The 70 kDa S6 Kinase: Enrichment and Characterization of a Sea Star Homologue, and the Role of p70^{S6K} in the Transformed Keratinocyte cell line Pam212 in Response to Photodynamic Therapy

by

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ABSTRACT

The 70 kDa ribosomal protein S6 kinase (p70^{S6K}) has been implicated in the control of cell proliferation and the onset of protein synthesis following mitogenic and hormonal stimulation. Due to the importance of this role in signal transduction we undertook to purify and characterize the echinoderm isoform of p70^{S6K}, and to evaluate the role of p70^{S6K} in a mammalian system. A rapid protocol was developed for the enrichment of a catalytically active isoform (p52^{S6K}) of the S6 kinase from oocytes of the sea star *Pisaster ochraceus*. The enzyme was purified ~50,000 fold from oocyte cytosol with a specific enzyme activity of 1.6 μmol per min per mg. The enriched preparation was characterized with respect to ion dependence, phosphotransferase activity and consensus sequence for substrate phosphorylation, determined to be RXXSXR, which was partially distinct from that reported for mammalian p70^{S6K}. Phosphoamino acid analysis of the 40S ribosomal protein S6 phosphorylated by partially purified p52^{S6K} yielded incorporation of ^{32}P exclusively on serine residues, while 2-dimensional phosphopeptide analysis revealed phosphorylation on at least 8 distinct tryptic peptides, consistent with previous studies of mammalian p70^{S6K}. Inhibition of sea star p52^{S6K} phosphotransferase activity after treatment with protein-serine/threonine phosphatases confirmed that p52^{S6K} was regulated by phosphorylation. The p52^{S6K} co-purified with the regulatory and catalytic subunits of protein phosphatase 2A and the heat shock protein 60.

A variety of cellular stresses are known to affect the activity of protein kinases. It was demonstrated that protein levels of sea star S6 kinase decreased in response to the stress of heat shock, in a time-dependent manner. In collaboration with Dr. Jing-Song Tao (Ph.D. candidate), I went on to evaluate how p70^{S6K} was affected in mammalian Pam212 keratinocytes in which stress pathways were activated by treatment with photodynamic therapy (PDT) with benzoporphyrin derivative mono-acid ring A (BPD-MA). We demonstrated that PDT caused a profound suppression of the phosphorylation and

phosphotransferase activity of $p70^{S6K}$ in cells maintained in serum-containing medium. In serum-starved Pam212 cells, PDT prevented the insulin-induced activation of phosphatidylinositol 3-kinase (PI 3-kinase), protein kinase B (PKB) and $p70^{S6K}$. PDT supressed the activation of $p70^{S6K}$ induced in response to anisomycin and UV irradiation. The activation of $p70^{S6K}$ by these stimuli was found to be independent of PI 3-kinase and PKB. Therefore, inhibition of $p70^{S6K}$ may represent a key signaling event mediating PDT-induced apoptosis.

The key novel findings of the research presented here include a substantial enrichment of an echinoderm homologue of $p70^{S6K}$, and analysis of the regulatory properties of the kinase. We also show, for the first time, that $p70^{S6K}$ is profoundly inhibited in response to PDT, although we do not at present understand the mechanism of PDT-induced signaling.

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List of Abbreviations

aa	amino acid(s)
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-indolyl phosphate
BME	beta-mercaptoethanol
BPD-MA	benzoporphyrin derivative mon-acid ring A
BSA	bovine serum albumin
CSAID	cytokine-suppressive anti-inflammatory drugs
DMEM	Dulbecco's minimum essential medium
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DTT	dithiolthreitol
EDTA	ethylenediaminetetraacetic acid (disodium salt)
EGF	epidermal growth factor
EGTA	ethylene bis(oxyethylenenitrilo) tetraacetic acid
ERK1/2	extracellular signal regulated protein kinase 1/2
FBS	fetal bovine serum
FRAP	FKBP-rapamycin associated protein
GDP	guanosine diphosphate
GTP	guanosine triphosphate
Grb-2	growth receptor binding-2
GSK-3	glycogen synthase kinase-3
GST	glutathione-S-transferase
H_2O_2	hydrogen peroxide
HSP27/60	27/60 kDa heat shock protein
HTP	hydroxylapatite
IRS-1	insulin receptor substrate-1
JNK	c-jun amino-terminal kinase
LPS	lipopolysaccharide
µg	microgram
µl	microlitre
µM	micromolar
mg	milligram
ml	millilitre
M	molar
MAPK/ERK	mitogen-activated protein kinase/extracellularly-regulated kinase
MAPKAP	mitogen-activated protein kinase activated protein kinase
MEK	MAPK/ERK kinase
MEKK	MAPK/ERK kinase kinase
MOPS	morpholinopropanesulfonic acid
NBT	nitro blue tetrazolium
ng	nanogram
nm	nanometer
PAK	p21-associated protein kinase

PAP	potato acid phosphatase
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PDK1	3-phosphoinositide-dependent protein kinase-1
PDT	photodynamic therapy
PI	phosphatidylinositol
PI(4,5)P$_2$	phosphatidylinositol-4,5-bisphosphate
PH	pleckstrin homology
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKI	peptide inhibitor of protein kinase A
PLCγ	phospholipase C gamma
pM	picomolar
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
PP2A	protein phosphatase 2A
ROS	reactive oxygen species
rpm	revolutions per minute
SAPK	stress-activated protein kinase
SDS	sodium dodecyl sulphate
SH2/SH3	src homology 2/3 domain
SOS	son-of-sevenless
TBS	Tris buffered saline
TOR	target of rapamycin
Tris	tris(hydroxymethyl)aminomethane
TTBS	TBS containing 0.05% Tween 20
Tween 20	polyoxyethylene-20-sorbitan monolaurate
UV	ultraviolet

List of Amino Acid Codes

Name	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glycine	Gly	G
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

For my mom, and Robert, with love........

Chapter 1: General Introduction

The millions of cells that make up all living organisms are derived from a single fertilized cell. The genetic program within our DNA orchestrates the differentiation of cells into tissues and organs that respond to a vast variety of hormones and extracellular signals. All cells contain hundreds of enzymes that play key roles in the transmission and integration of signals from outside the cell. Protein kinases are among these key signaling molecules. These enzymes, along with protein phosphatases, mediate the reversible phosphorylation of target proteins, thus affecting the subsequent activity of these signaling components.

The research contained in this thesis examined the role of a specific protein kinase, the 70 kDa S6 kinase ($p70^{S6K}$). This enzyme mediates the onset of protein synthesis required for cell division to occur and as such has an important role in cell proliferation and cell survival. In the Introduction, I will briefly discuss the structure and function of protein kinases and the current state of knowledge in the field of signal transduction. I will then elaborate on the mechanisms by which $p70^{S6K}$ may be regulated, from receptors on the cell surface, via adaptor proteins and protein phosphorylation/dephosphorylation cascades. I will briefly discuss the models systems used in these studies, and present a rationale and research objectives for the work presented.

1.1   Signal Transduction

Cells respond to a number of extracellular signals that mediate growth, survival, differentiation, proliferation, homeostasis or apoptosis. The signals are transmitted across the cell membrane, through the cytoplasm and into the nucleus, resulting in regulation of gene expression. Divergent signals are integrated, processed and relayed through the cell by thousands of different proteins. Reversible protein phosphorylation has become widely recognized as a major mechanism of signal transmission within the cell and protein kinase cascades form a highly integrated network through which signals received at the cell surface are transduced through the cytoplasm and into the nucleus where they mediate events required for growth and cell division. At least a third of all cellular proteins undergo phosphorylation, and although some of these phosphorylation events are likely without consequence, this covalent modification affects biological activity in many cases.

Many diseases are the result of the disruption of signaling pathways, from mutations in receptors, constitutive activation or inhibition of protein kinases, or deregulation of other downstream signaling molecules and transcription factors. These diseases include proliferative disorders such as cancers (Aaronson 1991), as well as diabetes (Reaven 1988), amyotrophic lateral sclerosis (ALS) (Krieger *et al.,* 1996), Huntington's disease (Tanaka *et al.,* 1993), and Alzheimer's disease (Saitoh *et al.,* 1991). Many mitogen and oncogene-regulated protein-serine/threonine kinases have been identified and characterized in a variety of cell systems, and the roles that these kinases play in signaling pathways are currently an active area of research.

1.1.1 Protein kinase structure and function

Protein kinases are enzymes that add the gamma phosphate from adenosine triphosphate (ATP) to target proteins and enzymes. The phosphorylation event may activate or inhibit the activity of target proteins. In this way, protein kinases, and protein phosphatases which cleave the phosphate from target proteins, can transduce and integrate signals from outside the cell.

All protein kinases share certain features required for their catalytic activity. A readily identifiable catalytic domain comprising about 250 - 300 amino acid residues, corresponding to about 30 kDa characterizes this family of proteins (Hanks and Quinn 1991). The homologous nature of the catalytic domains implies that they all fold into similar three-dimensional core structures, and catalyze the phosphotransferase reaction according to a common mechanism. Indeed, solution of the crystal structures of several protein kinases has demonstrated that the kinase domain folds into a two-lobed structure with the deep cleft between the lobes acting as the site of catalysis (Knighton *et al.,* 1991A and B; De Bondt *et al.,* 1993; Zhang *et al.,* 1994). Much of the diversity of signaling through protein kinases stems from regulation of the activity and substrate specificity of the catalytic domain by other regions of the protein, or by other enzymes and co-factors. The location of the catalytic domain within the protein is not fixed, but it often lies near the carboxyl terminus while the amino terminus often plays a regulatory role.

The two best described classes of protein kinases are those specific for serine/threonine and those that phosphorylate tyrosine residues. There is also evidence for a third class of protein kinases that are specific for histidine residues (Hanks and Quinn 1991). Although both serine/threonine and tyrosine kinase family members have very similar catalytic domain primary structures, certain short amino acid stretches can be used to predict the kinase specificity (Hanks 1987). Comparisons of the primary

sequences of kinase catalytic domains has allowed identification of eleven major conserved subdomains, separated by regions less well conserved. The conserved regions have been found to be important in regulating catalysis, either directly, as components of the active site, or indirectly by contributing to secondary structure and the formation of the active site. Non-conserved regions likely mediate secondary and tertiary structure, allowing essential conserved regions to come together.

Twelve amino acid residues within the eleven subdomains have been identified that are nearly invariant (conserved in over 95% of known sequences), throughout the family members. According to the numbering used in the primary structure of protein kinase A (PKA), these are equivalent to Gly-50 and Gly-52 in subdomain I, Lys-72 in subdomain II, Gly-91 in subdomain III, Asp-166 and Asn-171 in subdomain VI, Asp-184 and Gly-186 in subdomain VII, Glu-208 in subdomain VIII, Asp-220 and Gly-225 in subdomain IX, and Arg-280 in subdomain XI. The sequences within subdomains VI, VIII and IX have been particularly well conserved among the individual members of the different families, and thus have been targetted most often in cloning strategies aimed at identifying new members. Subdomain I appears to play a role in anchoring the non-transferable phosphates of ATP. The invariant Lys-72 in subdomain II is the best characterized cataytic domain residue and is essential for maximum kinase activity. This residue is required to anchor and orient ATP by interacting with the α- and β-phosphates and has been exploited to create catalytically inactive mutants of active kinases by altering this residue, usually to alanine. Subdomains VI and VII also play roles in ATP binding. Subdomain VIII contains the highly conserved Ala-Pro-Glu (APE) sequence that appears to play a major role in substrate recognition. In addition, many protein kinases are known to be activated by phosphorylation of residues in subdomain VIII. Subdomain VIII may maintain the kinase in an inactive state by occluding the substrate binding site, and the inhibition may be reversed by a conformational change in the kinase mediated either by autophosphorylation or phosphorylation by activating upstream

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kinases. Protein kinases can be regulated by a variety of mechanisms, some of which will be discussed in more detail in the following sections. It is important to keep in mind that the criteria are by no means mutually exclusive, and an individual protein kinase may be regulated by one or more of these mechanisms. First, many protein kinases are regulated by second messengers such as cAMP, phosphatidylinositol (3,4,5) trisphosphate (PIP$_3$), and Ca^{2+}, that are generated in response to extracellular ligands binding to cell surface receptors. A common feature of this class of protein kinase is the presence of autoinhibitory or pseudosubstrate regions that maintain the kinase in an inactive conformation in the absence of second messenger. Second, some protein kinases (for instance, the cyclin-dependent protein kinases) are composed of catalytic and regulatory subunits which must interact for kinase activity. Third, some kinases (notably receptor tyrosine kinases) are activated by ligand-induced dimerization and cross-phosphorylation on tyrosine residues. Fourth, many protein kinases are themselves regulated by phosphorylation by upstream or downstream components of the signaling pathways, or by autophosphorylation. In addition, many kinases now appear to be regulated, at least in part, by subcellular localization, nuclear translocation, or by interaction with other signaling components such as small G-proteins.

1.1.2 Receptor-mediated signal transduction

Cell growth and differentiation are regulated by a vast number of extracellular signals including growth factors, hormones, neurotransmitters and cytokines. The ligands interact with high-affinity receptors on the cell surface and the signal is perpetuated via signaling cascades through the cell cytoplasm to the nucleus. Heterotrimeric G-protein coupled receptors, receptor tyrosine kinases and cytokine receptors share the ability to transduce signals from the outside to the inside of the cell. These receptors utilize small guanine nucleotide-binding (G) proteins, cytoplasmic serine/threonine or tyrosine protein

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kinases, lipid kinases, phospholipases and various adaptor proteins to relay and integrate the signal.

Although the targets of the receptors may have quite different biochemical activities and biological functions, they often contain related sequences that mediate protein-protein interactions. These sequences include Src-homology-2 (SH2), Src-homology-3 (SH3), pleckstrin homology (PH) and C2 domains. The domains fold into functional modules independently of the rest of the protein and are found in many different signaling molecules. SH2 domains, first identified as modules contained in the Src tyrosine kinase, recognize short peptide motifs containing phosphotyrosine residues, while SH3 domains, also originally identified in Src, interact with proline-rich regions in target molecules (Anderson *et al.,* 1990; Ren *et al.,* 1993). The conserved features lie within more variable regions, allowing for specificity and the high affinity with which the individual SH2 or SH3 domains bind their target proteins. PH domains, first identified in pleckstrin, are less well-characterized, but interact with phospholipids and may serve to link signaling proteins with membranes (Harlan *et al.,* 1994). The C2 domain is present in conventional and novel PKC isoforms. This domain has been shown to be responsible for binding both Ca^{2+} and acidic phospholipids (Sutton *et al.,* 1995). As a number of proteins with C2 domains do not bind Ca^{2+}, notably the novel PKCs, presumably this domain plays a further role in signaling via interaction with lipid molecules (reviewed in Newton 1995).

Thus, binding of a growth factor to a specific receptor tyrosine kinase induces receptor dimerization and tyrosine cross-phosphorylation which in turn recruits SH2 and PH domain-containing proteins. Similarly, some members of the cytokine receptor superfamily, while not containing intrinsic tyrosine kinase activity, recruit cytoplasmic tyrosine kinases which relay the signal through many of the same molecules as above. Analogously, binding of ligand to G-protein coupled receptors leads to dissociation of βγ subunits from α subunits upon exchange of GTP for GDP. The βγ subunits subsequently

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interact downstream with many of the same intracellular signaling molecules and adapter proteins.

Whatever the mechanism of transmembrane signaling, it is becoming increasingly clear that the pathways converge on the same effectors and cytoplasmic signal transduction cascades, and may use functionally analagous mechanisms for the co-ordination and integration of signaling. The transmission of signals appears to utilize specific protein-protein interactions to localize key signaling molecules to the appropriate subcellular location where they interact with substrates, effectors or other downstream components. Specifically, post-receptor signaling leads to activation of the small G-protein Ras and phosphatidylinositol 3-kinase (PI 3-kinase) and on to the activation of a number of cytoplasmic protein-serine/threonine kinases. Many of these kinases are arranged in cascades, one of which leads to activation of mitogen-activated protein kinase (MAP kinase) family members and another that results in activation of protein kinase B (PKB) and the 70 kDa S6 kinase (p70^{S6K}).

1.1.3 MAP kinase pathways

The first MAP kinase cascade to be elucidated involves activation of the isoforms known as extracellular signal regulated protein kinase 1 and 2 (ERK1 and ERK2) following stimulation with mitogens such as epidermal growth factor (EGF) or platelet derived growth factor (PDGF) (reviewed by Cobb et al., 1991). One of the proteins that binds to activated receptor tyrosine kinases is the SH2 domain-containing adaptor protein Grb-2 (growth receptor binding-2). Grb-2 acts as a scaffold for binding other proteins through two SH3 domains that flank the SH2 domain (Schlessinger 1994) (Fig. 1). A proline-rich region of the guanine nucleotide exchange protein Son-of sevenless (SOS) associates with the SH3 domains of Grb-2, causing translocation to the membrane, and allowing interaction with Ras (Aronheim et al., 1994).

Fig. 1. Essential components of the Map kinase signaling pathways: A schematic illustration of some of the key post-receptor signaling molecules. Three well-characterized MAP kinase pathways include activation of extracellular signal-regulated (ERK1/2) protein kinases via growth factor dependent signaling, activation of the stress-activated (SAPK) pathways, or activation of the p38 signaling pathway. The SAPK and p38 pathways are activated by overlapping stress agents such as UV irradiation, toxins, heat shock or oxidative stress. Known links are in solid lines, while possible connections are in dotted lines. See text for details.

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Although the basic scheme of post-receptor activation of Ras is often depicted as a linear pathway, the reality is much more complex. For instance, although the PDGF and EGF receptors can bind Grb-2 directly, they also bind and phosphorylate other SH2-containing proteins such as Shc and the Syp tyrosine phosphatase, and Grb-2 itself can directly bind Shc and Syp. The activation of Ras is accomplished via the exchange of GDP for GTP, and Ras proteins contain an intrinsic GTPase activity that hydrolyzes GTP, thus abrogating the signal (Chardin *et al.,* 1993; Gale *et al.,* 1993). The primary effector of Ras signaling is the protein serine/threonine kinase Raf1. Ras-Raf1 binding serves to localize Raf1 to the plasma membrane where it is activated by as yet unclear events including phosphorylation and interaction with membrane lipids and certain isoforms of the 14-3-3 polypeptides (Marais *et al.,* 1995; Vojtek *et al.,* 1993). A number of other proteins have been shown to interact with Ras in a GTP-dependent manner, including the p110 subunit of PI 3-kinase and MEKK1, and it appears that Ras acts as a GTP-regulated switch recruiting a variety of signaling molecules to the membrane depending on the context of signals received at the cell surface. Thus, there is a complex network of interactions that integrate Ras activation with other signaling events and modulate downstream events.

The mitogenic activation of Raf1 leads to activation of MEK1/2 which in turn activates ERK1 and ERK2 via phosphorylation of tyrosine and threonine residues in a conserved TEY motif (Ahn *et al.,* 1992). Activated ERK1/2 translocates to the nucleus where it mediates phosphorylation of various transcription factors such as Elk-1, Ets1/2 and ATF-2 involved in gene transcription leading to proliferation, differentiation or survival (Marais *et al.,* 1993; Coffer *et al.,* 1994). The three enzyme signaling module (Raf1→ MEK→ ERK1/2) is considered the archetype of at least three other signaling pathways that are conserved in organisms as diverse as yeast, sea star, amphibians, and mammals.

Cells respond not only to physiological cues leading to cell growth, differentiation or proliferation, but also to environmental stresses such as UV irradiation, metabolic toxins, osmotic or heat shock, and DNA damage. Environmental assaults of this type often lead to growth arrest or apoptosis in damaged cells. The signaling cascades that mediate the stress response are related to, but distinct from the ERK1/2 pathway, as these agents stimulate activation of the ERK isoforms only poorly, but potently activate the closely related stress-activated protein kinases (SAPKs, also known as c-Jun amino-terminal kinases (JNKs)). In addition, SAPKs are strongly activated by cytokines such as tumor necrosis factor alpha (TNFα) and interleukin-1 (IL-1β) (Minden *et al.*, 1994; Kallunki *et al.*, 1994; Sluss *et al.*, 1994). This pathway is composed of a distinct signaling module comprising MEKK1 phosphorylation of SEK1/MEK4 which activates the SAP kinases via phosphorylation of tyrosine and threonine residues in a TPY motif, analogous to the activation of ERK1/2 (Yan *et al.*, 1994; Lin *et al.*, 1995) .

While Ras and Raf1 are upstream activators of the ERK1/2 signaling cascade, these proteins do not activate the SAPKs. The pathway is induced by a wide variety of agents, and it is likely that many of these act through independent targets. For instance, UV irradiation induces cysteine cross-linking leading to dimerization of receptors and other proteins. Inflammatory cytokines like IL-1 act via cell surface receptors that mediate their signals through activation of sphingomyelinase and production of the second messenger ceramide. Two Ras related GTP-binding proteins of the Rho family, Rac1 and cdc42 have been shown to activate SAPKs (Minden *et al.*, 1995; Coso *et al.*, 1995A; Brown *et al.*, 1996). These two molecules do not activate MEKK1 directly but have been implicated in activation of p21-activated kinase-1, 2 and 65 (PAK1, PAK2 and PAK65) (Martin *et al.*, 1995) leading to induction of the SAPK pathway (Coso *et al.*, 1995B). Members of the PAK family bind GTP-Rac and Cdc42 and are activated by them in a GTP-dependent manner, however the details of this activation remain to be clarified (Martin *et al.*, 1995; Manser *et al.*, 1994). Rac1 and cdc42 have also been

shown to be involved in regulation of the actin cytoskeleton, cell adhesion, morphology, motility and cytokinesis, although the precise signaling pathways remain to be elucidated (reviewed by Amano *et al.,* 1998).

Activated SAPKs also translocate to the nucleus where they phosphorylate transcription factors such as c-Jun and ATF-2, leading to the transcription of genes that mediate growth arrest or apoptosis. c-Jun is a component of the AP-1 transcription factor, which is composed of homo- and hetero-dimers of c-Jun and Fos. c-Jun is basally phosphorylated in the carboxyl terminus, and is transcriptionally inactive in resting cells. In response to mitogenic stimulation, c-Jun becomes dephosphorylated on these sites, allowing interaction with Fos and the activation of transcription. A different set of amino terminal residues are the target for phosphorylation by SAPKs. These sites (Ser-63 and Ser-73) lie near the δ transactivation domain and their phosphorylation by SAPK's greatly enhances the transactivation activity of these proteins in response to stress-related agonists (Franklin *et al.,* 1993).

The existence of multiple MAP kinase pathways in yeast led to the search for novel mammalian components of these signaling pathways and a mammalian homologue of a yeast protein involved in the response to high osmolarity (HOG1) was cloned. The mammalian kinase, p38 is relatively poorly characterized but appears to be activated by similar agonists as the SAPKs. However, p38 is not activated by SEK1, but is activated by MEK3/6 phosphorylation on tyrosine and threonine residues in a TGY motif (Han *et al.,* 1996; Raingeaud *et al.,* 1996). Many of the transcriptional events mediated by this pathway appear to be via the phosphorylation and activation of MAPKAP kinase-2 which is a direct target of p38. MAPKAP kinase-2 phosphorylates the same sites in both CREB and ATF-2 that are targeted by protein kinase A (PKA). The p38 family is activated by lipopolysaccharide (LPS), a known cytokine inducer, and is potently inhibited by a series of bio-active, anti-inflammatory pyridinyl-imidazole compounds called cytokine-suppressive anti-inflammatory drugs (CSAIDs). Since p38 is also activated by

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inflammatory cytokines, it is likely to be involved in the induction and response to these agonists. The use of these specific inhibitors and activators should lead to a more complete characterization of the p38 pathway.

Thus, the MAP kinase isoforms are grouped into subfamilies based on sequence similarity, mechanism of upstream regulation, and activation by different MEKs. Most MAPK family members require phosphorylation on tyrosine and threonine in specific motifs, but ERK3 lacks this dual phosphorylation motif, and lies in an as yet uncharacterized pathway (Cheng et al., 1996). More than a dozen MAPK family members have now been cloned, and the variation in specificity within the signaling modules may result from integration of different extracellular and upstream cues.

1.1.4 PI 3-kinase /PKB/p70 S6 kinase pathways

Another major signaling cascade that has been described entails signaling through phosphatidylinositol 3-kinase (PI 3-kinase), and on to the activation of protein kinase B (PKB) and the 70 kDa S6 kinase (p70^{S6K}) (Fig. 2). A role for activation of the PI 3-kinase pathway has been established in mitogenic signaling, intracellular vesicle trafficking, and cytoskeletal regulation (Carpenter and Cantley 1996). Activation of PI 3-kinase and PKB inhibits the induction of apoptosis by serum withdrawal (Kennedy et al., 1997), ultraviolet irradiation (Kulik et al., 1997), and the c-myc protein (Kaufmann-Zeh et al., 1997), implying a role in transducing survival signals.

Membrane lipids not only play a structural role in maintaining membrane integrity, but are also actively involved in signaling processes as second messengers. One of the best characterized examples is the hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P$_2$) by phospholipase Cγ (PLCγ), giving rise to diacylglycerol and inositol (1,4,5)-trisphosphate (IP$_3$), which causes release of calcium from intracellular stores and protein kinase C (PKC) activation (Divecha and Irvine 1995). The PI 3-kinase
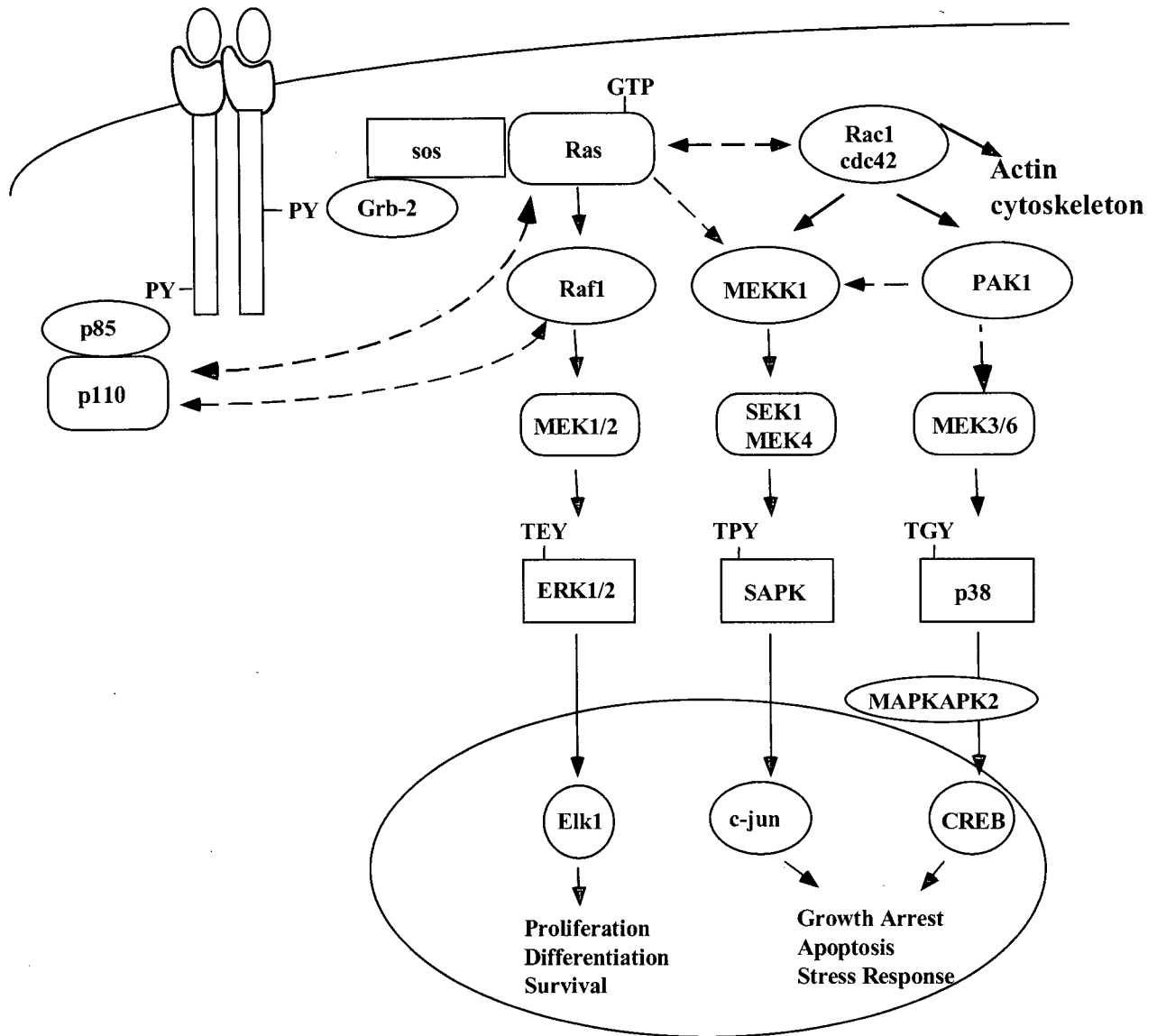
12
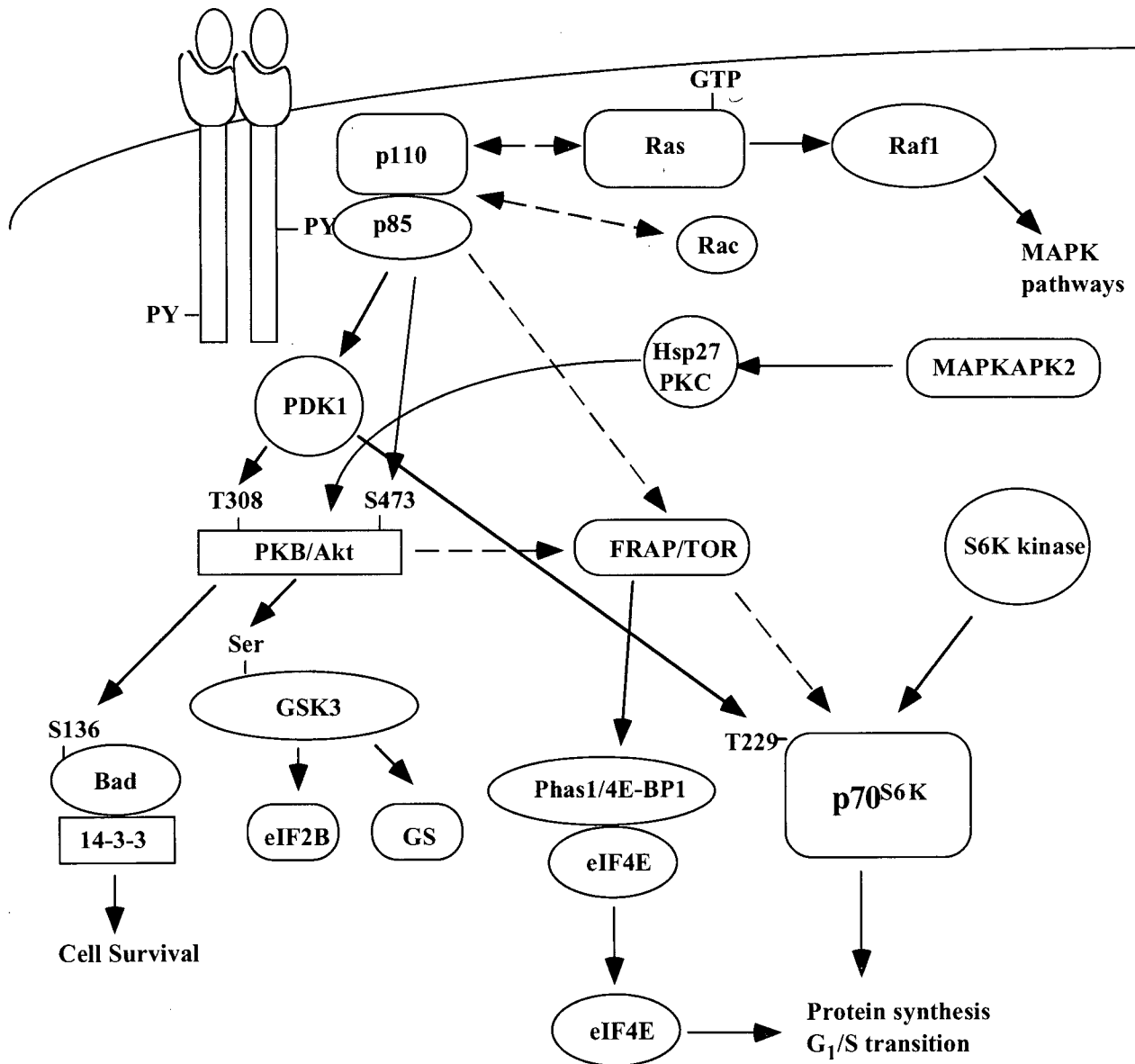
Fig. 2. Essential components of the PI 3-kinase/PKB/p70^{S6K} signaling pathway:
A schematic illustration of some of the key post-receptor signaling molecules. PI 3-kinase activates PDK1 which phosphorylates and activates PKB. PI 3-kinase is also upstream of FRAP/TOR, but the interactions are undefined. Known links are in solid lines, while possible connections are in dotted lines. See text for details.

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family members phosphorylate the hydroxyl group at the 3' position on the inositol ring and convert phosphatidylinositol (PI), PI(4)P, and PI(4,5)P$_2$ to PI(3)P, PI(3,4)P$_2$, and PI(3,4,5)P$_3$, respectively, within seconds to minutes in response to mitogenic stimulation. These products are not substrates for PLCγ and are now known to act as second messengers downstream of PI 3-kinase. PI(3,4)P$_2$ and PI(3,4,5)P$_3$ interact with protein targets and modulate their cellular localization and/or activity. Protein modules, such as PH and C2 domains that can bind lipids, are found in many of the downstream molecules that are modulated by the PI 3-kinase lipid products (Shaw 1996; Salim *et al.*, 1996).

Functional analysis of the various isoforms of PI 3-kinase has been facilitated by the existence of a number of specific inhibitors, as well as the availability of dominant negative or constitutively active mutants. One of the most commonly used inhibitors is the fungal metabolite, wortmannin, which inhibits PI 3-kinase at nanomolar concentrations by binding covalently to the p110 catalytic subunit (Yano *et al.*, 1993). Another inhibitor, LY294002 acts at micromolar concentrations by competitive inhibition of the ATP binding site (Vlahos *et al.*, 1994). These inhibitors and various mutants have helped to clarify not only the activation of the different isoforms of PI 3-kinases, but have proved to be useful reagents for analysis of the downstream signaling components of this family of lipid kinases.

There are multiple isoforms of PI 3-kinases, belonging to different families depending on substrate specificity and mechanism of activation. The class I enzymes are heterodimeric proteins composed of separate regulatory and catalytic subunits that utilize PI, PI(4)P and P(4,5)P$_2$ as substrates. The 110-kDa catalytic subunits, p110α and p110β both bind 85-kDa regulatory subunits and are stimulated by both receptor and nonreceptor protein tyrosine kinases. The p85 regulatory subunits contain an amino terminal SH3 domain followed by a proline-rich sequence, a breakpoint cluster region (BCR) homology domain, a second proline-rich domain, and two SH2 domains (Pleiman *et al.*, 1994; Klippel *et al.*, 1993). The regulatory subunits contain putative coiled-coil domains that

mediate stable dimerization with the p110 catalytic subunit which contains a p85 binding region, a ras-binding domain, and the catalytic core (Dhand *et al.*, 1994).

Regulation of the PI 3-kinase is complex, likely involving a number of second messengers and other signaling components. The p110 subunit has been shown to bind and be activated by ras-GTP *in vitro* (Kodaki *et al.*, 1994). The p85 SH3 domain binds to proline-rich peptides while the p85 proline-rich sequences bind to the SH3 domains from Fyn and Lyn (Booker *et al.*, 1993). The BCR homology domain binds to GTP-bound Cdc42, and the SH2 domains bind phosphotyrosine containing proteins (Songyang *et al.*, 1993; Zheng *et al.*, 1994). Each of these binding events activates PI 3-kinase *in vitro*, but it is unclear whether the interactions are redundant or additive (Pleiman *et al.*, 1994; Zheng *et al.*, 1994).

Very little is known about negative regulation of the PI 3-kinase isoforms. The family members, in addition to their lipid kinase activity, possess an intrinsic protein serine/threonine kinase activity. Autophosphorylation of PI 3-kinase on serine has been shown to down-regulate it's lipid kinase activity (Carpenter *et al.*, 1993; Dhand *et al.*, 1994B). A recent report demonstrated that c-Abl, a cytoplasmic tyrosine kinase that plays a role in DNA damage-induced apoptosis (Yuan *et al.*, 1997A), binds the p85 subunit and inhibits PI 3-kinase activity *in vitro* and in irradiated cells (Yuan *et al.*, 1997B). Since transforming variants of c-Abl activate PI 3-kinase (Varticovski *et al.*, 1991; Skorski *et al.*, 1995), c-Abl may play a role in the regulation of cell fate by down-regulation of PI 3-kinase.

There is controversial evidence implicating a role for PI 3-kinases in activation of the various MAP kinase pathways. The class I family of PI 3-kinases (p110α-p85α) have been shown to interact with Ras in a GTP-dependent manner (Kodaki *et al.*, 1994; reviewed by Marte and Downward 1997). Several direct targets of the lipid products of PI 3-kinase have been identified that may also link this pathway to MAP kinases. Members of the calcium-independent, novel PKC family, PKCε, PKCδ, and PKCη have

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been shown to be activated *in vitro* by PI(3,4)P$_2$ and PI(3,4,5)P$_3$ (Toker *et al.*, 1994), and

these PKC isoforms have been implicated in activation of the MAP kinase pathway via

phosphorylation of Raf1 (Kolch *et al.*, 1993; Troppmair *et al.*, 1994). Another report has

shown that a membrane-targeted form of PI 3-kinase can activate SAPK/JNK, and that a

dominant negative mutant of PI 3-kinase can block EGF-induced activation of JNK

(Logan *et al.*, 1997). Treatment with wortmannin indicates that the effect of PI 3-kinases

on the MAP kinase pathway may be cell- and tissue-specific and may depend on the

strength of the signal (Duckworth and Cantley 1997). Interpretation of this data requires

caution, however, as wortmannin may not be specific for PI 3-kinase and may in fact

directly inhibit other enzyme activites including the FKBP binding protein FRAP (also

known as target of rapamycin (TOR)), MAP kinase, and p70^{S6K} (Cheatham *et al.*, 1994;

Brunn *et al.*, 1996; Brunn *et al.*, 1997).

One of the first targets of PI 3-kinase to be identified was the product of the

retroviral oncogene *v-akt*, also known as protein kinase B (PKB). The kinase is activated

by mitogens and growth factors in a PI 3-kinase-dependent manner and is inhibited in

response to agents that block PI 3-kinase. Nevertheless, PKB can also be activated by PI

3-kinase-independent pathways (Konishi *et al.*, 1996).

PKB contains an amino terminal PH domain, a central catalytic core and a

carboxyl terminal regulatory domain. The PI(3,4)P$_2$ generated by PI 3-kinase directly

binds to the PH domain of PKB and activates its phosphotransferase activity (Frech *et al.*,

1997). Franke *et al.* (1997A) also demonstrated that binding of the PH domain to

PI(3,4)P$_2$ led to dimerization of PKB and subsequent activation of the kinase. PKB

activity is also regulated by phosphorylation, and the *in vivo* phosphorylation sites have

been identified as Thr-308 and Ser-473 (Franke *et al.*, 1997B). Thr-308 lies in the

activation loop between subdomains VII and VIII of the catalytic domain, nine residues

upstream of the APE motif that is conserved in protein kinases. This is a similar position

to the activating phosphorylation sites found in many other protein kinases, including the

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MAP kinase isoforms. Ser-473 is close to the carboxyl terminus in a motif found in other kinases that are activated by second messengers (Pearson *et al.*, 1995). Both sites require phosphorylation for PKB to be fully active (Alessi *et al.*, 1996).

A novel PH domain-containing protein, 3-phosphoinositide-dependent protein kinase-1 (PDK1) has recently been detected and purified (Alessi *et al.*, 1997B). PDK1 is activated specifically by $PI(3,4)P_2$ and $PI(3,4,5)P_3$ and activates protein kinase B (PKB) via phosphorylation of Thr-308. It is possible that reports showing that PKB is activated directly by $PI(3,4)P_2$ may have been the result of contamination of PKB preparations with trace PDK1 activity. PDK1 was recently cloned by two groups, and its function has been further clarified (Stephens *et al.*, 1998; Alessi *et al.*, 1997A and B). PDK1 also contains a PH domain that tightly binds $PI(3,4,5)P_3$, although the enzyme is constitutively active, and is not stimulated by insulin nor inhibited by wortmannin. The current model is that the PH domain of PKB normally restricts access of PDK1 to Thr-308, but is displaced when PKB binds $PI(3,4,5)P_3$, allowing phosphorylation to take place. PI 3-kinase is necessary for the translocation of PKB to the membrane and the phosphorylation of Ser-473. A recent study reported that Ser-473 was phosphorylated *in vitro* by the integrin-linked kinase (ILK), and that a kinase-deficient form of ILK severely inhibited Ser-473 phosphorylation *in vivo* (Delcommenne *et al.*, 1998). Thus, the regulation of PKB is complex and is mediated by multiple components. It is clear that an intact PH domain is absolutely required for activation and may cause translocation of the kinase to the plasma membrane where it can be further activated by phosphorylation. Indeed, altering the subcellular localization of PKB by attaching the myristoylation/ palmitoylation motif from the Lck tyrosine kinase (m/p-PKB), led to a 60-fold increase in PKB activity that could not be further stimulated by growth factors (Andjelkovic *et al.*, 1997). The m/p-PKB was found to be phosphorylated on Thr-308 and Thr-473 in the absence of mitogenic stimulation, and the phosphorylation and activation did not require the

presence of an intact PH domain, supporting the model of the PH domain serving as a membrane-targeting module.

PKB can also be activated by cellular stresses such as heat shock and hyperosmolarity, both of which activate the p38/HOG1 pathway via a wortmannin-insensitive (ie. PI 3-kinase independent) pathway (Konishi et al., 1996). Heat shock induces association of PKCδ with the PH domain of PKB, and the kinase also associates with PKCζ (Konishi et al., 1994). PKB is also activated by oxidative and chemical stressors, and associates with a small heat shock protein (HSP-27) in response to these stresses (Konishi et al., 1997). Overexpression of HSP-27 in embryonic stem cells has been shown to lead to differentiation, while underexpression caused apoptosis in these cells (Mehlen et al., 1997). HSP-27 is known to be phosphorylated by MAPKAP kinase-2 in response to cellular stresses (Ciocca et al., 1993), but the relationship between MAPKAP kinase-2, HSP-27, PKB and various PKC isoforms is as yet unclear. Heat shock has also been shown to activate PI 3-kinase, c-Src, and $p70^{S6K}$ in NIH 3T3 fibroblasts (Lin et al., 1997).

Until recently, the only known *in vivo* substrate for PKB was glycogen synthase kinase-3 (GSK-3). GSK-3 phosphorylates and inactivates glycogen synthase (GS) in the absence of insulin stimulation. Upon insulin stimulation, PKB phosphorylates GSK-3 on a single regulatory amino-terminal serine residue in a PI 3-kinase dependent manner (Cross et al., 1995). This phosphorylation inhibits GSK-3 activity, resulting in the dephosphorylation and activation of several metabolic and gene-regulatory proteins including GS, involved in the insulin response (reviewed by Welsh et al., 1996). GSK-3 also phosphorylates and inactivates the initiation factor eIF2B, and this inhibition is abolished with insulin treatment, leading to the onset of protein synthesis (Welsh and Proud 1993).

It has long been known that activation of PI 3-kinase and PKB promotes cell survival and blocks apoptosis, but the signals acting downstream have been difficult to

characterize. Apoptosis is a highly ordered mechanism by which cell death is induced in development, oncogenesis and the immune response (Gerschenson and Rotello 1992). Two families of proteins appear to play an essential role in the induction of apoptosis. The first is the family of cysteine proteases related to the interleukin 1β-converting enzyme (ICE) (Martin and Green 1995). Activation of the ICE protease cascade is a necessary event in the execution of apoptosis. The second family is the Bcl-2 family, members of which can either promote or inhibit apoptosis. The ratio of death-promoting (ie. Bax, Bad) to death-inhibiting (ie. Bcl-2, Bcl-x_L) factors determines the ultimate fate of the cell (Oltvai and Korsmeyer 1994). The mechanism by which Bcl-2 members function is not well understood, but may include the formation of ion channels and regulation of the ICE protease cascade (Stellar 1995). PKB phosphorylates a member of the Bcl-2 family (Bad), at Ser-136 *in vitro* and *in vivo* inhibiting Bad-mediated death induction (Datta *et al.,* 1997; del Peso *et al.,* 1997). Bad resides in the cytosol, and phosphorylation by PKB leads to its sequestration by the tau form of 14-3-3 proteins, inhibiting its interaction with Bcl-x_L and therefore promoting cell survival (Zha *et al.,* 1996).

The ability of PKB to promote cell survival may reflect its oncogenic potential. PKB can synergize with the Raf1/MAP kinase pathway to cause transformation of NIH 3T3 cells (Marte *et al.,* 1997) and may also drive cell-cycle progression through induction of E2F transcriptional activity. In Ras-transformed epithelial cells, the ability of the Ras oncogene to promote cell-survival is mediated through a PI 3-kinase/PKB-dependent pathway (Khwaja *et al.,* 1997), and it is likely that PKB plays a role in mediating survival signals in human cancers resulting from mutation and activation of Ras.

Several other proteins are known to lie downstream of PI 3-kinase and PKB though the precise interactions between them are unclear. These include a PI 3-kinase family member, the FKBP binding protein (FRAP, also known as target of rapamycin (TOR)), the elongation initiation factor binding protein, 4E-BP1 (also known as PHAS-

1), and p70^{S6K}. Much of the detail regarding these proteins has been established by use of the immunosuppressant drug rapamycin which blocks activation of FRAP, 4E-BP1 and p70^{S6K} by all known stimuli and causes cell cycle arrest by delaying or abrogating the G1 phase of the cell cycle. Agents such as wortmannin and LY294002 that inhibit PI 3-kinase and PKB also block activation of p70^{S6K} (King *et al.,* 1997; Cheatham *et al.,* 1994), providing a framework to identify other components of this signaling pathway.

1.2   70 kDa S6 kinase

Hyperphosphorylation of the 40S ribosomal protein S6 is one of the most dramatic and consistent responses of many cell types to a variety of mitogenic stimuli. The sites in the S6 protein are phosphorylated sequentially *in vivo* in the order Ser-236>Ser-235>Ser-240>Ser-244>Ser-247 (Krieg *et al.,* 1988; Martin-Perez and Thomas 1983). The enzyme responsible for phosphorylation of the S6 protein, the mitogen-activated 70-kDa S6 kinase (p70^{S6K}) plays a key role in cellular growth control mechanisms by coordinating protein synthesis via regulation of the S6 protein and the activity of 4E-BP1. The p70^{S6K} also plays an important role in the progression of cells from G1 to S phase of the cell cycle (Chung *et al.,* 1992). Due to the role this enzyme plays in cell cycle control and the initiation of protein synthesis, much attention has focused on the key question of how the kinase activity is regulated.

1.2.1   Structure of p70^{S6K} - functional domains

The p70^{S6K} has been purified or cloned and sequenced from a variety of sources including rat liver (Price *et al.,* 1989; Kozma *et al.,* 1989; Kozma *et al.,* 1990), vanadate-stimulated Swiss 3T3 cells (Jenö *et al.,* 1989), and rabbit liver (Harmann and Kilimann 1990). The two isoforms, p70^{S6K} and p85^{S6K} are generated from alternative

translation sites of the same transcript (Grove *et al.*, 1991; Reinhard *et al.*, 1992) and are identical except for a 23 amino acid extension present at the amino terminus of the p85^{S6K} isoform. Although most researchers use the p70^{S6K} form when describing the regulatory residues, some investigators use the p85^{S6K} isoform, adding 23 amino acids to the numbering system (Alessi *et al.*, 1998). The extension targets the p85^{S6K} form to the nucleus while the p70^{S6K} form is predominantly cytoplasmic. The nuclear form likely plays a key role in the regulation of cell growth, as the S6 protein is present in free form in the nucleus and becomes phosphorylated at the same sites in response to mitogenic stimulation as the cytoplasmic form (Franco and Rosenfeld 1990). Since protein synthesis does not take place in the nucleus, the function of phosphorylated S6 protein in the nucleus remains obscure.

The enzyme contains a single catalytic domain most closely related to the amino terminal catalytic domain in p90^{rsk} and to second-messenger-regulated kinases. In addition, p70^{S6K} contains an amino terminal acidic domain, and a pseudosubstrate sequence resembling the region in S6 that is phosphorylated in response to mitogenic stimulation (Fig. 3A). The pseudosubstrate sequence lies on the carboxyl side of the catalytic domain and acts as an autoinhibitory domain, possibly by interaction with the amino terminal acidic domain (Banerjee *et al*, 1990; reviewed by Pullen and Thomas 1997). This region contains four proline-directed mitogen-induced phosphorylation sites. The kinase also contains a number of *in vivo* phosphorylation sites in the kinase domain and linker region, that are flanked by large, aromatic amino acids. The regulation and activation of p70^{S6K} is complex, requiring multiple phosphorylation events and interactions with additional proteins.
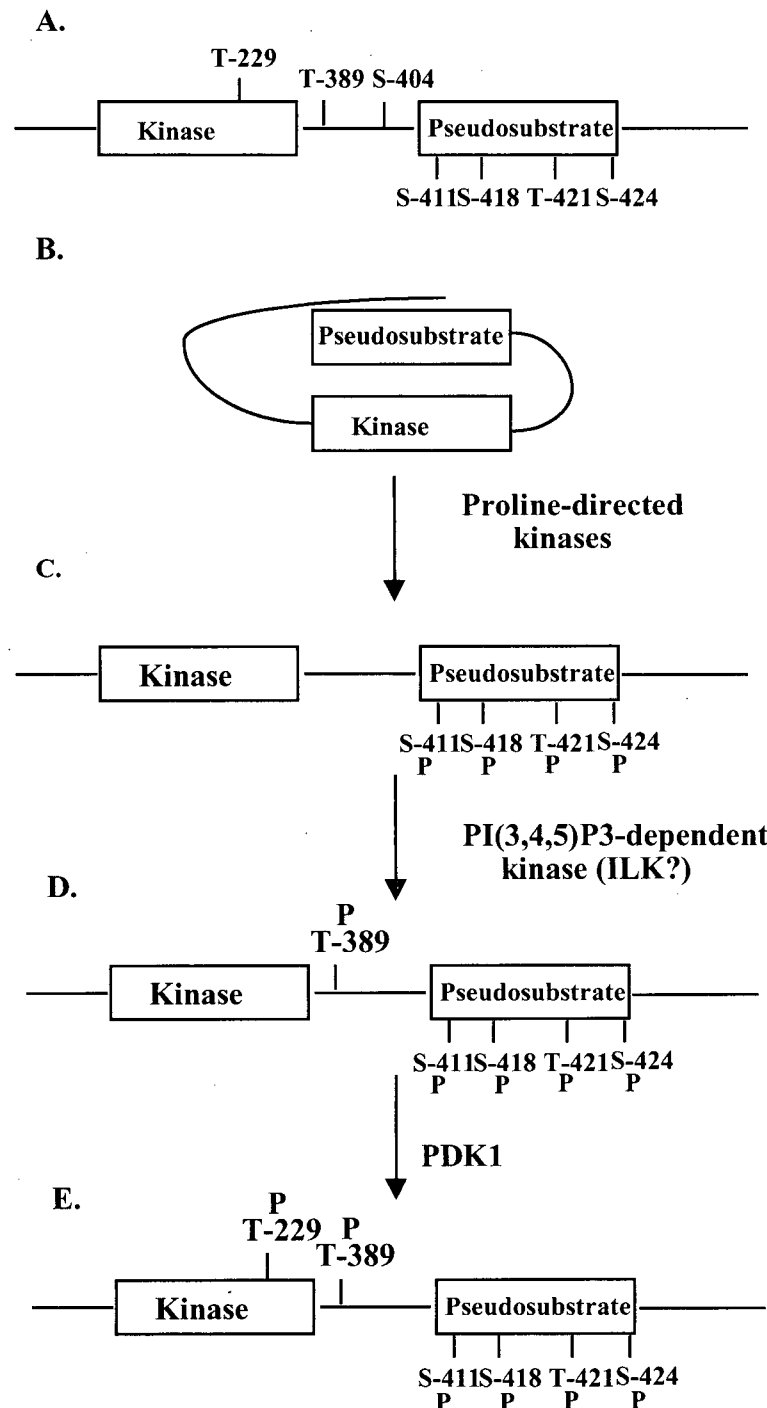
Fig. 3. Structure and regulation of p70[S6K]: A schematic drawing of the S6 kinase showing regions essential for catalytic activity. The various domains and phosphorylatable sites are shown (A). The kinase is maintained in an inactive form by interaction of the pseudosubstrate region with the kinase domain (B). The kinase is activated by multiple phosphorylation events by more than one kinase, on proline-directed sites (C), and by PI(3,4,5)P$_3$ dependent kinases (D, E). See text for details.

1.2.2 Regulation and activation of p70^{S6K}

In all types of quiescent cells p70^{S6K} is basally phosphorylated and mitogenic stimulation induces increased phosphorylation on multiple sites leading to activation of its phosphotransferase activity. Four of the basally phosphorylated, mitogen-induced sites (Ser-411, Ser-418, Thr-421 and Ser-424) lie in the carboxyl terminus within the pseudosubstrate/autoinhibitory domain (Ferrari et al., 1992). Mutation of these residues to alanine inhibits activation of the kinase (Han et al., 1995). Mutation of these sites to acidic residues increases kinase activity (Ferrari et al., 1993) but not fully, supporting the notion that other phosphorylation events and interactions are required for full activation.

Although the mitogen-induced sites contain recognition determinants for proline-directed kinases, MAP kinase fails to activate p70^{S6K} after phosphorylation of these sites in vitro (Mukhopadhyay et al., 1992), and it is now well established that p70^{S6K} activation does not lie on the Ras→Raf→MEK→MAP kinase pathway (Ballou et al., 1991). A growing body of evidence has implicated PKC, PI 3-kinase and PKB as upstream signaling molecules in p70^{S6K} activation in insulin-, PDGF-, EGF-, and IL2-treated cells (reviewed by Chou and Blenis 1995).

The immunosuppressant drug rapamycin, a bacterial macrolide, has become one of the most important and useful tools for dissecting the pathway leading to p70^{S6K} activation and its regulation. Rapamycin blocks activation of p70^{S6K} by all known mitogens by preventing the phosphorylation of a specific subset of sites including Thr-229, Thr-389, Ser-404 and Ser-411 (Ferrari et al., 1993). The rapamycin-sensitive sites are not basally phosphorylated but become highly phosphorylated in response to mitogenic stimulation. Except for Ser-411, these sites are flanked by large, aromatic residues, and the principal inhibitory site has been identified as Thr-389 in the linker region coupling the catalytic and autoinhibitory domains (Pearson et al., 1995). This residue is highly conserved among kinases of the second messenger family and is

analogous to Ser-473 in PKB. Mutation of Thr-389 to an acidic amino acid (T389E) confers high basal activity and rapamycin resistance on the kinase (Pearson et al., 1995). Mutation of this residue to alanine (T389A) fully inactivates the kinase (Pearson et al., 1995). Thr-229 is also a highly conserved residue, analogous to Thr-308 in PKB, that lies within the kinase activation loop and is essential for phosphotransferase activity (Pearson et al., 1995; Weng et al., 1995A). Emerging evidence indicates that Thr-389 phosphorylation modulates Thr-229 phosphorylation (Pullen and Thomas 1997). The T389E mutant elevates Thr-229 phosphorylation while T389A blocks it. Mutation of Thr-229 to alanine or glutamic acid ablates kinase activity and does not potentiate Thr-389 phosphorylation (Pearson et al., 1995). Since p70^{S6K} and PKB are distantly related, Alessi et al. (1997C) and Pullen et al. (1998) tested whether PDK1 could phosphorylate p70^{S6K} at Thr-229 and found that indeed it could. Furthermore, they found that a dominant negative form of PDK1 blocked activation of p70^{S6K} in cells.

The small Rho family G proteins, rac-1 and cdc42 can also bind to the hypophosphorylated form of p70^{S6K} in vitro, in a GTP-dependent fashion, and GTPase deficient forms can activate p70^{S6K} activity in vivo (Chou and Blenis 1996). Nevertheless, it is not yet known if these molecules interact directly with p70^{S6K} or if other signaling intermediates are required.

Although there has been considerable difficulty in identifying the upstream kinases that phosphorylate Thr-389 and Thr-229, as well as other interacting proteins, a model of the coordinated and heirarchical activation of p70^{S6K} has been proposed (Pullen and Thomas 1997; Downward 1998). In its hypophosphorylated form, the pseudosubstrate domain is thought to bind to the amino terminal, and occlude the substrate binding site (Fig. 3B). Deletion of the amino terminal prevents kinase activation and also prevents phosphorylation of Thr-229 and Thr-389 in response to mitogenic stimulation (Weng et al., 1995B; Dennis et al., 1996). The deletion can be rescued by removal of the pseudosubstrate region (Pearson et al., 1995; Dennis et al.,

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1996) or by mutation of the proline-directed sites in this region to acidic residues (Pullen and Thomas 1997). In both instances, the rescue is accompanied by phosphorylation of Thr-389 and Thr-229 (Pearson *et al.,* 1995; Dennis *et al.,* 1996). These data indicate that the amino terminal and the pseudosubstrate region cooperate in signaling to Thr-389, and deletion of the amino terminal may prevent binding of a necessary effector molecule such as rac-1 or cdc42. Thus, $p70^{S6K}$ activation may proceed by binding of an effector molecule at the amino terminal which releases the interaction with the pseudosubstrate region. This conformation would then be stabilized by phosphorylation of the proline-directed sites in this region (Fig. 3C). Subsequent phosphorylation of Thr-389 by an as yet unidentified $PI(3,4,5)P_3$-dependent kinase (possibly PDK2 or ILK) (Fig. 3D) and phosphorylation of Thr-229 by PDK1 (Fig 3E) would lead to full activation of the kinase phosphotransferase activity.

The similarities in the regulation of PKB and $p70^{S6K}$ are striking. In the inactive state, both are maintained in a conformation precluding access by the constitutively active PDK1. Binding of $PI(3,4,5)P_3$ to the PH domain of PKB causes a conformational change, while phosphorylation of the pseudosubstrate region of $p70^{S6K}$ by proline-directed kinases and subsequent phosphorylation of Thr-389 enable PDK1 access to its target site.

1.2.3 FRAP and 4E-BP1

Rapamycin does not directly interact with $p70^{S6K}$, but binds to FK506-binding protein 12 (FKBP12) and the complex interacts with FRAP/TOR upstream of $p70^{S6K}$ (Brown *et al.,* 1994; Sabatini *et al.,* 1994). FRAP is structurally related to the catalytic subunit of PI 3-kinase and DNA-dependent protein kinases (Keith and Schreiber 1995), and is thought to function as a lipid or protein kinase (Hunter 1995). Mutant FRAP proteins which lack the ability to bind FKBP/rapamycin complexes are resistant to the effects of rapamycin (Stan *et al.,* 1994; Brown *et al.,* 1995). These mutant forms of

FRAP confer at least partial rapamycin resistance to p70^{S6K}, and a further mutation that inactivates the FRAP catalytic domain abrogates the rescue of p70^{S6K} (Brown et al., 1995). The same FRAP mutants can also protect 4E-BP1 from rapamycin-induced dephosphorylation and such protection again requires the presence of an active catalytic domain (Hara et al., 1997). Rapamycin-induced p70^{S6K} dephosphorylation and inactivation are paralleled by dephosphorylation of S6 (Chung et al., 1992) and 4E-BP1 (Beretta et al., 1996).

In normal resting cells, 4E-BP1 is tightly bound to the elongation initiation factor eIF4E, inhibiting cap-dependent translation (Pause et al., 1994). In response to mitogenic stimulation, 4E-BP1 becomes phosphorylated on five proline-directed sites in a rapamycin-dependent manner (Fadden et al., 1997). The phosphorylation of 4E-BP1 leads to its release from eIF4E, allowing the initiation factor to then interact with the eIF4G subunit of the mRNA cap-binding protein complex (Mader et al., 1995). The p70^{S6K} does not phosphorylate either the free or eIF4E-bound form of 4E-BP1 in vitro and is likely not involved in this event in intact cells (Diggle et al., 1996). It has recently been shown that anti-FRAP immunoprecipitates contain kinase activity that can phosphorylate the proline-directed sites in free 4E-BP1, but it is not yet clear whether this activity can also phosphorylate 4E-BP1 bound to eIF4E, as must occur in vivo (Brunn et al., 1997). Other evidence indicates that the rapamycin-dependent signaling events immediately upstream of 4E-BP1 and p70^{S6K} are distinct, but require a common proximal activator (von Manteuffel et al., 1997).

Several studies have reported dissociation of p70^{S6K} from the known components of the PI 3-kinase pathway. PI 3-kinase, but not p70^{S6K}, is required for cytokine-regulated hemopoietic cell survival (Scheid et al., 1996), and PKB, but not p70^{S6K}, mediates growth-factor induced neuronal cell survival (Dudek et al., 1997). Activation of PKC by phorbol ester treatment leads to activation of p70^{S6K} in a PI 3-kinase-independent manner (Chung et al., 1994), and in a PKB-independent manner (Reif et al., 1997).

1.2.4 Protein translation and the cell cycle

The translation of mRNA into protein is a complex process, controlled at multiple levels. Translation initiation, which involves recognition of the capped mRNA, binding of ribosomal subunits, and selection of the start codon, is generally the rate-limiting step (Pain 1996). The phosphorylation of initiation factors and ribosomal proteins modulates the rate of initiation and plays a central role in the control of protein synthesis (Proud 1992). Rapamycin treatment has been shown to significantly inhibit translation of a select group of genes, including those encoding ribosomal proteins (S3, S6, S14 and S24), translation elongation factors (eEF1A and eEF2), and a secreted peptide growth factor, insulin-like growth factor II (IGF-II) (Terada *et al.,* 1994; Neilsen *et al.,* 1995; Jefferies and Thomas 1994). The inhibition is due to structural elements in the 5' untranslated region (UTR) of certain genes, and reflects the specific nature of the inhibition (Jefferies *et al.,* 1994). This class of mRNA transcripts contains an oligopyrimidine tract at the transcriptional start site (5'-TOP) which plays a key role in the regulation of their translation (Levy *et al.,* 1991). These mRNAs undergo translational up-regulation in response to stimulation leading to activation of initiation and elongation factors, as well as increasing synthesis of ribosomal proteins and other ribosomal components for enhanced translational activity. The 40S ribosomal protein S6 may play a role as it lies in the mRNA-binding site of the ribosome, and is phosphorylated in a rapamycin-sensitive manner (Jefferies and Thomas 1996). S6 remains the only established physiological substrate for $p70^{S6K}$ with mitogenic stimulation leading to multiple, ordered phosphorylation of the S6 protein at five serines located in the carboxyl terminus (Bandi *et al.,* 1993; Krieg *et al.,* 1988). Thus, dominant negative and rapamycin-insensitive mutants of $p70^{S6K}$ have been used to examine the role of the kinase in the control of 5'-TOP mRNA translation (Jefferies *et al.,* 1997). Expression of the dominant negative mutant blocked activation of wild-type $p70^{S6K}$, as well as the activation of 5'-TOP mRNA translation by serum. The expression of the rapamycin-

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insensitive T389E mutant also caused 5'-TOP mRNA translation to become insensitive to rapamycin treatment. These results support the likelihood of a role for p70^{S6K} in regulation of 5'-TOP mRNA translation, though the details remain to be clarified.

p70^{S6K} also plays an important role in cell cycle control but the details are unclear. Rapamycin causes cell cycle delay or arrest by prolonging or abrogating the G1 phase of the cell cycle. In spite of the fact that rapamycin totally abolishes p70^{S6K} activity in all cell types examined, its effect on G1 progression varies with cell type. Complete blockage occurs in mature T-cells (Calvo *et al.,* 1992), but only some G1 delay occurs in NIH 3T3 cells (Morice *et al.,* 1993). The variation in effect on G1 progression is parallelled by changes in cyclin E and A expression (Morice *et al.,* 1993) and rapamycin treatment in at least some cell types inhibits the activation of specific cyclin-dependent kinases (Nourse *et al.,* 1994).

Thus, the importance of p70^{S6K} for cell cycle progression may be at least partially cell- and tissue-dependent. p70^{S6K} function is required for G1 progression and the kinase activity remains high throughout G1 (Lane *et al.,* 1993). Microinjection of antibodies directed against p70^{S6K} abolished serum-induced entry into S phase of the cell cycle, and abrogated the activation of protein synthesis (Lane *et al.,* 1993). Microinjection of neutralizing antibodies specific for the p85 isoform into the nucleus can block cell cycle progression and this block can be reversed by co-injection of the p70^{S6K} isoform (Reinhard *et al.,* 1994).

1.3   Photodynamic Therapy and Apoptosis

Photodynamic therapy (PDT) is a new therapeutic procedure being used to treat human malignancies, macular degeneration and psoriasis. PDT is a combination of light and light sensitive agents (porphyrins) in an oxygen-rich environment and is based on the dye-sensitized photo-oxidation of biological macromolecules. A photosensitive dye or

agent is delivered to the target tissue via intravenous injection (IV) or topical application. The porphyrins are selectively retained by rapidly growing tissues (e.g., cancer or psoriasis), and are activated by light. Lasers are used as the primary light source since laser light is monochromatic (one wavelength), coherent (parallel light waves allowing precise focusing), and intense (allowing for shorter treatment times). Activation is followed by the generation of toxic molecules such as singlet oxygen and other oxygen free radicals. The reactive oxygen species (ROS) generated during the excitation of the photosensitizer are thought to be responsible for the toxic effects and tissue destruction (Girotti 1990). The ROS are highly reactive and can interact with proteins, lipids, nucleic acids and other cellular components. Cellular responses to PDT include expression of stress-responsive proteins (Gomer *et al.,* 1991A; Gomer *et al.,* 1988; Curry and Levy 1993), release of cytokines (Henderson and Donovan 1989; Evans *et al.,* 1990), and induction of apoptosis (Agarwal *et al.,* 1991; Agarwal *et al.,* 1993).

Apoptosis is a morphologically and biochemically distinct form of cell death that contributes to cell turnover under normal conditions. The apoptotic process can be divided into at least three functionally distinct phases: initiation, effector and degradation. The initiation phase is heterogeneous and involves different signaling pathways depending on the type of apoptotic stimuli. The effector phase involves activation of cascades of proteases in a mitochondria-dependent manner. The degradation phase is characterized by changes in the cytoplasmic redox state and nuclear apoptosis (Kroemer *et al.,* 1997; Nicholson and Thornberry 1997). Protein kinase pathways are likely to be involved in the initiation phase of apoptosis. The SAPKs have been implicated in apoptosis induced by a variety of agents including FAS-ligation (Juo *et al.,* 1997), environmental stress (Zanke *et al.,* 1996), UV irradiation (Chen *et al.,* 1996) and withdrawal of growth factors (Xia *et al.,* 1995). PI 3-kinase and PKB have also been shown to be involved in apoptosis induced by growth factor withdrawal and UV irradiation (Kulik *et al.,* 1997; Dudek *et al.,* 1997). Treatment of cardiac myocytes with

sodium arsenite also induced stress responses and apoptosis. Interestingly, this treatment caused activation of the p38 isoform of MAP kinase, PI 3-kinase, and p70^{S6K}, but did not activate PKB, JNK, ERK1 or ERK2 (Wang and Proud 1997).

A common feature of cells in the effector phase is the activation of caspases, a family of aspartic acid-directed proteases (Alnemri *et al.,* 1996). Caspase-mediated proteolysis of specific proteins results in an irreversible commitment of cells to undergo apoptosis. Raf1 and PKB have been shown to be cleaved in a caspase-dependent manner during the apoptotic response induced by Fas activation and UV irradiation (Widmann *et al.,* 1998). Cleavage of Raf1 and PKB inhibited their kinase activity which may partially explain why the ERK kinase and PI 3-kinase pathways become inhibited during the progression of apoptosis.

ROS have also been implicated in the induction of apoptosis. For instance, apoptosis may be induced by generation of ROS or by the depletion of endogenous antioxidants (Sandstrom *et al.,* 1994; Ratan *et al.,* 1994), and conversely, can be inhibited by exogenous or endogenous antioxidants (Chang *et al.,* 1992; Schulze-Osthoff *et al.,* 1992). Furthermore, there may be a direct relationship in that apoptosis has been correlated with increases in intracellular ROS levels. As mentioned previously, the Bcl-2 family members play a role in regulation of apoptosis and Bcl-2 itself can block the increase in ROS levels associated with apoptosis (Hockenbery *et al.,* 1993; Veis *et al.,* 1993), and can inhibit ROS-induced apoptosis (Hockenbery *et al.,* 1993). Nevertheless, it seems that ROS are not essential for apoptosis to occur as anaerobically cultured cells deprived of IL-3 undergo apoptosis and Bcl-2 can protect them (Jacobson and Raff 1995). Although it is unclear precisely what role ROS play in apoptosis, PDT treatment generates ROS, leads to apoptosis, and antioxidants can protect keratinocytes against the effects of PDT. Thus, analysis of the molecular role of ROS and apoptosis in PDT will lead to a clearer understanding of the signaling systems involved in this response of cells to stresses of all types.

1.4 Rationale and Research Hypotheses

1.4.1 <u>Rationale</u>

The phosphorylation of ribosomal protein S6 plays a key role in the onset of specific protein synthesis required for cell growth and proliferation (Jefferies and Thomas 1996). During sea star oocyte maturation, dramatic changes in translation rates occur in preparation for the oocyte to become fertilized, and many protein kinases, including previously undefined S6 kinases, become activated. The $p70^{S6K}$ is intimately connected with the translational requirements through phosphorylation of the S6 protein in response to widely divergent stimuli (Bandi *et al.*, 1993; Krieg *et al.*, 1988). I therefore undertook the purification and biochemical characterization of an S6 protein kinase from mature sea star cytosolic extract in order to identify, and establish the regulatory properties of this kinase and to develop a clearer picture of the signaling processes involved in meiotic maturation.

The PI 3-kinase pathway is strongly activated by mitogenic stimulation and has been implicated in suppression of apoptosis (Yao and Cooper 1995; Scheid *et al.*, 1995). Photodynamic therapy with benzoporphyrin derivative mono-acid ring A (BPD-MA) is thought to induce apoptosis via generation of reactive oxygen species (ROS). Therefore, the investigation of the role of $p70^{S6K}$ and the PI 3-kinase/PKB signaling components in PDT-treatment in a mammalian model system was undertaken.

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1.4.2 Objectives

1. To purify the ribosomal S6 kinase that became activated during sea star oocyte maturation.

2. To characterize the purified S6 kinase biochemically.

3. To investigate the regulation of the purified S6 kinase.

4. To investigate the function of the purified S6 kinase.

5. To investigate the effect of PDT treatment on p70^{S6K} and on the potential upstream regulators, PI 3-kinase and PKB.

Chapter 2: Materials and Methods

2.1 Materials

Pisaster ochraceus were collected by all members of the laboratory from beaches in the Vancouver area. β-glycerol phosphate, EGTA, EDTA, MOPS, β-methylaspartic acid, sodium orthovanadate, ATP, methylsulfonyl fluoride, aprotinin, leupeptin, benzamidine, dithiothreitol (DTT), Coomassie brilliant blue, L-tyrosine agarose, poly-L-lysine agarose, heparin agarose, Q Sepharose, L-arginine agarose, recombinant human insulin, recombinant human EGF, phorbol 12-myristate 13-acetate (PMA), phosphatidylinositol-4,5-bisphosphate (PI(4,5)P$_2$) and the peptide inhibitor of protein kinase A (PKI) were bought from Sigma. Phosphatidylinositol (PI) was from Avanti Polar Lipids. P-81 phosphocellulose filter paper was from Whatman. [γ-^{32}P]ATP, [γ-^{32}P]azido-ATP, Tris-HCl and glycine were from ICN. Brij 35, nitro blue tetrazolium (NBT), 5-bromo-4-chloro-indolyl phosphate (BCIP), hydroxylapatite (HTP), β-mercaptoethanol (BME) and affinity-purified goat anti-rabbit IgG alkaline phosphatase conjugates were bought from Bio-Rad. Anisomycin, wortmannin and rapamycin were purchased from Calbiochem. Histones H2B, H2A and H1 were from Boehringer Mannheim. Bromophenol blue was from Schwarz-Mann Biotech. Protein A- Sepharose beads were from Pharmacia. Protran™ Pure Nitrocellulose membrane was from Schleicher & Schuell, and Polyscreen® PVDF membrane was from Biotechnology Systems. TEMED, ammonium sulfate, silver nitrate and sodium chloride were from Fisher Scientific. The S6 peptides were synthesized at the Biomedical Research Centre, University of British Columbia. The 40S ribosomal subunits were prepared from rat liver by a procedure modified from that of Kreig *et al.* (1988). Molecular weight standards for gel electrophoresis and gel filtration were prepared in our laboratory by a procedure modified from that of Griffith (1972). Anti-rabbit polyclonal antibodies produced in our laboratory were affinity-purified on peptide-agarose columns.

Potassium dichromate, glycerol and all other routine buffer chemicals were from BDH. Liposomally formulated benzoporphyrin derivative mono-acid ring A (BPD-MA) was supplied by QLT PhotoTherapeutics, Inc. (Vancouver, B.C.). Aliquots were reconstituted with distilled water at 1.5 mg/ml and stored in the dark at 4°C. Further dilutions were carried out immediately prior to experimental use with phosphate-buffered saline (PBS).

The S6 peptide column was prepared by swelling 5.0 g CNBr-activated Sepharose 4B beads for 15 min in 1 mM HCl. The beads were washed several times with 200 ml aliquots of the same solution on a sintered glass filter (porosity G3). The gel was washed with coupling buffer (0.1 M NaHCO₃, pH 8.3, 0.5 M NaCl) and incubated with 48 mg of the S6 peptide (AKRRRLASLRASTSKSESSQK) in coupling buffer overnight at 4°C. The gel was transferred to buffer to block remaining active groups (0.2 M glycine, pH 8.0) for 2 h at room temperature. The blocking agent was then washed away with coupling buffer and the column was stored at 4°C.

2.2 Preparation of mature sea star oocyte cytosol

Oocytes that were arrested in the G_2/M border prior to mitosis were isolated from sea stars as described previously (Meijer *et al.*, 1984). The immature oocytes were induced to undergo maturation by the addition of the hormone 1-methyladenine for ~2 h at 14°C. At the end of this period over 90% of the oocytes had undergone maturation as judged by the onset of germinal vesicle breakdown (GVBD). Subsequently, a 35% (v/v) oocyte homogenate was prepared in chilled homogenization Buffer A (50 mM β-glycerol phosphate, 20 mM MOPS, 2 mM EDTA, 5 mM EGTA, 1 mM sodium vanadate, 0.25 mM DTT, 1 mM PMSF, 1 mM benzamidine, 0.5 mM β-methyl aspartic acid, pH 7.2) by 2 x 30s bursts at the 18,000 setting using a Brinkmann 3000 Polytron Homogenizer. The homogenate was centrifuged at 12,000 x g for 10 min and the supernatant subsequently centrifuged at 100,000 x g for 30 min. The resulting cytosolic (supernatant) fraction was

stable for over one year when stored at -70°C and the frozen extract was used for all subsequent experiments.

2.3   Enrichment of the 40S ribosomal S6 protein kinase

Cytosol (270 ml, ~15 mg/ml protein) was thawed and diluted 1:1 with chilled 1.6 M $(NH_4)_2SO_4$ in Buffer B (50 mM Tris, 0.5 mM EDTA, 0.1 mM EGTA, 0.25 mM DTT, 1 mM β-methylaspartic acid, pH 7.2). The slurry was gently stirred over ice for 20 min, then centrifuged at 10,000 g for 10 min in a pre-cooled Beckman rotor. The supernatant was applied at 0.5 ml/min to two 25-ml L-tyrosine agarose columns equilibrated in 0.8 M $(NH_4)_2SO_4$. The columns were washed with 50 ml of 0.8 M $(NH_4)_2SO_4$ and the proteins were eluted on an FPLC (Fast Protein Liquid Chromatography) system (Pharmacia) with a 400 ml linear 0.8-0.0 M $(NH_4)_2SO_4$ gradient at a flow rate of 1 ml/min. Fractions (5 ml) were collected and assayed for phosphotransferase activity towards a synthetic peptide substrate modeled after the C-terminus of S6 (AKRRRLSSLRAGGRR). The peak activity fractions were pooled, diluted 1:10 with Buffer B and applied at 0.5 ml/min to one freshly poured 25-ml hydroxylapatite (HTP) column equilibrated in Buffer B. The loaded HTP column was washed with 50 ml of Buffer B, and eluted with a 400 ml linear gradient 0.0-0.35 M K_2HPO_4/KH_2PO_4 in Buffer B at a flow rate of 1 ml/min. Fractions (5 ml) were collected, and assayed for phosphotransferase activity. The peak activity fractions were then pooled and frozen at -70°C for later use. To complete one purification, eight of the pooled HTP fractions (ie. 16 L-tyrosine agarose elutions) were combined for the following steps which were completed over two days. The fractions were thawed slowly over ice, diluted 1:4 with Buffer B and applied to a 25-ml heparin agarose (used as a reverse column as S6 kinase does not bind to this resin) and a 25-ml poly-L-lysine agarose column linked in series. The S6 kinase was eluted from the poly-L-lysine column with a 400 ml linear 0.0-1.0 M NaCl in Buffer B gradient at a flow rate of

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1 ml/min. Fractions (5 ml) were collected and those containing S6 kinase phosphotransferase activity were then pooled, diluted 1:3 with Buffer B and applied to a 25-ml Q Sepharose anion exchange column equilibrated in Buffer B. The column was washed with 50 ml Buffer B, and the S6 kinase activity was eluted with a 300 ml linear gradient of 0.0-0.80 M NaCl in Buffer B at 1 ml/min. Fractions (4 ml) were collected, peak phosphotransferase activity fractions were pooled, diluted 1:3 with Buffer B and loaded on to a 10-ml L-arginine agarose column equilibrated in Buffer B. The column was eluted with a 120 ml linear gradient of 0.0-0.8 M NaCl in Buffer B, and fractions (2 ml) were collected. The fractions were assayed for kinase activity, peak fractions were pooled, diluted 1:3 with Buffer B, and applied to a 1-ml MonoQ column. The column was eluted with a 20 ml linear gradient of 0.0-0.8 M NaCl in Buffer B, and fractions (250 μl) were collected. The fractions were assayed for S6 kinase activity, pooled and frozen at -70°C for future use.

2.4   Kinase assays, autophosphorylation and protein assays

Ribosomal protein S6 kinase phosphotransferase activity was assayed by a procedure described previously (Sanghera *et al.,* 1992). Briefly, 5 μl of extract were assayed in a final volume of 25 μl containing 0.40 mg/ml of the S6 peptide in assay dilution buffer (ADB: 25 mM β-glycerol phosphate, 20 mM MOPS, pH 7.2, 5 mM EGTA, 2 mM EDTA, 20 mM $MgCl_2$, 0.25 mM DTT, 5 μM β-methylaspartic acid and 1 μM PKI) and 50 μM [γ-^{32}P]ATP. The reaction was incubated for 5 min at 30°C, then 20 μl were removed and spotted onto a 1.5 cm^2 piece of Whatman P-81 phosphocellulose paper. The papers were washed extensively (15x) in 1% (w/v) H_3PO_4, transferred into 6-ml plastic vials containing 0.5 ml of Ecolume (ICN) scintillation fluid, and the incorporated radioactivity was measured in a Wallac (LKB) scintillation counter. Autophosphorylation of the S6 kinase was performed in a final volume of 50 μl

containing 5 mM MgCl$_2$, 12 mM MOPS, pH 7.2 and 50 μM [γ-^{32}P]ATP. The reaction

was incubated for 30 min at 30°C, and terminated by the addition of SDS sample buffer

(125 mM-Tris-HCl, pH 6.8, 4% SDS, 0.01% Bromophenol Blue, 10% β-mercaptoethanol

and 20% glycerol). Protein was determined by the method of Bradford (1976) using

bovine serum albumin as a standard.

2.5   Electrophoresis, silver staining and immunoblotting

SDS/PAGE was performed on 1.5 mm thick gels, with acrylamide at 11% (w/v)

(unless indicated otherwise) in the separating gel and 4% (w/v) in the stacking gel, in the

buffer system described by Laemmli (1970). Samples were boiled for 5 min in the

presence of SDS sample buffer and electrophoresed for ~17 h at 10 mA. The separating

gels were silver-stained by the method of Merril *et al.* (1981) or for autoradiography, gels

were exposed to Dupont reflection film at room temperature. For immunoblotting,

SDS/PAGE gels were soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20%

methanol) for 5 min, sandwiched with a nitrocellulose membrane and transferred at 300

mA for 3 h. The nitrocellulose membrane was stained for proteins with Ponceau S, and

blocked for 2 h at room temperature in Tris-buffered saline (TBS: 150 mM NaCl, 20 mM

Tris-HCl, pH 7.5) containing 5% skim milk. The membrane was washed 3 x 5 min with

TBS containing 0.05% Tween 20 (TTBS) before incubation with p70^{S6K} specific

antibodies (in TTBS with 0.05% sodium azide 1:500 dilution) overnight at room

temperature. The next day, the membrane was washed extensively (5 x 5 min) with

TTBS before incubation with the secondary antibody (goat anti-rabbit IgG coupled to

alkaline phosphatase in TTBS with 0.05% sodium azide) for 2 h at room temperature.

The membrane was washed 2 x 5 min with TTBS then briefly with Alkaline Phosphatase

Buffer (AP: 0.1 M NaHCO$_3$ 10 mM MgCl$_2$, pH 9.8) before incubation with BCIP/NBT

color development solution (3% NBT in 70% DMF and 1.5% BCIP in 100% DMF in 100

ml AP buffer). The color reaction was terminated by successive washes in distilled water. Antibodies used for the experiments described included anti-peptide antibodies prepared in our laboratory based on the amino terminal (residues 24-40), subdomain three (residues 127-153) and carboxyl terminal (residues 509-525) of human p70^{S6K}. In addition, an anti-peptide antibody based on the carboxyl terminal (residues 483-502) of rat p70^{S6K} was purchased from Santa Cruz. Anti-peptide antibodies against HSP-60 were from StressGen. Anti-peptide antibodies to the regulatory subunit of PP2A, anti-PI-3 kinase (N-SH2) monoclonal antibody and anti-rat PI-3 kinase (p85 subunit) polyclonal antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY).

2.6   Gel filtration chromatography of sea star S6 kinase

A pre-packed Superose 12 or Superdex 200 (Pharmacia) gel filtration column was equilibrated overnight with pre-filtered, 150 mM NaCl in Buffer A at a flow rate of 0.2 ml/min. Two hundred μg each of BSA (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen (25 kDa) were loaded on to the column in 200 μl of 150 mM NaCl in Buffer A via a 200 μl injection loop. The standards were eluted isocratically with the same buffer at a flow rate of 0.2 ml/min. Fractions (250 μl) were collected and protein concentrations were measured by Bradford assay as described above. An aliquot of the purified S6 kinase (250 μl) was loaded and eluted as above, and assayed by filter paper with the S6 peptide as a substrate. The size of the purified kinase was estimated by linear regression based on the elution of the standards.

2.7   Regulation of sea star S6 kinase by ions and inhibitors

Purified S6 kinase was assayed as described above in a final volume of 30 μl containing 5 μl enzyme, 5 μl compound, 15 μl cocktail and 5 μl ATP. The ions and

inhibitors were titrated, and the concentration that caused half-maximal inhibition (IC_{50}) was determined.

2.8   Photoaffinity labelling of sea star S6 kinase with [γ-^{32}P]azido-ATP

The purified S6 kinase (150 μl) was incubated with 10 mM $MgCl_2$ and 100 μM [γ-^{32}P]azido-ATP (20,000 cpm/pmol) at 0°C. The sample was irradiated with UV light (254 nM) at a path length of 2 cm for 5 min. The reaction was terminated by addition of SDS-sample buffer, the sample was electrophoresed and the gel was autoradiographed. The control mixture was incubated under similar conditions in the absence of photoactivation.

2.9   Phosphoamino acid analysis

The purified p52^{S6K} was incubated with purified 40S ribosomes (or other substrates), electrophoresed on SDS/PAGE (as described above), and transferred to PVDF membrane for 3 h at 300 mA. The radio-labelled ribosomal protein S6 band (or other protein band) was excised from the membrane and digested in 300 μl of constant-boiling HCl at 105°C for 1h. The acid-hydrolyzed sample was dried under vacuum (Speed Vac), washed with water, re-dried, and then resuspended in 5 μl of electrophoresis buffer containing pyridine/acetic acid/water (1:10:189 by vol.). The sample was spotted on to a cellulose thin layer chromatography (t.l.c.) plate next to phosphoserine, phosphothreonine and phosphotyrosine standards (1 mg/ml each), and the plate was electrophoresed for 45-60 min at 1000 V with cooling. The plate was dried, sprayed with ninhydrin solution (1%) and baked in a 90°C oven for 10 min. The plate was then exposed to x-ray film.

2.10   Two dimensional phosphopeptide mapping

Two dimensional tryptic-phosphopeptide map analysis of the phosphorylated S6 protein or MBP was performed by excision of the radio-labelled protein band from SDS/PAGE gels, digestion of the protein with TPCK-treated trypsin in 1 ml of 50 mM NH_4HCO_3, pH 8.0 at $37^{\circ}C$ for 24 h. The samples were then dried under vacuum (Speed Vac), washed with 4 x 200 μl of water, re-dried, and then resuspended in 5 μl of electrophoresis buffer (1% NH_4HCO_3, pH8.9). The sample was spotted in the center of a cellulose t.l.c. plate 1.5 cm from the bottom. Electrophoresis was performed in the long dimension at 600 V for 40 min. After drying, the plate was chromatographed in the second dimension in butanol/pyridine/acetic acid/water (13:10:2:8 by vol). The plate was dried overnight and exposed to x-ray film.

2.11   Substrate specificity of sea star S6 kinase

Synthetic peptides were patterned after the C-terminal sequence in the S6 protein known to be phosphorylated by $p70^{S6K}$ (Bandi *et al.,* 1993). The peptides were synthesized on an A.B.I. 430A peptide synthesizer and cleaved from the resin by hydrofluoric acid. Purity of the peptides was demonstrated by reverse-phase HPLC, and the identity confirmed by ion spray mass spectrophotometry analysis (model API-III). Due to the small size of the peptides and the high purity as confirmed by reverse-phase HPLC, they did not require further purification after lyophilization. The peptides were carefully dried before weighing on an analytical balance. In the experiments shown here, the peptides were solubilized as stock solutions of 2.0 mM in ADB, and serial dilutions from 1 mM to 2 μM final peptide concentrations in a final volume of 25 μl were assayed at least three times in the presence of enriched kinase and 50 μM $[\gamma-^{32}P]ATP$. For each

peptide, K_m was determined using the Michaelis-Menton rate equation, and V_{max} was estimated using Lineweaver-Burke double-reciprocal plots of dose-response data.

2.12   Protein micro-sequencing, alignment and identification of sequenced fragments

Approximately 2.1 ml of purified MonoQ p52^{S6K} were concentrated by centrifugation in a Centricon 10 tube to a final volume of ~500 μl (~2 h at 4000 rpm). This was further centrifuged in a microfuge Centricon 10 tube to a final volume of ~150 μl (~15 min at 12,000 rpm). Fifty μl of SDS sample buffer were added, the sample was boiled 5 min and electrophoresed on 11% PAGE. A CD Immobilon membrane was pre-equilibrated in methanol for 15 min followed by equilibration in transfer buffer for 15 min. The proteins were transferred in transfer buffer containing 10% methanol for 3 h at 300 mA, and then for a further hour at 200 mA. The CD membrane was stained with 10 ml of quick stain solution A for 1 min, and then with 25 ml of quick stain solution B for 3 min (Immobilon-CD, Millipore). This negative stain is compatable with sequencing techniques. The membrane was washed for 1 min in distilled water, and the protein bands were excised, cut into small pieces and placed in 500 μl Eppendorf tubes containing tryptic cleavage buffer (0.1 M Tris, 1 M NaCl, 2 mM $CaCl_2$, 5% acetonitrile (ACN) pH 8.2 containing 0.25 μg bovine trypsin tosylphenylalanylchloromethane (TPCK) treated XII, Sigma/20μl) for 5 h at 37°C. After 5 h, 0.25 μg trypsin in 20 μl cleavage buffer containing 2 M urea were added, and the samples were incubated a further 15 h at 37°C. The supernatant was removed, the samples were washed with 20 μl 1% TFA, which was pooled with the supernatant and injected directly onto HPLC. Peaks were collected directly from the HPLC elution, and were analyzed by mass and sequenced in the laboratory of Dr. Ruedi Aebersold (University of Washington, Seattle) according to previously published protocols (Patterson *et al.*, 1992; Hess *et al.*, 1993).

2.13   Inactivation of sea star S6 kinase by phosphatase treatment

Purified protein phosphatase 2A (PP2A) was the generous gift of Drs. David Brautigan and Jian Chen (University of Virginia, Charlottesville). The enzyme activity was ~1.4 units per ml (1 unit will release 1 nmol of P_i per min from phosphate-labelled phosphorylase). Potato acid phosphatase (PAP) was prepared in our laboratory as active enzyme linked to protein A-Sepharose beads. The purified $p52^{S6K}$ was pre-incubated in the presence of active PP2A or PAP. The PP2A reaction was terminated by the addition of okadaic acid, while the PAP reaction was terminated by centrifugation of the beads and removal of the supernatant for assay. Phosphotransferase activity of the dephosphorylated purified $p52^{S6K}$ was assayed with the S6 peptide substrate as described above.

2.14   Immunoprecipitation

Unless indicated otherwise, immunoprecipitations were carried out with 500 μg protein to which 500 μl NEFT buffer (1.0 M NaCl, 5 mM EDTA, 50 mM Tris HCl, 50 mM NaF pH 7.4) containing 6% NP40 (6%NEFT) was added. The PI 3-kinase immunoprecipitations were pre-cleared with 30 μl protein A-Sepharose beads for 30 min prior to immunoprecipitation. The samples were incubated with 5 μl of antibody for 4 h, after which 30 μl of protein A-Sepharose beads were added to precipitate the adsorbed proteins. The immunoprecipitates were washed twice with 6%NEFT buffer, once with NEFT buffer and once with ADB. Immune complex assays were carried out by addition of 30 μl substrate in ADB, 10 μl $MgCl_2$ and 50 μM [γ-^{32}P]ATP, followed by incubation at 30°C for 30 min. Aliquots (20 μl) were spotted onto filter papers, washed and counted as described above. SDS-sample buffer (30 μl) were then added to the protein A-

Sepharose beads and the remaining sample was electrophoresed, transferred and immunoblotted as described above.

2.15   Immunoprecipitation of S6 phosphotransferase activity by anti-PP2A antibody

S6 kinase was partially purified via the tyrosine agarose/HTP/polylysine/Q Sepharose steps, and a 1 ml aliquot was pre-cleared with 100 μl of 1:1 slurry of protein A Sepharose beads for 15 min at 4°C. The beads were pelleted by centrifugation for 2 min at 12,000 and the supernatant was incubated with 10 μl of anti-PP2A A subunit antibody overnight at 4°C. A 1:1 slurry of protein A Sepharose beads (40 μl) were added and the mixture incubated for a further 1.5 h at 4°C. The samples were washed once with 6%NEFT buffer, once with NEFT buffer and once with Buffer B. To the precipitated proteins were added 30 μl ADB, 4 μl purified 40S ribosomes and 50 μM [γ-^{32}P]ATP, and the samples were incubated at 30°C for 35 min. The reaction was terminated by addition of 40 μl SDS sample buffer, the samples were boiled for 5 min and electrophoresed on a 12.5 % PAGE gel. The gel was transferred to a nitrocellulose membrane and exposed to film overnight.

2.16   Procedure for heat shock-treatment of sea star oocytes

Oocytes from individual *P. ochraceous* were suspended in natural sea water preheated to 35°C. Samples of the oocyte suspension were removed immediately (zero timepoint) and at various indicated times, pelleted by centrifugation, the sea water removed, and the oocytes quick frozen in a dry ice/ethanol bath. Frozen oocytes were stored at -70°C until processing. Due to the treatment, cell volumes changed during the time courses. To maintain equivalent protein concentrations, the oocytes were homogenized in a standard total volume. To each aliquot of packed cells were added 5X

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homogenization buffer (900 µl). The samples were diluted to a final volume of 6 ml and homogenized by 2 x 30 s bursts with a sonicator. The extracts were centrifuged at 40,000 rpm for 30 min, the supernatants divided into aliquots (1 ml) and frozen at -70°C.

2.17   Expression and purification of GST-S6 kinase from baculovirus

The baculoviral expression and purification of the GST-S6 kinase was carried out by Jun Yan (B.Sc., Research technician) following a protocol developed by PharMingen Canada (Instruction Manual for Baculovirus Expression Vector System, Section 4.6). *Spodoptera frugiperda* cells (sf9) (Invitrogen) cells were grown in Hink's TNM-FH insect medium supplemented with 10% FBS at 27°C. Three µg of prepared transfection plasmid pAcG2T-S6K were co-transfected with 0.5 µg of linear AcNPV BaculoGold DNA (PharMingen) into sf9 cells using the cationic liposome CellFectin mediated procedure, as described (GibcoBRL). Sf9 cells were incubated with the media from the four days post-transfected plates to amplify recombinant virus. The products from recombinant virus-infected sf9 cells were identified by immunoblotting with the anti-p70^{S6K} C/T antibody.

Approximately 500 ml of High Five cells (Invitrogen, ~5 x 10^6 cells/ml) in a 1L spinner flask were infected with the baculovirus constructs with an MOI of 3~10. After 3 days, the cells were harvested by centrifugation at 1,000 x g for 15 min. The cell pellet was washed once, resuspended in 20 ml ice-cold lysis buffer, and disrupted with a Dounce homogenizer. Debris was pelleted at 20,000 x g for 30 min and 100 µg DNAse I was added to the decanted supernatant. The cell supernatant was loaded onto a glutathione-agarose column (5 ml bed volume) at 4°C. The column was washed with 3 column volumes of STE-500 buffer (50 mM Tris pH 7.4, 500 mM NaCl and 1 mM EDTA) followed by 4 column volumes of STE-50 buffer (50 mM Tris pH 7.4, 50 mM

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NaCl and 1 mM EDTA). The beads were transferred into Eppendorf tubes, adjusted to 50% concentration with PBS and stored at -70°C.

2.18   Stimulation of GST-S6 kinase with PMA

The stimulation experiments were performed by Jun Yan (B.Sc., Research technician). About 350 ml of infected cells were incubated as above for 3 days at 27°C. The cells were pelleted in 50 ml Falcon tubes by spinning at 2,000 rpm at room temperature for 5 min, and resuspended in Excell-400 media to a total volume of 20 ml. The cells were treated with 25 or 50 nM PMA for 15 or 25 min. After stimulation, the cells were placed on ice and pelleted at 2,000 rpm at 2°C for 5 min, washed 2 x with PBS and frozen at -70°C. The fusion proteins were purified on GST-bead columns as described above.

2.19   Microinjection studies

The microinjection experiments were performed by Harry Paddon (B.Sc., Research technician). Sea stars, *Pisaster ochraceus*, were kept in the laboratory in a 100 gallon refrigerated aquarium at a constant temperature of 10°C. They were fed mussels and barnacles collected locally at low tide. For microinjection, oocytes were removed fresh from the gonad by insertion of a 16 guage needle into the arm of a sea star and gently aspirating a small volume which typically contained a few thousand oocytes. The oocytes were placed in 10 ml of filtered normal sea water (NSW) and kept on ice. Oocytes were sorted visually with a dissecting microscope (WILD M3B) at 16x power. At this magnification oocytes (~150 microns diameter) were inspected and healthy, immature oocytes were picked up individually by hand with a pulled capillary pipette and transferred to the microinjection slide. Normally, about 20 oocytes were in the 100 µl

NSW on each slide. The NSW was localized within a ring of rubber cement ~1 cm diameter made with a PAP pen (The Binding Site). The glass slides were kept at 12-13°C on a 3/8" thick aluminum plate on a thermoelectric cold plate from Thermoelectrics Unlimited Inc. Evaporation was minimized by using blue plastic Falcon caps to cover the seawater.

Microinjection of the oocytes was done using a Leitz Labovert F5 inverted microscope with differential interference contrast optics. The microscope was flanked by right and left Leitz micromanipulators which held the injection needles and holding pipettes respectively. During the microinjection the slide was kept at 10-12°C using a refrigerated microscope stage based on a Peltier element receiving variable direct current 12 volts, 0-2 amps. The microinjection needles were made on a needle puller (model P-80 Brown-Flaming micropipet puller supplied by Sutter Instrument Co., U.S.A.) set at 335°C, Gas 75, velocity 10, pull 7 from 1.0 mm glass capillaries with filament, from World Precision Instruments Inc.

The holding pipettes were made from the same pulled needles which were broken, heated and manipulated with a microforge to produce a blunt tip with an inside diameter of 10-20 microns. The microinjection needles were backfilled using pulled capillary pipettes into which approximately 1-2 µl of sample was aspirated. Once the pulled capillary was threaded down the back of the injection needle, the sample was expelled into the needle tip. The capillary was withdrawn from the needle and the needle was inserted into an Eppendorf needle holder connected by Tygon tubing to an Eppendorf 5242 microinjector. This allowed control of pressurized nitrogen to cause expulsion of the sample from the needle into the oocyte. The volume of the oocytes was approximately 2500 picolitres and 50-200 picolitres of sample was injected. The volume was judged by eye and the instrument settings to produce an injection of a certain volume that varied with each needle and during the course of the injections as the needle frequently became plugged with cell debris and needed to be changed.

2.20 Cell culture, stimulation and preparation of Pam212 cell lysates

All the tissue culture, stimulation and preparation of Pam212 keratinocyte cell lysates was performed by Dr. Jing-Song Tao (Ph.D. candidate) at QLT PhotoTherapeutics Inc. (Vancouver, B.C.). All of the subsequent experiments involving Pam212 keratinocytes were carried out as a collaboration between myself and Dr. Jing-Song Tao (Ph.D. candidate). All the immunoprecipitations, enzyme assays, lipid kinase assays and immunoblots were performed at the Kinetek site, with each of us contributing equally.

The spontaneous transformed murine keratinocyte cell line Pam212, was kindly provided by Dr. Stephen E. Ullrich (University of Texas, Houston, TX). The cell line was maintained in Dulbecco's minimum essential medium (DMEM) (Gibco) supplemented with 5% fetal bovine serum (FBS, Sigma), and antibiotics (100 U/ml penicillin, 100 U/ml streptomycin). Cultures were incubated at 37°C in 5% CO_2. For stimulation, 5×10^5 Pam212 cells were seeded to 100 mm tissue-culture dishes in 22 ml of medium and incubated for 72 h without further manipulation. After 72 h, the volume of medium was adjusted to 20 ml, and the cells were stimulated by the indicated treatment and incubated for appropriate time periods. The medium was aspirated, and the cells were washed twice with ice-cold PBS containing 1 mM Na_3VO_4. The cells were lysed by scraping them off in 800 μl of homogenizing buffer (20 mM MOPS, pH 7.2, 5 mM EGTA, 1% (W/V) Nonidet P-40, 1 mM DTT, 75 mM β-glycerol phosphate, 1 mM Na_3VO_4 and 1 mM PMSF) and sonication for 30 s. The insoluble material was pelleted by centrifugation (10,000 x g) for 15 min at 4°C and the supernatant removed, divided into aliquots, quickly frozen in liquid nitrogen and stored at -80°C.

The protein concentration of the keratinocyte lysate was determined using the bicinchoninic acid assay (Pierce, IL). The assays were performed on flat bottom 96-well tissue culture plates (Falcon) by pipetting 5 μl of variously diluted cell extracts into a

well. Protein standards were serial dilutions of bovine serum albumin (BSA). A 100 μl BCA solution was added to individual wells containing test samples and the protein standard. The plate was then incubated at 37°C for 3 h. The optical density was determined in a Dynatech MR 5000 microtiter plate reader at 565 nm (Dynatech Laboratories).

For serum starvation, a culture of 75% subconfluently growing Pam212 cells were transferred to DMEM supplemented with 0.5% FCS. The cultures were incubated at 37°C in 5% CO_2 for 36 h. The cells were then transferred to serum-free DMEM and incubated for an additional 12 h before treatment.

2.21   *In vitro* PDT treatment and UV irradiation of Pam212 cell lysates

BPD-MA at indicated concentrations was added to the medium for 12 h prior to the light treatment. The cells were then exposed to red light at a fluence of 2 J/cm^2. The light source was a light box equipped with 2 panels of General Electric red fluorescent tubes with an emission spectrum between 620 to 700 nm. UV irradiation was performed by exposing cells in 100 mm tissue culture dishes with 5 ml of medium to a General Electric germicidal lamp in a biosafety cabinet at a distance of 50 cm from the UV source for 4 min. After PDT or UV treatment, cells were incubated at 37°C in the dark for various periods of time prior to cell lysis. The entire procedure was carried out in a semi-dark room.

2.22   Insulin, EGF and anisomycin treatment of Pam212 cell lysates

Following 12 h serum-starvation, 100 nM insulin, 100 ng/ml EGF or 40 μg/ml anisomycin was added to the culture medium. The cells were returned to the 37°C incubator for an additional period of time as indicated before the cell extracts were

prepared. For inhibitor treatment, serum-starved Pam212 cells were pretreated with wortmannin or rapamycin at the indicated concentrations for 15 min prior to stimulation.

2.23 _In vitro_ lipid kinase assay

Anti-PI-3 kinase-p85 (N-SH2) immune complexes were resuspended in 50 μl of reaction buffer containing 30 mM HEPES, pH 7.4, 30 mM MgCl$_2$, 50 μM ATP, 0.2 mM adenosine, 10 μCi [γ-^{32}P]ATP, and 10 μg PI or a mixture of lipids including 2.5 μg PI, 2.5 μg PI(4,5)P$_2$ and 5 μg phosphatidylserine. The lipids were dispersed by sonication in 30 mM HEPES. The reactions were carried out at room temperature for 15 min and terminated by addition of 100 μl 1 N HCl and 200 μl chloroform/methanol (1:1, v/v). Lipids were extracted from the chloroform layer and separated by thin-layer chromatography on silica gel G plates impregnated with 1% potassium oxalate as previously described (Matter _et al.,_ 1992). PI(3)P and PI(3,4,5)P$_3$ were visualized by autoradiography and quantitated by determining ^{32}P activity in the spots in a liquid scintillation counter.

2.24 Statistical analysis

As indicated in the results section, some data were expressed as arithmetic mean±standard error of the mean (S.E.). Bars refer to standard deviation (S.D.). Data from 3 to 5 independent experiments (assuming normal distribution) were pooled and subjected to statistical analysis using analysis of variance followed by Bonferroni multiple comparison test (α=0.05). *, statistically significant difference between samples derived from variously treated cells and that from untreated control cells. *, statistically significant difference between samples derived from variously treated cells and that from insulin- or growth factor-treated cells.

Chapter 3: Results and Discussion: p70 S6 kinase in *Pisaster ochraceus*

3.1 Introduction and Model System

The meiotic maturation of sea star oocytes is a useful system for investigation of cellular regulatory mechanisms linked to protein synthesis, since during this period dramatic quantitative and qualitative changes in the synthesis of protein occurs. Isolated sea star oocytes are arrested at meiotic prophase I, and can be induced to resume meiosis when exposed to the natural hormone 1-methyladenine at a concentration of 1 μM or more for a cumulative period of time. A large germinal vesicle and its prominent nucleolus are visible in the immature oocyte, and maturation can be followed and correlated to the breakdown of the germinal vesicle (GVBD) (Fig. 4), normally within 2 hours.

There are many advantages associated with the oocyte system, particularly the ability to obtain a large amount of an essentially pure population of oocytes at the same developmental stage. Induction of synchronous maturation by the simple addition of hormone allows large amounts of mature sea star extracts to be obtained and used subsequently for analysis and protein purification. The culture and treatment of the oocytes can be performed under non-sterile conditions in the absence of expensive media. The large size of the oocytes (~150 microns diameter) allows micromanipulation such as microinjection to be carried out routinely. The activation of undefined S6 kinases has been previously described during meiotic maturation of frog and sea star oocytes (Cicirelli *et al.,* 1988; Pelech *et al.,* 1987; Meijer *et al.,* 1987) and this system was used herein to attempt to identify, purify and characterize maturation-induced S6 kinase activity.

0 min   25 min   35 min   60 min
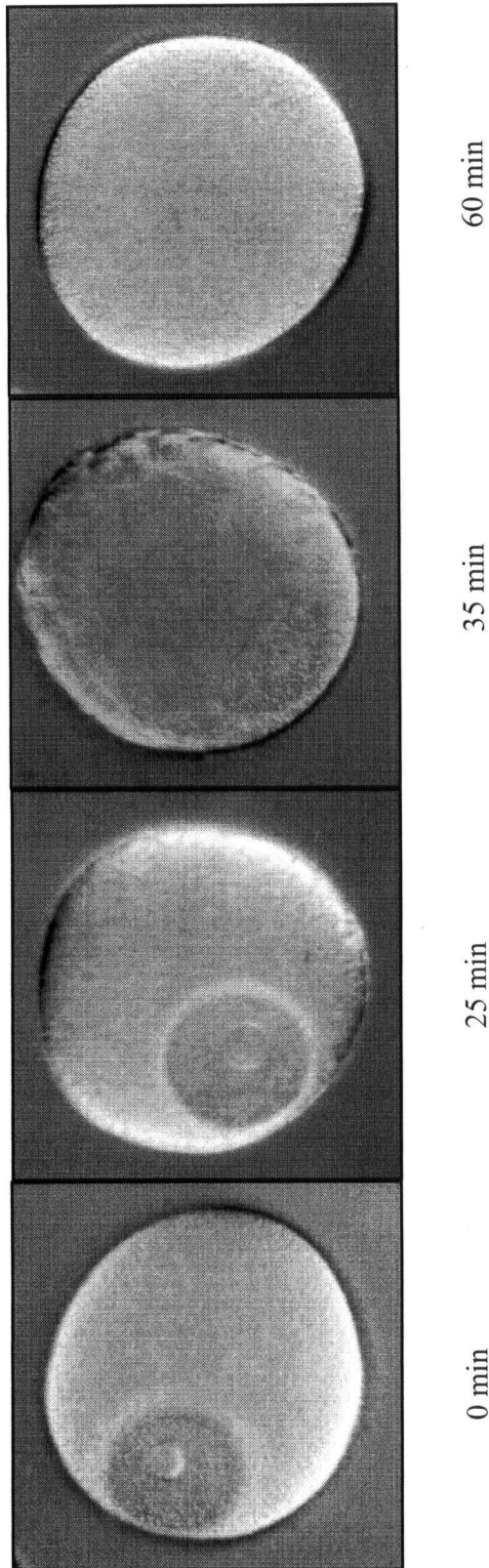
Fig. 4. 1-Methyladenine-induced maturation of P. ochraceus oocytes: Oocyte maturation can be clearly followed with the breakdown of the germinal vesicle. Panel A shows the immature oocyte with its clearly defined germinal vesicle membrane and nucleolus within. In panel B, 25 min post-induction, very little change is apparent. Within the next 10 minutes, the germinal vesicle starts to break down, with other changes visible in the cytoplasm. Panel D shows the first fully mature oocyte, 60 min after 1-methyladenine addition. With germinal vesicle breakdown complete, the mature oocyte appears homogeneous. By 90 minutes, the majority of oocytes in the population (>90%) have reached the mature state.

3.2 Identification and Enrichment of Active S6 Kinase from Mature Sea Star Oocytes

3.2.1 Identification of S6 phosphotransferase activity in mature sea star cytosolic extract

Cytosolic extracts were prepared from mature sea star oocytes treated for 90 min with 1-methyladenine or from immature oocytes that were arrested at the beginning of prophase. The extracts were fractionated by anion exchange chromatography on a MonoQ (Pharmacia) column and screened with a panel of commonly used substrates of serine/threonine protein kinases. Using a 40S ribosomal S6 protein C-terminal peptide, we detected a phosphotransferase activity that was markedly stimulated in cytosolic extracts as compared to extracts from immature oocytes (Fig. 5A). The MonoQ fractions containing S6 kinase phosphotransferase activity were probed with anti-peptide antibodies against the p70^{S6K} amino terminal (Fig. 5B), the p70^{S6K} carboxyl terminal (Fig. 5C) and subdomain III of p70^{S6K}. All three antibodies recognized a protein at ~70 kDa, the characteristic size of the p70^{S6K}, notably in fraction 26 which contained the peak phosphotransferase activity. The three antibodies also immunoreacted with a protein at ~52 kDa. MonoQ fractionation provided a partial purification, but there were certainly more than one kinase present in the fractions. Therefore, to further identify and characterize the kinase that was responsible for the S6 peptide phosphotransferase activity, I undertook to attempt its purification.

B. a-S6K-NT

69 kDa —
52 kDa —

22 23 24 25 26 27 28 29 30

C. a-S6K-CT

69 kDa —
52 kDa —

22 24 26 28 30 32 34 36 38

D. a-S6K-III

69 kDa —
52 kDa —

22 24 26 28 30 32 34 36 38

MonoQ fraction number

A.

S6 phosphotransferase activity (pmol per min/ml)

MonoQ fraction number
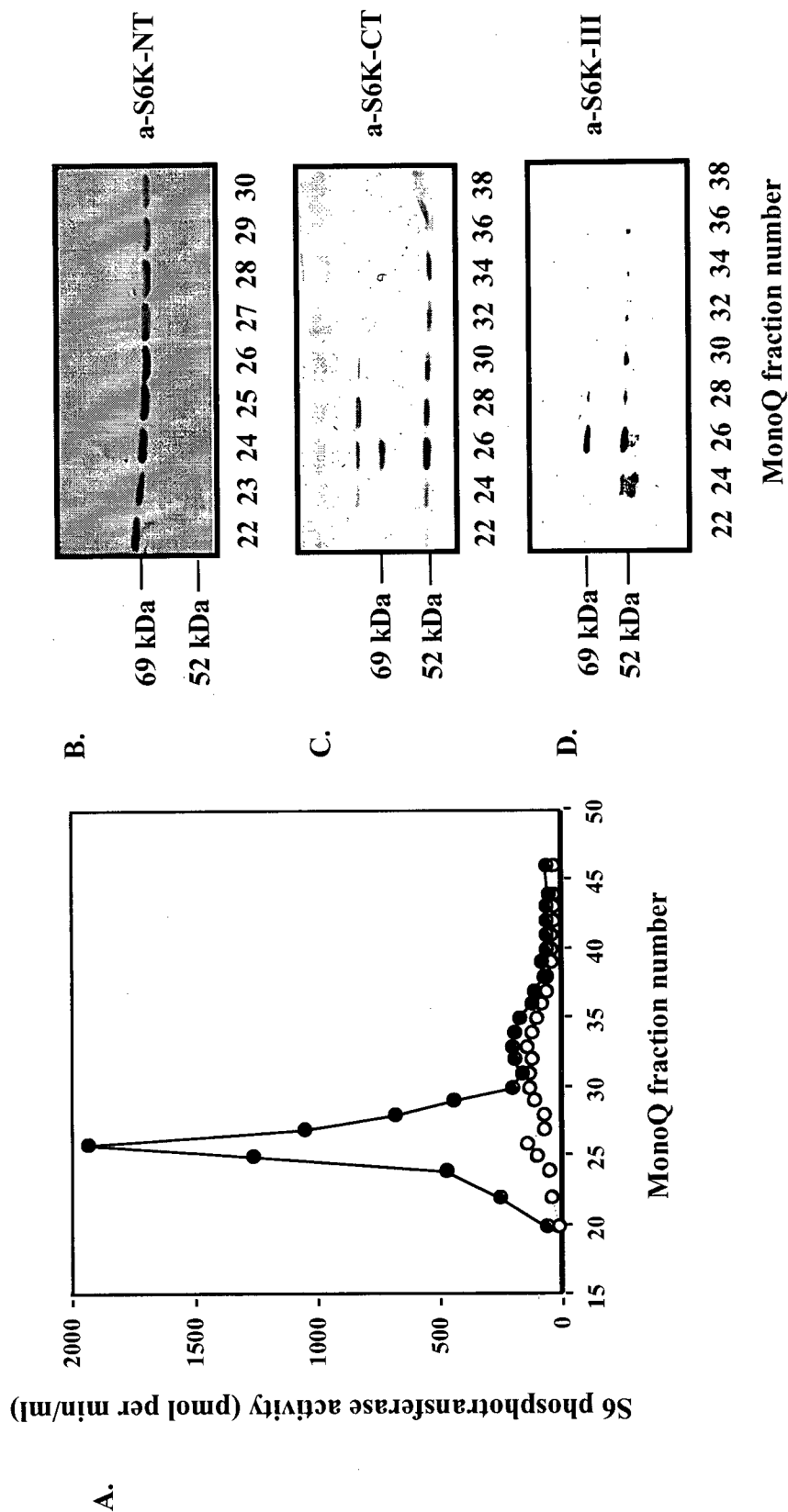
Fig. 5. Identification of S6 phosphotransferase activity in mature sea star extract: Crude immature (○) and mature seastar extracts (●) (5 mg protein) were fractionated on a MonoQ ion exchange column with a linear NaCl gradient of 0 to 0.8 M. The fractions were assayed for phosphotransferase activity against a synthetic peptide (AKRRRLSSLRASTSKSESSQK) based on the carboxyl terminal sequence in the 40S ribosomal protein S6 known to be phosphorylated by p70^{S6K} (A). The S6 kinase activity eluted from the column at approximately 0.3 M NaCl. The data shown is representative of three separate MonoQ fractionations. The fractions were immunoblotted with anti-peptide antibodies generated against the amino terminal of p70^{S6K} (B), the carboxyl terminal of p70^{S6K} (C) and subdomain III of p70^{S6K} (D).

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3.2.2   Enrichment of a 40S ribosomal protein S6 kinase from mature sea star oocytes by
        sequential column chromatography

Column chromatography matrices with a vast array of biochemical properties are commercially available. The columns allow for protein separation and purification based on the particular biochemical characteristics of the protein of interest. Some of the more common resins include ion exchangers, hydrophobic interaction, affinity, and size exclusion or gel filtration. During the course of the purification attempted here, many columns with differing biochemical properties and affinities were tested for retention of kinase activity, separation of contaminating proteins, and binding capacity. For each column tested, eluants were assayed for enzyme activity as well as protein concentration to ensure that separation of the enzyme of interest from contaminating proteins was accomplished. Table 1 lists the columns assayed, the elution buffers and retention of activity, as well as the relative purification attributed to the column. The reactive dye columns, which basically act as affinity matrices, were tested many times, both as early and late steps in the purification protocol. However, the columns that bound the S6 peptide phosphotransferase activity and provided adequate separation were not useful for purification at early stages due to low capacity, or at later stages due to the fact that nearly all contaminating proteins co-eluted. Some of the columns demonstrated differential binding depending on the point in the purification they were tested, presumably due to changes in protein concentrations. For instance, the heparin agarose column bound S6 peptide phosphotransferase activity when used early on in the procedure but did not bind this activity when used at later stages. This could be due to the presence of more than one kinase in the fractionations from earlier on in the purification. ATP-agarose was thought to be useful for purification, as this resin binds ATP-binding proteins. Unfortunately, similarly to the reactive dye columns, this column

Table 1

<u>Summary of columns tested for S6 kinase purification</u>

Column tested	Eluted with	S6K bound	Relative activity	Relative purification
Arginine-agarose	0-1.0 M NaCl	yes	+++	+
ATP-agarose	0-1.0 M NaCl	yes	+	NA
Butyl-Sepharose	0-0.4 M NaCl	no	NA	
Cellex P	0-0.8 M NaCl	no	NA	++
Cibacron blue 3GA	0-2.0 M NaCl	no	NA	
Heparin agarose	0-0.8 M NaCl	early-yes	+	++
Heptyl-Sepharose	0-0.4 M NaCl	no	NA	
Histidine-$_L$-agarose	0-1.0 M NaCl	no	NA	
Hydroxylapatite (HTP)	0-0.35 M KHPO4	yes	+++	+++
MonoS (pH 6.2)	0-0.8 M NaCl	yes	+	+
MonoS (pH6.8)	0-0.8 M NaCl	yes	+	+
Octyl-Sepharose	0-0.4 M NaCl	no	NA	
p11 phosphocellulose	0-0.8 M NaCl	no	NA	
Phenyl-$_L$-Sepharose	0-3% Brij	no	NA	
Polylysine (pH 6.2)	0-0.8 M NaCl	yes	+++	++
Polylysine (pH 7.2)	0-1.0 M NaCl	yes	+++	+++
Q Sepharose (pH 6.5)	0-1.0 M NaCl	yes	+	+
Q Sepharose (pH 7.2)	0-0.8 M NaCl	yes	+++	++
Q Sepharose (pH 8.0)	0-1.0 M NaCl	yes	+++	-
Reactive blue 4	0-2.0 M NaCl	yes	+	+
Reactive blue 72	0-2.0 M NaCl	yes	+	+
Reactive brown 10	0-2.0 M NaCl	no	NA	
Reactive green 19	0-2.0 M NaCl	no	NA	
Reactive green 5	0-2.0 M NaCl	no	NA	
Reactive red 120	0-2.0 M NaCl	no	NA	
Reactive yellow 3	0-2.0 M NaCl	yes	++	+
Reactive yellow 86	0-2.0 M NaCl	no	NA	
S6 peptide column	0-1.0 M NaCl	yes	++	++
Superose 12	150 mM NaCl		++	+
Tyrosine-$_L$-agarose	0.8-0 M NH$_4$SO$_4$	yes	++	++
Yellow Sepharose	0-1.0 M NaCl	no	NA	

had low capacity, and when used as a later step in the purification, S6 peptide phosphotransferase activity co-eluted with many contaminating proteins.

A column that did not bind the protein of interest but did bind other contaminating proteins could be used as a negative column to effect a substantial purification. I initially used a Cellex P phosphocellulose exchanger as a negative column as it bound many other proteins but the S6 kinase activity washed through the column. However, this matrix is no longer produced and the substitute, p11 phosphocellulose exchanger, exhibited different properties when used during the purification. The p11 column did not bind S6 kinase (similarly to Cellex P), but bound far fewer contaminants, and was therefore not an efficient purification step.

Representative purifications are shown in Fig. 6 and Fig. 7. One partial purification consisted of six chromatography steps (Fig. 6A) and the peak MonoQ fractions were stained with silver (Fig. 6B) and autophosphorylated (Fig. 6C) as part of the characterization of the phosphotransferase activity. Hydroxylapatite, the initial column used, has a high capacity and is often used as an early step in protein purification protocols. The resin is a crystallized form of calcium phosphate and the biochemical interaction with proteins is an electrostatic binding to calcium and phosphate sites on the matrix. The column is normally eluted with an increasing phosphate gradient, in this case potassium phosphate. The peak phosphotransferase fractions were pooled, diluted to reduce the conductivity of the sample, and applied to a p11 phosphocellulose exchanger linked in series with a poly-L-lysine column.

As mentioned above, the S6 kinase activity did not bind to the p11 phosphocellulose column and the activity washed through on to the poly-L-lysine column. As lysine is a positively charged amino acid, the column primarily acts as an anion exchanger, although it also exhibits some affinity characteristics. The peak phosphotransferase activity fractions eluted from the poly-L-lysine were pooled, diluted with buffer and applied to another anion exchanger, Q Sepharose. The peak fractions

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A. Purification procedure

Hydroxylapatite

↓

p11

↓

Polylysine

↓

Q Sepharose

↓

S6 peptide column

↓

MonoQ

B. Silver stain

106 k—
80 k—
49 k—

36 38 40 42 44* 46 48 50 52 54

MonoQ fraction number

C. Autophosphorylation

106 k—
80 k—
49 k—

36 38 40 42 44* 46 48 50 52 54
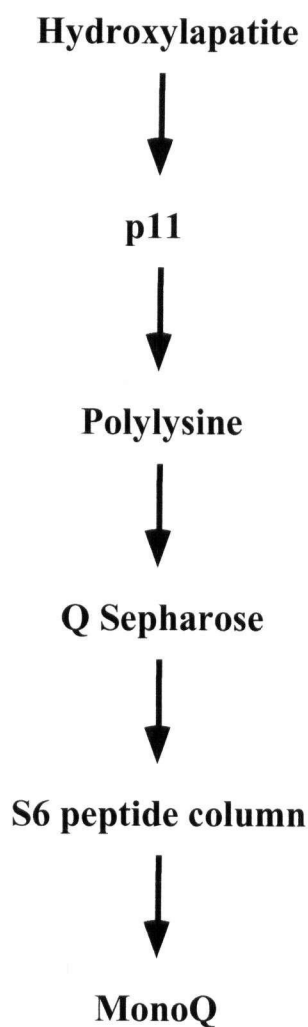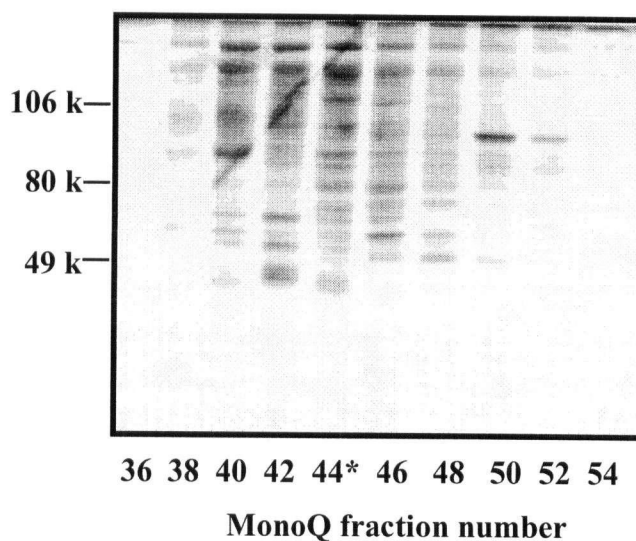
MonoQ fraction number
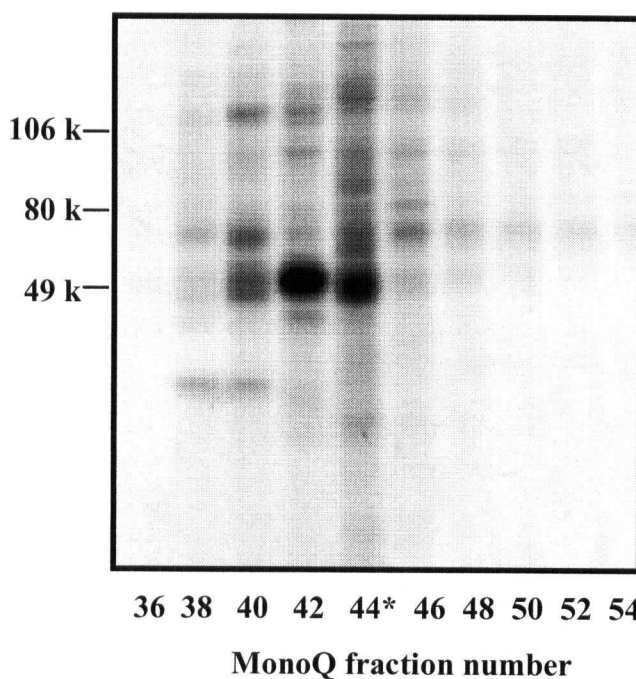
Fig. 6. Representative S6 kinase partial purification: The kinase was chromatographed over several steps (A) and the final MonoQ fractions were subjected to staining with silver (B) and autophosphorylation (C). The peak activity fraction was fraction number 44 (*) (See Fig. 7F), and the phosphotransferase activity was over 8000 pmol per minute per ml in a five minute assay.
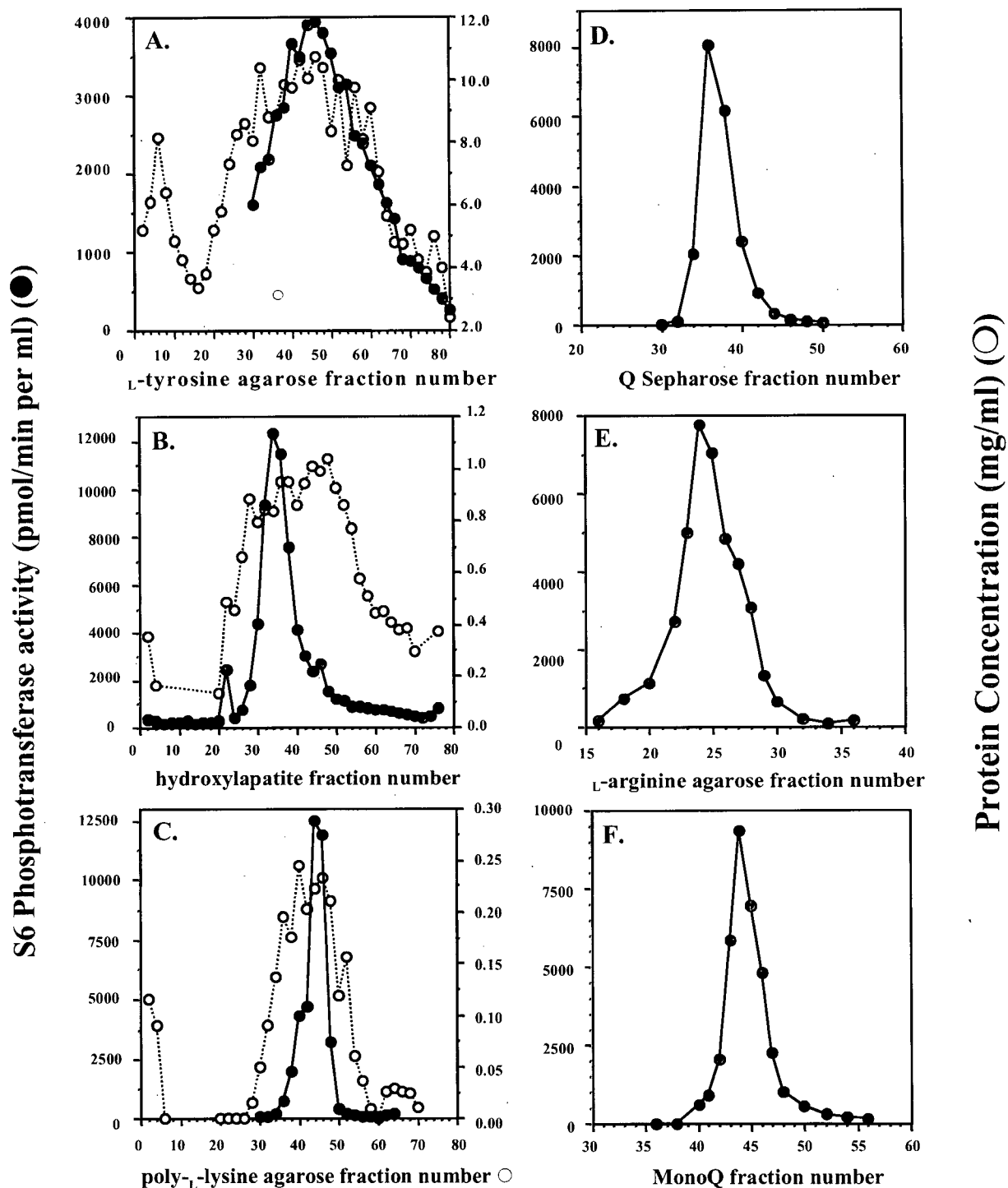
Fig. 7. Enrichment of a 40S ribosomal Protein S6 kinase: The S6 kinase activity (●) was purified to near homogeneity over a series of chromatography columns. The tyrosine agarose (A), hydroxylapatite (B) and polylysine agarose (C) fractionations include protein concentrations measured by Bradford assay (○). The protein levels were too low to measure in the final three purification steps: Q Sepharose (D), arginine agarose (E) and MonoQ (F). See Materials and Methods for the complete purification protocol.

from this column were loaded on to an affinity column prepared by linking the S6 peptide substrate to agarose beads (see Materials and Methods). This column, while exhibiting strong binding, co-eluted many of the contaminating proteins with the S6 kinase activity, and was not effective for purification. The fractions containing S6 peptide kinase were pooled and concentrated on a MonoQ anion exchange column. The peak activity fractions were electrophoresed on 11% SDS-PAGE and either stained with silver (Fig. 6B) or subjected to autophosphorylation (Fig. 6C). Many protein kinases undergo autophosphorylation, and often this intramolecular modification has an effect on kinase phosphotransferase activity. It can be seen in Fig. 6C that proteins at around 50-55 kDa in fractions 42 and 44 underwent strong phosphorylation. It is evident from Fig. 6B that there were still many contaminating proteins in the peak fractions, and it was impossible to unambiguously determine which protein corresponded to the S6 peptide kinase.

The final enrichment of the S6 kinase phosphotransferase activity is outlined in Fig. 7 and the purification table is presented in Table 2. The optimized procedure (detailed in Materials and Methods) was completed over 2 days. As the S6 kinase activity was not labile, it was not significantly decreased by the length of purification treatment which ranged up to 4 days. The initial ammonium sulfate precipitation produced a substantial purification as approximately 20% of the total protein was precipitated at 0.8 M $(NH_4)_2SO_4$, while the S6 kinase remained in the soluble fraction. This treatment raised the ionic strength of the cytosolic fraction sufficiently to allow the kinase to bind to the hydrophobic L-tyrosine agarose resin (Fig. 7A). Hydroxylapatite (HTP) fractionation also provided significant purification as the kinase activity eluted from the column at ~0.10-0.21 M K_2HPO_4/KH_2PO_4 (Fig. 7B). Heparin-agarose was used to bind contaminating proteins, and the wash-through material containing the S6 kinase activity was directly applied to poly-L-lysine agarose. The phosphotransferase activity eluted from the poly-L-lysine agarose column at approximately 0.46 - 0.54 M NaCl (Fig. 7C). The S6 kinase activity was loaded on to an anionic Q Sepharose column, and eluted between 0.33 and

Table 2

Enrichment of S6 kinase from mature sea star oocytes

Column Step	Volume (ml)	Total Activity (nmol/min)	Protein (mg)	Specific Activity (nmol/min/mg)	Recovery (%)	Purification (-fold)
homogenate	720	350	10800	0.032	100	1.0
L-tyrosine agarose	640	286	6000	0.048	82	1.5
hydroxylapatite	320	240	320	0.75	69	23
poly-$_L$-lysine agarose	35	175	7	25	50	772
Q Sepharose	28	130	1.5	87	37	2,690
$_L$-arginine agarose	10	56	0.05	1120	16	34,600
MonoQ	1.25	8	0.005	1600	2	50,000

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0.40 M NaCl (Fig. 7D). Prior to this step, protein concentrations had been measured by Bradford (1976) assay, but due to low levels of protein present for the latter part of the purification, protein concentrations were estimated from pooled fractions.

As the consensus sequence for phosphorylation of the S6 protein by known p70^{S6K} isoforms contains several arginine residues, it was thought that an $_L$-arginine agarose column might effectively act as an affinity matrix. The S6 kinase activity eluted from this column between 0.26 and 0.32 M NaCl (Fig. 7E). Finally, pooled fractions from the $_L$-arginine agarose column were concentrated on a MonoQ column from which the S6 kinase phosphotransferase activity eluted between 0.30 and 0.35 M NaCl (Fig. 7F). Five MonoQ fractions (250 µl) containing the peak of S6 kinase phosphotransferase activity were pooled, aliquoted into 100 µl samples and immediately frozen at -70^{0}C. The averaged results of three separate purifications are shown in Table 2. Beginning with ~11 g of cytosolic protein, ~5 µg of the enzyme was enriched over 50,000 fold with a final recovery of ~2% of the starting activity.

For rapid and convenient screening, column fractions were assayed with a cocktail containing a peptide based on the known p70^{S6K} phosphorylation sites in the S6 protein. The specificity of phosphorylation was enhanced in the kinase assays by the addition of a variety of inhibitors and activators. The peptide inhibitor (PKI) of protein kinase A (PKA) was routinely added to all assays. In addition, assays were performed in the presence or absence of calcium and lipid co-factors to ensure that protein kinase C (PKC) isoforms were not present in the fractions. The concentration of [γ-^{32}P]ATP in the kinase assays was assessed, with 50 µM [γ-^{32}P]ATP final concentration found to be optimum (Data not shown). The peptide substrate is able to be phosphorylated by other kinases, while the 40S ribosomal protein S6 is selective for S6 kinases. Therefore, to ensure that the kinase activity being enriched was due to S6 kinase activity, the sea star enzyme preparation was assayed in the presence of purified 40S ribosomes. The S6 kinase was purified by a slightly different protocol (Fig. 8A), and assayed in the presence of 5 µg of

A. Purification procedure

Hydroxylapatite

↓

Ammonium
sulfate ppt.

↓

Tyrosine agarose

↓

Polylysine

↓

Q Sepharose

↓

S6 peptide column

↓

Superdex 200

↓

MonoQ
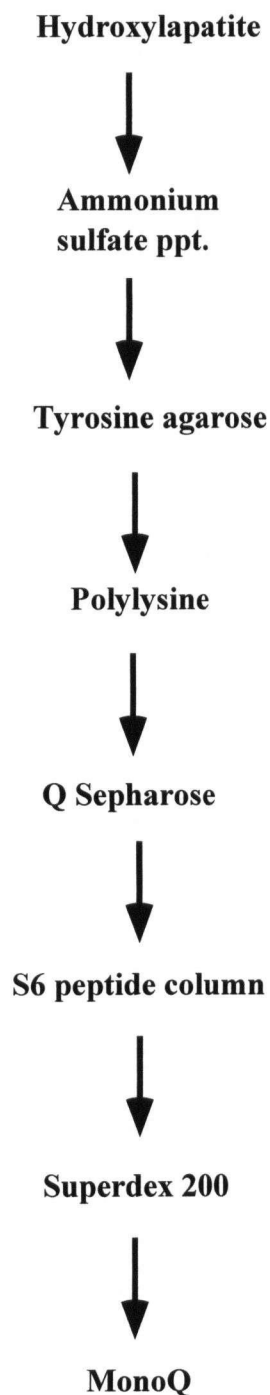
B. Phosphorylation of ribosomal protein S6

MonoQ fraction number
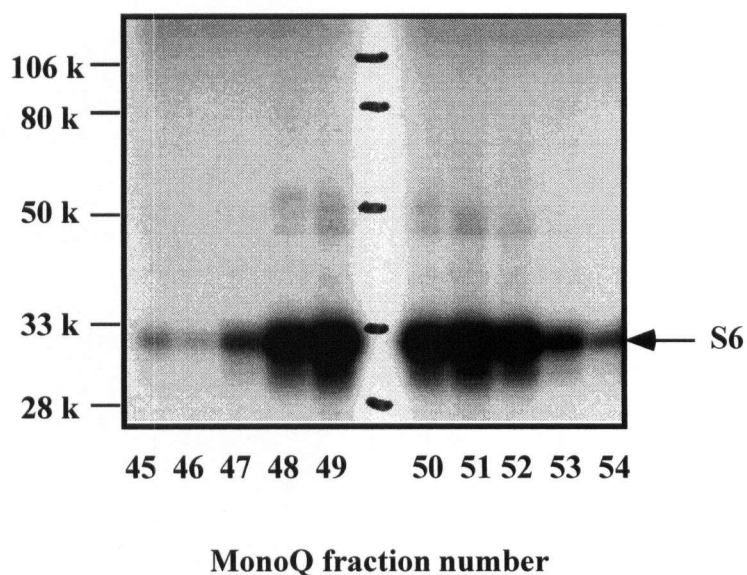
C. Silver stain of ribosomal protein S6

MonoQ fraction number
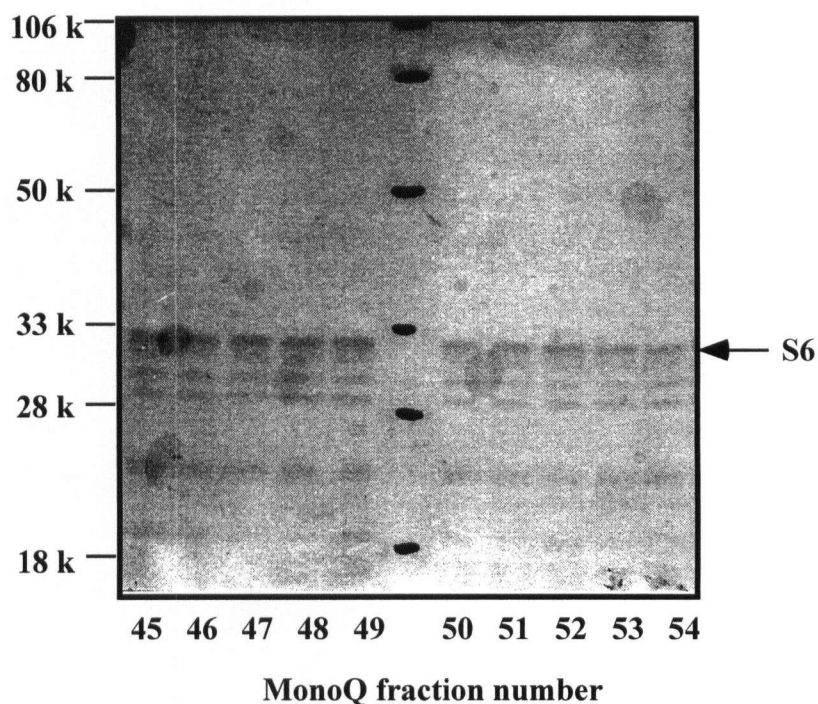
Fig. 8. Phosphorylation of 40S ribosomal proteins by the sea star S6 kinase: The S6 kinase was enriched over several column chromatography steps (A). The kinase was incubated in the presence of 5 mg purified 40S ribosomes, and ADB for 30 min at 30°C. The samples were electrophoresed on 11% SDS-PAGE, transferred and exposed to film (B). The same fractions were stained with silver (C). The data shown was representative of three separate experiments.

purified 40S ribosomes, electrophoresed and the gel exposed to film (Fig. 8B). The same fractions were stained with silver (Fig. 8C), demonstrating similar amounts of total protein in each fraction. The fractions containing the peak phosphotransferase activity in filter paper assays with the peptide substrate (fractions 49 and 50) also contained the peak phosphotransferase activity against the S6 protein (~33 kDa) (Fig. 8B). Additional lower molecular weight protein bands were likely degradation products of the purified 40S ribosomes. A phosphorylated protein at about 50 kDa can also be seen in panel B, though the incorporation of phosphate was significantly less than that incorporated into the ribosomal proteins. This phosphorylation could be due to autophosphorylation of the kinase, or it could be due to phosphorylation of a contaminating protein present in the enriched preparation.

3.2.3   Immunological identification of sea star S6 kinase as homologue of $p70^{S6K}$

The MonoQ fractions containing the enriched enzyme were electrophoresed on SDS-PAGE (11%) and the proteins were visualized by staining with silver (Fig. 9A). Several distinct protein bands coincided with the peak of S6 kinase activity, and these had apparent molecular masses of ~60 kDa, 55 kDa and 52 kDa. The major protein at ~52-55 kDa in the enriched preparation was identified as $p70^{S6K}$ by immunoblotting with a polyclonal antibody directed against the amino terminus of the mammalian enzyme (Fig. 9B). Several immunoreactive protein bands ranging in size from ~50 to 55 kDa were apparent, perhaps reflecting differences in phosphorylation state or possibly, proteolyzed fragments of the $p70^{S6K}$. The same fractions did not immunoreact with the carboxyl terminal or subdomain III antibodies (data not shown). The enriched preparation was probed with a number of antibodies directed against a variety of protein kinases including casein kinase 2 (CK2), ribosomal S6 kinase (Rsk), ERK 1/2, p38 HOG1, SAPK, and PKCα, β and γ. None of these antibodies demonstrated any immunoreactivity with the enriched fractions (Data not shown).

A. Silver stain

B. anti-S6K-N/T immunoblot

C. Autoradiogram
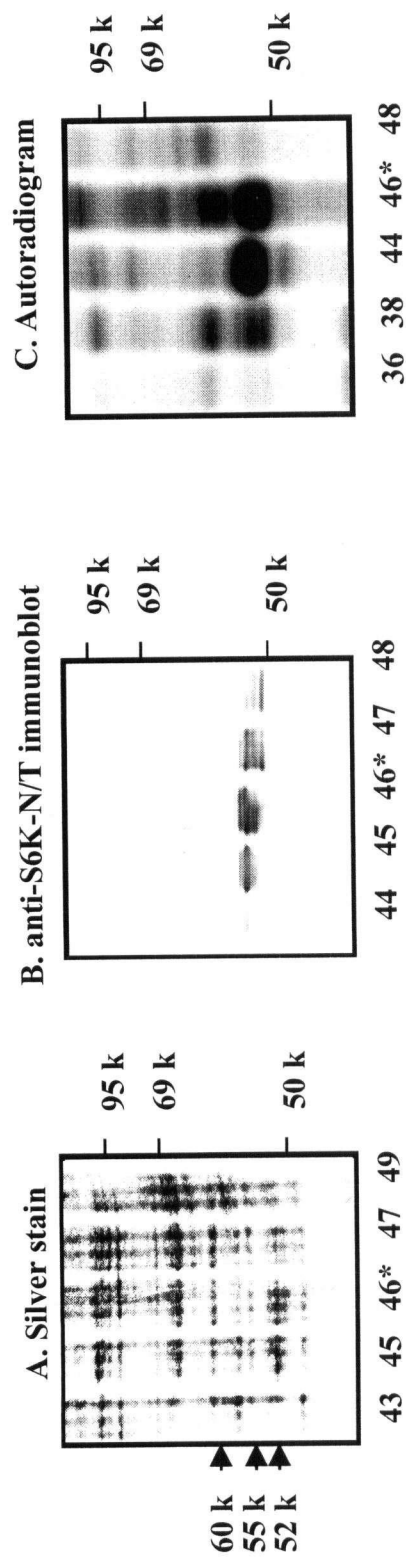
Fig. 9. Silver stain, immunoblot and autophosphorylation of enriched preparation of sea star S6 kinase: The monoQ fractions containing S6 peptide phosphotransferase activity were electrophoresed on SDS-PAGE (11%), and stained with silver (A). The major protein bands are marked with arrows. The same fractions were immunoblotted with the p70^{S6K} N/T antibody (B), and subjected to autoradiography (C). Migration positions of molecular weight standards are shown. The data shown were representative of at least three separate experiments.

Many kinases have been shown to undergo autophosphorylation and this intramolecular event often correlates with changes in phosphotransferase activity (Ahn *et al.,* 1992; Cobb *et al.,* 1991). An autophosphorylation reaction of the purified kinase showed that protein bands at about 52-55 kDa became strongly phosphorylated as did several other, higher molecular weight proteins (Fig. 9C). Again, this phosphorylation could be due to autophosphorylation of the kinase, but could also be due to cross-phosphorylation of, or by, contaminating proteins.

Size exclusion, or gel filtration chromatography is a form of separation based on the molecular size of proteins. The matrix contains pores of specific sizes that exclude proteins larger than that size. The elution is performed isocratically, usually in a salt buffer, and is thought to maintain protein complexes. Gel filtration analysis of the purified kinase yielded a single peak of phosphotransferase activity with an apparent molecular size of 50-55 kDa (Fig. 10).
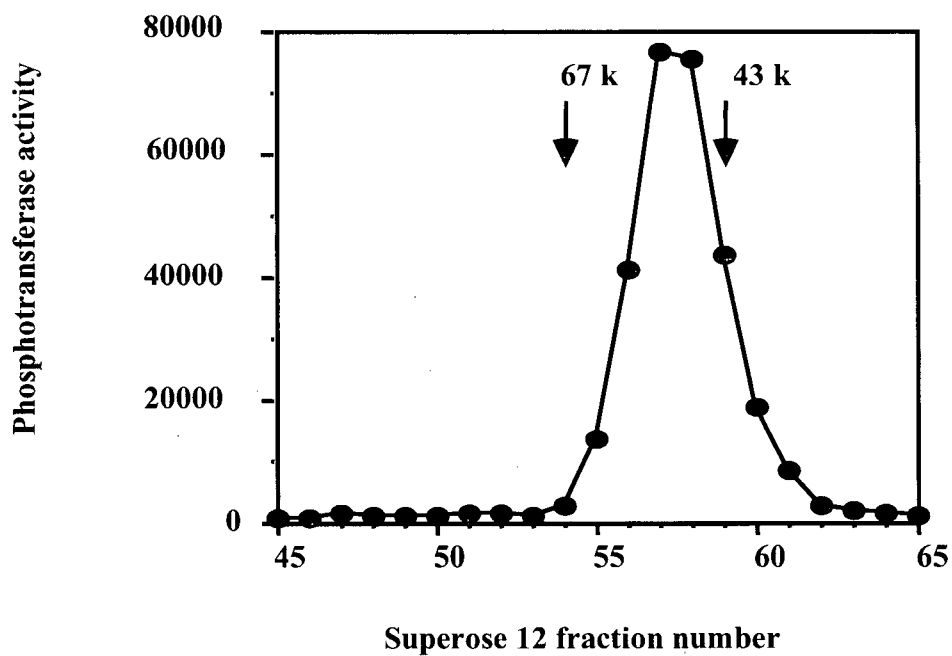
Fig. 10. Gel filtration chromatography of the enriched sea star S6 kinase:
The purified kinase was chromatographed over a Superose 12 gel filtration
column. The column was eluted with 150 mM NaCl, and the fractions were
assayed for phosphotransferase activity with the peptide substrate. Standards
applied to the column (200 μg each) were BSA (67 kDa), ovalbumin (43 kDa)
and chymotrypsinogen (25 kDa). The data shown was representative of three
separate experiments.

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3.3 Characterization of Active S6 Kinase from Mature Sea Star Oocytes

3.3.1 Immunological identification of partially purified sea star S6 kinase

The ~52-55 kDa enriched sea star S6 kinase immunoreacted with an amino terminal antibody, but was not recognized by antibodies generated against other regions of the kinase (Fig. 9). Further, antibodies directed against carboxyl and internal sequences of p70^{S6K}, as well as the amino terminal antibody recognized ~52-55 kDa proteins in MonoQ fractionated sea star extracts (Fig. 5). Therefore, immunoblot analysis of a partially purified form of the kinase was carried out. After two column steps (tyrosine agarose and hydroxylapatite) proteins were visualized by staining with Coomassie brilliant blue (Fig. 11A). Several protein bands were apparent, especially a major protein at ~75 kDa and another at ~60 kDa. Polyclonal antibodies against the amino terminal (Fig. 11B), carboxyl terminal (Fig. 11C) as well as subdomain III (Fig. 11D) of the p70^{S6K} recognized ~75 kDa and ~60 kDa proteins at this earlier stage of the purification. An ~75 kDa protein was also recognized by the carboxyl terminal antibody in the MonoQ fractionated sea star extract (Fig. 5). Slight immunoreactivity was also seen at ~52 kDa with the antibodies against the carboxyl terminal (Fig 11C) and subdomain III (Fig. 11D). Thus, it was possible that several distinct, homologous proteins were present in sea star, and that the purification procedure had effectively purified the ~52-55 kDa form.

A. Coomassie Blue stain

97 k
67 k
51 k

B. anti-p70^S6K N/T

97 k
67 k
51 k

C. anti-p70^S6K C/T

97 k
67 k
51 k

D. anti-p70S6K III

97 k
67 k
51 k
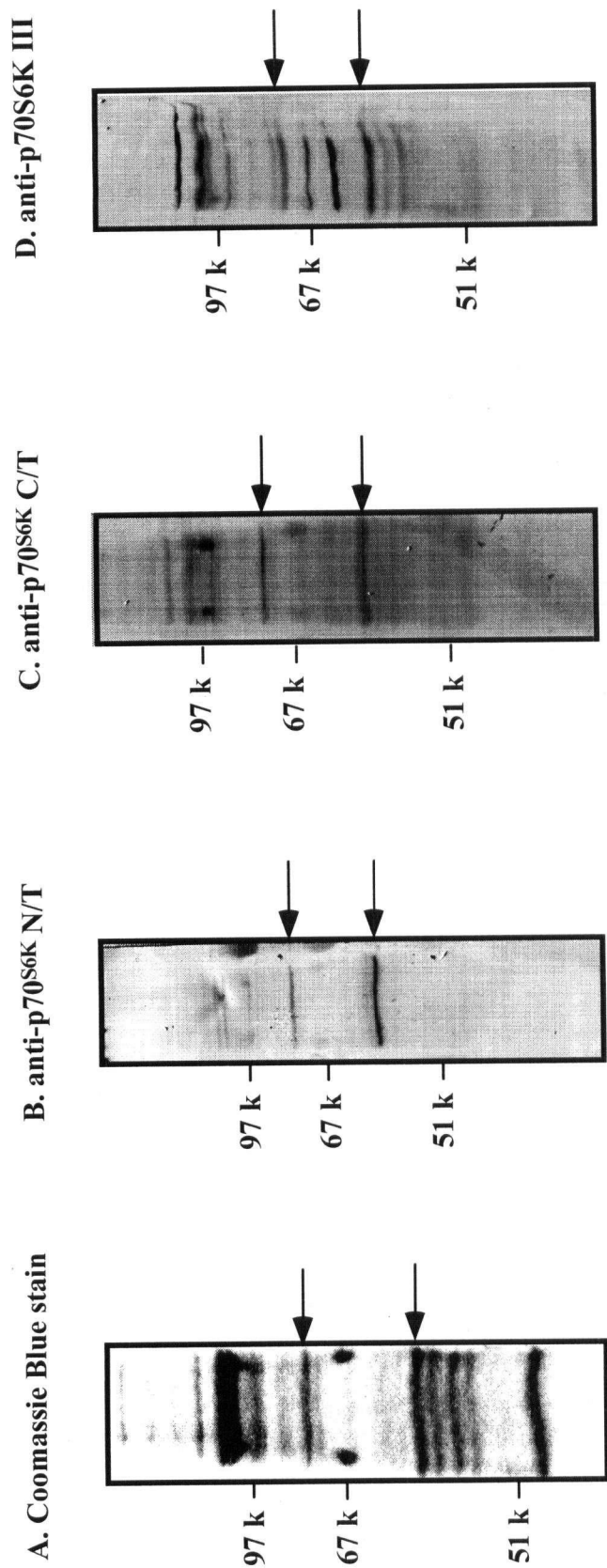
Fig. 11. Immunological identification of partially purified sea star S6 kinase: A partially purified preparation of the S6 phosphotransferase activity (tyrosine agarose and hydroxylapatite steps) was visualized with Coomassie Brilliant Blue staining (A) and immunoblotting with anti-p70^S6K N/T (B), anti-p70^S6K C/T (C) and anti-p70S6K subdomain III (D) antibodies.

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3.3.2 Dose- and time-dependence of sea star S6 kinase against the S6 peptide

To optimize assay conditions for further analyses of the enriched kinase, its phosphotransferase activity with respect to concentration of peptide substrate and length of incubation was investigated. The phosphotransferase activity of the purified kinase was tested in filter paper assays with the S6 peptide at varying concentrations (Fig. 12A). At final concentrations below about 0.06 mg/ml, the phosphotransferase activity of the purified kinase was decreased, while concentrations over about 1.0 mg/ml were found to inhibit the kinase phosphotransferase activity. This inhibition could be due to steric hindrance caused by high peptide concentrations. It could also be due to the presence of contaminating kinases or phosphatases in the enriched preparation. The same phenomenon was also noted during the substrate specificity studies outlined in Section 3.3.8. The optimum concentration for activity was around 0.40 mg/ml, and this concentration of peptide was used for the following experiments unless noted otherwise. Dose response assays were carried out multiple times to obtain the data detailed in Section 3.3.8 characterizing the substrate specificity of the enriched kinase preparation. The time course of incubation with substrate was plotted as counts per min as well as pmol/min per mg (Fig. 12B). The optimum incubation time was from 5 to 10 min, and a 10 min incubation time was used for subsequent experiments.
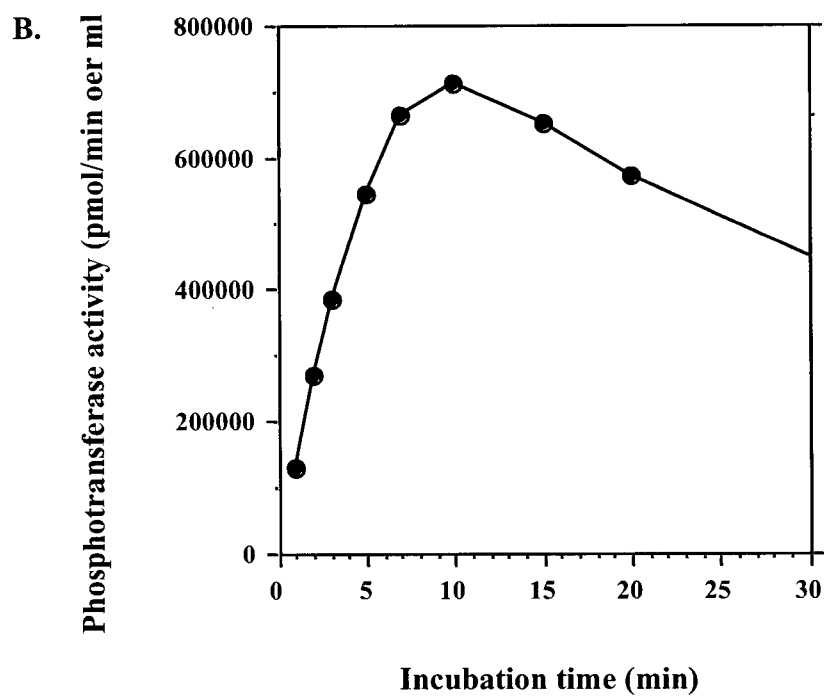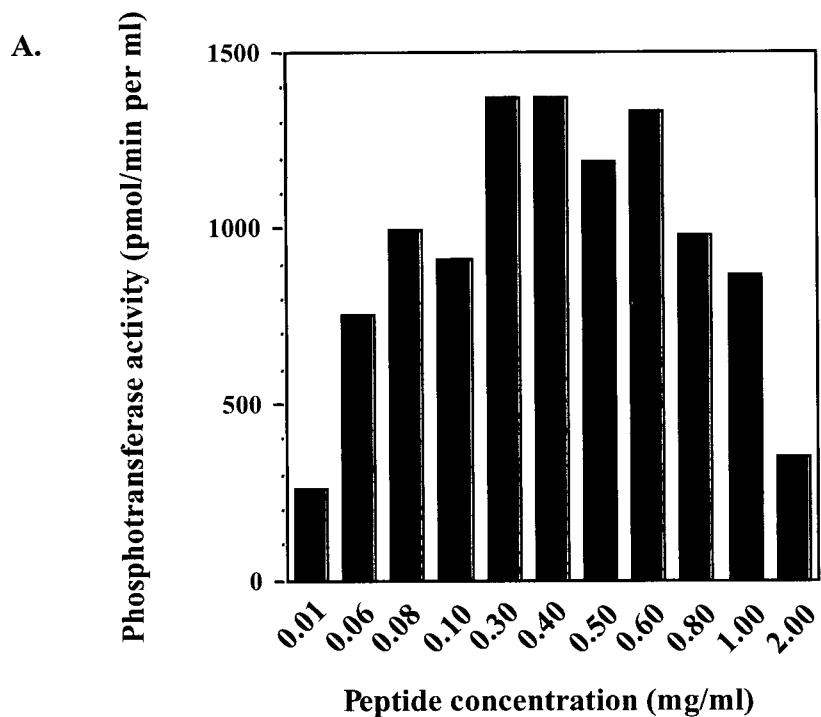
Fig. 12. Concentration dependence and time course of the purified sea star S6 kinase: S6 kinase was incubated for 5 min in the presence of various concentrations of S6 peptide substrate (A), and with 0.40 mg/ml S6 peptide substrate for different incubation times (B).

3.3.3 Regulation of sea star S6 kinase by a variety of ions and inhibitors

All protein kinases require metal co-factors, usually magnesium, for phosphotransferase activity. The enriched $p52^{S6K}$ utilized either magnesium or manganese for the phosphotransferase reaction. To further characterize the requirement of $p52^{S6K}$ for various ions and the effect of inhibitors on kinase activity, the enriched kinase was incubated in the presence of a variety of molecules, and the concentration that caused 50% maximal inhibition (IC_{50}) was determined (Table 3). $p52^{S6K}$ was inhibited by sodium fluoride but was relatively insensitive to high concentrations of β-glycerol phosphate, EGTA, dithiothreitol, spermine, heparin, NaCl and metal ions such as Mn^{2+}, Zn^{2+} and Ca^{2+}.

$p70^{S6K}$ was first purified from the livers of cycloheximide-treated rats (Price *et al.*, 1989; Kozma *et al.*, 1989) and from vanadate-stimulated Swiss 3T3 cells (Jenö *et al.*, 1989). Both purified enzymes had a molecular size of ~70 kDa. Ferrari *et al.* (1991) have characterized the $p70^{S6K}$ that was purified and cloned from the livers of rats treated with cycloheximide. They found that the requirement for Mg^{2+} could not be substituted by Mn^{2+}, and that Mn^{2+} actually inhibited the $p70^{S6K}$ activity at an IC_{50} of 100 μM. In addition, they found that Zn^{2+} was a potent inhibitor of $p70^{S6K}$ activity, with an IC_{50} of 2 μM. The enzyme was inhibited by protamine, and sodium fluoride, similarly to the enriched $p52^{S6K}$ (see Table 3 and Fig. 16). The S6 kinase purified from Swiss 3T3 cells was found to be inhibited by Mn^{2+} (Jenö *et al.*, 1989).

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Table 3

Summary of various inhibitors of purified sea star S6 kinase

Compound	Concentrations tested	IC$_{50}$
Zinc	2.5 - 150 mM	15 mM
Calcium	2.5 - 250 mM	40 mM
Manganese	2.5 - 250 mM	15 mM
DTT	5.0 - 100 mM	>100 mM
EGTA	35 - 485 mM	300 mM
Spermine	0.5 - 40 mM	>40 mM
NaCl	0.2 - 5.0 M	>5 M
NaF	15 - 300 mM	60 mM
β-Glycerophosphate	60 - 800 mM	>800 mM
Heparin	2 - 200 mg/ml	>200 mg/ml
cAMP	1 - 20 μM	>20 μM

3.3.4 Phosphorylation of 40S ribosomal proteins by sea star S6 kinase

Although the S6 peptide substrate was very useful for quickly and efficiently assaying the S6 kinase phosphotransferase activity, the physiological substrate is the intact S6 protein. Therefore, the enriched sea star kinase was further characterized with respect to phosphotransferase activity against the 40S ribosomal protein S6. The kinase was incubated in the presence of varying amounts of purified 40S ribosomes, electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose and exposed to film (Fig. 13). A protein evident at ~31 kDa corresponded to the predicted size of the intact S6 protein. The incorporation of phosphate into the S6 protein correlated with increasing quantity of purified 40S ribosomes added to the incubation mixture. The autoradiograph of the ribosomal proteins clearly showed phosphorylation of a protein at ~50 kDa similar to that seen in Fig. 8.

3.3.5 Photoaffinity labelling of sea star S6 kinase with [γ-^{32}P]azido-ATP

Autophosphorylation reactions were carried out as part of the general characterization of the enriched protein kinase. Several proteins became phosphorylated during an autophosphorylation reaction (Fig. 9), and a protein at ~50 kDa became phosphorylated during incubation of the purified kinase with purified 40S ribosomal proteins (Fig. 8 and Fig. 13). It was unclear whether the kinase itself became phosphorylated, or if the signal seen was due to cross-phosphorylation of contaminating proteins in the preparation. To distinguish between these possibilities, photolabelling was carried out in the presence of [γ-^{32}P]azido-ATP. [γ-^{32}P]azido-ATP cross-links to ATP-binding proteins such as protein kinases so that the kinase cannot release the ATP.

The enriched sea star S6 kinase was incubated in the presence of [γ-^{32}P]azido-ATP at 0°C (Fig. 14). The mixture was kept in the dark, and the [γ-^{32}P]azido-ATP was

40S ribosome concentration (units)
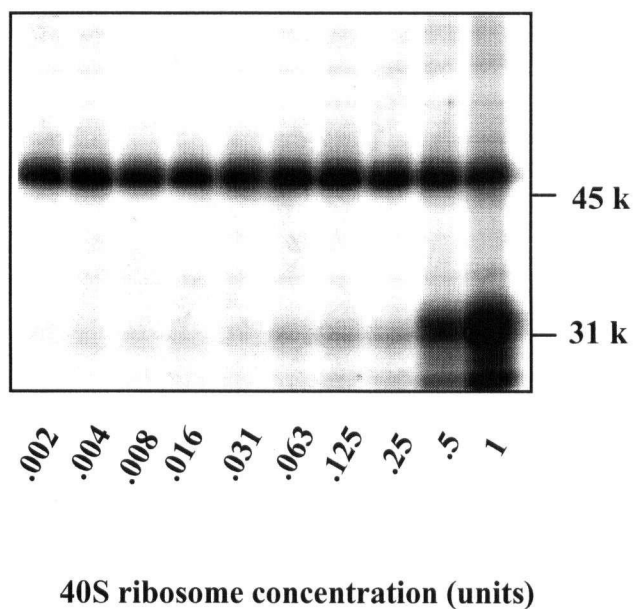
Fig. 13. Concentration response of enriched S6 kinase with 40S ribosomal proteins:
Varying amounts of purified 40S ribosomal proteins were incubated with the enriched
kinase. The proteins were subsequently separated by SDS-PAGE (10%), transferred to
nitrocellulose, and exposed to film. The 40S ribosomal subunits were standardized
and measured as 100 U/ml reading the absorbance at 260 nM, with 1 unit equivalent
to 5 mg of protein.
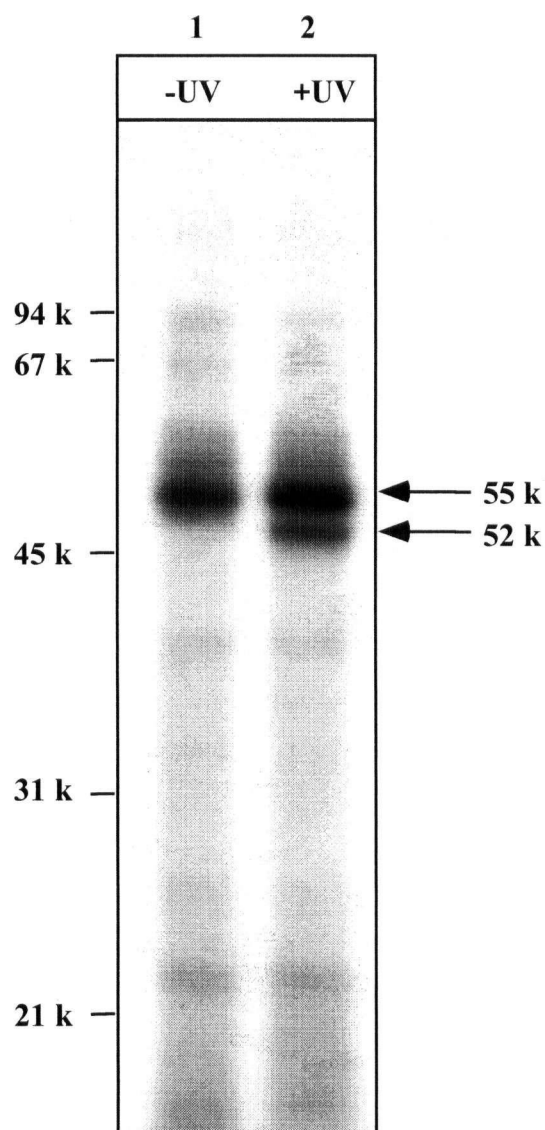
Fig. 14. Photoaffinity labelling of enriched S6 kinase with [γ-^{32}P]azido-ATP: The enriched sea star kinase was incubated at 0°C with 10mM MgCl$_2$ and 100μM [γ-^{32}P]azido-ATP in the absence (lane 1) or presence (lane 2) of UV light at 254 nm at a distance of 2 cm. The reaction was terminated after 5 min by the addition of SDS sample buffer, and the samples were electrophoresed on 11% SDS-PAGE, and exposed to film. The data shown was representative of two separate experiments.

activated by a 5 min exposure to UV radiation. The reaction was terminated with SDS

sample buffer, and the proteins were electrophoresed, and the gel exposed to film. A

protein of about 52 kDa could be seen in the $[\gamma\text{-}^{32}P]$azido-ATP-treated sample, that was

not present in the control sample. Based on immunoblot results, the ~52 kDa protein was

identified as p52^{S6K}, while the phosphorylated protein at ~55 kDa was identified as

protein phosphatase 2A (PP2A). The data regarding identification of PP2A will be

discussed in detail in Section 3.4.

3.3.6   <u>Phosphoamino acid and 2 dimensional phosphopeptide mapping of the 40S</u>

<u>ribosomal protein S6 phosphorylated by sea star S6 kinase</u>

The S6 protein is known to become hyperphosphorylated in reponse to many

various types of stimuli. The S6 phosphorylation sites consist of a series of five serine

residues in a short, carboxyl region of the protein that are phosphorylated in a sequential

manner (Bandi *et al.,* 1993; Kreig *et al.,* 1988; Martin-Perez and Thomas, 1983).

Although other kinases can phosphorylate some of the S6 sites *in vitro*, only p70^{S6K} has

been shown to phosphorylate all five sites in the order that they are phosphorylated *in*

vivo (Franco and Rosenfeld 1990). I therefore used the enriched sea star S6 kinase to

phosphorylate a preparation of purified 40S ribosomes and the S6 protein was isolated

prior to phosphoamino acid and phosphopeptide analysis.

Purified 40S ribosomes (50 µg) were incubated with 50 µM $[\gamma\text{-}^{32}P]$ATP and

enriched p52^{S6K} at 30°C for 30 min. The proteins were separated by SDS-PAGE,

transferred to PVDF membranes, and subjected to phosphoamino acid (Fig. 15A) and 2D

phosphopeptide analysis (Fig. 15B). The S6 protein was phosphorylated exclusively on

serine residues, consistent with other reports for mammalian p70^{S6K} (Bandi *et al.,* 1993;

Kreig *et al.,* 1988). The 2D phosphopeptide map generated by the tryptic digest of the

phosphorylated S6 protein produced at least 8 distinct radio-labeled spots (Fig. 15B)

A. Phosphoaminoacid analysis of the S6 protein phosphorylated by enriched p52 ^{S6K}
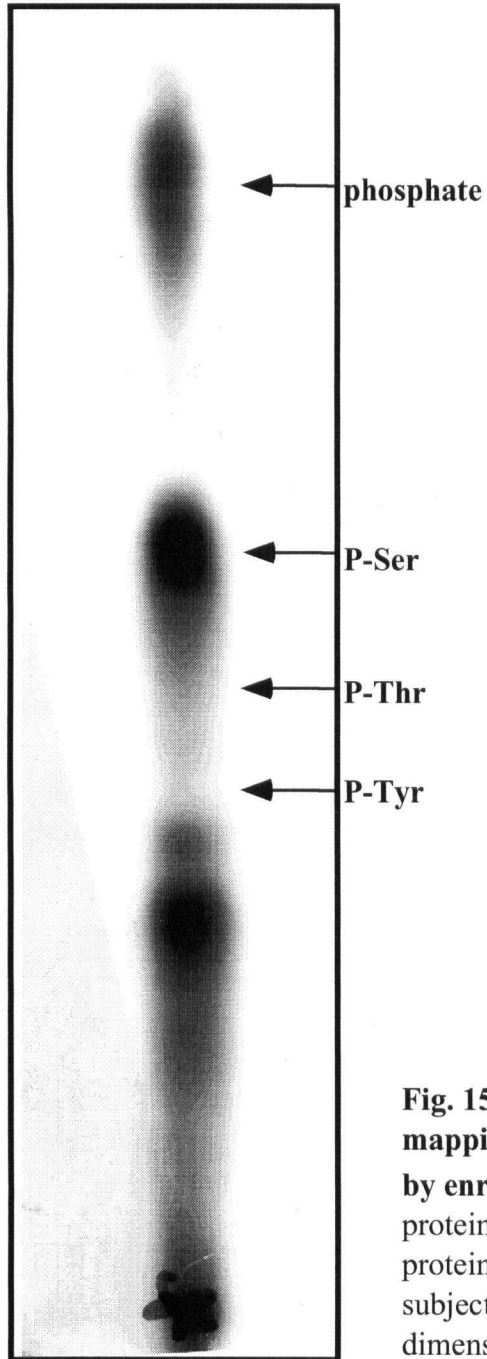
B. Two dimensional tryptic map of the S6 protein phosphorylated by enriched p52 ^{S6K}
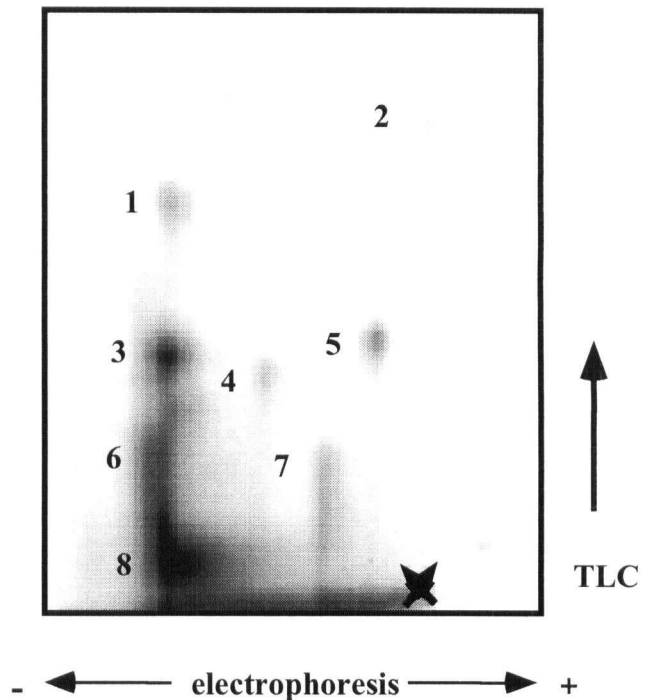
Fig. 15. Phophosphoamino acid and 2D phosphopeptide mapping of the 40S ribosomal protein S6 phosphorylated by enrched sea star S6 kinase: Fifty μg of 40S ribosomal proteins were incubated with the enriched kinase. The proteins were separated by electrophoresis and were subjected to phosphoamino acid analysis (A) and 2-dimensional phosphopeptide analysis (B). The data shown were representative of two separate experiments.

indicating several phosphorylation sites. Treatment of the most highly phosphorylated form of S6 from serum- or growth factor-stimulated cells with trypsin has been shown to generate up to 10-11 major phosphopeptides (Martin-Perez and Thomas, 1983; Martin-Perez *et al.*, 1984), so it is possible that I have not induced full phosphorylation of the S6 protein with the sea star enzyme. The incubation time was 30 min and it is possible that further incubation was required for full phosphorylation to occur.

3.3.7   Phosphotransferase activity of sea star S6 kinase against a variety of exogenous substrates

The enriched sea star S6 kinase was analyzed by *in vitro* filter paper assays with respect to its phosphotransferase activity against a variety of common exogenous serine/threonine kinase substrates (Fig. 16). Two separate preparations were assayed and the kinase activity was measured as a percentage of the phosphotransferase activity of the enriched enzyme against the S6 peptide substrate. Myelin basic protein (MBP) (~85%) and histone H2A (HH2A) (~45%) were both efficient substrates for the kinase while histone H2B (~10%), histone H1 (~15%), casein (~5%) and protamine (~15%) were not efficient substrates. The $p70^{S6K}$ purified from cycloheximide-treated rats was found to phosphorylate histone H2B, glycogen synthase, and a number of hepatic nonhistone nuclear proteins in addition to efficiently phosphorylating the S6 protein (Price *et al.*, 1989). The enzyme that was purified from Swiss 3T3 cells was found to phosphorylate the S6 protein, but did not phosphorylate casein, histones 2A and 3S, or phosvitin.

To further analyze the phosphotransferase activity, MBP, HH2A and HH1 were phosphorylated by the enriched kinase, electrophoresed, and the gel was exposed to film (Fig. 17A). The phosphate-containing protein bands were excised, and subjected to phosphoamino acid analysis (Fig. 17B). MBP and H2A were phosphorylated exclusively on serine residues, while HH1 was phosphorylated on both serine and threonine residues.

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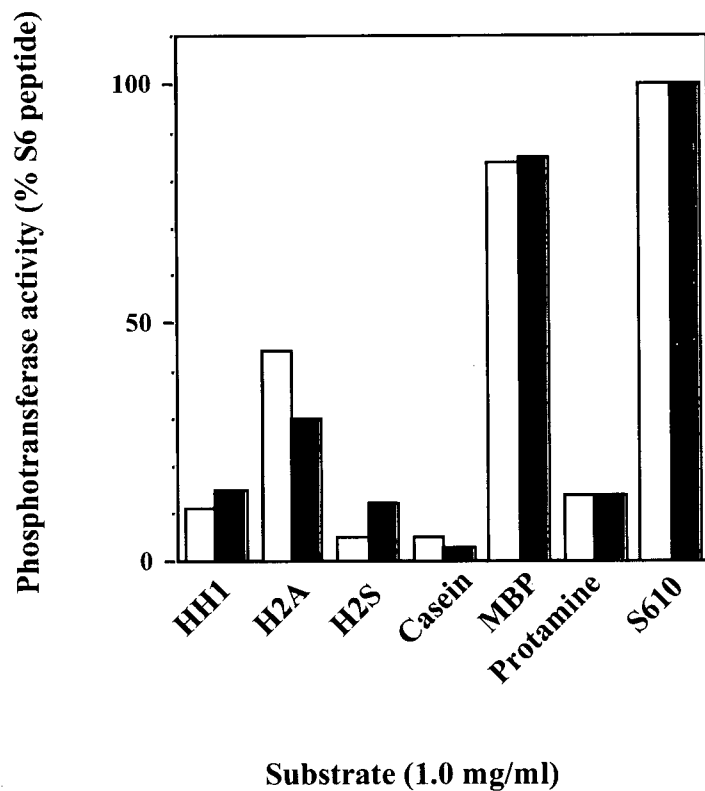
Fig. 16. Phosphotransferase activity of enriched sea star S6 kinase against a variety of exogenous substrates: The enriched kinase was incubated in the presence of a series of common protein kinase substrates, and the phosphotransferase activity was quantitated as a percentage of that of the S6 peptide. All the proteins were assayed at a final concentration of 1.0 mg/ml except the S6 peptide which was assayed at a final concentration of 0.4 mg/ml. The open and solid bars represent results from two separate purifications.

B. Phosphoamino acid analysis
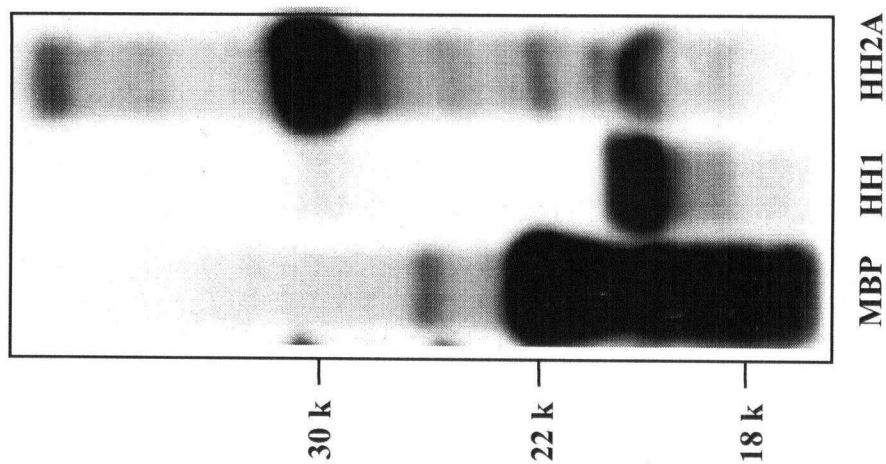
A. Phosphorylation by S6 kinase
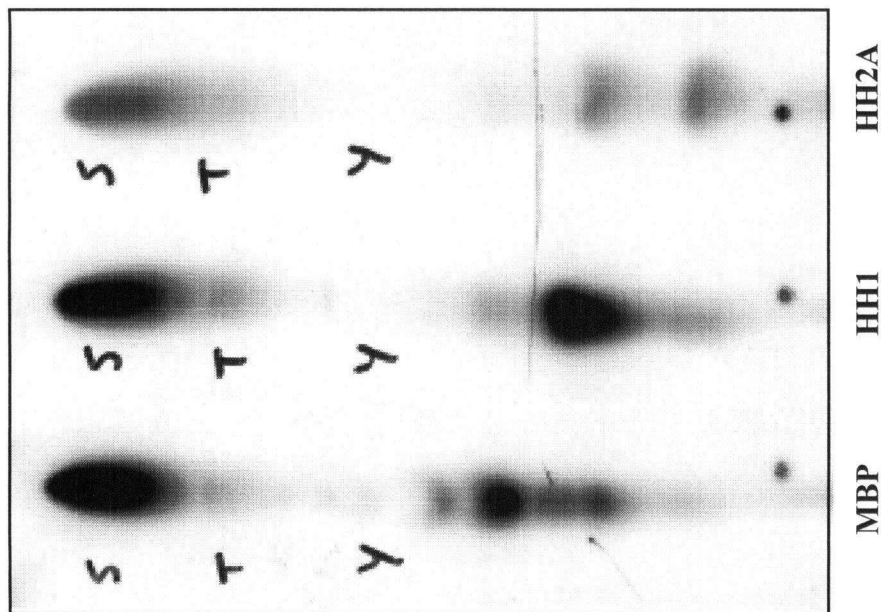
Fig. 17. Phosphorylation of exogenous substrates with enriched sea star S6 kinase: Myelin basic protein, histone H2A and histone H1 were incubated in the presence of the enriched sea star S6 kinase. The proteins were separated by electrophoresis, transferred, and the gel was exposed to film (A). The phosphorylated protein bands were subjected to phosphoamino acid analysis (B). S,T and Y correspond to the migration positions of phosphoserine, phosphothreonine and phosphotyrosine, respectively. The data was representative of two experiments.
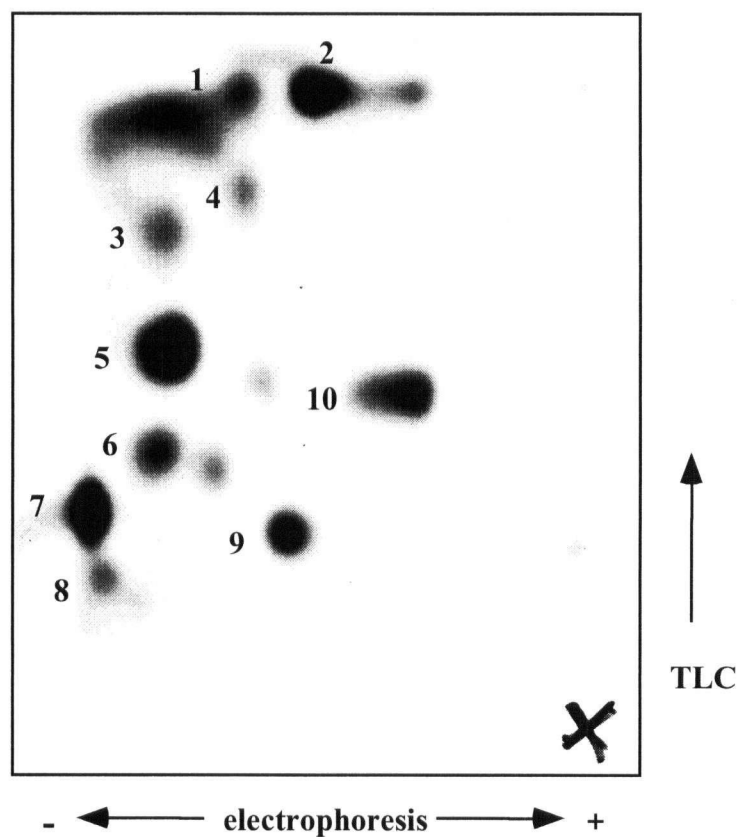
Fig. 18. 2 dimensional tryptic phosphopeptide map of MBP phosphorylated by enriched sea star S6 kinase: Myelin basic protein was incubated in the presence of enriched S6 kinase. The proteins were separated by electrophoresis, transferred and the protein bands were subjected to 2 dimensional tryptic phosphopeptide analysis.

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Phosphorylated MBP was further analyzed by 2-dimensional phosphopeptide digestion, and at least ten distinct phosphorylated spots were evident (Fig. 18). This was the first demonstration that MBP is a reasonable substrate for S6 kinase *in vitro*.

3.3.8 Substrate specificity of sea star S6 kinase

The consensus sequence of optimal peptide substrates for mammalian $p70^{S6K}$ has been determined (Flotow and Thomas, 1992) and the minimal sequence required for recognition was deduced to be R(R)RXXSX, where X could be any amino acid. The purified mammalian kinase had an apparent K_m for 40S ribosomal subunits of 0.25 μM, and the apparent K_m for a 32 amino acid synthetic peptide substrate was 2.5-fold higher. The enriched sea star $p52^{S6K}$ was therefore analyzed using synthetic peptides based on the C-terminus of the S6 protein. The peptides were constructed by step-wise alteration of specific amino acid residues surrounding the phosphorylation site. Each peptide was assayed a minimum of three times as described in the Material and Methods Section, the outlying values were discarded, and the K_m values were estimated from linear Lineweaver-Burke plots (Table 4). As mentioned in Section 3.3.2, several of the peptide substrates actually inhibited the phosphotransferase activity at higher concentrations. It is possible that this observation was due to the presence of other kinases or phosphatases (see Section 3.4.1) in the enriched preparation.

Kinetic analyses of the various peptides provided some intriguing differences from the previously reported consensus sequence for $p70^{S6K}$ isoforms. Flotow and Thomas (1992) found that the arginine residues in the -3 and -5 positions were essential for activity, while arginine in the -4 position was not as important. In our analyses, we found that an arginine residue in the -3 position was critical for kinase activity (K_m and V_{max} of 0.30 μM and 3.7 nmol/min per mg), but that arginine in the -4 position was dispensable and an additional arginine at the -5 position was actually inhibitory (K_m and

Table 4

Summary of substrate specificity of purified sea star S6 kinase

Peptide Sequence	$K_m(\mu M)$ (apparent)	V_{max} (nmol/min per mg)	V_{max}/K_m
1. AKRRRLS**S**LRA**S**TSKSE**S**SQK	0.22	7.3	33
2. RRRLS**S**LRA**S**TSKSE**S**SQK	0.19	5.7	30
3. RRLS**S**LRAGGRR	0.27	3.6	13
4. RLA**S**LRAGGRR	0.30	3.7	12
5. RRLA**S**LRAGGRR	0.31	3.5	11
6. AAQKRP**S**QRTKYLA	0.34	3.6	11
7. RRLA**T**LRAGGRR	3.91	23.8	6.1
8. RRLA**S**ERAGGRR	0.40	1.4	3.6
9. RRRLA**S**LRAGGRR	0.77	1.8	2.4
10. AKRRRA**S**A	0.79	0.9	1.1
11. AKRRRL**S**-amide	2.07	1.0	0.5
12. RRLA**S**RAGGRR	8.40	0.7	0.08

V_{max} of 0.77 μM and 1.8 nmol/min per mg) (compare peptide number 4, 5 and 9 in Table 4).

In addition, it was found that an arginine residue at the +2 position enhanced kinase activity (compare peptide number 5 with 12). Replacement of the hydrophobic leucine at the +1 position with an acidic residue (compare peptide number 5 with 8) was also inhibitory to kinase activity. The sites in the S6 protein are phosphorylated sequentially *in vivo* in the order Ser-236>Ser-235>Ser-240>Ser-244>Ser-247 (Krieg *et al.,* 1988; Martin-Perez and Thomas, 1983), so peptides with Ser-235 changed to alanine were tested (compare peptide number 3 with 5), and it was confirmed that Ser-236 was also preferentially phosphorylated by p52^{S6K}. Another interesting observation was that changing Ser-236 to threonine (compare peptide number 5 with 7) resulted in K_m and V_{max} being affected in opposite directions. The S6 protein becomes phosphorylated strictly on serine residues *in vivo*, and clearly threonine was not efficiently phosphorylated by p52^{S6K} in our assay. The consensus sequence for phosphotransferase activity of the enriched preparation was therefore found to be RXXSXR where the amino acid in the +1 position cannot be an acidic residue. The apparent K_m and V_{max} values for the best peptide substrates were approximately 0.2 μM and 6-7 nmol/min per mg respectively. It is certainly possible that substrate phosphorylation by S6 kinase is synergistic such that phosphorylation of one serine leads to increased phosphorylation of subsequent sites. This effect could explain why some serine residues that do not conform to the ideal consensus sequence can be phosphorylated in the S6 protein.

3.3.9 Inactivation of sea star S6 kinase by dephosphorylation

The enriched p52^{S6K} was preincubated in the presence of protein phosphatase 2A (PP2A) or potato acid phosphatase (PAP) on protein A-Sepharose beads. The phosphatase reactions were inactivated by the addition of okadaic acid (PP2A) or by pelleting the beads by centrifugation (PAP), and kinase activity was measured in the presence of the peptide substrate (Fig. 19). Control samples were assayed in the presence of p-nitrophenylphosphate (p-NPP) to ensure that no residual phosphatase activity remained after addition of the inhibitor or removal of the PAP beads. The kinase became dephosphorylated and inactivated by pretreatment with either PP2A and PAP. The S6 kinase purified from liver of cycloheximide-treated rats was also completely deactivated by incubation with PP2A in both autophosphorylation and 40S S6 protein phosphorylating activities (Price *et al.,* 1990).
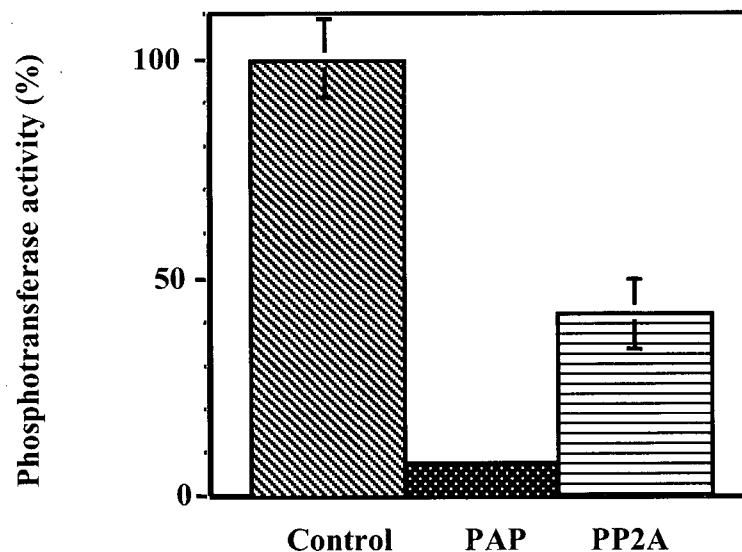
Fig. 19. Enriched S6 kinase is dephosphorylated and inactivated by potato acid phosphatase and protein phosphatase 2A: Enriched S6 kinase was pre-incubated in the presence of PAP or PP2A, then assayed for phosphotransferase activity against the S6 peptide substrate. The experiment shown here was the average of three separate assays (+/-SEM)

3.4 Complex Formation of Active Sea Star S6 Kinase

3.4.1 Identification of co-purifying proteins by micro-sequencing and immunoblotting

As previously shown, several distinct protein bands were apparent in the enriched enzyme preparation by silver staining. Three of these had apparent molecular masses of ~60 kDa, 55 kDa and 52 kDa. To produce enough material to allow micro-sequencing of the proteins, a large-scale purification was completed. An equivalent of five complete purifications (~3.5 liters) of mature cytosolic sea star extracts were used to generate approximately 35 μg of protein. The proteins were separated by electrophoresis, transferred to CD membrane and treated as outlined in the Materials and Methods section. The micro-sequencing was carried out in the laboratory of Dr. Ruedi Aebersold, first at the Biomedical Research Center (Vancouver, B.C.) and later at the University of Washington (Seattle). Micro-sequence analysis identified the ~60 kDa protein as HSP-60, an abundant heat shock protein that co-purified with the kinase (Table 5). Four separate peptides were sequenced (T4, T5, T6 and T7). Of these, T4 was identical in 10 of 12 amino acids to the published sequence of human HSP-60. T5 was identical in 3 of the 3 amino acids that were unambiguous, T6 was identical in 8 of 8 residues, and T7 was identical in 6 of 8. In addition, the ~55 kDa protein and the ~52 kDa protein were identified as the regulatory subunit of protein phosphatase 2A (PP2A) by protein microsequence analysis (Table 5). The T1 peptide was a perfect match to *Drosophila* PP2A alpha subunit in all 16 amino acids sequenced, while the T2 peptide was identical in 8 of 11 residues. The T3 peptide was identical in 9 of 10 residues to the *porcine* PP2A beta subunit.

The same samples were subsequently stained with silver (Fig. 20A) and immunoblotted with antibodies directed against HSP-60 (Fig. 20B) and PP2A (Fig. 20C). The immunoblots confirmed the identification and presence of HSP-60 and PP2A in the

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Table 5

Alignment and Identification of Sequenced Fragments

Band ID Protein MW	Peptide Number	Sequence	Identification
Band 1-52 kDa	T1	GEYNVYSTFQSHEPEF	PP2A-ALPHA
"	T2	MFEEPEDPSNR	PP2A-ALPHA
Band 2-55 kDa	T3	LSSGDWFTSR	PP2A-BETA
Band 3-60 kDa	T4	IGGSSDVEVNEK	HSP60
"	T5	XXXFXDN	HSP60
"	T6	GANPVEIR	HSP60
"	T7	VQDAMNAT	HSP60

Band 1 Alignment with PP2A-ALPHA

PP2A ^{61}ANPRRGEYNV YSTFQSHEPE FDYLKSLEIE EKINKIRWLQ QKNPVHFLLS^{110}
T1　　　　　　GEYNV YSTFQSHEPE F

PP2A ^{181}YSSSKGTIRL CDMRSAALCD RHSKQFEEPE NPTNRSFFSE IISSISDVKL^{230}
T2　　　　　　　　　　　　　　　　MFEEPE DPSNR

Band 2 Alignment with PP2A-BETA

PP2A ^{121}PSDLEAHFVA LVKRLASGDW FTSRTSACGL FSVCYPRVSS AVKAELRQYF^{170}
T3　　　　　　　　　LSSGDW FTSR

Band 3 Alignment with HSP60

HSP60 ^{121}RSIAKEGFEK ISKGANPVEI RRGVMLAVDA VIAELKKQSK PVTTPEEIAQ^{170}
T6　　　　　　　　　GANPVEI R

HSP60 ^{301}KAPGFGDNRK NQLKDMAIAT GGAVFGEEGL TLNLEDVQPH DLGKVGEVIV^{350}
T5　　　　XXXFXDN

HSP60 ^{391}NERLAKLSDG VAVLKVGGTS DVEVNEKKDR VTDALNATRA AVEEGIVLGG^{440}
T4/T7　　　　　　　　　IGGSS DVEVNEK　　VQDAMNAT

88
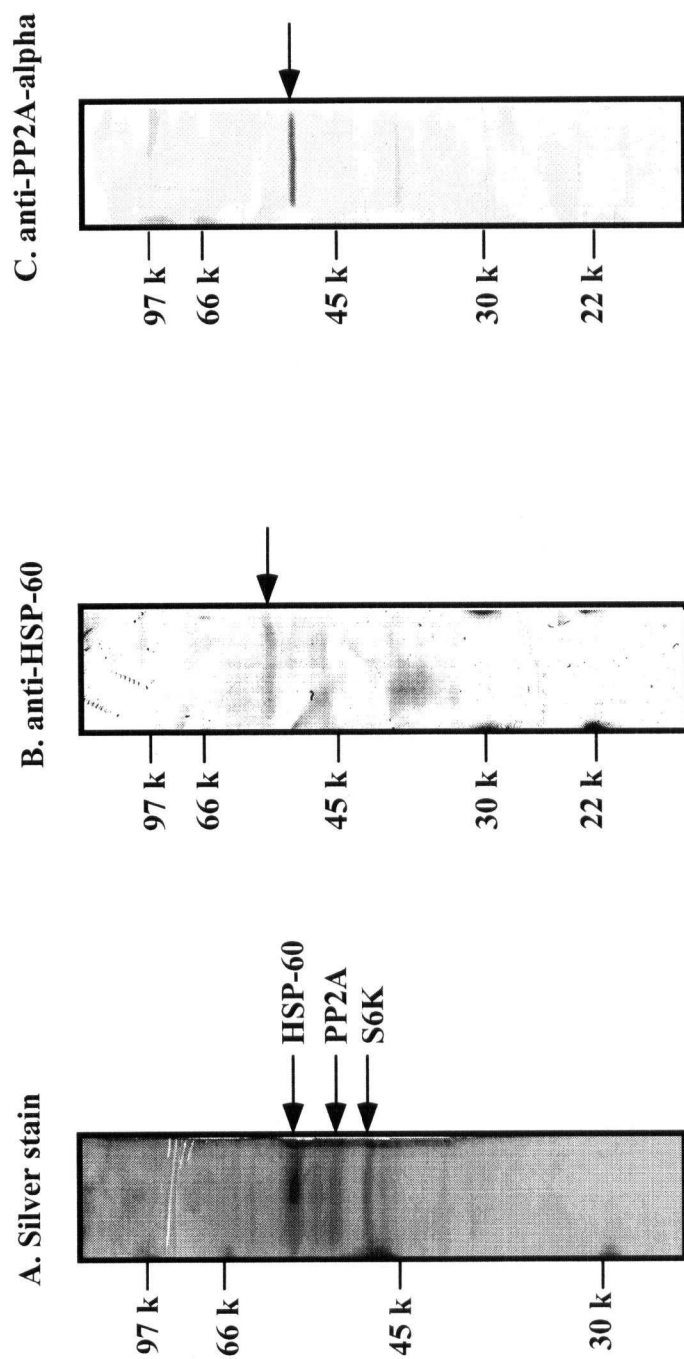
Fig. 20. Immunological identification of PP2A and HSP-60 in enriched sea star S6 kinase preparation: The enriched S6 kinase preparation was electrophoresed on 11% SDS-PAGE, and either silver stained (A) or transferred to nitrocellulose and probed with antibodies directed against the catalytic and regulatory subunits of PP2A (B) and HSP-60 (C). The data shown was representative of two separate experiments.

enriched preparation. In spite of repeated attempts, we were unable to obtain sequence data for a kinase in the preparation. We speculate that the sea star S6 kinase was masked by the presence of contaminating proteins in the final sample, especially PP2A. As the sequence data identified both the ~52 kDa and the ~55 kDa proteins as regulatory subunits of PP2A, it was possible that PP2A was much more abundant than the kinase in the enriched preparation, and thus prevented its identification by micro-sequencing. (See Discussion).

3.4.2   Co-immunoprecipitation of sea star S6 kinase, HSP-60 and PP2A

It was found that the S6 kinase co-purified over a number of column chromatography steps with an HSP protein and PP2A. We therefore speculated that these proteins may form a multi-unit complex *in vivo*. It was also possible that the kinase activity, or the activity of PP2A or HSP-60 might be regulated in some way by the interaction. Co-immunoprecipitation experiments were undertaken to determine if the $p70^{S6K}$ was interacting with other proteins. The S6 kinase was first immunoprecipitated from mature sea star oocyte extract with the N/T antibody, and probed with the same antibody to confirm that the antibody could immunoprecipitate the kinase (Fig. 21A). The antibody immunoprecipitated a protein at ~65 kDa. No protein bands were detected at ~52-55 kDa, possibly due to the presence of the immunoglobulin (Ig) band. It was also possible that the the 52 kDa form of the S6 kinase was simply a degradation product of the p70 isoform, which was not present in the mature extract, but was produced during the purification protocol.

The same immunoprecipitates were then probed with anti-HSP-60 antibody. The S6 kinase and a protein of ~80 kDa were co-immunoprecipitated and the ~80 kDa protein was recognized by antibody directed against HSP-60 (Fig. 21B). The S6 C/T antibody

C. anti-PP2A IP

50 k

33 k

S6

28 k

Control IP

Incorporation of phosphate

B. anti-p70^{S6K} N/T IP

97 k

HSP-60

69 k

47 k

Control IP

anti-HSP-60 blot

A. anti-p70^{S6K} N/T IP

97 k

69 k S6K

47 k

Control IP

anti-p70^{S6K} N/T blot
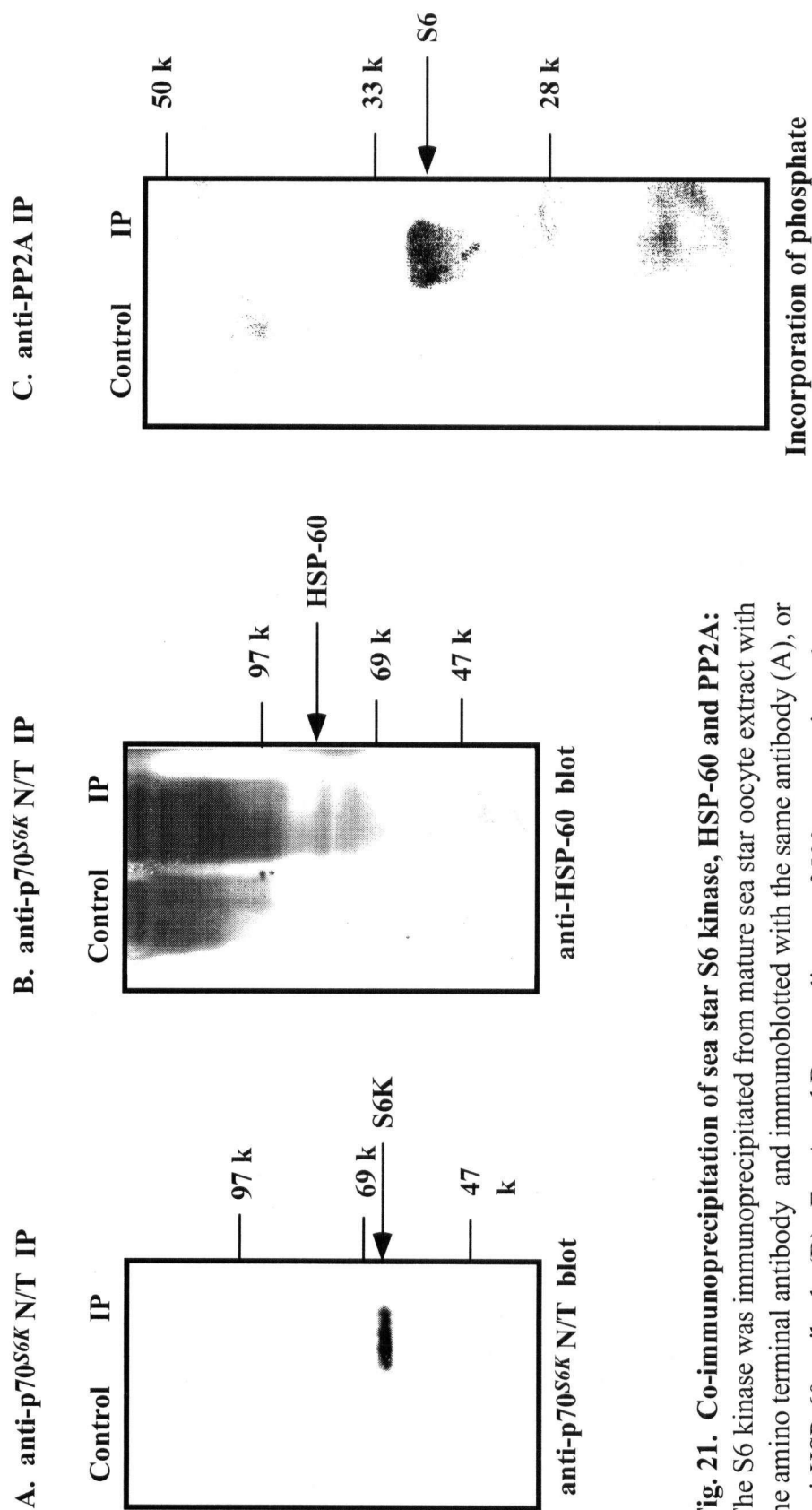
Fig. 21. Co-immunoprecipitation of sea star S6 kinase, HSP-60 and PP2A:
The S6 kinase was immunoprecipitated from mature sea star oocyte extract with the amino terminal antibody and immunoblotted with the same antibody (A), or anti-HSP-60 antibody (B). For A and B, two aliquots of 500 μg protein each were immunoprecipitated in the presence of 5% SDS, and the samples were pooled prior to the washes. Anti-PP2A alpha antibody was incubated with crude, mature sea star extract. The immunoprecipitate was assayed for kinase activity against 40S ribosomal protein S6 and phosphorylation was visualized by exposure to film (C).

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and the subdomain III antibody failed to immunoprecipitate the S6 kinase in sea star

(Data not shown).

Protein phosphatase 2A (PP2A) was then immunoprecipitated from partially

purified sea star extract with antibody directed against the regulatory alpha subunit (Fig.

21C). The immunoprecipitates were assayed in the presence of 40S ribosomes and were

shown to contain phosphotransferase activity against the 40S ribosomal S6 protein under

these conditions. There was no phosphotransferase activity seen in the control lane

(antibody and protein A Sepharose beads in the absence of sea star extract). We were

unable to obtain immunoreactivity of PP2A in $p70^{S6K}$ immunoprecipitates, or

immunoreactivity of $p70^{S6K}$ in PP2A immunoprecipitates.

It was previously demonstrated that a protein at ~55 kDa became phosphorylated

when the enriched sea star preparation was incubated in the presence of ATP (Fig. 8, 9

and 13). This protein was distinguished from $p52^{S6K}$ by incubation in the presence of

azido-ATP (Fig. 14), by immunoreactivity with the PP2A antibody (Fig. 19), and by

micro-sequencing (Table 5). The ~55 kDa PP2A protein band was subjected to

phosphoamino acid analysis (Fig. 22). Incorporation of phosphate was seen exclusively

on serine residues, demonstrating that PP2A was a substrate for and was phosphorylated

by active $p52^{S6K}$ in vitro. These findings supported the possibility that PP2A, HSP-60

and $p52^{S6K}$ might be associated in a high molecular weight complex. It is important to

note however, that the gel filtration data (Fig. 10) was inconsistent with the presence of a

multi-protein complex, as the peak of phosphotransferase activity eluted from the column

had an estimated molecular mass of ~50-55 kDa. It is possible that unidentified

molecule(s) required for maintenance of the complex had been purified away during the

purification procedure, leaving only the highly active kinase (See Discussion). It is also

possible that the PP2A and HSP-60 were simply contaminating proteins that had co-

purified with the kinase.
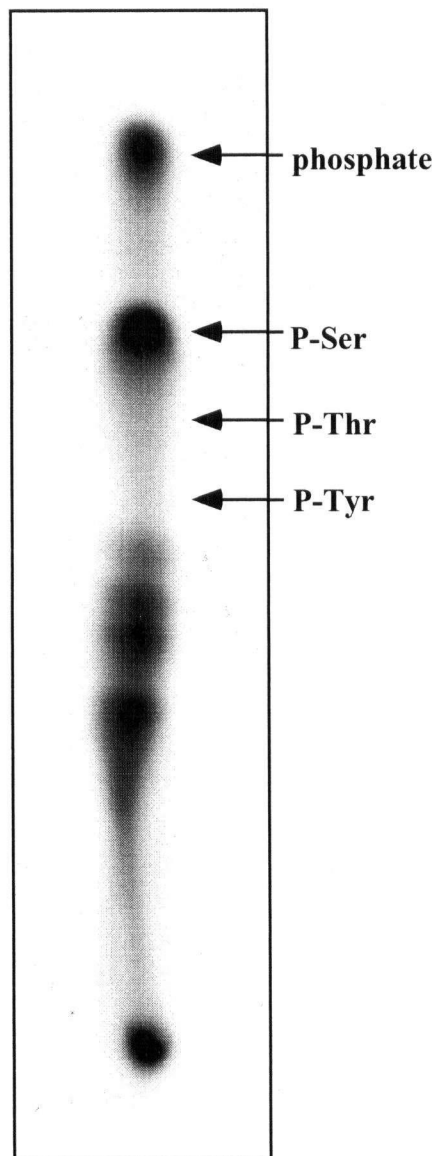
Fig. 22. Phosphoamino acid analysis of p55 phosphorylated by enriched sea star p52^{S6K}: The enriched S6 kinase preparation was incubated in the presence of ATP. The proteins were separated by SDS-PAGE, transferred to PVDF and the phosphorylated 55 kDa protein was subjected to phosphoamino acid analysis.

3.5 Functional Studies of Sea Star S6 Kinase

3.5.1 Immunoprecipitation of active S6 kinase from immature and mature sea star oocytes

In Section 3.2, I described the enrichment of an active S6 kinase from mature sea star oocytes and demonstrated that it was the sea star cognate of $p70^{S6K}$. It was possible that the activation of $p70^{S6K}$ was generated by proteolysis during the purification procedure. Therefore, to compare activity from immature and mature sea star oocyte extracts, the S6 kinase was immunoprecipitated with an antibody directed against a carboxyl terminal region of the $p70^{S6K}$ (Santa Cruz), and the amino terminal (N/T) antibody. The immunoprecipitate formed with the C/T antibody was electrophoresed on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the same antibody (Fig. 23A). An immunoreactive protein was apparent at ~70 kDa in both immature and mature oocyte extracts, although there appeared to be slightly more immunoprecipitated kinase in the mature oocyte extract. Immunoprecipitations with both antibodies were then immunoblotted with the N/T antibody (Fig. 23B) and protein bands were detected at ~70 kDa in both immunoprecipitates. It appeared that there was increased immunoprecipitated kinase present in mature oocyte extract as compared to immature. Immunoblots of crude mature and immature oocyte extracts showed that the same amount of protein was present in each sample, so it is unclear why more immunoprecipitatable S6 kinase was present in the mature samples as compared to the immature. No immunoreactive proteins were seen at ~52 kDa, but if $p52^{S6K}$ was immunoprecipitated, the protein would be obscured by the presence of the immunoglobulin (Ig) protein band at ~52-55 kDa.

The immunoprecipitates were assayed with the S6 peptide substrate and the phosphotransferase activity was plotted as counts per min (Fig. 23C). The mature
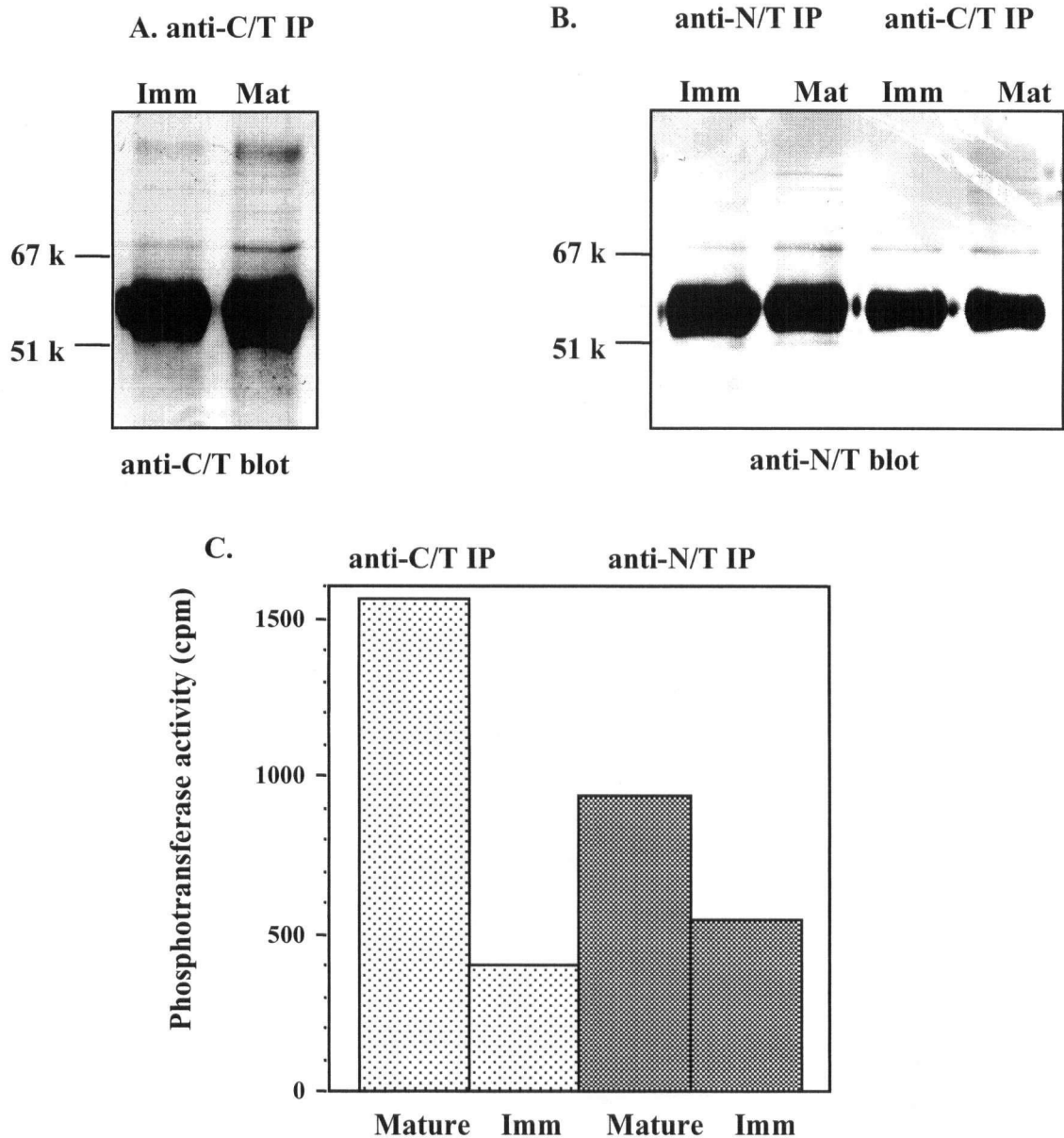
Fig. 23. Immunoprecipitation of active p70^{S6K} from immature and mature sea star oocytes:
A sample of 125 µl of crude immature or 90 min post 1-methyladenine-treated sea star
cytosol was added to 125 µl high salt NEFT buffer containing 6% NP-40. The S6 kinase
was immunoprecipitated with 5 µl of antibody as outlined in Materials and Methods.
The proteins were electrophoresed on 12.5% gels, transferred to nitrocellulose and
immunoblotted with anti-C/T (A) or anti-N/T (B) antibodies. The immunoprecipitates
were assayed in duplicate using the S6 peptide substrate, and the results were pooled
for determination of phosphotransferase activity (C). The data shown were from a single
experiment.

oocyte extract contained about 2- to 3.5-fold of the phosphotransferase activity of the immature oocyte extract, consistent with the kinase being activated during sea star oocyte maturation. However, the increase in activity could be due to the fact that there was more immunoprecipitated kinase in the mature oocyte extract as compared to immature. The cytosolic extract used for the immunoprecipitation experiments was from more recent sea star preparations than the extract used for the previous experiments, and the S6 peptide kinase activity in these extracts had been inconsistent. It was therefore possible that the S6 kinase was already partially activated in the immature oocyte extract, or conversely, the S6 peptide kinase activity may have been reduced in the mature oocyte extracts.

3.5.2 Immunoblot analysis of heat shock-treated immature and mature sea star extracts

Heat shock proteins interact with various cellular proteins in response to stresses such as osmotic shock and irradiation. For instance, PKB has been shown to interact with heat shock proteins in response to stress-stimuli (Konishi *et al.*, 1997). As an abundant heat shock protein (HSP-60) was found to co-purify with the sea star S6 kinase, it was possible that HSP-60 and p70^{S6K} may form a complex *in vivo*. To investigate this possibility, a time course of heat shock treatment was undertaken.

Oocytes from individual *P. ochraceus* were resuspended in natural sea water preheated to 35°C. At various times, aliquots were removed and frozen at -70°C. The aliquots were fractionated on Resource Q, assayed for kinase phosphotransferase activity and probed with a variety of antibodies. The peak kinase activity fractions immuno-reacted with all three p70^{S6K} antibodies (Fig. 24A). The subdomain III antibody recognized a protein at ~63 kDa that appeared to increase in expression with length of incubation time, as well as a ~65 kDa protein that decreased in amount with increasing time at 35°C. The C/T antibody also recognized the ~65 kDa protein, as did the N/T antibody. We concluded that the ~65 kDa protein corresponded to p70^{S6K}, which became degraded during heat shock. The N/T antibody also recognized a protein at ~52 kDa, a size similar to that identified in the purified sea star kinase preparation.

The same fractions were probed with anti-HSP-60 antibodies, and a protein at ~65 kDa which was unaffected in expression levels during the heat shock period was seen (Fig. 24B). The same fractions were also probed with antibodies directed against the alpha subunit of PP2A (Fig. 24B). An immunoreactive protein could be seen at ~65 kDa which was also unaffected in expression levels during the heat shock treatment. These results demonstrated that PP2A and HSP-60 may be present in the same fractions as p70^{S6K}, and was consistent with the possibility that these proteins may interact *in vivo*.
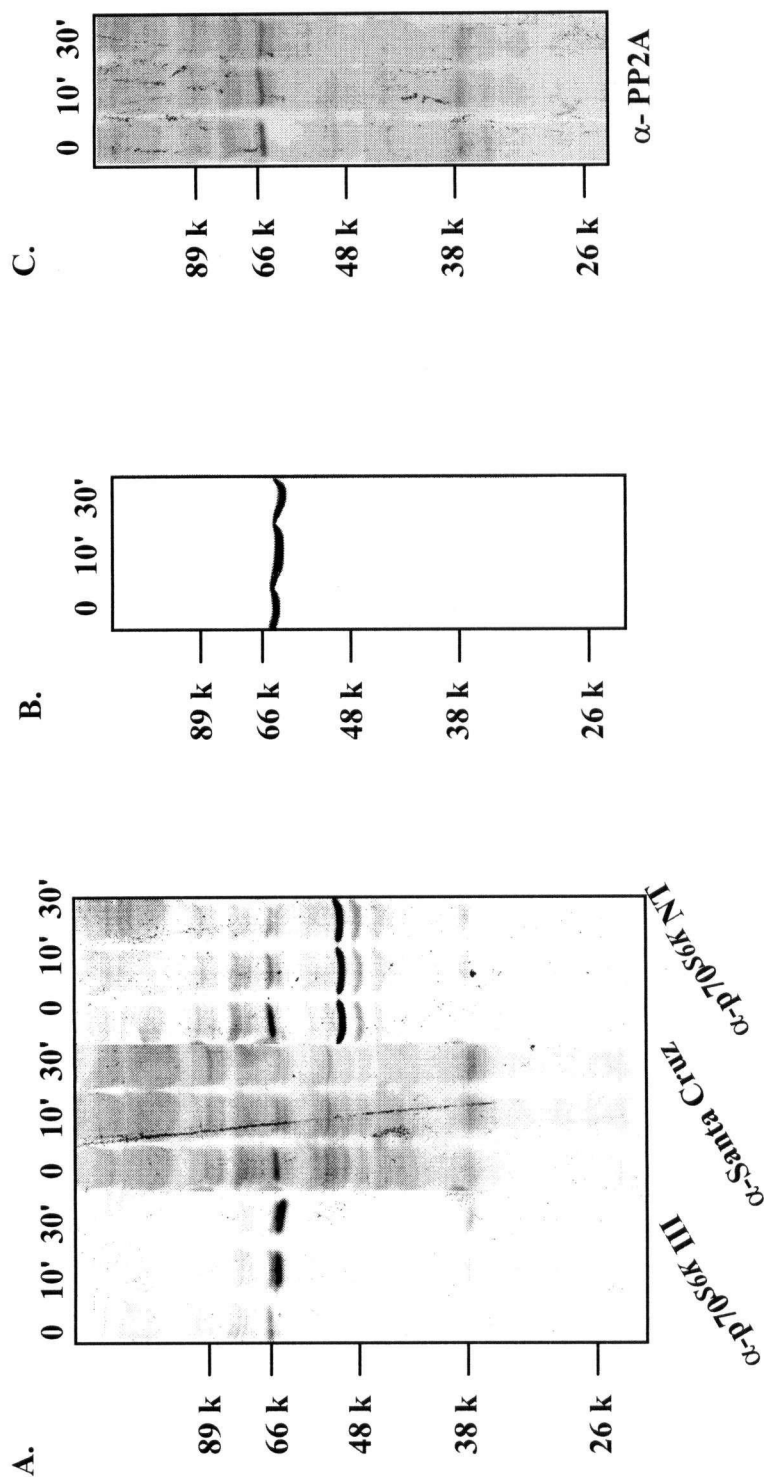
Fig. 24. Immunoblot analysis of heat shock-treated mature and immature sea star extracts:
Sea star oocytes were incubated at 35°C and aliquots were removed at 0, 10 and 30 min. The aliquots were fractionated by ResourceQ, assayed for S6 kinase activity and the peak phosphotransferase activity fractions were electrophoresed, transferred and immunoblotted. The samples were probed with three different antibodies directed against $p70^{S6K}$ (A). The same fractions were also probed with antibody directed against HSP-60 (B), and the regulatory subunit of PP2A (C). The data shown were from a single experiment.

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3.5.3 Expression of active recombinant S6 kinase from baculovirus

Yields of enriched, active kinase obtained from mature sea star oocytes were very low, and the process was labour intensive. The S6 kinase is highly conserved among various species, therefore we speculated that expressed kinase from other species could be used to screen sea star extracts to identify potential upstream regulators of $p70^{S6K}$, or for a variety of other biological experiments such as micro-injection. We obtained clones for human $p70^{S6K}$ from Dr. James Woodgett (Ontario Cancer Inst.). The $p70^{S6K}$ was expressed and purified from baculovirus infected Sf9 cells as detailed in the Materials and Methods section. The GST-S6K baculovirus/Hi5 cells were stimulated with 25 or 50 nM PMA for 25 or 15 min to further activate the $p70^{S6K}$ phosphotransferase activity. The PMA-treated GST-S6K was then electrophoresed, transferred and immunoblotted with the N/T antibody (Fig. 25A). The antibody recognized an immunoreactive protein at ~85 kDa, the expected size of the expressed GST-linked kinase. Protein levels in all the samples were approximately equivalent (data not shown).

To test the activity of the expressed kinase, the PMA-treated GST fusion protein was assayed in the presence of myelin basic protein (MBP) and the S6 peptide substrate (Fig. 25B). The kinase, at ~85 kDa, became phosphorylated during the incubation. In fact, incorporation of phosphate into the expressed kinase was enhanced in the presence of MBP (lanes 2 and 5 in Fig. 25B), and inhibited in the presence of the peptide substrate (lanes 3 and 6 in Fig. 25B). A possible explanation could be that in the presence of the preferred substrate (S6 peptide), there is less incorporation of phosphate into the kinase whereas in the presence of the less preferred substrate (MBP), more phosphate was incorporated into the kinase. In any event, the expressed kinase exhibited significant phosphotransferase activity against the substrates and will be useful for future experiments. The 25 min 25 nM PMA treatment produced the most highly active preparation of $p70^{S6K}$.

B.

GST-S6K

MBP

S6 peptide

S610
MBP
control

S610
MBP
control

50 nM PMA
15 min

25 nM PMA
25 min

89 k

66 k

48 k

38 k

26 k

19 k

A.

GST-S6K

S610
MBP
control

S610
MBP
control

50 nM PMA
15 min

25 nM PMA
25 min

89 k

66 k

48 k

38 k

Fig. 25. Expression of recombinant S6 kinase: Hi5 cells which were infected by GST-S6K baculovirus three days earlier, were stimulated with 25 or 50 nM PMA for 25 or 15 min, respectively. The GST-S6K protein was subsequently purified on a GST-bead column. The purified kinase was electrophoresed on 11% SDS-PAGE, transferred and immunoblotted (A), or incubated in the presence of myelin basic protein or the S6 peptide substrate, electrophoresed, transferred and exposed to film (B). The data shown were from a single experiment.

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3.5.4 Microinjection studies

The human form of p70^{S6K} was expressed in the baculovirus system. The kinase was cleaved from GST beads with thrombin, and prepared in PBS in the absence of proteases or phosphatase inhibitors. Although echinoderm and human are very divergent, the p70^{S6K} is known to be very highly conserved amongst species. We therefore speculated that the expressed human form of the kinase may have an effect on the maturation process in sea star. To test this hypothesis, preparations of the active human kinase and PBS controls were microinjected into immature sea star oocytes to determine the effect of the active kinase on maturation as identified by germinal vesicle breakdown (GVBD). Control cells were treated with 1-methyladenine, and underwent normal maturation in every set of experiments (data not shown). Comparison of PBS-injected oocytes with S6 kinase-treated oocytes showed that microinjection of purified, active S6 kinase had no obvious effect on maturation of the oocytes (Fig. 26). The same proportion of oocytes appeared to undergo normal maturation in treated and untreated samples (Table 6).

A.
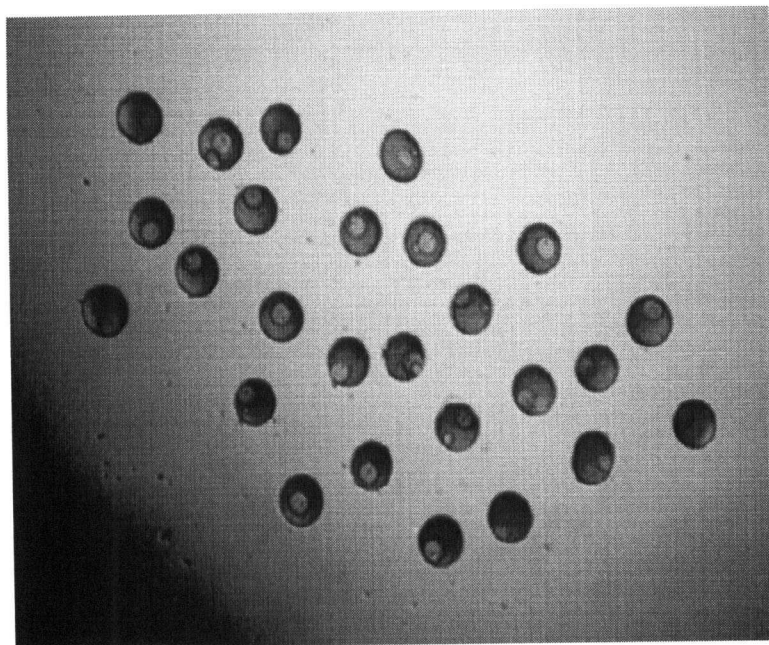
B.
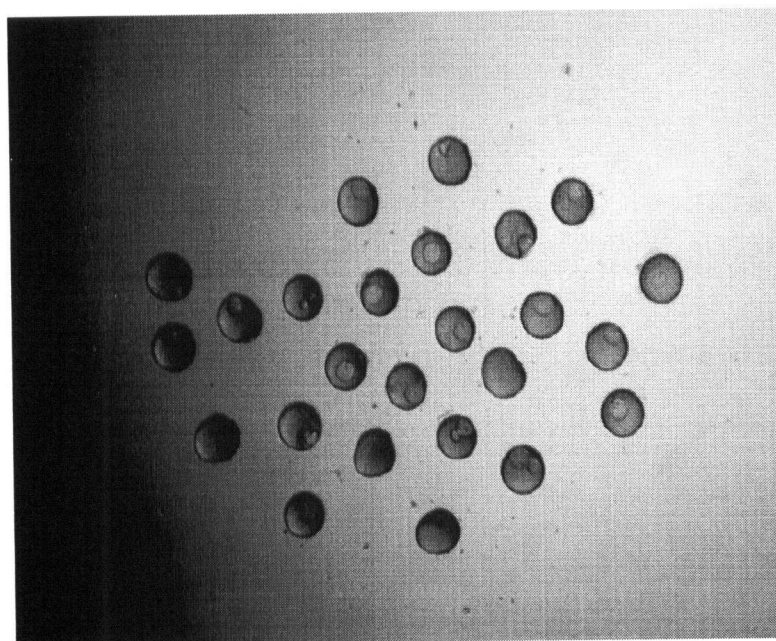
Fig. 26. Microinjection of p70^{S6K} into immature sea star oocytes: Microinjection was carried out as detailed in the Material and Methods Section. Immature sea star oocytes were microinjected with active, expressed S6 kinase (A) or PBS (B). The pictures were taken 3 1/2 hours post injection. In panel A, the maturation was 2/26 while in panel B the maturation was 3/24 oocytes.

Table 6

Microinjection of purified human p70^{S6K} into sea star oocytes

Date	Microinjected	Number of oocytes matured
09-12-1996	p70^{S6K}	2/30, 3/37, 5/38
	Buffer B + 0.4 M NaCl	0/24, 1/34
06-06-1997	p70^{S6K}	3/26, 1/38
	PBS	0/26
07-31-1997	p70^{S6K}	3/21, 5/24, 5/18, 2/26
	PBS	3/21, 5/21

Chapter 4: Results and Discussion: Photodynamic Therapy and it's Effect on the Phosphorylation and Activation State of $p70^{S6K}$

4.1 Introduction and Model System

In heat shock-treated sea star oocytes, the levels of $p70^{S6K}$ appeared to decline. In the next series of studies, we sought to investigate how $p70^{S6K}$ was affected in a mammalian cell system in which stress signaling responses were activated. It was previously reported that photodynamic therapy (PDT) with benzoporphyrin derivative mono-acid ring A (verteporfin) (BPD-MA) caused a marked, ROS-mediated activation of both stress-activated protein kinase (SAPK) and p38 HOG1 without affecting the activity of extracellularly regulated protein kinase 1 and 2 (ERK1 and ERK2) in the transformed keratinocyte cell line Pam212 (Tao *et al.*, 1996). Although $p70^{S6K}$ does not appear to be involved in the apoptotic pathway, much evidence has placed it downstream of the growth factor-induced activation of PI 3-kinase and PKB, both of which have been shown to block the induction of apoptotis (Kennedy *et al.*, 1997; Kulik *et al.*, 1997; Kaufmann-Zeh *et al.*, 1997). We were therefore interested in analyzing the PI 3-kinase/PKB/$p70^{S6K}$ pathway in Pam212 cells treated with PDT, to evaluate the effect of PDT on this pathway.

Keratinocytes are the major component of the epidermis that is affected by UV irradiation and PDT treatment. In response to these treatments, keratinocytes release cytokines such as TNFα, TGFβ, IL-10, IL-8 and GM-CSF. We have observed many similarities between UV and PDT in both cell culture and animal models, therefore, increased understanding of the signaling pathways in PDT-treated keratinocytes may improve our understanding of the actions of PDT and ROS on $p70^{S6K}$ and its regulation at the molecular level. We therefore undertook analysis of some of the signaling components in response to PDT in Pam212 cells.

4.2   Initial Observations: PDT Effect on p70^{S6K}

4.2.1   Effect of PDT on activation of p70^{S6K}

Initially, we immunoprecipitated active p70^{S6K} from crude Pam212 cell extracts

treated with anisomycin, UV irradiation and PDT (Fig. 27). The immune complexes

were assayed for phosphotransferase activity and electrophoretic mobility shift (see

Material and Methods). PDT treatment caused a profound suppression of S6 peptide

phosphotransferase activity in cells maintained in a medium supplemented with 5% FCS.

The enzyme activity of the PDT-treated samples was about 20% that of untreated

controls. The untreated control cells had a high basal S6 peptide activity that was

stimulated 2- to 3-fold by anisomycin and UV irradiation. Immunoblot analysis revealed

the presence of a high molecular weight, activated form of p70^{S6K} in the samples derived

from untreated cells, BPD-MA-treated, anisomycin-treated or UV-treated cells. This

activated form of p70^{S6K} was not detected in PDT-treated samples. These results

indicated that PDT inhibited the phosphorylation and phosphotransferase activity of

serum-activated p70^{S6K}.
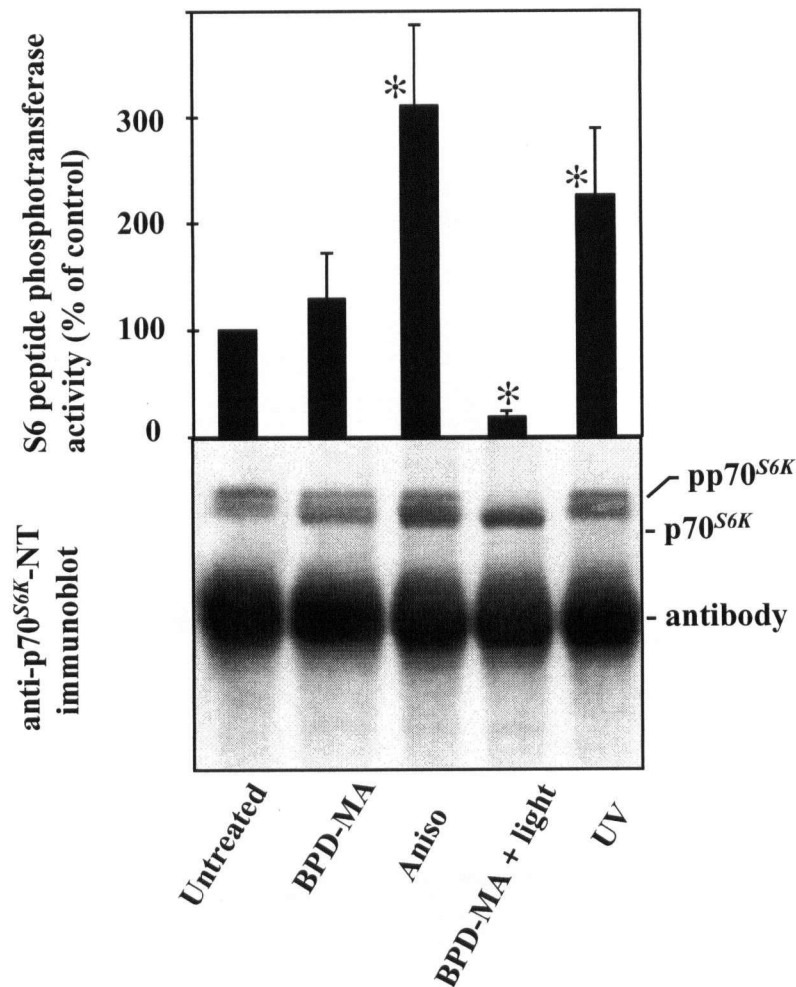
Fig. 27. Effect of PDT on activation of p70^{S6K} : Pam212 cells cultured in DMEM supplemented with 5% FCS were treated for 5 min with 200 ng/ml BPD-MA only, 40 mg/ml anisomycin, 200 ng/ml BPD-MA plus 2 J/cm^2 of light, or 4 min exposure of UV radiation. p70 S6 kinase was immunoprecipitated from crude cell lysates using anti-p70^{S6K} N/T antibody, and the immunoprecipitated kinase was incubated in the presence of the peptide substrate and assayed for phosphotransferase activity. The immunoprecipitates were electrophoresed, transferred and immunoblotted with the same antibody. Data from three independent experiments were pooled. Untreated controls exhibited an enzyme activity of about 10,000 counts per minute in a 10 minute incubation.. ✳ , statistically significant difference between samples derived from variously treated cells and from control cells. Bars, S.D.

4.2.2 Dose- and time-dependence of PDT effect on $p70^{S6K}$

We investigated the relationship between the dose of PDT and inhibition of the phosphorylation and phosphotransferase activity of $p70^{S6K}$. Pam212 cells maintained in a medium supplemented with 5% FCS were treated with various doses of PDT (Fig. 28). The light dose was fixed at 2 J/cm^2, and the BPD-MA dose varied from 5 to 250 ng/ml. Thirty min after treatment, cells were lysed, and $p70^{S6K}$ was immunoprecipitated from the crude cell lysates with anti-$p70^{S6K}$ C/T antibody (Santa Cruz). The immunoprecipitates were assayed for phosphotransferase activity using the S6 peptide as a substrate, and were immunoblotted with the same antibody. The results show a correlation between the dose of BPD-MA and the extent of inhibition of both the phosphorylation and the phosphotransferase activity of $p70^{S6K}$.

We went on to evaluate the time-dependence of the kinase activity to treatment with PDT. Pam212 cells were maintained as above, and crude cell lysates were prepared at different times following PDT at a dose of 200 ng/ml BPD-MA and 2 J/cm^2 of light (Fig. 29). $p70^{S6K}$ was immunoprecipitated, assayed and immunoblotted as above. The earliest inhibition of $p70^{S6K}$ was noted at 10 min following PDT treatment while maximum inhibition was observed 30 to 40 min following treatment. The duration of the inhibition persisted during the course of the experiment and there was no recovery of $p70^{S6K}$ activity up to 2 h after PDT treatment. Immunoblot analysis demonstrated that the change in kinase activity correlated with a shift in electrophoretic mobility. Thus, the inhibitory effect of PDT on the phosphorylation and phosphotransferase activity of $p70^{S6K}$ was both dose- and time-dependent.
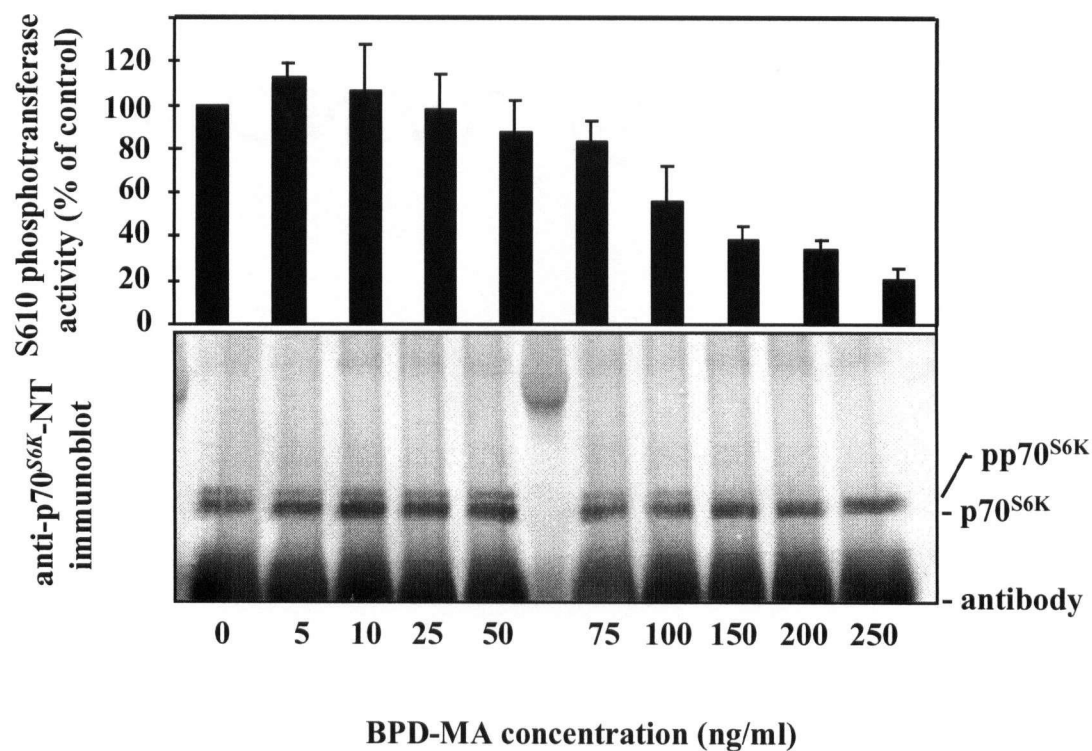
Fig. 28. BPD dose response of PDT inhibition of p70^{S6K}: Pam212 cells maintained in a medium supplemented with 5% FCS were pre-incubated with various doses of BPD-MA ranging from 5 ng/ml to 250 ng/ml for 12 h. Subsequently the cells were exposed to 2J/cm^2 red light. The whole cell extracts were prepared 30 min after PDT treatment. p70^{S6K} was then immunoprecipitated, assayed, and immunoblotted. Data from three independent experiments were pooled. Control activity was approximately 10,000 counts per minute in a 10 minute incubation. Bars, S.D.
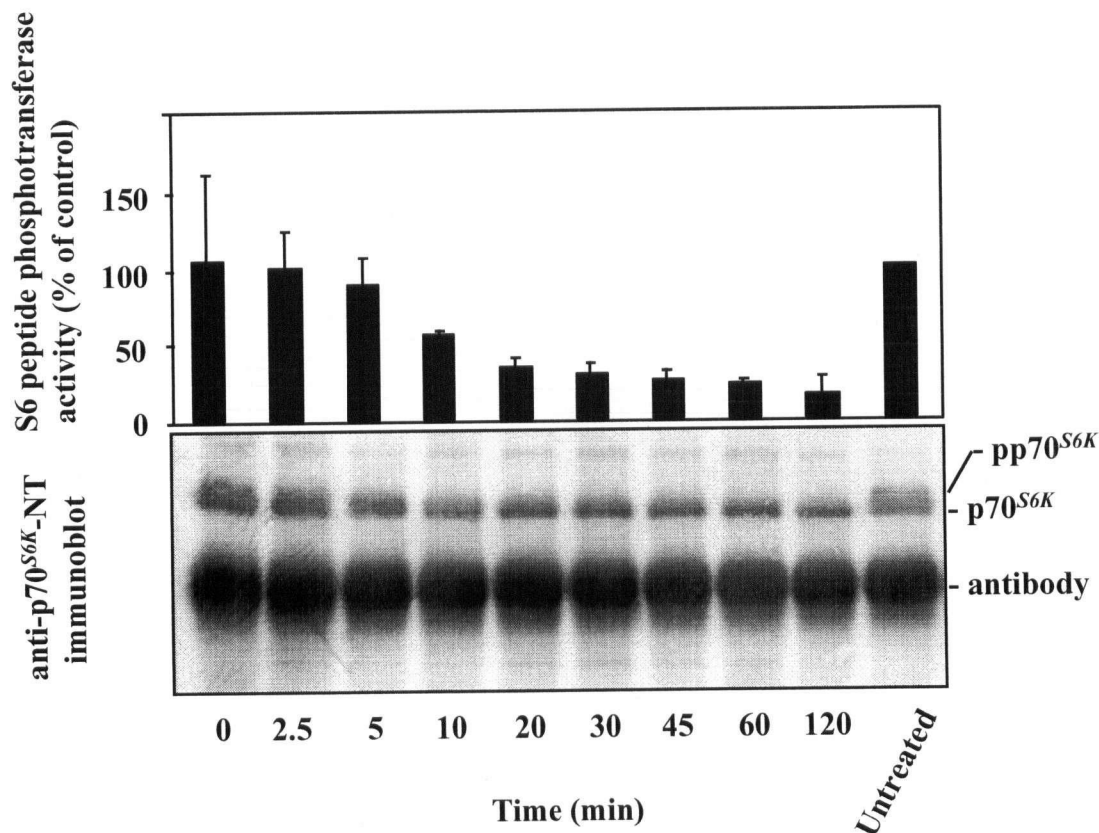
Fig. 29. BPD time course of PDT inhibition of p70^{S6K} : Pam212 cells maintained in a medium supplemented with 5% FCS were treated with 200 ng/ml BPD plus 2 J/cm^2 red light. After PDT, cultures were incubated at 37°C for additional periods of time ranging from 2.5 min to 2 h. p70^{S6K} was then immunoprecipitated, assayed, and immunoblotted. Data from 3 independent experiments were pooled. Untreated controls exhibited an enzyme activity of about 10,000 counts per minute in a 10 minute incubation. Bars, S.D.

4.3 Analysis of the PI 3-kinase /PKB/_p70^{S6K} Pathway in PDT Treatment

4.3.1 Effect of insulin on PI 3-kinase, PKB and p70^{S6K} in serum-starved Pam212 cells

It has been reported that mitogenic stimulation caused strong activation of PI 3-kinase, PKB and p70^{S6K} (Alessi *et al.*, 1996; Chung *et al.*, 1994; Mukhopadhyay *et al.*, 1992; Price *et al.*, 1990). There is also circumstantial evidence that PI 3-kinase and PKB may be upstream mediators of p70^{S6K} activation (Bourewijn *et al.*, 1995; Chung *et al.*, 1994). Since PDT had a profound inhibitory effect on p70^{S6K}, we were interested in the effect PDT may have on these upstream kinases. Initially, we investigated the effect of insulin stimulation on the three enzymes. Serum-starved Pam212 cells were treated with 100 nM of insulin for various periods of time (Fig. 30). PI 3-kinase, PKB and p70^{S6K} were immunoprecipitated from crude cell lysates, and assayed for activity. PI 3-kinase activity was measured by immune complex assay using PI as a substrate. This was followed by separation with thin-layer chromatography and radiography. The activity of PKB and p70^{S6K} was assayed in filter paper assays using HH2B or the S6 peptide as substrates. We observed a 2-fold increase in PI phosphotransferase activity 1 min following insulin stimulation. The peak activation was detected at 20 min post insulin treatment. A significant activation of HH2B phosphotransferase activity was noted at 5 min following insulin stimulation. A 5-fold peak activation was detected at 30 min post insulin treatment and the activation persisted during the course of the experiment. The time course of p70^{S6K} activation was similar to that of PKB. These results demonstrated that Pam212 cells responded to insulin stimulation in a manner consistent to that previously reported for other cell types. Therefore, we could use the Pam212 cell system to study the effect of PDT on this pathway.
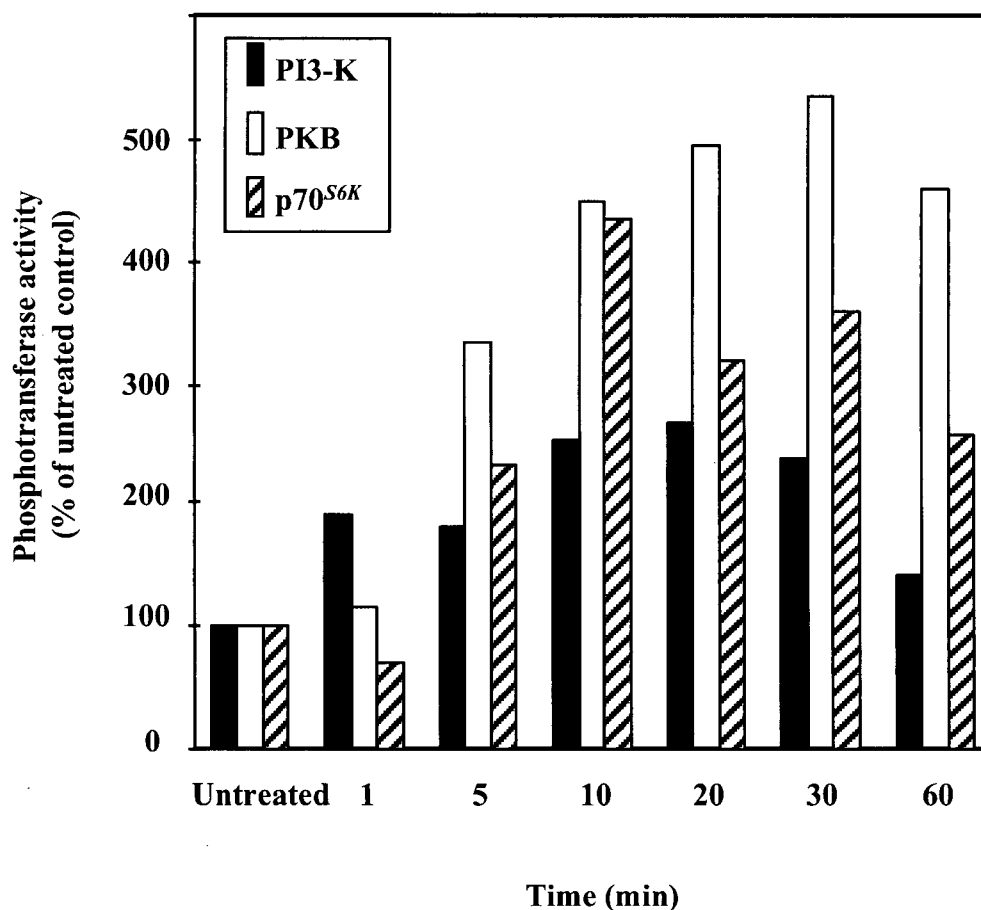
Fig. 30. Insulin-induced activation of PI3-K, PKB and p70^{S6K}. Pam212 cells were cultured in DMEM supplemented with 5% FCS until 75% subconfluent. The cells were then maintained in DMEM supplemented with 0.5% FCS for 36 h, then serum-starved for 12 h. The resting cells were stimulated with 100 nM insulin for various periods of time as indicated. Crude whole cell lysates were immunoprecipitated with antibodies directed against PI 3-K (N-SH2), PKB-PH, or p70^{S6K} N/T. The immune complexes were assayed using PI, HH2$_B$ or the S6 peptide substrate. The data shown were representative of two separate experiments. Untreated controls exhibited an enzyme activity of about 4,000, 500 and 2,000 counts per minute in a 10 minute incubation, for PI 3-kinase, PKB and p70^{S6K}, respectively.

4.3.2   Effect of PDT on insulin-induced activation of PI 3-kinase, PKB and p70^{S6K} in
           serum- starved Pam212 cells

The activity of PI 3-kinase was measured by immune complex assay using PI and
PI 4,5-P_2 as substrates (Fig. 31). There were 2-fold increases in PI and PI 4,5-P_2
phosphotransferase activity detected at 1 min post insulin treatment. Pre-incubation with
100 nM wortmannin completely abolished the insulin induced PI 3-kinase activity, in
agreement with previously published reports (Ui *et al.*, 1995). As expected, pre-
incubation with 25 ng/ml of rapamycin did not affect the insulin-induced activation of PI
3-kinase. However, pre-treatment with PDT totally inhibited activation of PI 3-kinase.
Immunoblot analysis indicated that the antibody immunoprecipitated approximately
equivalent amounts of protein.

PKB was assayed using HH2B as a substrate (Fig. 32). A 6-fold increase in
HH2B phosphotransferase activity was detected at 10 min post insulin treatment. The
insulin-induced HH2B phosphotransferase activity was partially inhibited by pre-
incubation with wortmannin. Rapamycin pre-treatment did not significantly affect the
insulin-induced activation of PKB. Like wortmannin, PDT at least partially inhibited
insulin-induced HH2B phosphotransferase activity. Immunoblot analysis of the
immunoprecipitated PKB demonstrated that the amount of protein immunoprecipitated
was approximately the same.

p70^{S6K} was assayed using the S6 peptide as a substrate (Fig. 33). An 8-fold
increase in S6 phosphotransferase activity was detected at 10 min post insulin treatment.
In addition, 8- to 10-fold increases in S6 phosphotransferase activity were detected at 10
min stimulation with 100 ng/ml of EGF. As expected, both wortmannin and rapamycin
completely blocked the inductive effect of insulin and EGF. Pretreatment with PDT
totally abolished insulin- or EGF-induced increase in S6 phosphotransferase activity.
Anti-p70^{S6K} immunoblot analysis demonstrated that the pattern of electrophoretic

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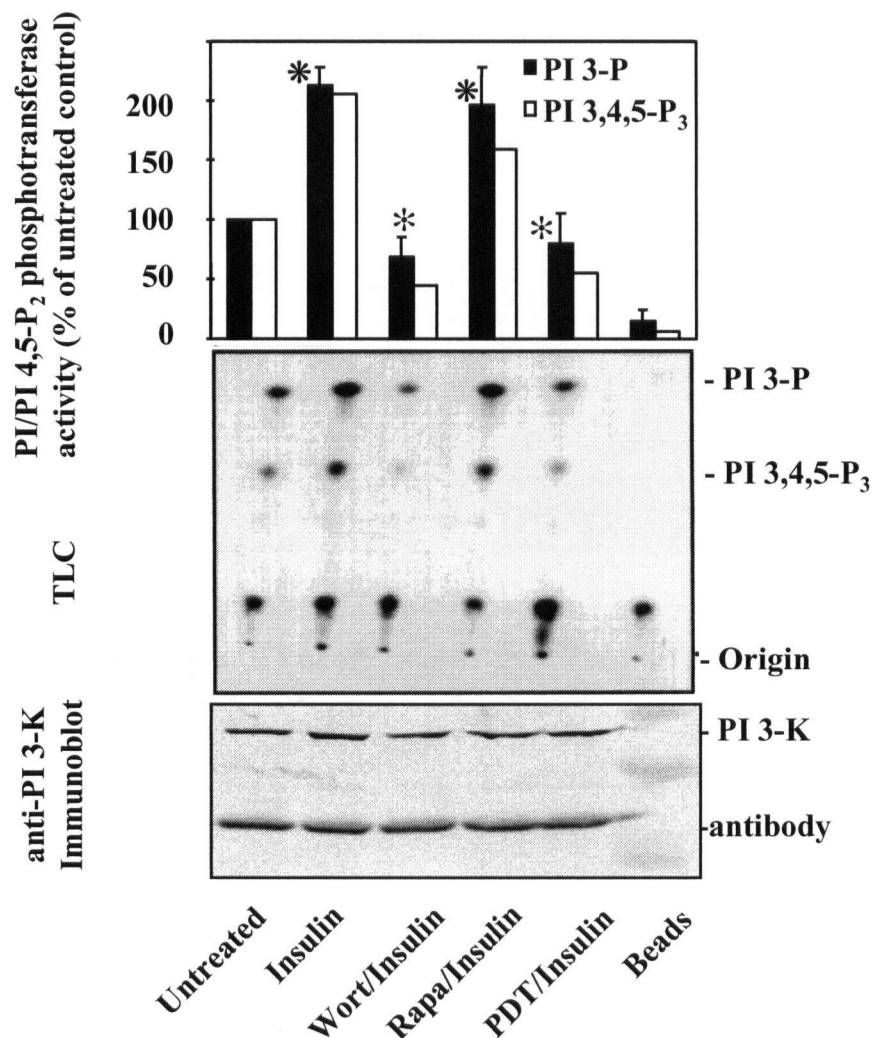
Fig. 31. PDT inhibits insulin-induced activation of PI 3-K: Serum-starved Pam212 cells were treated with 2 J/cm^2 of red light. Thirty min after PDT or following a 15 min pre-incubation with 100 nM wortmannin or 25 ng/ml rapamycin, cells were stimulated with 100 nM insulin for 1 min. The immunoprecipitates were assayed by TLC using PI as a substrate. Identical immunoprecipitates were electrophoresed, transferred and immunoblotted. Data from three independent experiments were pooled. Beads control represents protein A sepharose beads incubated with extract in the absence of antibody. Untreated controls exhibited an enzyme activity of about 4,000 counts per minute in a 10 minute incubation. ✱, statistically significant difference between samples derived from variously treated cells and that from untreated control cells. ✳ , statistically significant difference between samples derived from variously treated cells and that from insulin-treated cells. Bars, S.D.

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Fig. 32. PDT inhibits insulin-induced activation of PKB: Serum-starved Pam212 cells were treated with 2 J/cm^2 of red light. Thirty min after PDT or following a 15 min pre-incubation with 100 nM wortmannin or 25 ng/ml rapamycin, cells were stimulated with 100 nM insulin for 10 min. The immunoprecipitates were assayed using HH2B as a substrate. Identical immunoprecipitates were electrophoresed, transferred and immunoblotted. Data from three independent experiments were pooled. Beads control represents protein A sepharose beads incubated with extract in the absence of antibody. Untreated controls exhibited an enzyme activity of about 500 counts per minute in a 10 minute incubation. $\ast$, statistically significant difference between samples derived from variously treated cells and that from untreated control cells. $\ast$, statistically significant difference between samples derived from variously treated cells and that from insulin-treated cells. Bars, S.D.

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Fig. 33. Effect of PDT on growth factor-induced activation of p70^{S6K} : Serum-starved Pam212 cells were stimulated 10 min with 100 ng/ml EGF or 100 nM insulin. Cells were pre-incubated with 100 nM wortmannin, 25 ng/ml rapamycin for 15 min, or treated with PDT prior to growth factor stimulation. The immunoprecipitates were assayed, electrophoresed, transferred and immunoblotted. Data from three to five independent experiments were pooled. Untreated controls exhibited an enzyme activity of about 2,000 counts per minute in a 10 minute incubation. *, statistically significant difference between samples derived from variously treated cells and that from untreated control cells. ✳, statistically significant difference between samples derived from variously treated cells and that from growth factor-treated cells. Bars, S.D.

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mobility shift correlated with the results of the *in vitro* kinase assay. Both insulin and EGF stimulation resulted in an electrophoretic mobility shift that correlated with the activation of the kinase. From these results, we concluded that PDT caused a profound inhibition of insulin-induced activation of PI 3-kinase and PKB as well as the insulin- or EGF-induced activation of $p70^{S6K}$.

4.3.3 Effect of UV irradiation on insulin-induced activation of PI 3-kinase, PKB and p70^{S6K} in serum-starved Pam212 cells

UV irradiation has been shown to induce stress-response and apoptosis in many cell types (Chen *et al.,* 1996). Generation of ROS is thought to be the mechanism for the biological and biochemical effects of both UV and PDT (Zhang *et al.,* 1997; Kochevar, 1995; Girotti *et al.,* 1990). It was previously shown that the PDT-induced activation of SAPK and p38 HOG1 was at least partially mediated by ROS (Tao *et al.,* 1996). In addition, both ERK1 and ERK2 were strongly activated by treatment with H_2O_2 and we had previously observed activation of PI 3-kinase and PKB by treatment with H_2O_2 (Data not shown). Therefore, there may be common upstream regulators modulated by ROS in the signaling pathways mediated by UV and PDT. We therefore wanted to compare the effect of either UV irradiation or PDT on the insulin-induced activation of PI 3-kinase, PKB and p70^{S6K} in serum-starved Pam212 cells.

The cells were pretreated with PDT or 4 min UV irradiation. The dose and conditions of UV treatment were similar to those used to activate SAPK and p38 HOG1 (Tao *et al.,* 1996). At various times after UV irradiation, cells were stimulated with 100 nM insulin for 10 min before cell extracts were prepared. Immunoprecipitates were analyzed with *in vitro* kinase assays as previously described. When the cells were given insulin 5 min after UV irradiation, there was a similar level of inhibition of PI phosphotransferase activity as was observed following PDT treatment (Fig. 34). The inhibition became less pronounced with the increase in the interval between UV irradiation and insulin stimulation. However, UV irradiation alone, PDT alone, or PDT plus UV irradiation did not significantly affect the basal level of PI phosphotransferase activity.

UV irradiation did not inhibit the insulin-induced HH2B phosphotransferase activity of PKB (Fig. 35). Indeed, there was a potentiation of the insulin response

Fig. 34. Effect of UV irradiation on insulin-induced activation of PI 3-kinase: Serum-starved Pam212 cells were treated with 100 ng/ml BPD plus 2 J/cm^2 of red light, 4 min exposure to UV light, or PDT prior to UV irradiation. At various times after treatment, cells were stimulated with 100 nM insulin for 1 min. The immunoprecipitates were assayed by TLC using PI as a substrate. Identical immunoprecipitates were electrophoresed, transferred and immunoblotted. Data from three independent experiments were pooled. Beads control represents protein A sepharose beads incubated with extract in the absence of antibody. Untreated controls exhibited an enzyme activity of about 4,000 counts per minute in a 10 minute incubation. *, statistically significant difference between samples derived from variously treated cells and that from untreated control cells. ✻ , statistically significant difference between samples derived from variously treated cells and that from insulin-treated cells. Bars, S.D.
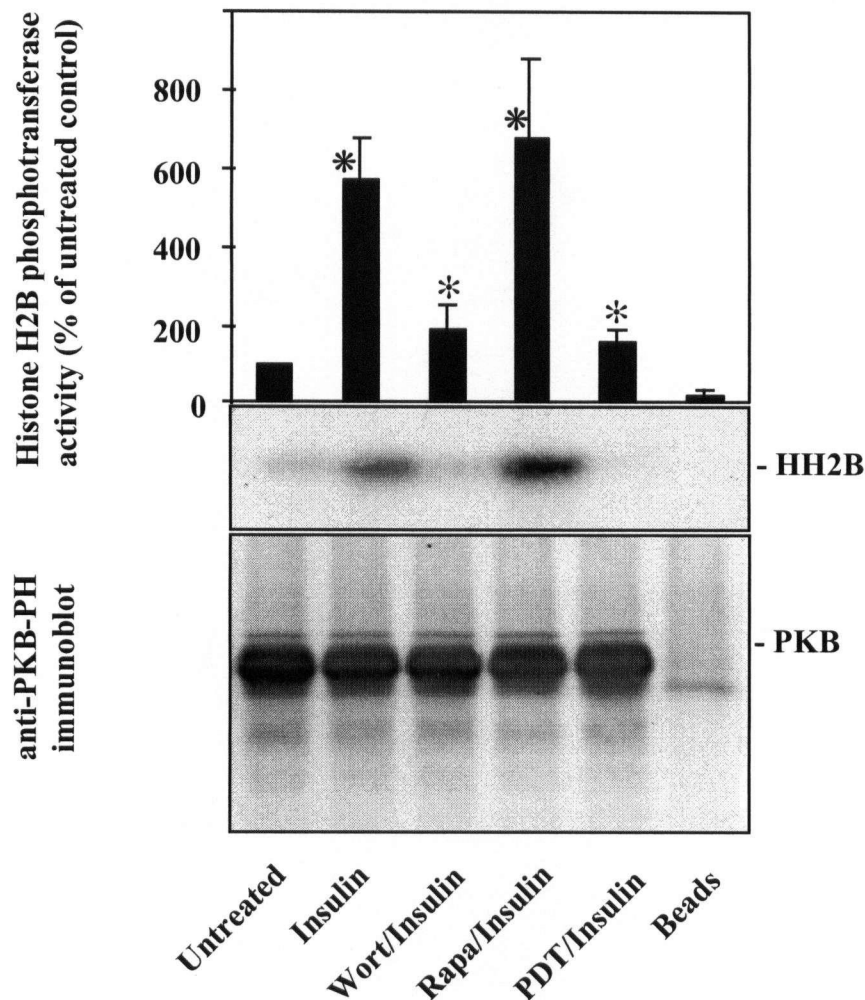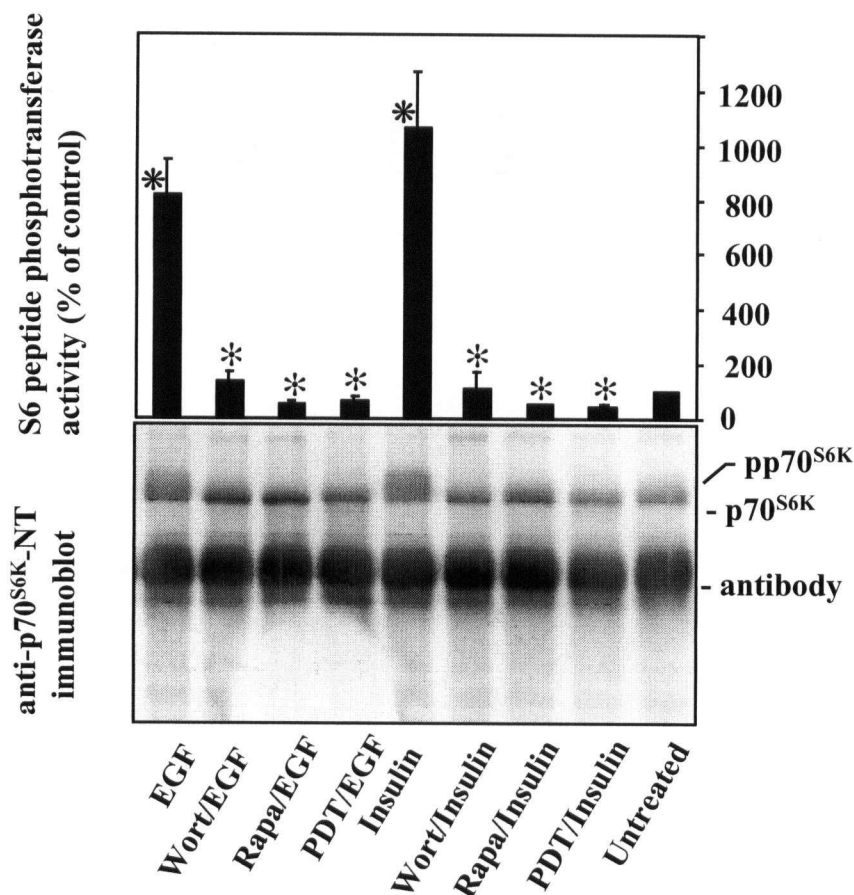
Fig. 35. Effect of UV irradiation on insulin-induced activation of PKB: Serum-starved Pam212 cells were treated with 100 ng/ml BPD plus 2 J/cm^2 of red light, 4 min exposure to UV light, or PDT prior to UV irradiation. At various times after treatment, cells were stimulated with 100 nM insulin for 10 min. Immunoprecipitates with anti-PKB-PH antibody were subsequently assayed using HH2B as a substrate. Identical immunoprecipitates were electrophoresed, transferred and immunoblotted. Data from three independent experiments were pooled. Beads control represents protein A sepharose beads incubated with extract in the absence of antibody. Untreated controls exhibited an enzyme activity of about 500 counts per minute in a 10 minute incubation. ✳, statistically significant difference between samples derived from variously treated cells and that from untreated control cells. ✳, statistically significant difference between samples derived from variously treated cells and that from insulin-treated cells. Bars, S.D.

between 30 and 60 min following UV irradiation. Like that of PI 3-kinase, the phosphotransferase activity of PKB was not significantly affected by UV irradiation alone, PDT alone, or PDT plus UV irradiation without insulin stimulation.

Except for a partial inhibition observed in cells treated with insulin 5 min after UV irradiation, the effect of UV on insulin-induced S6 phosphotransferase activity in the immunoprecipitates was not significant (Fig. 36). However, the phosphotransferase activity of the immunoprecipitates was increased 2- to 3-fold in the cells treated with UV irradiation alone. The UV-induced S6 phosphotransferase activity was completely abolished by pretreating the cells with PDT. Immunoblot analysis demonstrated the presence of the high molecular weight, activated form of p70^{S6K} in the samples derived from insulin-treated cells. This high molecular weight species was not detected in the samples derived from the cells pretreated with PDT. The pattern of electrophoretic mobility correlated with the *in vitro* kinase assay results.

These findings indicated that the effect of UV irradiation on insulin responses was different from that of PDT. Pretreatment with UV irradiation inhibited insulin-induced PI 3-kinase activation. However, insulin-induced PKB activiation was potentiated in cells stimulated with insulin 30 min after UV irradiation. UV irradiation did not significantly affect insulin-induced p70^{S6K} activation, but UV irradiation alone induced 2- to 3-fold activation of p70^{S6K}. The UV-induced activation of p70^{S6K} was blocked by pre-treating the cells with PDT. Therefore, although the effects of UV irradiation and PDT on insulin-induced activation of PI 3-kinase were similar, they were very different with respect to the insulin-induced activation of PKB and p70^{S6K}.
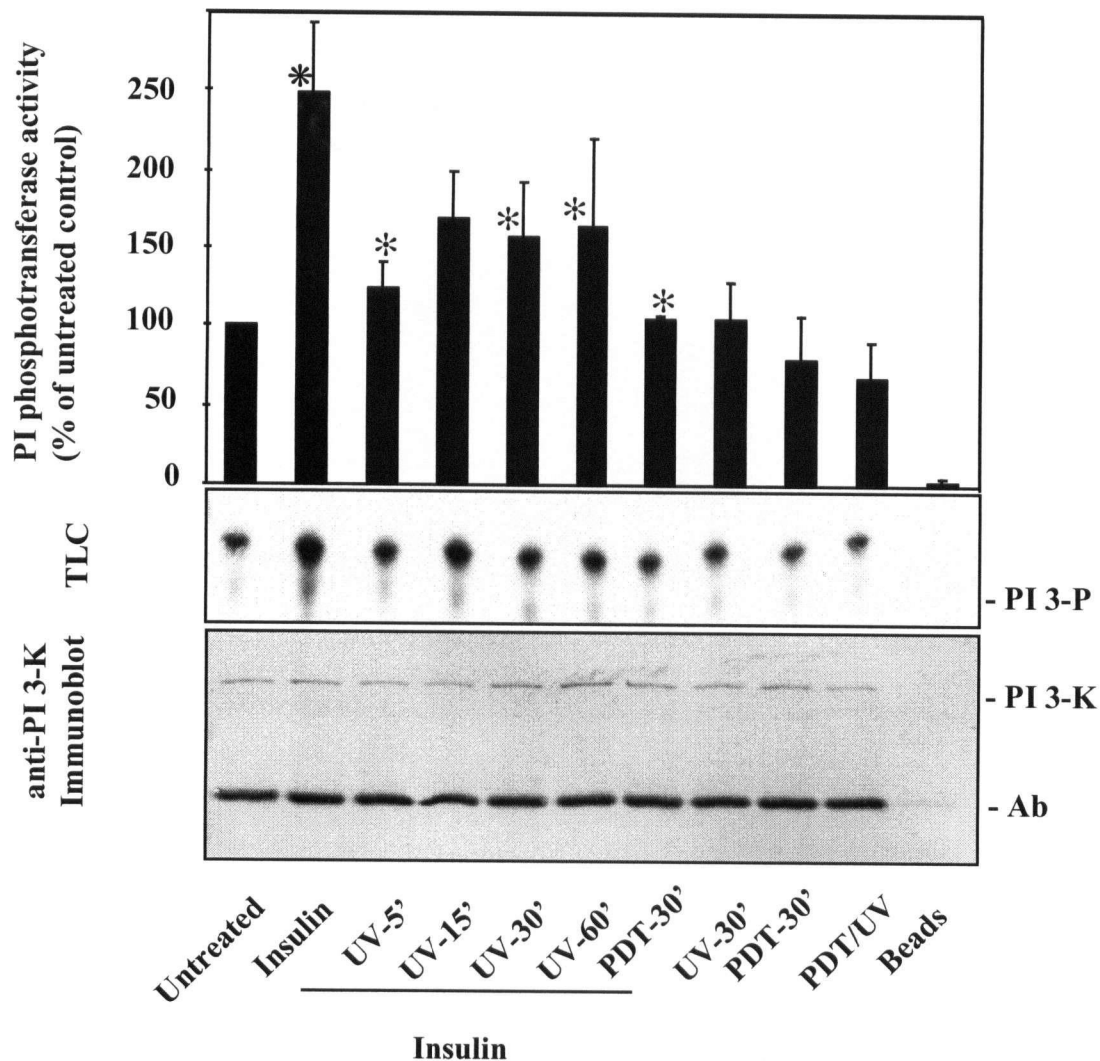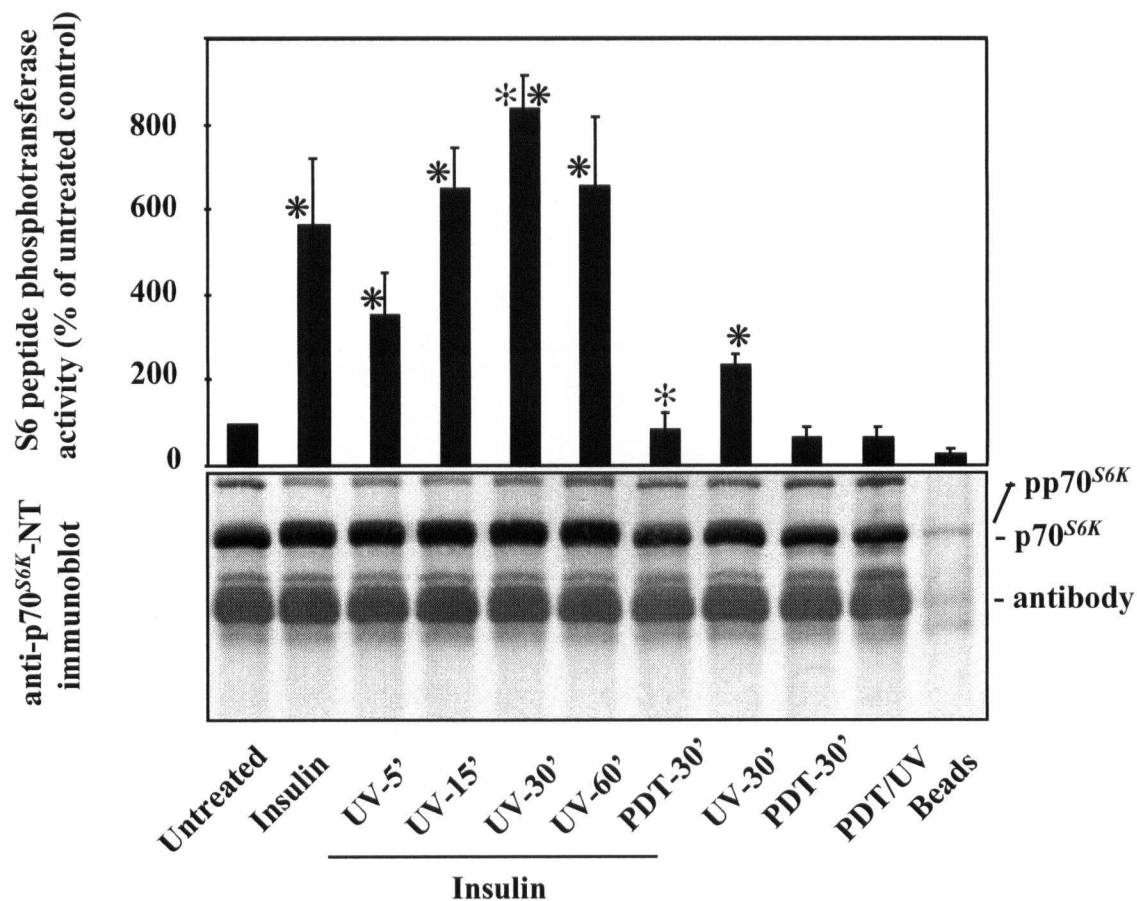
Fig. 36. Effect of UV irradiation on insulin-activation of p70^{S6K}: Serum-starved Pam212 cells were treated with 100 ng/ml BPD plus 2 J/cm^2 of red light, 4 min exposure to UV light, or PDT prior to UV irradiation. At various times after treatment, cells were stimulated with 100 nM insulin for 10 min. The immunoprecipitates were assayed using the S6 peptide as a substrate. Identical immunoprecipitates were electrophoresed, transferred and immunoblotted. Data from three independent experiments were pooled. Beads control represents protein A sepharose beads incubated with extract in the absence of antibody. Untreated controls exhibited an enzyme activity of about 2,000 counts per minute in a 10 minute incubation. ✱, statistically significant difference between samples derived from variously treated cells and that from untreated control cells. ✶ , statistically significant difference between samples derived from variously treated cells and that from insulin-treated cells. Bars, S.D.
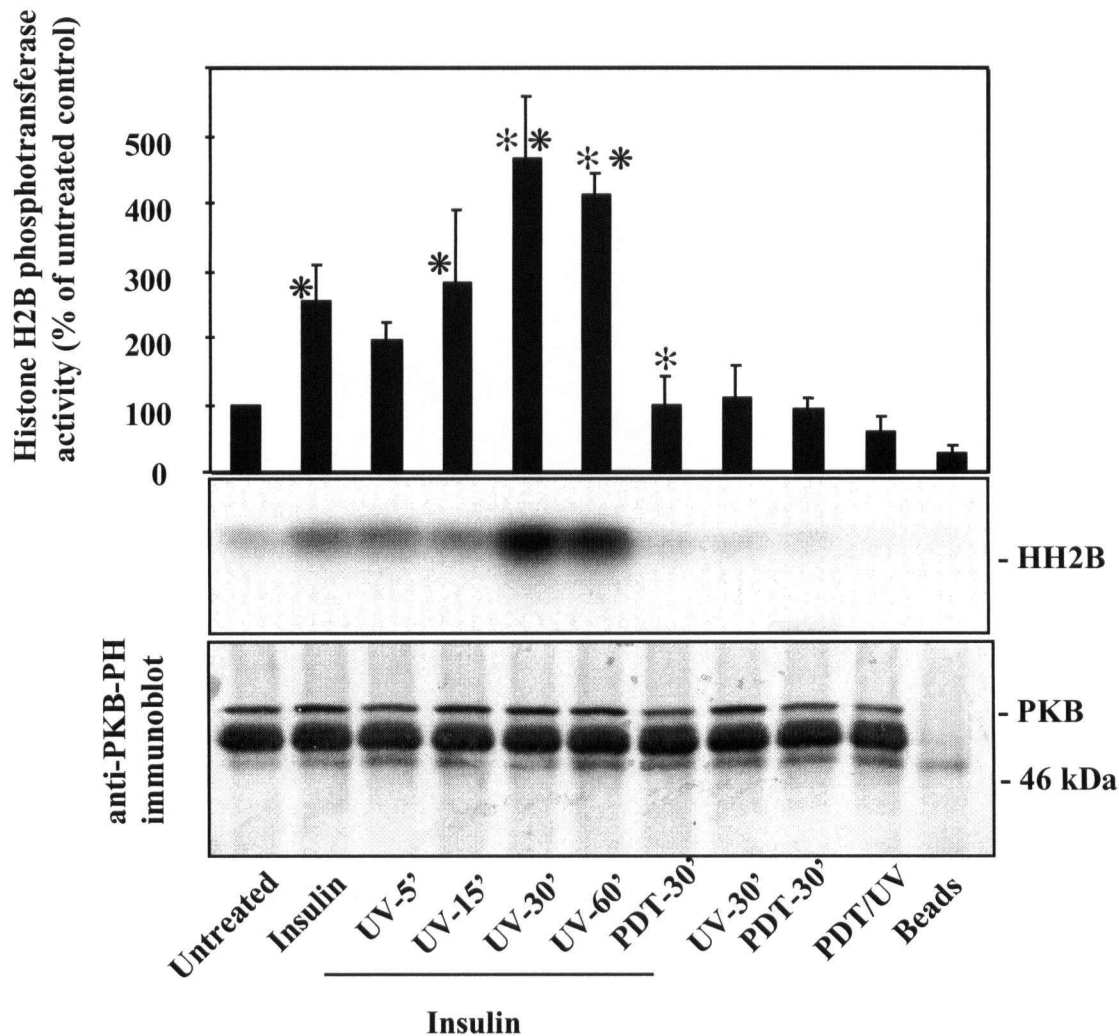
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4.3.4 Effect of PDT on anisomycin-induced activation of p70^{S6K}

We have demonstrated that PDT caused profound inhibition of growth factor-induced activation of PI3-kinase, PKB and p70^{S6K}. It is unknown whether the inhibitory effect of PDT on p70^{S6K} phosphotransferase activity was a consequence of inhibition of the activity of PI 3-kinase, PKB, or other proximal targets upstream of p70^{S6K}. That the inhibition of UV-induced activation of p70^{S6K} by PDT was independent of PI 3-kinase and PKB favored the second possibility. To address the question of whether PDT inhibited upstream targets other than those in the PI 3-kinase /PKB pathway, we investigated the effect of PDT treatment on anisomycin-induced activation of p70^{S6K}.

Serum-starved Pam212 cells were treated with 40 µg/ml of anisomycin for various periods of time up to 90 min (Fig. 37A). PI 3-kinase, PKB and p70^{S6K} were immunoprecipitated from the crude cell extracts and assayed *in vitro* as described previously. There was no significant increase in PI phosphotransferase activity in the PI 3-kinase immunoprecipitates for the duration of the experiment. Similarly, there was no significant change in the phosphotransferase activity of the immunoprecipitated PKB. However, 1 min after anisomycin treatment, there was a marked, 2.5-fold increase in the S6 phosphotransferase activity of p70^{S6K}. The anisomycin-induced activation of p70^{S6K} peaked at 20 to 30 min post anisomycin treatment and lasted for the duration of the experiment. These results indicated that anisomycin-induced p70^{S6K} activation was independent of PI 3-kinase and PKB.

We then investigated the effect of PDT on the anisomycin-induced activation of p70^{S6K} (Fig. 37B). Thirty min treatment with 40 µg/ml anisomycin induced a 9-fold increase in S6 phosphotransferase activity of p70^{S6K}. The activation was completely blocked when cells were pre-treated with PDT or 25 ng/ml rapamycin. The activation of p70^{S6K} was also largely inhibited by pretreatment with 100 nM wortmannin. Immunoblot analysis revealed the presence of the high molecular, activated form of p70^{S6K}

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Fig. 37. PDT inhibits anisomycin-induced activation of p70^S6K: Serum-starved Pam212 cells were stimulated with 40 mg/ml anisomycin for various periods of time as indicated (A). The data presented were representative of two separate experiments. Untreated controls exhibited an enzyme activity of about 4,000, 500, and 10,000 counts per minute in a 10 minute incubation, for PI 3-kinase, PKB, and p70^S6K, respectively. Serum-starved Pam212 cells were treated with 100 ng/ml BPD plus 2 J/cm² red light, 15 min pre-incubation with 100 nM wortmannin or 25 ng/ml rapamycin prior to 20 min stimulation with 40 mg/ml anisomycin (B). Data from three independent experiments were pooled. Untreated controls exhibited an enzyme activity of about 2,000 counts per minute in a 10 minute incubation. Crude whole cell lysates were immunoprecipitated and assayed as previously described. The immune complexes were electrophoresed, transferred and immunoblotted as described. ∗, statistically significant difference between samples derived from variously treated cells and that from untreated control cells. ✳, statistically significant difference between samples derived from variously treated cells and that from anisomycin-treated cells. Bars, S.D.

in the samples derived from insulin-treated cells. This high molecular weight species was not present in the samples derived from cells pretreated with wortmannin, rapamycin or PDT.

These results support the conclusion that the inhibitory effect of PDT on anisomycin-induced $p70^{S6K}$ activation was independent of the PI 3-kinase and PKB pathway.

Chapter 5: General Discussion, Future Directions and Conclusions

5.1   Introduction

$p70^{S6K}$ is a mitogenically activated protein-serine/threonine kinase whose only

known physiological substrate is ribosomal protein S6 (Pullen and Thomas 1997). The

kinase mediates increased protein translation of 5'TOP (terminal oligopyrimidine tract)

mRNAs. This group represents a small family of transcripts essential for the onset of

protein synthesis, such as ribosomal subunits and translation initiation factors. In spite of

the fact that the number of individual genes containing 5'TOP sequences is small, these

RNAs are predicted to make up 15-20% of total cellular RNA (Meyuhas *et al.*, 1996), and

it is likely that the kinase that plays a major role in the induction of these genes is

essential for mitogenesis. Therefore, a clearer understanding of the role of $p70^{S6K}$ in

signaling and during the maturation process will contribute to our understanding of these

processes. This study was undertaken to attempt to purify and characterize the sea star

homologue of $p70^{S6K}$, and to use the purified kinase to attempt to clarify the role the

kinase plays in maturation in sea star oocytes.

The signaling events leading to the activation of $p70^{S6K}$ are complex, and have

been difficult to unravel. We had previously noted that photodynamic therapy (PDT)

with benzoporphyrin derivative mono-acid ring A (verteporfin) (BPD-MA) led to

activation of the SAPK and p38 HOG1 pathways in a transformed murine keratinocyte

cell line, Pam212 (Tao *et al.*, 1996). In this system, $p70^{S6K}$ activity was profoundly

inhibited in response to PDT. We therefore undertook an investigation of the role of

$p70^{S6K}$ as well as PKB and PI 3-kinase in Pam212 cells treated with PDT.

5.2   p70^{S6K} in *Pisaster ochraceus*

5.1.1   Identification of p52^{S6K} as a homologue of p70^{S6K} and regulation of the kinase activity

I have described, for the first time, the partial purification of a ribosomal S6 kinase from an echinoderm source. The kinase was identified by screening immature and mature sea star extracts that had been fractionated by MonoQ chromatography. An S6 peptide phosphotransferase activity that became activated during maturation was enriched through a series of chromatography steps. The seven column-step procedure yielded extremely active protein kinase phosphotransferase activity, although protein yields were very low. Immunoblot analysis of purified proteins from early stages of the purification produced immunoreactive bands at ~70-75 kDa and ~52 kDa with three different antibodies directed against the amino terminal, carboxyl terminal and internal sequences of p70^{S6K}. During progressive chromatography steps, the antibodies immunoreacted with proteins at ~70 kDa as well as at ~52 kDa with the smaller form of the protein immunoreacting much more strongly with the amino terminal antibody. In the purified preparation, a protein at ~ 52 kDa was recognized by the amino terminal antibody only. I interpreted these findings to indicate that two distinct isoforms of p70^{S6K} are present in sea star, and that the purification procedure detailed in this study substantially enriched the p52^{S6K} isoform.

As discussed earlier, previously characterized isoforms of p70^{S6K} contain a pseudosubstrate, auto-inhibitory region in the carboxyl terminus that likely regulates activity. It is possible that this region was proteolytically cleaved during the purification procedure, leading to purification of a hyperactivated form of the kinase. This would be consistent with the observed result (see Section 3.3.5) that the partially purified kinase did not undergo a significant amount of autophosphorylation. Indeed, if the kinase was

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already maximally activated, it would not be expected to undergo further autophosphorylation. Although the model of proteolytic activation during purification is one explanation for the observed results, it is also possible that a distinct 52 kDa isoform of p70^{S6K} had been enriched. This will ultimately be established by molecular cloning of the 52 kDa isoform from sea star oocytes. This was not undertaken during the course of this study due to the difficulty in obtaining an mRNA library in sea star.

The sea star p52^{S6K} phosphorylated the S6 peptide substrate as well as the 40S ribosomal protein S6 and the phosphotransferase activity was stable for months at -70°C, allowing it to be analyzed over a period of time. p52^{S6K} was further characterized with respect to ion and co-factor dependence and it was found that the kinase was inhibited by fluoride, but relatively insensitive to most other common treatments, similar to other published reports for p70^{S6K} (Jenö et al., 1989; Ferrari et al., 1991), as discussed in Section 3.3.3.

The sea star p52^{S6K} efficiently utilized myelin basic protein and the 40S ribosomal protein S6 as substrates, and phosphoamino acid analysis of the S6 protein showed that the kinase phosphorylated S6 exclusively on serine. Two-dimensional phosphopeptide mapping of the 40S ribosomal proteins revealed phosphorylation of 8 distinct tryptic peptides, fewer than that of previously identified p70^{S6K} isoforms (Bandi et al., 1993; Martin-Perez and Thomas, 1983; Martin-Perez et al., 1984). This result was likely due to insufficient incubation time of the kinase with its substrate as discussed in Section 3.3.6.

I went on to examine the substrate specificity of echinoderm p52^{S6K} using a series of synthetic peptides modeled after the sites in the S6 protein known to be phosphorylated by p70^{S6K} (R^{232}RLS*S*LRAS*TSKS*ESS*QK). The sites in p70^{S6K} are phosphorylated in a sequential manner in vivo, in the order Ser-236>Ser-235>Ser-240>Ser-244>Ser-247 (Krieg et al., 1988; Martin-Perez and Thomas, 1983). Enzyme kinetics for K_m were determined using the Michaelis-Menton rate equation and V_{max} was estimated using

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Lineweaver-Burke double-reciprocal plots of dose-response data. Kinetic analyses of the various peptides (see Table 4 and Section 3.3.8) provided some intriguing differences from previously reported consensus sequences for p70^{S6K}. Of particular interest was the finding that an arginine residue in the +2 position enhanced kinase activity, unlike previous reports (Flotow and Thomas 1992). This finding may at least partially explain why Ser-236 is the first residue to be phosphorylated by the p70^{S6K} *in vivo*. The initial phosphorylation event may then allow phosphorylation of the other sites to proceed.

Unfortunately, in spite of many attempts, we were unable to obtain micro-sequence data for the purified sea star kinase. One possible explanation for this was the fact that the enzyme was present in such small quantities. Two proteins that were present in the final purification, PP2A and HSP-60, are much more abundant in cells than protein kinases. It is possible, therefore, that these two proteins physically masked the presence of the kinase, preventing us from obtaining sufficient quantities for micro-sequencing to be successful. Nevertheless, the basic biochemical characterization of the kinase activity supports its identification as an echinoderm homologue of p70^{S6K}. The kinase was recognized by antibodies to p70^{S6K}, the substrate specificity was similar to, though not identical to published reports and the phosphotransferase activity was active against 40S ribosomal subunits and was inactivated by dephosphorylation. It would be desirable in the future, to attempt to produce enough material to obtain micro-sequencing data. One potential strategy would be to utilize 2-dimensional gel electrophoresis to better separate the protein contaminants from the kinase. The recent availablility of more sensitive antibodies will also facilitate identification of the kinase band on immunoblots.

5.1.2 Multi-protein complex formation

Interestingly, the two proteins that co-purified with p52^{S6K} were identified by immunoblot analysis and by direct protein micro-sequencing, as a heat shock protein (HSP-60) and the regulatory subunit of PP2A (Table 5 and Fig. 19). Further, I have co-immunoprecipitated S6 kinase phosphotransferase activity with anti-PP2A specific antibodies, and co-immunoprecipitated S6 kinase with HSP-60 (Fig. 21). In addition, PP2A in the purified preparation was phosphorylated on serine residues, consistent with its phosphorylation by the p52^{S6K} in the preparation (Fig. 22). These results raise the intriguing possibility that the sea star S6 kinase may form a multi-protein complex *in vivo* and the activity of the S6 kinase may be regulated by it's association with PP2A and/or HSP-60. In collaboration with Dr. Brian Wadzinsky (Vanderbilt University, Nashville), we have also observed that purified rat brain PP2A complexes with p70^{S6K} (unpublished observations).

Immunoblot analysis of heat shock-treated sea star oocytes demonstrated that the sea star S6 kinase, HSP-60, and PP2A were present in the same fractions and that the protein levels of S6 kinase seemed to decrease in response to this treatment (Fig. 24). Co-purification of the three proteins over seven different column chromatography steps, combined with the co-immunoprecipitation studies, as well as the results of the heat shock-treatment immunoprecipitations support the possibility that these proteins interact *in vivo*.

However, gel filtration analysis of the enriched kinase (Fig. 10), contradicts this hypothesis since the phosphotransferase activity eluted at a molecular size of about 50-55 kDa. Although gel filtration is thought to be a gentle process that can maintain proteins and complexes in native form, it could be that the protein-protein interactions within the complex were weak, and the components may have become denatured during this step. It was also possible that an essential component for maintenance of the integrity of the

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complex had been purified away during a previous purification step. Alternatively, it is also possible that the S6 kinase/PP2A complex interacted with the gel filtration resin, eluting with a lower apparent molecular weight. It was also possible that the larger isoform ($p70^{S6K}$) was required for interaction with PP2A, HSP-60 or both. This could explain why co-immunoprecipitation of S6 kinase with HSP-60 and immunoprecipitation of phosphotransferase activity against the 40S ribosome with anti-PP2A antibodies needed to be carried out using either crude, cytosolic sea star extract or partially purified (two or three steps) sea star S6 kinase.

Several potential lines of investigation could be pursued from these results. Future experiments could evaluate which regions of $p70^{S6K}$ are important for binding to PP2A and HSP-60, and which subunits or regions of PP2A and HSP-60 are critical for binding to $p70^{S6K}$. A series of $p70^{S6K}$ point and deletion mutants could be created, expressed, and tested for their ability to bind to PP2A and HSP-60 purified from other sources. In addition, we speculate that the formation of PP2A complexes with $p70^{S6K}$ may be reduced or altered in human tumours. Immunological techniques could be used to evaluate quantitiative changes of these proteins in human tumours as compared to matched, normal tissue. Functionally, the effect of $p70^{S6K}$ association with PP2A and HSP-60 on it's phosphotransferase activity could be evaluated. It would be expected that the association of PP2A with $p70^{S6K}$ would decrease the catalytic activity of $p70^{S6K}$. This could be tested by assessing the activities of the various active, recombinant components through enzyme assays and gel electrophoresis.

5.2.3   Functional aspects of $p70^{S6K}$ signaling

Decreased activity of $p70^{S6K}$ has been implicated as playing a role in G1 arrest (Calvo *et al.*, 1992; Morice *et al.*, 1992). Presumably, this blockage is at least partially due to the decrease in protein translation in response to decreased protein S6

phosphorylation by p70^{S6K}. During sea star oocyte maturation, dramatic increases in the rate of protein translation occur, and it is likely that the activation of p70^{S6K} during maturation plays a critical role in the onset of increased translation. Active, human p70^{S6K} was expressed using the baculovirus expression system and the active kinase was microinjected into immature sea star oocytes to measure the effect of this treatment on the maturation process. This had no discernible effect on the maturation process and injection of active p70^{S6K} did not induce maturation in the absence of 1-methyladenine. As p70^{S6K} activation is a relatively late event in the signaling pathway, many other cellular events would be expected to be required to support the processes leading to maturation. It seems likely that a number of alterations or pertubations in signaling pathways would be necessary to disrupt such an important process, and cells undoubtedly have other redundant routes of signal transmission.

To identify an upstream activator of p70^{S6K}, immature sea star extract could be fractionated on MonoQ and fractions assayed with expressed, inactive p70^{S6K}. This would permit identification of fractions containing potential p70^{S6K} kinases, which could then be purified and characterized. Further, the inactive, expressed kinase, or a dominant-negative S6 kinase could be microinjected into oocytes to assess whether such a construct could block or delay oocyte maturation.

As the phosphorylation of the S6 protein is an integral and obligatory step in the initiation of translation of specific proteins required for mitosis, the purification of p52^{S6K} described here will provide the means to further dissect the pathways leading to cell division and growth in a variety of systems. In addition, studies could be conducted to determine the role, if any, of HSP-60 and PP2A in the regulation of p70^{S6K} or p52^{S6K}. Specific kinase-phosphatase complexes may represent a novel means by which cellular signaling may be regulated.

5.3   PI 3-kinase/PKB/ p70^{S6K} Signaling in PDT

5.3.1   Effect of PDT

Although the molecular events by which PI 3-kinase activates PKB are rapidly being uncovered (Cohen *et al.,* 1997), the signaling events leading to activation of FRAP, p70^{S6K}, and 4E-BP1 have proved much more difficult to unravel. Removal of the amino and carboxyl termini of p70^{S6K} generates a rapamycin-resistant mutant that retains sensitivity to wortmannin (Dennis *et al.,* 1996). This indicates that FRAP and PI 3-kinase signal to p70^{S6K} via different routes. FRAP may be constitutively active, with downstream regulation of p70^{S6K} in response to mitogenic stimulation provided by signaling from PI 3-kinase. Recent data indicates that different kinases phosphorylate 4E-BP1 and p70^{S6K}. First, the rapamycin-sensitive sites in p70^{S6K} are flanked by large, aromatic amino acids, while the rapamycin-sensitive sites in 4E-BP1 are proline-directed sites (Pearson *et al.,* 1995). Furthermore, overexpression of p70^{S6K} blocks reporter p70^{S6K} activation and prevents phosphorylation of the rapamycin-sensitive sites (Von Manteuffel *et al.,* 1997). Significantly, overexpression of p70^{S6K} also blocks phosphorylation of the rapamycin-sensitive sites in 4E-BP1 (Von Manteuffel *et al.,* 1997), indicating that the overexpressed p70^{S6K} acts by sequestering an upstream activator, possibly FRAP. This hypothesis would be consistent with the notion that although FRAP lies upstream of p70^{S6K}, and may be required for its activation, another kinase responsible for phosphorylation of the sites surrounded by bulky aromatic amino acids is also required for full p70^{S6K} activity. Two recent publications have identified PDK1, known to activate PKB, as also activating p70^{S6K} by phosphorylation of Thr-229 (Pullen *et al.,* 1998; Alessi *et al.,* 1998). The kinase that phosphorylates p70^{S6K} at Thr-389 remains to be identified, although a recent report suggests that this site may be regulated by ILK (Delcommenne *et al.,* 1998). To further investigate the role of p70^{S6K}, PKB and PI 3-kinase, we carried out

analysis of photodynamic therapy (PDT) with benzoporphyrin derivative monoacid ring A (BPD-MA) treatment in a transformed murine keratinocyte cell line, Pam212.

We have shown for the first time that PDT caused a dose- and time-dependent inhibition of serum-induced $p70^{S6K}$ activation in Pam212 cells. PDT also blocked insulin-induced activation of PI 3-kinase, PKB and $p70^{S6K}$ in serum-starved cells. In addition, PDT blocked anisomycin-induced activation of $p70^{S6K}$ in these cells.

We analyzed the phosphotransferase activities of PI 3-kinase, PKB and $p70^{S6K}$ by immunoprecipitation followed by *in vitro* kinase assays using standard substrates. PI 3-kinase activity was assessed by demonstrating incorporation of phosphate into PI and PIP_2 respectively, while PKB phosphotransferase activity was assessed by incorporation of phosphate into HH2B. The $p70^{S6K}$ phosphotransferase activity was measured both by incorporation of phosphate into the S6 peptide substrate, as well as by immunoblot analysis of the well established electrophoretic mobility shift due to increased phosphorylation during activation of the kinase. For the analysis of the effects of PDT on insulin, anisomycin and UV treatment of Pam212 keratinocytes we included control samples derived from the cells treated with well established pharmacological inhibitors of these kinases of interest.

Rapamycin is a specific inhibitor of FRAP that blocks the activation of FRAP and $p70^{S6K}$ by all known mitogens (Brown *et al.,* 1994; Sabatini *et al.,* 1994). In Pam212 cells, rapamycin inhibited the activation of $p70^{S6K}$ induced by all stimuli used, including serum, insulin, EGF, and anisomycin. As expected, rapamycin did not inhibit the activities of PI 3-kinase or PKB in these experiments. Wortmannin is an inhibitor of members of the PI 3-kinase family. This compound acts by binding covalently to the p110 subunit and inhibits PI 3-kinase at nanomolar concentrations (Yano *et al.,* 1993). In Pam212 cells, wortmannin inhibited growth factor-induced activation of PI 3-kinase, PKB and $p70^{S6K}$. Wortmannin also inhibited the anisomycin-induced activation of $p70^{S6K}$, though not to the same extent as it did the growth factor-induced activation.

Significantly, the results from immune complex assays indicated that the anisomycin-induced activation of p70^{S6K} was independent of PI 3-kinase and PKB, although the activation of p70^{S6K} by anisomycin was wortmannin-sensitive. This can be explained by the fact that wortmannin may have other cellular targets, including FRAP (Brunn *et al.*, 1996). Since FRAP may be an upstream component of growth factor-induced activation of p70^{S6K}, it is possible that the inhibitory effect of wortmannin on anisomycin-induced activation of p70^{S6K} is a consequence of the inhibition of FRAP.

We demonstrated high basal activity of p70^{S6K}, which was abolished by PDT, in Pam212 cells maintained in serum-containing medium. PDT treatment also blocked agonist-induced activation of p70^{S6K} in serum-starved cells. As the signaling events leading to activation of p70^{S6K} are unclear, we investigated the role of two potential upstream signaling proteins, PI 3-kinase and PKB. We found that PDT also inhibited the insulin-induced activation of PI 3-kinase and PKB in these cells. It was unclear, however, whether the inhibitory effect of PDT was exerted directly on the individual kinases, or if it resulted from an upstream inhibition. To address this question, we investigated the effect of anisomycin on the three proteins. We found that anisomycin caused a strong activation of p70^{S6K}, in a PI 3-kinase and PKB independent manner. PDT completely blocked the anisomycin-induced activation of p70^{S6K}. Thus, the PDT effect could be either direct, on p70^{S6K} or on upstream kinases other than PI 3-kinase and PKB. We have also observed that UV irradiation and PMA activated p70^{S6K} in a PI 3-kinase and PKB independent manner, and the activation was blocked by PDT. PMA is known to activate PKC, and thus PKC may be upstream of p70^{S6K} activation in this system. It will be interesting to explore the relationship between PKC and p70^{S6K} in anisomycin- and UV-induced p70^{S6K} activation. Future investigations should characterize the effect of PDT on various isoforms of PKC and the role of PKC in anisomycin- and UV-induced activation of p70^{S6K} in Pam212 cells.

The fact that PDT inhibited p70^{S6K} by all agonists used indicated that PDT may act on proximal targets or may act directly on p70^{S6K} by promoting dephosphorylation of serine/threonine residues required for activation of the kinase. Similarly, we could not conclude whether PDT effects on PKB and PI 3-kinase were direct or a consequence of inhibition above the level of PI 3-kinase. The data from the UV experiments provided some hints as to the answer to this question. We observed a significant inhibition of insulin-induced PI 3-kinase activation when the growth factor was given 5 min post UV treatment. The inhibition of PI 3-kinase decreased with increasing incubation time, yet this inhibition did not lead to a corresponding inhibition of PKB. Instead, there was a significant potentiation of the insulin-induced PKB activation, implying that the potentiation effect of UV irradiation on PKB was independent of PI 3-kinase activation.

One explanation of these results is that UV irradiation could activate upstream kinases other than PI 3-kinase that act synergistically with insulin on PKB. A potential candidate for such a kinase is p38 HOG1. PKB has been shown to be activated by heat and osmotic shock in a PI 3-kinase independent manner (Konishi *et al.,* 1996). UV, heat and osmotic shock are also known to activate p38 HOG1 (Kyriakis and Avruch, 1996). Therefore, in light of our results here, PKB may be activated via a p38 HOG1 dependent pathway. In this case, we should have seen a similar potentiation effect with PDT, since PDT also caused strong activation of p38 HOG1 (Tao *et al.,* 1996), but we did not. Instead, insulin-induced activation of PKB was blocked when cells were given insulin 30 min later. Therefore, the effect of PDT on PKB may be mediated through two separate mechanisms, inhibition of PI 3-kinase, or a direct inhibition of PKB or its proximal activating kinase, PDK1.

In Pam212 cells, UV irradiation and PDT inhibited insulin-induced activation of PI 3-kinase. The inhibition appeared to be more profound with PDT, but the mechanism of the inhibition is unknown. PDT targeted events may include ligand-binding-induced activation of the insulin receptor tyrosine kinase, phosphorylation of IRS-1 and

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recruitment of signaling molecules, including PI 3-kinase to the membrane. Alternatively, growth factor-induced activation of Ras may be targeted by PDT. Further analysis of these upstream signaling events will facilitate our understanding of the mechanism of PDT-mediated inhibition of PI 3-kinase.

In conclusion, our results demonstrated that PDT caused an inhibition of growth factor-induced activation of PI 3-kinase, PKB and $p70^{S6K}$. PDT also inhibited agonist-induced activation of $p70^{S6K}$ in a PI 3-kinase, PKB independent manner. At present we have no experimental data indicating possible targets of PDT. We do not know if the inhibitory effects of PDT are direct or a consequence of upstream inhibition, although the evidence implies that the inhibition may be exerted independently on multiple kinases. One model in support of such a notion would be PDT activation of a serine/threonine protein phosphatase that was capable of dephosphorylating and inactivating all three kinases. Consistent with this hypothesis, the activation of PKB and $p70^{S6K}$ require multiple serine and threonine phosphorylations, although such a mechanism has not been proposed for PI 3-kinase activation.

Future investigations in this system should include a thorough analysis of the various PKC isoforms and the PDT effect on the anisomycin-induced activation of $p70^{S6K}$. One of the major difficulties that we have encountered in this series of experiments was that we had no assay for, or immunoblotting or immunoprecipitating antibodies against FRAP. Such antibodies would be of considerable use in unraveling the links between PKC, PKB, FRAP, 4E-BP1 and $p70^{S6K}$.

5.3.2 Specificity of the PDT effect

The PDT-induced inhibition of PI 3-kinase, PKB and $p70^{S6K}$ in Pam212 cells was not observed in cells treated with drug in the absence of light, or with light in the absence of drug. This indicated that the observations were the results of reactive oxygen species (ROS)-mediated photodynamic effects. It had been shown that H_2O_2 strongly activated ERK1 and ERK2 and we had previously observed that H_2O_2 also activated PI 3-kinase and PKB (Tao *et al.*, 1996). We have shown here that the PDT effect is mediated differently than that of UV irradiation, which activated $p70^{S6K}$ and potentiated the effect of insulin on PKB. As the UV effect is also dependent on ROS, the effects of PDT reported here were not likely to be due to the ubiquitous effects of ROS, but specific properties of PDT with BPD-MA.

Possible explanations for the unique properties of PDT with BPD-MA may be understood in terms of the characteristics of the photosensitizer. BPD-MA is a hydrophobic molecule that is localized in the cytoplasm in association with membranous structures (Margaron, 1998). The subcellular localization of many signaling molecules permits their interaction with substrates and other regulatory proteins. Similarly, the localization of BPD-MA, and a local increase in ROS may be responsible for the specificity of the photodynamic effect. To establish the role of subcellular localization of the photosensitizer in mediating the effects of PDT, direct evidence including functional interactions and physical co-localization of the signaling components needs to be obtained using biochemical and imaging methods. It will also be of interest to discover if the signaling components of this pathway undergo oxidation-mediated modifications during PDT.

5.3.3 PDT in apoptosis

The inhibitory effect of PDT on PI 3-kinase, PKB and p70^{S6K} was observed within a dose range of PDT between 50 to 100 ng/ml BPD-MA plus 2 J/cm^2. At this dose range, significant cytotoxicity was observed in apoptosis assays and antioxidant treatment provided some protection against PDT-induced cell death (data not shown).

Both PI 3-kinase and PKB have been implicated as playing a key role in prevention of apoptosis in response to survival signals (Kulik *et al.,* 1997; Kennedy *et al.,* 1997; Kaufmann-Zeh *et al.,* 1997). The role that p70^{S6K} plays in prevention or induction of apoptosis is unclear. Recent work that I performed in collaboration with Michael Scheid (Ph.D. candidate) at the Jack Bell Institute provided the first report that p70^{S6K} is not required for PI 3-kinase mediated cell survival in hemopoietic cells (Scheid *et al.,* 1996). We showed that rapamycin was unable to induce apoptosis in cells stimulated with cytokines. Inhibition of PI 3-kinase with wortmannin or LY294002 was able to induce apoptosis in these cells, providing the first evidence of a bifurcation of the signaling pathways from PI 3-kinase. A second collaborative work with Dr. John Jackson at the University of British Columbia also dissociated the p70^{S6K} from the PI 3-kinase pathway (Tudan *et al.,* submitted). In this study, we showed that in neutrophils activated by calcium pyrophosphate dihydrate crystals, p70^{S6K} was activated via a PI 3-kinase independent and PKC dependent pathway.

Therefore, the available evidence indicates that p70^{S6K} does not play a role in PDT-mediated induction of apoptosis. Nevertheless, the fact that PDT activated stress-activated pathways and inhibited PI 3-kinase, PKB and p70^{S6K} is intriguing. It is possible that PDT triggers a pro-apoptotic signal leading to the activation of SAPKs and the inhibition of PI 3-kinase and PKB survival signals. The PDT effects on these kinases may arise as signaling effects that occur in parallel with apoptosis. To test these possibilities, the susceptibility to apoptosis of cells expressing mutant SAPKs which are

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insensitive to PDT, or mutant PKB and $p70^{S6K}$ which are resistant to PDT-mediated inhibition should be tested. Pharmacological inhibitors of the SAPKs and MEKKs could also be employed in these studies.

5.4   Conclusions

PI 3-kinase, PKB, FRAP and $p70^{S6K}$ appear to represent a branch of a larger signaling pathway that is involved in cell growth, survival, and the initiation of protein synthesis. Several components of the pathway appear to be required for prevention of apoptosis, while others appear to be involved in the translation of proteins required for growth or proliferation. The interactions between these proteins are complex, especially in the case of $p70^{S6K}$. Some of the direct upstream activators are as yet uncharacterized, and it is clear that the regulation and activation of $p70^{S6K}$ is complicated and requires multiple signaling events and interactions with other regulatory proteins. The analysis of $p70^{S6K}$ has been facilitated recently by the availability of immunoprecipitating antibodies, specific inhibitors, and expression of recombinant, active kinase.

The key novel findings of the research presented here include the first protocol for purification of an echinoderm homologue of $p70^{S6K}$, and identification of distinct differences in substrate specificity from previously reported isoforms. In addition, I provide evidence supporting the possibility of multi-complex formation comprising $p70^{S6K}$, PP2A and HSP-60. Finally, we show for the first time, that $p70^{S6K}$ is profoundly inhibited in response to PDT, although we do not at present understand the mechanism of PDT-induced signaling. Our observations here will contribute to our understanding of the role of $p70^{S6K}$ in sea star oocyte maturation, and the mechanism of action of PDT in murine keratinocytes.

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