IFN-γ-induced tyrosine phosphorylation of AxV was responsible for promoting its association with the IFN-γR2 subunit. Consistent with this conclusion, using immunoprecipitation and anti-phosphotyrosine immunoblotting, we were unable to find evidence for tyrosine phosphorylation of AxV in IFN-γ-treated cells (data not shown). Another possibility to consider is that serine phosphorylation of AxV could have been responsible for modulating its interaction with the R2 subunit, as has been shown for the phosphorylation-dependent interaction
of Axl with the glucocorticoid receptor in hormone treated cells (145). Additional studies will be required to address this question.

The inducible association of Axl with IFN-γR2 could also have been brought about by a conformational change in the protein regulated by calcium binding. It has been clearly established that calcium influences the association of annexins with other proteins (146;147). For example, Satoh et al., 2002 (146) found an interaction between apoptosis-linked gene-2 protein and AxlXI using the yeast two hybrid system. Furthermore, using GST–AxlXI and an overlay assay, this interaction was found to be calcium-dependent. AxlV has been also been shown to bind to the β5-integrin receptor (147). Moreover, using Far Western blotting in the presence and absence of calcium chelators, this interaction was shown to be calcium-regulated. An increase in intracellular calcium in human foreskin fibroblasts was shown to bring about the re-distribution of several annexin family members, including AxlV, from the cytosol to the plasma membrane which may promote associations with plasma membrane proteins including transmembrane receptors (148). With respect to the influence of calcium upon the interaction of AxlV with IFN-γR2, it has been reported that treatment of cells with IFN-γ can result in rapid and transient increases in cytosolic, intracellular free calcium (149). In fact, it has been suggested that a rise in intracellular free calcium in response to IFN-γ may bring about activation of calmodulin kinase II leading to serine phosphorylation of Stat1 (149). The lysis buffer that we used to solubilize the cells prior to immunoprecipitation of IFN-γR2 did not contain the calcium chelators EDTA and EGTA, thereby favoring the
potential for calcium binding to AxV. Thus, based upon what is known about the ability of calcium to influence the interactions of AxV with other proteins and the evidence that IFN-γ can bring about changes in intracellular free calcium, a reasonable model to be tested is that the inducible association of AxV with R2 is calcium regulated. In this model, conformational changes in the structures of either AxV, IFN-γR2 or both in response to calcium could explain this ligand-induced association.

Annexin V regulates IFN-γ signalling

The observation that the interaction of AxV with IFN-γR2 was inducible in response to ligand suggested the possibility that AxV may modulate IFN-γ signalling. Investigating the role of AxV in IFN-γ signalling required a model cell system that was both responsive to IFN-γ and amenable to transfection for delivery of either antisense oligonucleotides or siRNA expression vectors. Given the difficulties associated with high efficiency transfection of macrophages (64;67), either THP-1 or U937 cells were not attractive initial choices. Rather, we chose human embryonic kidney 293T cells which fulfilled the two criteria outlined above. Thus, 293T cells have been used before as a model of IFN-γ signalling (34;150;151) and they are amenable to transfection (114;152;153). An additional advantage of 293T cells, particularly in respect to siRNA, is that these cells lack TLR-3 and consequently, dsRNA-dependent protein kinase R does not become activated in 293T cells in
response to the presence of siRNA sequences (154). This is a useful property since PKR has been implicated in IFN-γ signalling (155) and activation of PKR could have complicated interpretation of experiments designed to examine the effects of AxV levels in IFN-γ cell regulation.

Initially, we verified that 293T cells responded to IFN-γ as expected (34; 150; 151) in terms of Stat1, Jak1 and Jak2 tyrosine phosphorylation and the results suggested that this should be a useful model to study IFN-γ signalling (Figure 30). AxV is a relatively abundant cellular protein and hence it seemed likely that a robust approach would be required to reduce its expression substantially. Given this concern, a strategy based upon siRNA or RNA interference (RNAi) was selected to reduce AxV levels in 293T cells. This decision was also influenced by the fact that the successful use of siRNA to silence both endogenous and exogenous genes in a 293T cells has been reported (114).

Following the introduction of double-stranded RNA (dsRNA) into target cells RNAi mediates sequence-specific post-transcriptional gene silencing. RNAi as an experimental strategy is based upon an ancient, natural regulatory mechanism widely distributed in nature -including plants, Caenorhabditis elegans, Drosophila, and mammalian cells- designed to protect cells from viral infection. RNAi is initiated when dsRNA is processed into short (< 30 bp) dsRNA duplexes called small-interfering RNA (siRNA) by dicer, a host RNAse. These short dsRNA duplexes are then incorporated into a multi-component nuclease complex referred to as the RNA-
induced silencing complex (RISC). Based upon the specific siRNA sequence, RISC targets cognate mRNAs for degradation (58;114). Recently, the specificity of the siRNA has been established using DNA microarrays (156).

The particular system chosen for RNAi was based upon use of the pSHAG vector to direct the production of small interfering RNAs (siRNAs) to silence AxV expression. One particular advantage of the pSHAG system is that the cassette that generates the siRNA can be readily transposed to a lentiviral vector to be used subsequently to achieve stable gene silencing in human or murine monocytic cell lines (Lee, JS, Z. Hmama, A. Mui and N.E. Reiner, J. Biol. Chem. In press). Of special interest, such an approach would be particularly promising for analyzing the role of S100A9 in IFN-γ signalling. Since the abundance of S100A9 is quite low in 293T cells (data not shown), an alternative system would be required where S100A9 expression is substantial such as in THP-1 cells [(123) and Figure 24] in order to examine the phenotype associated with silencing of S100A9.

To examine whether IFN-γ signalling is regulated by AxV, three siRNA constructs based upon the AxV mRNA sequence were designed using a web-based platform (http://katahdin.cshl.org). One of these sequences, AV.2.1 proved to be highly effective at bringing about 70-80% reduction in the AxV levels (Figure 31) without affecting the expression of control genes (actin, SHP-2 and AxII). Once AxV gene silencing was established, the conditions were set to analyze how AxVLo cells respond to IFN-γ. A phenotype of hyper-responsiveness to IFN-γ in AxVLo cells was
initially suggested by anti-phosphotyrosine blots of whole cell lysates. These showed increased tyrosine phosphorylation of a 90 kDa protein in IFN-γ-treated pSHAG AV2.1 transfected cells when compared with control pSHAG vector transfected cells (Figure 32). The size of this protein suggested that it might be Stat1. This inference was in fact supported by the finding of enhanced tyrosine phosphorylation of Stat1 in response to IFN-γ after immunoprecipitation of the protein and anti-phosphotyrosine blotting (Figure 33). Increased tyrosine phosphorylation of Stat1 was not explained by increased protein abundance (Fig. 6, panel B), but did correlate with enhanced activation of Jak2 in response to IFN-γ in cells with reduced expression of AxV (Figure 34).

One caveat in respect to the strategy of using siRNA to investigate the role of AxV in regulating cellular responses to IFN-γ is worth commenting upon at this point. It was recently reported that siRNA vectors can trigger an interferon response (157) thereby potentially complicating interpretation of any phenotype observed. One hallmark of such a response is the induction of expression of Stat1 (158). To control for this possibility, we monitored Stat1 levels in siRNA transfected cells and these did not change (Figure 33). Thus it seems unlikely that interpretation of the phenotype we observed in AxVLo cells would be complicated by the non-specific induction of an interferon response.

These results suggested that the interaction of AxV with IFN-γR2 controls activation levels of Jak2 and Stat1 and they are consistent with a model in which AxV
normally negatively regulates signalling through this pathway. One possible mechanism to explain this could involve the association of AxV with IFN-γR2 interfering with Jak2 binding because of either overlapping binding sites or steric hindrance. In this model, reduced levels of AxV would increase access of Jak2 to IFN-γR2 and enhance its activation -and secondarily that of Stat1- in response to IFN-γ. It is also possible that AxV might act to inhibit Jak2 activation either directly or indirectly by promoting the recruitment of a phosphotyrosine phosphatase to the receptor. In fact, the interaction between another annexin family member, AxII, and SHP-2 has been described (144). SHP-2 has also been implicated in Stat1 dephosphorylation on both serine and tyrosine residues (34). In a similar vein, annexin family members themselves have been assigned putative phosphatase activities (159) or have been shown to co-purify with a phosphatase activity (108).

While further research will be required to distinguish amongst these various possibilities, the model does allow certain predictions to be made. For example, if AxV normally controls the level of Jak2-Stat1 activation, then removing this influence should lead to enhanced transcriptional activation in response to IFN-γ, as proposed in the model depicted in Figure 37. The biological significance of increased Stat1 tyrosine phosphorylation in cells expressing reduced levels of AxV and treated with IFN-γ was examined by a series of approaches. One involved investigating transcriptional levels of IFN-γ-inducible genes by reverse transcriptase PCR. The data showing increased abundance of transcripts for EGR-1 and IFN-γR2 in IFN-γ-treated AxVLo cells (Figure 35) suggests that the model is correct.
IFN - γ- response in normal cells
- IFN-γ induces tyrosine phosphorylation of Jak1, Jak2, Stat1 and IFN-γR1 chain.
- IFN-γ induces recruitment of annexin V to IFN-γR2 chain and may regulate biological response.

Figure 37. Proposed model for the role of AxV in regulating IFN-γ-induced cell signalling and gene transcription. Left panel. Upon exposure of cells to IFN-γ, AxV is recruited to the R2 receptor subunit. The association of AxV with R2 controls the phosphorylation and activation states of Jak2 and Stat1α thereby regulating downstream responses. Right panel. Under conditions where the abundance of AxV is limiting.
A second approach to examine the impact of enhanced tyrosine phosphorylation of Stat1 in cells with reduced levels of AxV was to evaluate the IFN-γ-induced growth inhibition. Inhibition of cell proliferation is a hallmark of the interferon response (17;21;126) and the anti-proliferative response to IFN-γ has been shown to be dependent upon activated Stat1 (21). The latter study examined cells transfected with either wild type Stat1 and or Stat1 mutants. The introduction of wild type Stat1 enhanced the anti-proliferative effect of IFN-γ, as determined by decreased thymidine incorporation, whereas Stat1 mutants reversed IFN-γ-induced suppression of thymidine incorporation. Consistent with this paradigm, we observed that the anti-proliferative effect of IFN-γ was enhanced in cells expressing reduced levels of AxV (Figure 36). These findings provide additional direct evidence to show that AxV negatively regulates biological responses to IFN-γ and suggest that this may be related to effects on Jak-Stat1 activation.

Phosphorylated Stat1 homodimers translocate to the nucleus where they activate transcription of genes containing GAS elements (160). Therefore, a third potential approach that we considered to investigate the importance of AxV in regulating Stat1 phosphorylation involved use of a GAS luciferase reporter assay to examine directly Stat1-driven gene transcription (26). Surprisingly, the GAS luciferase reporter system showed that -in the absence of exposure to IFN-γ- AxV_Lo cells had significantly increased luciferase activity in comparison with control vector (pSHAG) transfected cells (data not shown). Whereas, control, pSHAG transfected cells showed an IFN-γ dose-dependent increase in GAS driven transcription, AxV_Lo
cells did not (data not shown). Thus, this system did not allow us to evaluate the impact of enhanced IFN-γ induced tyrosine phosphorylation of Stat1 on GAS-dependent transcription in cells with reduced AxV levels.

Nevertheless, these results suggested the possibility that AxV negatively regulates transcription through the GAS element and that once this control is removed, GAS-dependent transcription becomes constitutive and is no longer responsive to IFN-γ. Regarding mechanisms that may explain this finding, it has previously been shown that both tyrosine and serine phosphorylation of Stat1 are important to drive transcription through a GAS luciferase reporter gene (161). However, we found no evidence for induction of Stat1 tyrosine phosphorylation in cells expressing low levels of AxV in the absence of IFN-γ (Figure 33). We did not examine directly the possibility that Stat1 was serine phosphorylated in these cells, though this remains an interesting possibility in the light of the fact that AxV is a PKC inhibitor (65) and that PKC appears to be involved in mediating serine phosphorylation of Stat1 (162). Changes in serine and tyrosine phosphorylation of Stat1 have been associated with a band shift, where a slower migrating band is related to increased phosphorylation (161). In this regard, the fact that we did not detect a Stat1 band shift (Figure 33) mitigates somewhat against changes in phosphorylation accounting for increased basal GAS-luciferase activity in AxV_Lo cells. It is not possible to exclude this completely, however, since changes in levels of phosphorylation may have been below the levels of detection by band shift assay. In
future studies, Stat1 serine phosphorylation could also be directly assessed by the use of specific antibodies as reported previously (29).

Two other potential mechanisms are worthy of discussion. First, Stat1 homodimerization and complex formation with IRF-1 in the absence of phosphorylation has been reported (163) and has been proposed as a model of how Stat1 regulates constitutive gene expression. It seems that this would be another worthwhile possibility to examine in respect to constitutive increases in GAS activity in AxVLo cells. Alternatively, factors other than Stat1 may bind to the GAS sequence and drive gene expression. Contursi and co-workers (48), found that IFN-γ- induced GAS luciferase activity was severely impaired in ICSBP−/− macrophages, despite clear evidence of Stat1 activation. Transfection of ICSBP restored the ability of these cells to drive GAS luciferase activity. Cells deficient in one or another component of the Jak-Stat signalling pathway were able to drive GAS luciferase activity when transfected with an ICSBP construct, even in the absence of IFN-γ. These findings suggested that ICBSP may regulate gene expression through GAS promoters both constitutively and in response to IFN-γ under conditions where there is no functional Jak-Stat pathway. Interestingly, in this study ICSBP was show to require other factors to bind to the GAS sequence, suggesting that ICSBP by itself is not sufficient to drive GAS promoters. Whereas in theory, a role for ICSBP in driving enhanced transcription through the GAS element in cells with reduced levels of AxV is a possibility, RT-PCR results from this thesis did not show that ICSBP transcript abundance was increased in these cells (Figure 35). While not formally excluding
this possibility, this result suggests that in this system other proteins may be involved in regulating GAS luciferase activity in the absence of IFN-γ.

While the model of AxV involvement in IFN-γ signalling (Figure 37) suggests that AxV -through an interaction with the IFN-γR2 chain of the receptor- constrains signalling through the IFN-γ-activated Jak-Stat1 pathway, it does not preclude the possibility that this interaction may also allow AxV to influence other Stat1-independent signalling elements that are activated downstream of IFN-γR2. In this regard, it would be informative to map the interaction site of AxV on the receptor. A site directed mutagenesis approach targeting the R2 subunit in order to abrogate its interaction with AxV would likely yield a better understanding of the role of AxV in IFN-γ signalling, in an environment where the levels of AxV are unaltered. Combined with a cDNA microarray read out, this type of experiment could be particularly informative.

**S100A9- IFN-γR1 interaction**

The interaction of S100A9 with the IFN-γR1 chain identified in this thesis is of particular interest in light of the fact that efforts to identify binding partners for the S100A8-A9 complex have thus far been unsuccessful (164). With respect to the association between S100A9 and IFN-γR1, initial results suggested that this was inducible (Figure 24). A key question to investigate is what are the factors that
regulate the IFN-γ-inducible association of S100A9 with IFN-γR1. It has been reported that changes in concentration of intracellular calcium promote the translocation of S100A8/A9 dimers to the membrane in a monocyte model (165) and this could influence its association with IFN-γR1 or other receptors. There are several lines of evidence to support this possibility including: i) IFN-γ induces transient fluxes in cytosolic intracellular free calcium (149), ii) calcium induces the formation of hetero-dimers (~ 28 kDa in size) of S100A8/S100A9 (76) and iii) we found a 28 kDa complex that included S100A9 co-immunoprecipitated with the IFN-γR1 chain in a ligand-inducible manner (Figure 25). Taken together, these findings suggest that calcium may be an important mediator of the interaction between S100A9 and IFN-γR1.

Another possibility that we considered was that tyrosine phosphorylation of S100A9 might regulate its association with the receptor. Although we did not find direct evidence that S100A9 became tyrosine phosphorylated in response to IFN-γ, a 90 kDa tyrosine phosphorylated protein consistently co-immunoprecipitated with S100A9 in a ligand-inducible manner (Figure 27). This association was supported by other experiments using methionine-labelled THP-1 cells, where we also found a 90 kDa radiolabelled band co-immunoprecipitating with S100A9 in the IFN-γ-treated cells (data not shown). An interesting possibility to consider is that this protein promotes the association of S100A9 with the receptor and this would be an important focus for future studies.
An alternative model to explain the IFN-γ-inducible association between S100A9 and IFN-γR1 could involve effects of AxV. This possibility was suggested by reports indicating that some annexins have been shown to interact with S100 family proteins (54;166). For example, S100A1 and S100B were found to interact with AxVI in a calcium-dependent manner (166) and this association blocked the inhibitory effect of S100A1 and S100B on intermediate filament assembly. Also, when a GST-AxI N-terminal domain fusion protein was used in pull-down experiments, a novel calcium-dependent S100C-AxI interaction was found (167). Taken together with the finding that AxV is recruited to the IFN-γR in an IFN-γ-inducible manner (Figure 29), these results suggest the possibility that AxV may function as a scaffolding protein that links the R2 subunit with S100A9. An ideal model in which to test this hypothesis would be PMA-differentiated THP-1 cells where we have been able to corroborate these interactions by co-immunoprecipitations and where their biological significance could be tested.

At present, potential biological consequences of the association of S100A9 with the IFN-γ receptor in the context of IFN-γ signalling are not known. However, it is of interest to consider that S100A8/A9 complexes have been shown to modulate the activity of casein kinase I and II, enzymes involved in the regulation of the activity of topoisomerase I and RNA polymerases I and II (90). Furthermore, analysis of potential phosphorylation sites in the IFN-γ receptor revealed sites for casein kinase recognition (37). It has also been shown that inhibitors of serine threonine kinases, that could affect casein kinase activities, enhanced the
phosphorylation of the IFN-γ receptor, while selectively attenuating IFN-γ-induced gene expression in human peripheral blood monocytes (168). Casein kinase II has also been shown to be involved in IFN-γ-induced nuclear translocation of YB-1 (Y-box-binding protein) a transcriptional factor involved in gene repression (169). This suggests the possibility that a protein or protein complex –such as S100A9/A8– capable of modulating casein kinase activity at the IFN-γ receptor level could influence gene expression. In order to elucidate the role of S100A9 in IFN-γ signalling, an initial approach likely to be informative would involve the use of bone marrow-derived macrophages from control and S100A9 null mice, to examine the expression induced by IFN-γ. This would be done using microarrays followed by RT-PCR for confirmation. Once a functional read out of the interaction in terms of gene expression is obtained, it would be of interest to examine the mechanism of the S100A9-R1 interaction, in particular the role of calcium influx in mediating this interaction.
In summary, results reported in this thesis identified three novel components of IFN-γ signalling: TIP120a, AxV and S100A9. Of these three molecules, the role of AxV was examined in greatest detail. The results show that the predominant impact of this interaction is a negative regulation of IFN-γ signalling and this may represent a physiologic mechanism to control signalling through this pathway. As such, these findings add to what is already known about the complex regulation of IFN-γ signalling through other control elements such as SOCS-1 and the tyrosine phosphatases SHP-1 and SHP-2 (31;35;36).
XI. REFERENCES


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