IDENTIFICATION OF SUBSTRATES AND PATHWAYS REGULATED BY PAS KINASE

APPROVED BY SUPERVISORY COMMITTEE

Steve McKnight, Ph.D.

Michael Rosen, Ph.D.

Kosaku Uyeda, Ph.D.

Andrew Zinn, M.D., Ph.D.

DEDICATION

I would like to dedicate this work to my wonderful wife Stephanie and my three boys: Taylor, Tanner and Hunter – for their loving support and encouragement. Also to my parents who helped make this dream a reality.

IDENTIFICATION OF SUBSTRATES AND PATHWAYS REGULATED BY PAS KINASE

By

BRANDON LINN PROBST

DISSERTATION

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IDENTIFICATION OF SUBSTRATES AND PATHWAYS REGULATED BY PAS KINASE

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Brandon Linn Probst, Ph.D.

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Supervising Professor: Steven L. McKnight, Ph.D.

Abstract: PAS kinase, a serine/threonine protein kinase, is unique in that it comprises the only mammalian protein kinase regulated by a PAS domain. The interest of the McKnight laboratory in understanding the regulation and biological role of PAS kinase stems from knowledge that PAS domains typically function as sensors in other systems. My study primarily focused on unveiling physiological PAS kinase (PASK) substrates and interacting gene products to establish pathways regulated by PASK. First, we examined the function of PASK in the budding yeast, *S. cerevisiae*. An unbiased biochemical screen for putative PASK substrates from cellular extracts uncovered five polypeptides phosphorylated in a PASK-dependent manner. Two of the substrates identified are known translation factors and a third is an RNA-binding protein that was also found to be a high copy suppressor of the

psk1 psk2 double mutant phenotype. We further observed PASK to phosphorylate two enzymes in the pathway for the synthesis of glycogen: UDP-glucose pyrophosphorylase and glycogen synthase. Genetic and biochemical data provide evidence that both of these enzymes are inhibited by PASK-dependent phosphorylation.

We next examined the role of PASK in mammalian cells. With the exception of glycogen synthase none of the mammalian homologs of the yeast PASK substrates have been found to qualify as substrates for the mammalian PASK. To this end, a second unbiased, large-scale biochemical screen was employed using HeLa cell extracts to discover phosphorylation targets of the mammalian enzyme. This biochemical screen entailed the disruption of 150 liters of HeLa cells into a soluble extract and subsequent fractionation over seven chromatographic steps to generate roughly 1000 partially purified pools of protein. This effort led to the identification of enzyme substrates involved in protein synthesis and intermediary metabolism. Phosphorylation site mapping of these substrates identified the consensus motif, R-X-A/x-S*/T* as the optimal substrate for PASK.

Although it remains unclear as to the functional role of PAS kinase-dependent phosphorylation of these substrates, there appears to be a unifying theme in both yeast and mammalian systems. PAS kinase is a PAS-domain regulated enzyme controlling translation as this energetically expensive process is coupled to cellular energy metabolism.

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PRIOR PUBLICATIONS

Rutter J, **Probst BL** and McKnight SL. (2002). Coordinate Regulation of Sugar Flux and Translation by PAS Kinase. **Cell**. 111, 17-28.

Wilson WA, Skurat AV, **Probst BL**, de Paoli-Roach A, Roach PJ, and Rutter J. (2005). Control of Mammalian Glycogen Synthase by PAS Kinase. *In Press - PNAS.*

Probst BL, Xie S, Wu L, Michnoff CH, Rutter J and McKnight SL. (2005). Two Distinct HTS of PAS Kinase Yield Converging Insights to Enzyme Function. Manuscript in preparation.

Probst BL. (2005). MEKK1 Regulates PASK by both Phosphorylation and Induction of 14-3-3γ Interaction. Manuscript in preparation.

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LIST OF DEFINITIONS

AMPK – 5'-AMP Activated Protein Kinase

BSA – Bovine Serum Albumin

cAMP – Cyclic Adenosine Monophosphate

cDNA – Complimentary DNA

Cys – Cysteine

DNA – Deoxyribonucleic Acid

DTT – Dithiothreitol

E. coli – *Escherichia Coli*

EDTA – Ethylenediaminetetracetic Acid

FAD – Flavin-adenine Dinucleotide

FBS – Fetal Bovine Serum

FMN – Flavin-mono nucleotide

GSK3 – Glycogen Synthase Kinase 3

GST – Glutathione S-Transferase

H_2O_2 – Hydrogen Peroxide

HeLa – Human Cancerous Cervical Cell Line

HPLC – High Pressure Liquid Chromatography

HRP – Horseradish Peroxidase

kB – Kilobase

kD – Kilodalton

MALDI – Matrix Assisted Laser Desorption Ionization Mass Spectrometry

$MgCl_2$ – Magnesium Chloride

MLCK – Myosin Light Chain Kinase

mRNA – Messenger RNA

NaCl – Sodium Chloride

NaF – Sodium Fluoride

$NaVO_4$ – Sodium Orthovanadate

NES – Nuclear Export Signal

NLS – Nuclear Localization Signal

NMR – Nuclear Magnetic Resonance

OD – Optical Density

ORF – Open Reading Frame

PAGE – Polyacrylamide Gel Electrophoresis

PASK – PAS Kinase

PCR – Polymerase Chain Reaction

PKA- Protein Kinase A

RNA – Ribonucleic Acid

SDS- Sodium Dodecyl Sulfate

Ser – Serine

Sf9 – *Spodoptera frugiperda* Cells

SH2 – Src Homology Domain 2

SH3 – Src Homology Domain 3

Thr – Threonine

TOF – Time of Flight

UDP – Uridine Diphosphate

β-ME – Beta-mercaptoethanol

CHAPTER ONE
Introduction

PROTEIN KINASE MEDIATED SIGNAL TRANSDUCTION

Protein Kinases

Intracellular signal transduction pathways are key regulatory events that control nearly every decision made by living cells. Reversible phosphorylation of tyrosine, serine and threonine residues on proteins is the most common mechanism in the regulation of intracellular signaling pathways. These signaling pathways govern metabolic decisions, assembly and disassembly of macromolecular complexes, cell growth and differentiation, cell death, membrane transport and intercellular communication. It is estimated that one-third of all intracellular proteins are phosphorylated at a given time and condition in the cell (Johnson and Hunter, 2005). This correlates to approximately 20,000 phosphoprotein states (Johnson and Hunter, 2005). Phosphorylation and dephosphorylation are catalyzed by specific enzymes called protein kinases and phosphatases, respectively. Protein kinases are one of the largest protein superfamilies (Hanks and Hunter, 1995) and the human kinome consists of 518 members (Manning et al., 2002). While this single superfamily is fairly complex, protein kinases share a conserved catalytic domain of approximately 270 amino acids and employ a conserved mechanism to move phosphate from ATP to macromolecular substrates (Hanks et al., 1988).

REGULATION OF PROTEIN KINASES

Despite the preservation of conserved catalytic domain and mechanism, sequence and structural differences, both in the core and flanking regions, allow each kinase to be tuned in order to respond to a distinct stimulus and phosphorylate a distinct set of substrates. Most of

these differences involve conformational changes that allow the kinase to exist in tightly controlled “on” or “off” states. Typical regulatory mechanisms include autophosphorylation, post-translational modifications, allosteric mechanisms, second messengers, feedback inhibition, spatial and temporal localization.

For many kinases, activation requires phosphorylation of the activation loop. For protein kinase A (PKA), the crystal structure demonstrates that the active kinase has a pocket of positively charged residues (RD pocket) that interacts with the phospho-residue in the activation loop (Knighton et al., 1991). This allows adoption of a distinct conformation that cannot not be seen in inactive kinase structures (Zhang et al., 1994). In some cases phosphorylation of the activation segment allows it to change conformation in such a way that the activation loop positions itself for substrate binding. In other cases, rearrangement of the activation loop upon phosphorylation relieves autoinhibitory mechanisms such as blockage of ATP or substrate binding sites.

Some kinases contain multiple phosphorylation sites, many of which are not in the activation loop. Such phosphorylation events can occur both N- and C-terminal to the primary activation site and exhibit distinct roles in kinase activity. Other kinases have multiple phosphorylation events within the activation loop. For example, the insulin receptor kinase has three sites within the activation loop, and autophosphorylation occurs in discrete increments. This allows for a stepwise increase in activity (Favelyukis et al., 2001). Similarly, the extracellular signal-regulated kinase 2 (ERK2) contains a second phosphorylation site, C-terminal to its activation loop. This secondary site is crucial for ATP binding, and phosphorylation of this site increases the k_{cat} ~1000-fold relative to the unphosphorylated state (Prowse et al., 2001).

Given the vast diversity that exists among the regulatory mechanisms controlling the activity of protein kinases, it is not hard to imagine that multiple mechanisms exist, other than phosphorylation that positions the activation loop to an "on" state. This mechanism is seen in which a separate regulatory subunit, cyclin A, is allowed to interact with cyclin-dependent kinase 2. Such interaction positions the activation loop to accommodate a low-activity conformation that can be fully activated by both cyclin binding and phosphorylation of the loop (Jeffrey et al., 1995) (Figure 1-1A). C-terminal Src kinase (Csk) does not require activation loop phosphorylation. It possesses SH2 and SH3 domains that interact with an alpha helix adjacent to the magnesium-binding site that stabilizes the activation loop (Ogawa et al., 2002) (Figure 1-1B). Casein kinase 1 (CK1) has a long extension of the activation loop that allows for extra surface contacts to be made. This stabilizes the loop by making additional contact anchoring points so that neutralization of the RD pocket is not needed (Bossemeyer, 1995). Phosphorylase kinase (PhK) is another kinase that does not require active site phosphorylation. In lieu of phosphorylation it contains a glutamate residue at the would be primary phosphorylation site in phosphorylation-dependent enzymes, thereby mimicing a phosphoresidue by binding to the RD pocket (Owen et al., 1995).

Phosphorylation is not the only posttranslational mechanism used to regulate protein kinases. In the last few years, considerable evidence has demonstrated a physiological role for oxidation of protein kinases and phosphatases. Mitogen activated protein kinase kinase 1 (MEKK1), an upstream activator of the SAPK/JNK pathways, is directly inhibited by cysteine alkylation. Further analysis revealed, both *in vivo* and *in vitro*, that MEKK1 was directly glutathionylated at a conserved cysteine that resulted in compete inhibition of catalytic activity.

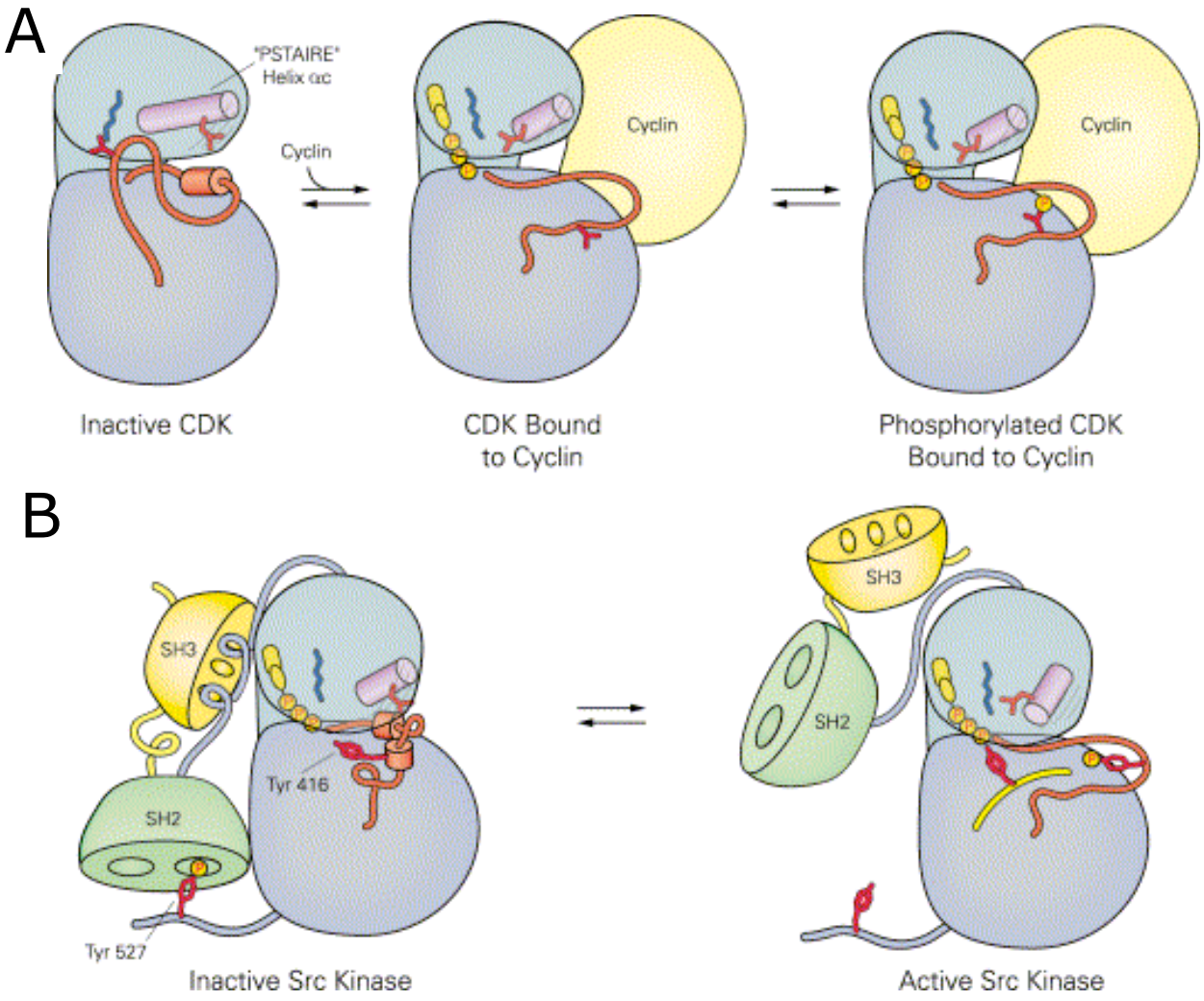


Figure 1-1 The Regulation of Cyclin-Dependent Kinase (CDK) and Src Tyrosine Kinase In the absence of cyclin, the αC helix of CDK is rotated so as to move a crucial catalytic glutamate out of the active site. This is correlated with an inhibitory conformation of the activation loop. Cyclin binding reorients the αC helix so as to place the glutamate within the active site. The activation loop adopts a near-active conformation upon cyclin binding, and its subsequent phosphorylation further stabilizes the active form. In Src, intramolecular interactions between the phosphorylated tail and the SH2 domain, and between the SH2-kinase linker and the SH3 domain, stabilize inhibitory conformations of both helix αC and the activation loop. The conformation of αC in the off state is quite similar to that seen in CDK. Disengagement of the SH2 domain by dephosphorylation of the tail (at Tyr527) allows the αC helix to move into an active conformation. Figure taken from (Huse and Kuriyan, 2002).

The modified cysteine residue lies in the glycine rich loop of the kinase domain, a region that is critical for ATP coordination and binding (Cross and Templeton, 2004). Similarly, elegant work by Taylor's group has shown that that Cys-199 of PKA, two residues downstream from Thr-197, the primary site of phosphorylation in the activation loop, could be subjected to oxidation via alkylation, glutathionylation or internal disulfide bond formation (Humphries et al., 2005). Each of these events leads to increased dephosphorylation of Thr-197 and inactivation of the enzyme.

Additionally, ubiquitin and SUMO proteins also covalently modify protein kinases (Lu et al., 2002). With the increasingly large number of recently discovered posttranslational modifications, such as palmitoylation, hydroxylation, farnesylation, glycosylation, citrullination, nitrosylation, prenylation, methylation, acetylation and many more, it will not be surprising if these mechanism are also used to regulate the activity of protein kinases.

Another protein kinase regulatory paradigm is the use of allosteric ligands to control autoinhibitory domains. These autoinhibitory domains interact either in *cis* or *trans* with the catalytic domain and maintain the enzyme in an inactive state. Binding of the ligand then alters the conformation of the autoinhibitory domain in such a way that enzyme is activated. Regulation of this type has been described in many kinases such as calmodulin-dependent kinases (CaMK), PKA and PKC. In all cases a conserved autoinhibitory domain folds back on the catalytic domain perturbing the ATP-binding site (Goldberg et al., 1996). In the case of CaMKs, binding of Ca^{2+}-bound calmodulin to a ligand-binding pocket induces structural rearrangements in the regulatory region that relieves kinase inhibition. Similar mechanisms occur for PKA and PKC in response to cAMP levels and phorbol esters/diacylglycerol respectively.

SUBSTRATE SPECIFICITY

Consensus motifs

Protein kinases exert their regulation on other proteins via covalent addition of a phosphate group to a hydroxyl moiety of a serine, threonine or tyrosine residue. While some kinases can be quite specific and have only a few distinct substrates others can be quite promiscuous and phosphorylate multiple substrates. For example, Casein kinase 2 (CK2) a ubiquitous, serine/threonine kinase implicated in virus infection, cell survival and neoplasia has greater than 307 documented substrates (Meggio and Pinna, 2003). On the other hand phosphorylase kinase has only 3 known substrates (Pearson and Kemp, 1991). What determines substrate specificity? Historically, studies on phosphorylase kinase and protein kinase A (PKA) revealed that the primary determinant to be a recognition sequence (Kemp et al., 1975; Zetterqvist et al., 1976) surrounding the phosphorylatable residue. This concept has since led to the identification of consensus motifs for many protein kinases and has greatly increased our ability to identify kinase substrates and phosphorylation sites. Furthermore, the use of synthetic peptides, matching a given protein kinase consensus motif, have been used in drug discovery screens to identify small molecule antagonists and agonists of specific protein kinases.

Of the 307 CK2 substrates identified, 175 have the phosphorylation sites mapped. Since CK2 often phosphorylates each substrate on more than one residue, that corresponds to 308 mapped phosphorylation sites (Meggio and Pinna, 2003). Analysis of these sites reveals multiple acidic residues located mostly downstream from the phosphorylatable residues, with the one at n+3 position being the most important (Marin et al., 1986; Meggio et al., 1984). For protein kinase B (PKB/AKT), R-X-R-X-X-S*/T* is the primary consensus motif, where X is any amino acid. Table 1 shows a number of kinases and their respective consensus motif(s). It is

Protein Kinase	Motif[a]	Ratio S:T[b]	Frequency[c]
Calmodulin-dependent protein kinase II	X**R**XXS*/T*	15:5	13/20
	X**R**XXS*/T*V		6/20
Casein Kinase I	**S(P)**XXS*/T*	8:1	5/9
Casein Kinase II	S*/T*XX**E**X	28:2	23/30
	S*/T*XX**D**X		3/20
cAMP-dependent protein kinase	**R**XS*	40:6	21/46
	RRXS*		12/46
	RXXS*		11/46
	KRXXS*		2/46
cGMP-dependent protein kinase	**R/K**XS*/T*	7:3	9/10
	R/KXXS*/T*		8/10
	R/KR/KXS*/T*		7/10
	R/KXXXS*/T*		5/10
	S*/T*X**R/K**		2/10
Glycogen synthase kinase-3	S*XXX**S(P)**	10:2	6/12
Growth-associated histone H1 kinase	S*/T***PXK/R**	7:8	6/15
	K/RS*/T***P**		5/15
	S*/T***PK/R**		4/15
Phosphorylase kinase	**K/R**XXS***V/I**	3:10	3/3
Protein kinase C	S*/T*X**K/R**	31:6	20/37
	K/RXXS*/T*		13/37
	K/RXXS*/T*X**K/R**		7/37
	K/RXS*/T*		10/37
	K/RXS*/T*X**K/R**		6/37
Mitogen-activated protein kinase	**P**XS*/T***P**	ND	ND
	XXS*/T***P**		ND
Myosin II heavy chain kinase	**RG**XS*X**R**	ND	ND
Tyrosine Kinase			ND
EGF-receptor kinase	X**E/D**Y*X	NA	7/14
	X**E/D**Y***I/L/V**		5/14
Abl tyrosine kinase	**I/V/L**Y*XX**P/F**	NA	

Table 1-1 Consensus **phosphorylation sites: specificity motifs for protein kinases**
Table adapted from (Kennelly and Krebs, 1991; Pearson and Kemp, 1991). ND, not determined. NA, not applicable. [a] Asterisks indicate the phosphorylated residue. Specificity determinants are shown in bold type. [b] S:T ratio is the preference for serine vs. threonine for the total number of phosphorylation sites. [c] Frequency of use of that given consensus motif.

also abundantly clear that primary sequence alone is not sufficient to confer substrate specificity. Many kinases share the same or highly related substrate consensus motifs. It is also the case that a consensus sequence may not be accessible to the kinase in the context of the 3-dimensional structure of the substrate. Finally, several kinases have no clear recognition motif (Kennelly and Krebs, 1991). The answer for many kinases may lie in its mode of activation and/or regulation. CK2 has no additional subunits and is constitutively active. This constitutive "on" state may play a role in its pleiotropic nature. For others, substrate specificity is achieved by subcellular co-localization, interaction with substrates via a scaffolding protein, modular docking interactions and specific protein kinase–substrate-docking mechanism.

Docking-Site Interactions

Mitogen-activated protein kinase (MAPK), glycogen synthase kinase 3 (GSK3), and phosphoinositide-dependent protein kinase 1 (PDK1) are all protein kinases that rely on direct docking interactions with their substrates. Docking sites are distinct from the region surrounding the phosphorylation site, and docking interactions yield highly efficient concentrations of these proteins adjacent to the relevant protein kinase. The transcription factor c-Jun, a substrate for the MAPK c-Jun N-terminal Kinase (JNK), has a specific docking site that is required for binding to JNK (Kallunki et al., 1994). This docking site, termed δ-domain, has been found in many other transcription factors such bZIP and MADS transcription factors that are regulated by MAPKs (Sharrocks et al., 2000). δ-domains are typically situated less than 100 amino acids upstream from the phosphoacceptor sequences. Slight alterations in the primary amino acid sequence of this motif can contribute to specificity between different classes of MAPKs (Yang et al., 1998). In most cases δ-domains are required for efficient phosphorylation of substrates but several

examples have emerged where this domain serves to recruit MAPKs into various signaling complexes. For example, the δ-domain of JunB recruits JNK to phosphorylate its heterodimeric partner, JunD (Kallunki et al., 1996).

GSK3 requires a primed substrate prior to its ability to interact and phosphorylate a given residue on the same substrate. This priming event entails the phosphorylation of a serine, by another kinase, situated four amino acids C-terminal to the phosphorylation site (Fiol et al., 1987). The phosphorylated priming site fits directly into a phosphate binding pocket within the catalytic core of the protein (Dajani et al., 2001; ter Haar et al., 2001). It is also interesting to note that this same phosphate binding pocket is the means responsible for inhibition of the kinase. GSK3 can be phosphorylated on an N-terminal residue that can act as a pseudosubstrate and competitively block the docking to, and phosphorylation of, its substrates (Frame et al., 2001).

Scaffolding proteins link substrates to kinases

The associations of several protein kinases with a given substrate are controlled through association with various anchoring or scaffolding proteins. A Kinase Anchoring Proteins (AKAP) mediate intracellular targeting of PKA. AKAPs are a large, diverse family that consists of over 50 members (Tasken and Aandahl, 2004). AKAPs provide a high level of spatial specificity, targeting PKA to specific subcellular compartments via a PKA-binding domain and a unique targeting domain directing the PKA-AKAP complex to defined subcellular structures, membranes or organelles. In many instances these represent intracellular sites of cAMP production allowing for the rapid activation of PKA in response to signaling events that trigger cAMP production (Wong and Scott, 2004). AKAPs have three very important properties. First,

they have a PKA anchoring domain. Second, they have distinct localization domains. Third, they have the ability to bind both substrates and signaling molecules (Wong and Scott, 2004). The first demonstration of AKAP-mediated targeting of PKA to a specific substrate was shown when PKA-AKAP complex bound both AMPA and NMDA receptors (Rosenmund et al., 1994). This facilitates the efficient phosphorylation of these receptors in response to different synaptic signals. Protein kinase C (PKC) has also been found anchored in these complexes, allowing for the convergence of multiple phosphorylation and signaling events on the same substrate (Klauck et al., 1996) (Shih et al., 1999).

Protein kinase scaffolding complexes are also widely seen in MAPK signaling modules. In budding yeast, Ste5p is a scaffolding protein for Fus3p (MAPK) in the pheromone mating response pathway (Elion, 2000). In mammals, JNK-interacting proteins (JIP) are scaffolds that bind JNK, MKK7 and other kinases of the mixed-lineage protein kinase subfamily (MLK) (Davis, 2000). This type of scaffolding may be especially important for JNK and MKK7 in which dual phosphorylation on both a threonine and tyrosine is required for activation. Thus, the tethering of these molecules in an organized complex may lead to increased processivity and fidelity.

Subcellular localization

"Location, location, location" – is the most apt description of how protein kinases find and regulate their appropriate substrates. Protein kinases can be pre-positioned near their substrates before a stimulus, or undergo a spatial translocation to an anchoring protein only after receiving a proper stimulus. Translocation can increase the signal-to-noise ratio by keeping the kinase away from its substrate under basal conditions (Schulman, 2004). For example, AKAP

can target PKA to specific compartments via specialized targeting domains that are present on each anchoring protein. The diversity of these targeting sequences allows PKA to localized and interact with specific substrates at the mitochondria, plasma membrane, cytoskeleton and centrosome (Figure 1-2).

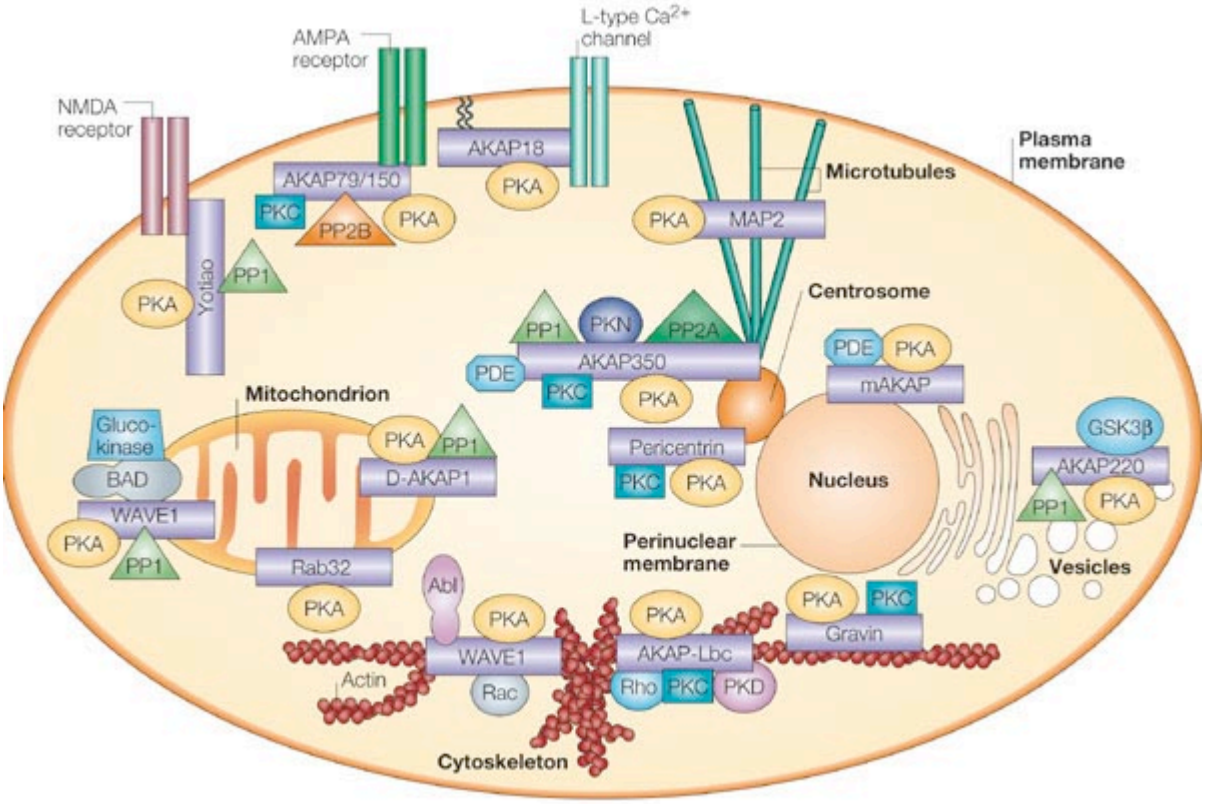


Figure 1-2 AKAP signaling complexes create focal points for signal transduction
A-kinase anchoring proteins (AKAPs) target protein kinase A (PKA) to specific compartments, including the plasma membrane, mitochondria, cytoskeleton and centrosome. Figure taken from (Wong and Scott, 2004).

Certain protein kinases use internal signal sequences such as nuclear localization sequences (NLS) or nuclear export sequences (NES) to regulate compartmentalization. Integrin-linked kinase 1 (ILK1), critical for differentiation and Wnt signaling, contains a NLS, which allows for entry into the nucleus. This NLS is situated near a caveolin-binding domain, so that

when bound to caveolin 1, the NLS is effectively masked and the ILK1 is retained in the cytoplasm (Chun et al., 2005). Calcium/calmodulin-dependent protein kinase II (CaMK II) also possesses an NLS but in this case nuclear localization is regulated by two phosphorylation events. Phosphorylation of either site on CaMK II, near the NLS, results in a block in nuclear targeting (Heist et al., 1998).

The pleckstrin homology domain (PH) consists of a stretch of 100-120 amino acids that was originally defined in the platelet protein pleckstrin. The PH domain is critical for many membrane targeted cellular signaling molecules. PH domains are composed of a core, seven-stranded β-sandwich structure, with three inter-strand loops that have been implicated in the specificity of substrate binding (Blomberg et al., 1999). Many studies have shown that these PH domains recognize and bind phosphoinositides with high specificity and affinity and allow efficient targeting to cellular membranes. Phosphoinositides can bind to the PH domain of PKB, inducing its translocation to the plasma membrane. At the plasma membrane PKB is able to associate with PDK1. These binding events also have been found to induce conformational changes that allow PDK1 to phosphorylate the activation loop of PKB (Chan et al., 1999).

SH2 and phosphotyrosine binding (PTB) domains were both initially identified as modules that recognize phosphorylated tyrosine in receptor tyrosine kinases (RTKs) and other signaling proteins. An example of the use of SH2 domains is observed in the cytokine-mediated JAK:STAT pathway, used to transmit extracellular signals to the nucleus. JAKs are receptor protein tyrosine kinases that are associated with cytokine receptors. STAT proteins are transcriptions factors that regulate the expression of a number of downstream genes. Upon cytokine binding JAKS become phosphorylated on tyrosine residues recruiting STAT proteins that bind to the phosphotyrosine residue via their SH2 domain (Ivashkiv and Hu, 2004). Once

recruited, STAT proteins themselves become phosphorylated, allowing for the dissociation from JAK and the formation of STAT homo- and hetero-dimers. STAT dimerization is required for nuclear translocation and DNA-binding (Ivashkiv and Hu, 2004).

Further studies have uncovered a large number of domains that function in a similar manner to localize and target cell-signaling events including protein phosphorylation. These include: PDB, SH3, PAZ, C2, WW, DD, MH2 and LIM domains.

CHAPTER TWO
Review of the Literature

PAS KINASE

Discovery of PAS Kinase

Phil Zhang and Charlene Liao (Tularik Inc., San Francisco, CA) originally identified the hPASK cDNA in a BLAST search of the GenBank database for proteins similar to the PAS domain of *Bradyrhizobium japonicum* FixL. Only one PAS kinase gene and/or family member exists in the human genome. This human PAS kinase gene has a single conserved ortholog in mice, flies, and other metazoans, and two such orthologs in the budding yeast *Saccharomyces cerevisiae*. Prokaryotic microorganisms and plants contain multiple related enzymes in which one or more PAS domains are fused to a kinase domain. These microbial and plant PAS domains are linked to histidine kinases and function as sensory molecules in two-component signaling systems.

Human PAS kinase is ubiquitously expressed and detectable in all tissues and cell lines investigated to date. Northern blot analysis performed on both adult and embryonic mouse tissue revealed a band of about 4.5 kb in all tissues examined (Hofer et al., 2001). A human multi-tissue dot blot, containing mRNA from 61 different human adult tissues, 7 fetal tissues and 8 cell lines yielded relatively uniform signal intensities for PASK mRNA (Hofer et al., 2001). A 2-4 fold higher expression level was seen in the caudate nucleus and the putamen of the brain, testes and prostrate while a 5-fold reduction was seen in the placenta (Hofer et al., 2001). In confirming the efficient gene targeting of the PASK knockout locus, northern blots of mouse organs were performed. These data confirmed that PASK mRNA expression is normally low in most tissues with the exception of an extremely strong signal from the testes. PASK expression in the testis

was greater than 90-fold higher than in the thymus, which had the next highest level of PASK expression (Figure 2-1D)(Katschinski et al., 2003). Using homologous recombination, Wenger's group replaced part of the PASK gene with a β-galactosidase reporter gene. PASK expression was found to be exceptionally high in post-meiotic germ cells during spermatogenesis (Wenger and Katschinski, 2005). Further analysis demonstrated β-galactosidase reporter gene activity in the luminal regions overlapping the cytoplasm of round or elongated spermatids while expression was not seen in the surrounding Sertoli or Leydig cells (Figure 2-1A-C)(Katschinski et al., 2003). *In situ* hybridization revealed that PAS kinase expression was absent in the testes at postnatal day 12 to 15, followed by a strong increased expression by postnatal day 25, and remaining high throughout adulthood (Katschinski et al., 2003).

Despite the strong expression pattern specific to post-meiotic germ cells, male fertility, sperm production and motility were not affected in PASK-deficient mice (Katschinski et al., 2003). Furthermore, the mice displayed no gross differences in development or behavior, and appeared to be indistinguishable to their wildtype littermates by all criteria investigated. It remains unclear, as such, as to the biological role of PAS kinase in mammals.

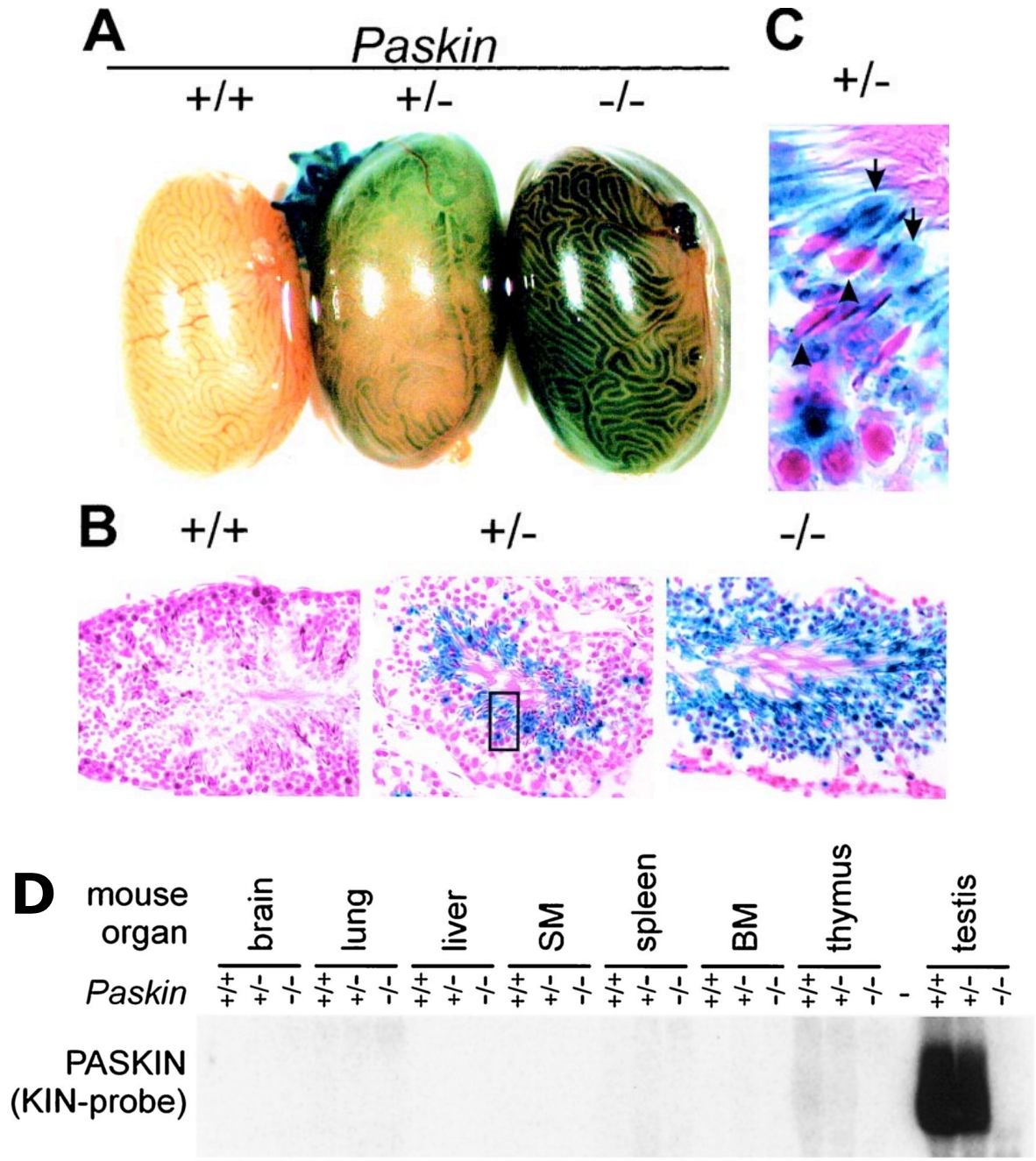


Figure 2-1 PASK expression in the testes
β-galactosidase activity in PASK knockout mice detected by X-gal staining of whole-mount testes (A) and testis cyrosections (B and C). (D) Northern blot analysis of total RNA isolated from the indicated tissues from wildtype (+/+), heterozygous (+/-) and null (-/-) mice. Figure adapted from (Katschinski et al., 2003).

Functional Domains of PASK

The human PASK enzyme is 1,323 amino acids in length with a predicted molecular weight of 142 kD. The enzyme contains two N-terminal PAS domains, a C-terminal serine/threonine kinase domain, and a 75 amino acid hydrophilic tail (Figure 2-2). The kinase domain contains all the hallmark residues of a serine/threonine kinase and belongs to the CaMK-like family of protein kinases (Manning et al., 2002).

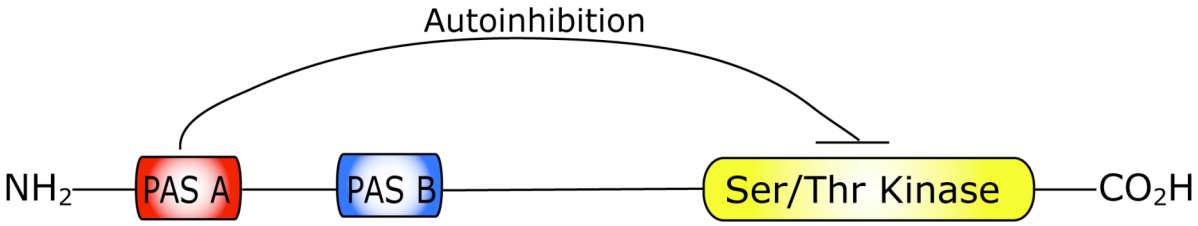


Figure 2-2 PAS kinase domain structure
Human, mice, fly, and yeast PASK exhibit 3 regions of conservation including 2 N-terminal PAS domains and a canonical serine/threonine kinase domain. PAS A is autoinhibitory toward the kinase domain.

PAS DOMAINS

PAS domains, deriving its name from the original founding members (Per-Arnt-Sim), have been identified in over 1100 proteins representing all kingdoms of life (Taylor and Zhulin, 1999). They are typically small modular domains comprising 100-150 amino acids and are classified based on their tertiary structure rather than primary sequence identity. Their unique structural flexibility has evolutionarily allowed them to be a signature of many regulatory proteins that are capable of sensing environmental conditions and transducing the appropriate biological response. The plasticity of these PAS domains can be seen in their functional capabilities. PAS domains are used for protein-protein interactions for both inter- and intra-molecular associations with macromolecules. Their hydrophobic cores can accommodate the

incorporation of organic cofactors or ligands. The ability to bind these different types of cofactors lies at the heart of the PAS domains' ability to act as a sensory module capable of quickly relaying vital signal transduction signals. For example, the binding of heme to the PAS domain of FixL allows *Rhizobia* to directly sense O_2 concentrations thus allowing it determine the best time to fix nitrogen (Gilles-Gonzalez, 2001). *Arabidopsis* NPH1, a PAS domain containing flavoprotein, utilizes FMN as a cofactor to sense blue light for phototropism (Christie et al., 1998). Plant photoactive yellow protein (PYP) uses its PAS domain to bind the cofactor 2-hydroxycinnamic acid to sense blue light (Borgstahl et al., 1995). Finally, other PAS domain containing proteins use FAD or 2Fe-2S clusters to sense the overall energy status or the cell, redox potential, and other signals diagnostic of the metabolic state of a cell (Bibikov et al., 2000; Fu et al., 1994).

PASK has two N-terminal PAS domains, PAS A and PAS B (Figure 2-2). Based on the Hidden Markov algorithm, the sequence corresponding to the PAS A domain strongly predicts a *bona fide* PAS domain with an E value of 4.8×10^{-8} (Rutter et al., 2001). PAS B has also been predicted to encode a PAS domain but with a much weaker E value (7.1×10^{-2}).

Structure of the PAS A domain of PAS kinase

Amezcua et al. used a fragment of PASK, residues 131-237, to solve the solution structure of the PAS A domain (Amezcua et al., 2002). As expected the domain adopts the typical α/β PAS domain fold, characterized by a helices flanking a five-stranded antiparallel β-sheet (Figure 2-3). It has a long flexible loop (FG loop), which corresponds to the regulatory loop in FixL that is critically involved in sensing and transmitting heme-bound molecular oxygen

(Gilles-Gonzalez, 2001). This suggests that this extended FG loop may play a role in transmitting the presence of a bound ligand if it is indeed the case that PASK is ligand regulated.

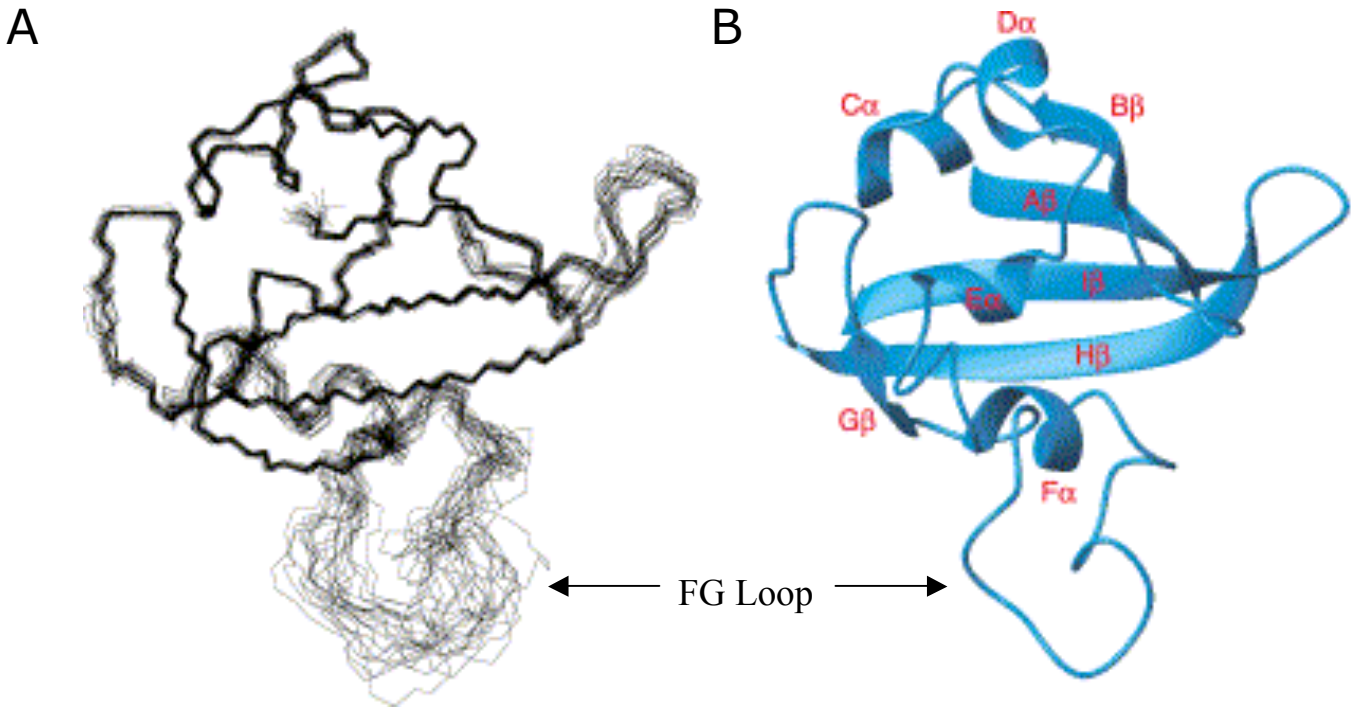


Figure 2-3 Solution structure of hPASK PAS A
A. Bundle of 20 lowest-energy structures for hPASK PAS A domain. B. Schematic ribbon diagram of the structure closest to the mean. Figure from (Amezcua et al., 2002).

Functionality of the PAS Domains

A truncated version of PASK (ΔNPASK), lacking residues 1-948, has been reported to exhibit approximately 5-fold higher k_{cat} than the full length enzyme (Rutter et al., 2001). This observation provided the initial evidence that the N-terminal region of PASK, which includes the two PAS domains, might be inhibitory towards the catalytic kinase domain. As previously mentioned, a number of kinases have autoinhibitory domains that can bind back in *cis* to regulate kinase activity. Purified PAS A domain was shown, in a dose dependent manner, to inhibit the

catalytic domain of the enzyme with an IC_{50} of 100 μM (Rutter et al., 2001). The specificity of this interaction was demonstrated when purified PAS A from NPAS2, or PAS B from EPAS1, could not inhibit ΔNPASK. Both of the PAS domains are well behaved in solution and adopt a similar fold as PAS A of PASK. Inversely, when the PAS A domain of PASK was added to kinase reactions containing either PKA or MLCK, no detectable change in kinase activity was observed (Rutter et al., 2001). These observations were interpreted to indicate that the inhibition caused by PAS A on the kinase domain of PASK is specific and does not act via a generalized, non-specific inhibitory mechanism. Furthermore, NMR studies have shown that the PASK PAS A interacts directly with the catalytic domain of the enzyme, suggesting its inhibitory effect is both specific and direct (Amezcua et al., 2002). Since PAS B of PASK did not express well, nor was soluble when purified as an independent entity, it is unclear if PAS B plays a similar or cooperative inhibitory role. Additionally, there are approximately 600 amino acids of uncharacterized sequence between PAS B and the kinase domain that may allow for more elaborate regulatory control (See Appendix A).

Biochemical Studies of PASK

hPASK protein purified from insect cells migrates on an SDS-PAGE with an apparent molecular mass of 145 kD (Rutter et al., 2001). Western blot analysis of endogenous PASK from HeLa cells also demonstrated PASK expression encodes for the full-length polypeptide. Gel filtration and gradient sedimentation of both crude endogenous PASK and purified recombinant PASK resulted in a molecular mass similar to what would be expected for the enzyme to exist as a monomer (Rutter et al., 2001). Immunolocalization studies in HeLa cells

reveals PASK is predominantly cytoplasmic with a tendency to predominate in the perinuclear region (Rutter et al., 2001).

Purified PASK was observed to become phosphorylated when exposed at room temperature to ATP and magnesium. Phosphorylation significantly elevates the activity of PASK by lowering its K_m for substrate without altering the catalytic rate constant of the enzyme. The observed autophosphorylating activity could be inhibited by an inactivating mutation, K1082R, within the ATP binding pocket. Mass spectrometry was employed to identify the sites of autophosphorylation generated by PASK in response to ATP. The major sites of phosphorylation are restricted to two short sequences, one corresponding to the activation loop of the catalytic domain and another corresponding to a serine-rich segment of the polypeptide located on the immediate carboxyl terminal side of the catalytic domain (Rutter et al., 2001). Mutational analysis of the phosphorylatable residues in these regions determined that Thr-1165 in the activation loop was one site of phosphorylation. This site aligns with Thr-172 in AMPK that must be phosphorylated to support catalytic activity (Stein et al., 2000). Consistent with this, mutation of T1165 to alanine completely eliminates PASK catalytic activity (Rutter et al., 2001). These data suggest that PASK can, in *trans*, autophosphorylate in a manner that mobilizes the activation loop of the enzyme and enhances the avidity of the enzyme for substrate.

Tryptic peptides from the second region of phosphorylation, located in the C-terminal tail of PASK, revealed five possible phosphorylation sites S1273, S1277, S1280, S1287 and S1289. A mutant in which all five serine residues were changed simultaneously to alanine exhibited catalytic activity and kinetic parameters indistinguishable from wildtype PASK (Rutter et al., 2001). Although Rutter et al. did not map which of these residues are the sites of

phosphorylation, our studies indicate that S1289 to be one them. The functional significance of these phosphorylation events remains unclear.

Studies in Yeast

The genome of the budding yeast, *Saccharomyces cerevesiae*, contains genes encoding a pair of protein kinases, orthologous to the single mammalian enzyme. One gene, designated *PSK1*, is located on the left arm of chromosome 1, corresponding to the open reading frame (ORF) YAL017W. The other, designated *PSK2* is located on the left arm of chromosome 15, corresponding to ORF YOL045W. PSK1 and PSK2 are more related in primary amino acid sequence to one another than either are to its metazoan orthologs (Rutter et al., 2002).

Deletion of either or both PSK genes yielded no obvious phenotype on normal growth conditions. Among more than 50 prototypical growth conditions tested (Hampsey, 1997), two specifically retarded the growth of the strain lacking both *PSK1* and *PSK2* genes. Wildtype yeast were able to grow to a more substantive degree than the *psk1 psk2* double mutant when plated on medium containing elevated levels of zinc and incubated at 38^{o}C. A similar phenotypic difference was seen on plates containing galactose, as opposed to glucose as the primary carbon source, when incubated at 38^{o}C (Rutter et al., 2002). Under either condition, psk1 strains grew at a reduced rate, psk2 strains at substantively reduced rates, and psk1 psk2 strains failed to grow at all (Figure 2-4) (Rutter et al., 2002). Rescue of PSK2 on a plasmid in the background of psk1 psk2 double mutant strain could restore its ability to grow near wildtype levels (Rutter et al., 2002).

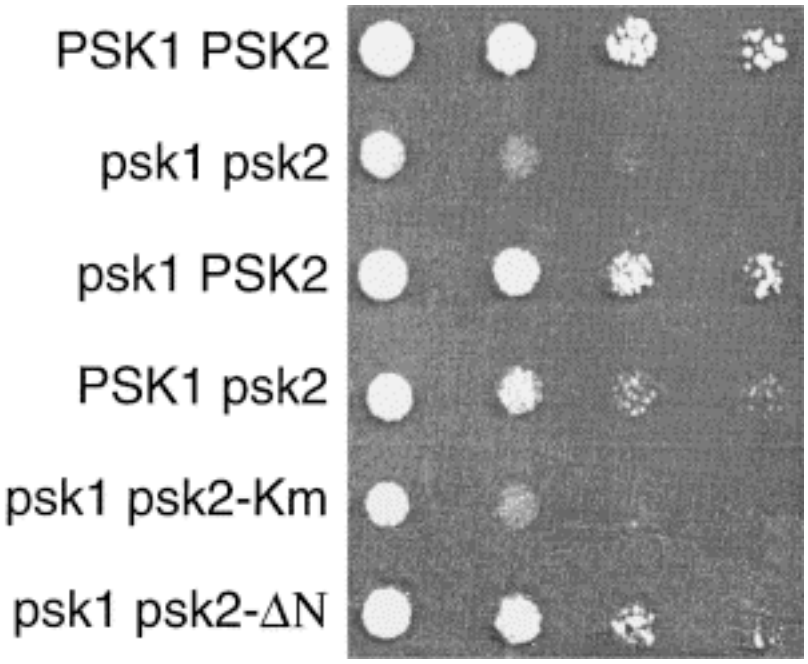


Figure 2-4 PSK activity is necessary for growth under gal^{ts}-restrictive conditions
Strains of the indicated genotype were grown to saturation in minimal 2% glucose medium lacking uracil and diluted in water. These diluted samples were spotted at 10,000, 2000, 400, and 80 cells per spot onto minimal medium plates lacking uracil with 2% galactose as carbon source. The plate was incubated for 6 days at 38°C. Figure from (Rutter et al., 2002).

Using the established phenotypic conditions, growth on galactose at restrictive temperatures, a high copy suppressor screen was used to search for genes that might overcome the growth deficit of psk1 psk2 double mutant cells. By transforming the psk1 psk2 strain with a yeast genomic library housed on a high copy plasmid, genes were identified that were capable of rescuing the ability for the double mutant to grow under these conditions. These studies led to the identification of approximately 50 high-copy suppressors of the *psk1 psk2* double mutant phenotype (Personal communication – Jared Rutter). Among these, Rutter et al. exclusively focused their attention on clones that were retrieved a minimum of two independent times, amounting to fifteen independent genes (Rutter et al., 2002). Three of the high copy suppressors

encoded proteins involved in sugar metabolism; including phosphoglucose mutase 1 (Pgm1p), phosphoglucose mutase 2 (Pgm2p), and Snf1 interacting protein (Sip1p) (Rutter et al., 2002). Pgm1p and Pgm2p are functionally redundant and are enzymes involved in the conversion of galactose to glucose (Boles et al., 1994). Sip1p has shown to be a positive regulator of the Snf1p kinase controlling the use of alternative carbon sources (Mylin et al., 1994). Since the screening and selection conditions entailed the replacement of glucose as a carbon source with galactose, and since the *PGM1&2* and *SIP1* gene products are involved in galactose utilization, it was originally believed that these high-copy suppressors do not represent relevant information to the biological role of PASK. A fifth gene isolated in the suppressor screen, designated *DDP1*, encodes diadenosine polyphosphate hydrolase (Cartwright and McLennan, 1999). It is not understood why over-expression of this enzyme suppresses the deleterious effects of the *psk1 psk2* double mutation on vegetative growth of *S. cerevesiae*.

The remaining genes that were retrieved in the high-copy suppressor screen encode products involved in RNA metabolism or translation (Rutter et al., 2002). These include: (i) the *DED1* and *DBP1* genes – both of which encode RNA helicases involved in translation initiation (Chuang et al., 1997; Jamieson and Beggs, 1991; Jamieson et al., 1991); (ii) *EDC1* – which encodes an enhancer of mRNA decapping (Dunckley and Parker, 1999) (iii) *RPR1* and *POP4* – which respectively encode the RNA and a protein component of RNaseP (Lee et al., 1991); and (iv) *RDN* which encodes the four RNA subunits of the ribosome (Venema and Tollervey, 1999). Additionally, the YDL189W ORF was recovered sixteen independent times in the high-copy suppressor screen. Although no function has been assigned to this gene, the encoded protein contains an R3H single-stranded nucleic acid binding domain (Grishin, 1998). This raises the possibility that this gene product may also be involved in RNA metabolism or protein synthesis.

Since most of the relevant high-copy suppressors encode products involved in RNA metabolism or translation, it is likely that PASK may somehow be involved in regulating protein synthesis.

Mammalian Studies

Aside from preliminary knowledge of certain regulatory mechanisms imposed upon PAS kinase, such as autophosphorylation and inhibition via the PAS domain (Amezcua et al., 2002; Rutter et al., 2001), almost nothing was known about the biological role(s) of mammalian PAS kinase. For this reason, we initiated an interrogation into the intracellular pathways regulated by mammalian PASK (Chapters 4 and 5) in parallel with our studies on yeast PAS kinase. During our search and characterization of putative mammalian PAS kinase substrates, da Silva Xavier et al. published a manuscript describing a role for PAS kinase in pancreatic islet β-cells (da Silva Xavier et al., 2004). They demonstrated that elevated glucose concentrations within the islet β-cells increased PAS kinase activity approximately 2-fold (Figure 2-5A). The activation of PASK in response to high glucose was followed by an accumulation of both PASK mRNA and protein (da Silva Xavier et al., 2004). Elevated concentrations of glucose in the pancreatic islet β-cells are known to induce the expression of the preproinsulin gene (Rhodes, 2000). PAS kinase was necessary for this event as microinjection of PASK cDNA or protein into the β- cell mimicked the glucose-induced up-regulation of the preproinsulin gene (Figure 2-5B and C)(da Silva Xavier et al., 2004). Conversely, silencing of PASK by RNAi suppressed glucose-mediated preproinsulin gene induction. PASK-mediated induction of the preproinsulin gene was specific as other known glucose induced genes remained unchanged (da Silva Xavier et al., 2004). The mechanisms by which glucose regulates PASK expression and its subsequent effects on the induction of the preproinsulin gene expression remain unclear.

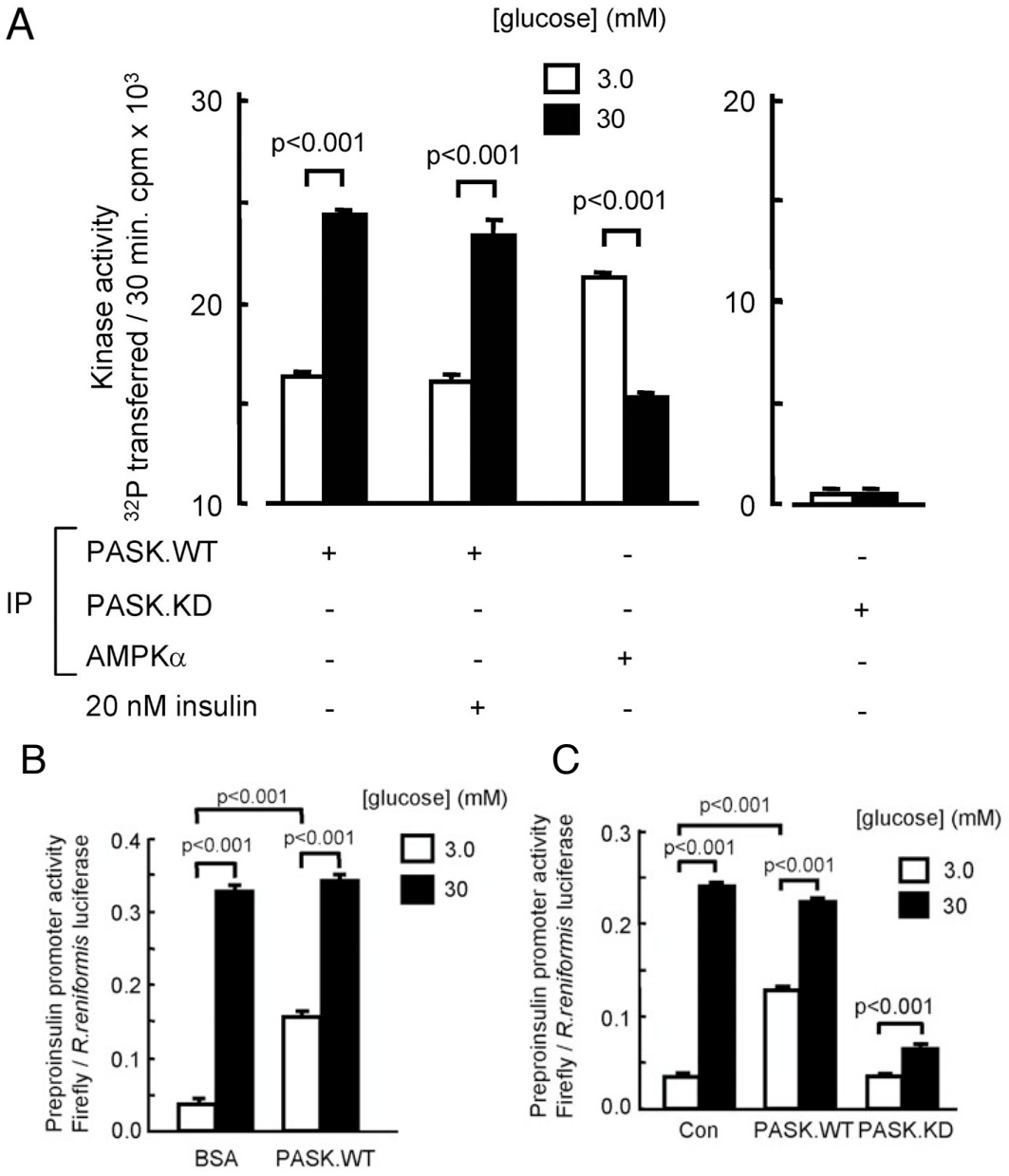


Figure 2-5 PAS kinase activity in the pancreatic β-cells
(A) MIN6 cells were transfected with empty vector (pcDNA3), WT PASK (pPASK.WT) or kinase-dead PASK (pPASK.KD), cultured in either low (3 mM) or high (30 mM) glucose for 1 hr. Enzymes were then immunoprecipitated and activity tested in kinase assays. MIN6 cells were co-microinjected with a lucficerase reporter gene fused to preproinsulin promoter plus either (B) BSA or wild-type PASK protein ; (C) pcDNA3 (con), pPASK.WT or pPASK.KD, prior to incubation followed by luciferase imaging. Figure adapted from (da Silva Xavier et al., 2004).

Furthermore, pancreatic islet β- cells are a very highly specialized group of fuel-sensing cells able to respond to elevated blood glucose concentrations via an increase in ATP production (Malaisse, 1983). With the ubiquitous expression of PAS kinase and the specificity of insulin production for pancreatic islet β- cells, it is possible that this observation represents a tissue- or cell-specific function of mammalian PAS kinase.

CHAPTER THREE
Results

BIOLOGICAL ROLE OF YEAST PASK

Abstract

PAS kinase is a serine/threonine kinase regulated in *cis* by a PAS domain. Using a biochemical screen for PAS kinase substrates, three translation factors were identified as direct phosphorylation targets. PAS kinase was also found to phosphorylate UDP-glucose pyrophosphorylase and glycogen synthase, the enzymes catalyzing the final steps in glycogen synthesis. Genetic, biochemical, and physiological data provide evidence that both of these enzymes are inhibited by PAS kinase-dependent phosphorylation, thereby down regulating carbohydrate storage. These studies provide evidence that PASK regulates a signaling mechanism that couples nutrient availability/storage and protein synthesis.

Introduction

Living organisms live in a realm of constant change. They are directly influenced by the environment in which they live and, as such, have acquired unique systems and abilities to respond to distinct environmental stimuli. Of utmost importance is the ability to acquire, store and mobilize the appropriate nutrients needed to support growth and fuel the energetically demanding processes vital to life. Most cells, such as yeast, require sugars to be brought into the cell where they are consumed via glycolysis and the TCA cycle for the generation of ATP. Alternatively, sugars can be stored for later use in the form of glycogen, trehalose, etc. Storage

and mobilization of these fuel storages are regulated by a number of cellular signaling pathways that are coupled to other processes in the cell. For example, protein synthesis consumes up to 5% of the human caloric intake (Matthews et al., 2000). The generation of ribosomes and the translation apparatus consumes roughly 60% of the total transcriptional activity in dividing eukaryotic cells. Given this very costly metabolic activity it is not surprising that it is tightly linked to the energy status of the cell and is closely monitored by a number of sensory molecules.

Materials and Methods

Synthetic Lethal Screen

JRY82 strain used in this study was derived from JRY40 and contained the following genetic markers (*psk1, psk2*, *ade2, ade3, ura3* and *leu2)*. JRY82 cells were transformed with a plasmid designated pJR984A-11. This is a single-copy, centromere-bearing plasmid that carries the *URA3, ADE3* and *PSK2* genes. JRY82 cells transformed with pJR984A-11 were mutagenized with EMS to levels that resulted in 90% lethality, plated onto YPD medium, and grown at 25^{o}C. Putative synthetic lethal mutants were re-streaked to confirm the non-sectoring phenotype. Mutants that rely on the *PSK2*-bearing plasmid were further identified by streaking cells on culture plates supplemented by 5-FOA. Under these culture conditions URA3-expressing cells are not be able to grow, providing evidence that they faithfully maintain the pJR984A-11 plasmid. As a final step, the suppressive activity of the *PSK2* gene was confirmed by transforming individual mutants with a construct bearing the yeast *LEU2* and *PSK2* genes. This test assesses whether an alternative source of the *PSK2* gene restores the sectoring phenotype, and eliminates the possibility of plasmid integration or other potential artifacts.

Kinase Substrate Screen and Assay

Soluble, cytoplasmic *S. cerevisiae* protein extracts were generated as described (Buzan and Frieden, 1996). The S100 was applied separately to MonoQ, MonoS, Heparin Sepharose (all Amersham Pharmacia), Bio-Gel hydroxyapatite (Bio-Rad), and Ni-NTA (Qiagen) chromatographic columns. In all cases, with the exception of Ni-NTA, this was done using an Amersham Pharmacia FPLC system, followed by shallow gradient elution using conditions appropriate for each resin. For Ni-NTA, the S100 was batch bound and bump eluted from a gravity-flow column. Fractions collected were subjected to a standard kinase assay using Psk2p enzyme under conditions previously described for human PASK (Rutter et al., 2001). Following incubation for 30 min at 25°C, kinase reactions were terminated with the addition of SDS sample buffer and subjected to SDS-PAGE. Gels were subsequently stained with Coomassie Blue, dried, and analyzed by autoradiography. Proteins observed to be phosphorylated in a Psk2p-dependent manner were cut from the gel and identified by mass spectrometry following in-gel trypsinolysis (Rosenfeld et al., 1992). The *in vitro* substrates identified in this screen were isolated from the following fractions: Sro9p—approximately 250 mM NaCl elution from MonoQ following Ni-NTA agarose; Ugp1p—approximately 300mM NaCl elution from MonoQ following Ni-NTA agarose; Caf20p—approximately 350 mM NaCl elution from MonoQ following Ni-NTA agarose; Tif11p—approximately 400 mM NaCl elution from MonoQ of crude protein extract.

Phosphorylation sites were mapped for each substrate protein by subjecting the recombinant protein to phosphorylation by Psk2p followed by SDS-PAGE and in-gel trypsinolysis (Hellman et al., 1995; Rosenfeld et al., 1992). Tryptic peptides were fractionated by reverse-phase HPLC on a Beckman ODS C_{18} column using a Beckman System Gold Model 126 HPLC system, eluting with a linear gradient of 0%–40% acetonitrile in 0.1%TFA/H_20. Fractions

(0.5 ml) were collected in deep well 96-well plates and subjected to scintillation counting using the Top Count Microplate Scintillation Counter (Packard Bioscience, Meriden, CT). The fraction corresponding to the peak of [^{32}P] radioactivity was subjected to mass spectroscopy as described (Vassilev et al., 2001). Experimentally determined masses were compared with the theoretical tryptic digest using the web-based MS-digest tool (http://prospector.ucsf.edu; (Clauser et al., 1999)). The identification of peptides by mass spectrometry was confirmed by automated Edman sequencing.

Glycogen and glycogen synthase assays

Glycogen measurements were performed as described (Parrou and Francois, 1997). 20 OD of culture was rapidly harvested, spun down and pellet frozen in liquid nitrogen. The pellet was thawed and resuspended in 0.25 ml of 0.25 M Na_2CO_3 and incubated at 95ºC for 4 hr, followed by the addition of 0.15 ml of 1 M acetic acid and 0.65 ml of 0.2 M sodium acetate [pH5.2]. Glycogen was hydrolyzed by *A. niger* amyloglucosidase (Sigma) (1.2 U/ml final) under constant shaking for 16 hr at 57ºC, followed by centrifugation for 5 min at 14,000g. The supernatant was assayed for glucose using the Sigma glucose assay kit (510A).

Glycogen synthase activity was assayed as described (Thomas et al., 1968) with the following exceptions. Uridine diphospho-D-[6-^{3}H]glucose (specific activity of 0.1 mCi/mmol) was used and assays conducted for 20 min at 30ºC. Additionally, we passed the extracts over Microspin G-25 columns (Amersham Pharmacia) prior to assay to remove low molecular weight molecules. One unit of activity is described as that sufficient to catalyze the transfer of 1 μmol of ^{3}H-glucose to glycogen per minute at 30ºC. Assays were carried out in the absence and presence of 6.7 mM glucose-6-phosphate, and the resulting (-/+ G-6-P) glycogen synthase

activity ratio is used an indicator of glycogen synthase phosphorylation state.

Results

Synthetic Lethal Screen

To resolve the biological role of PASK including the identification of interacting genes, we employed a genetic screen designed to identify genes that, when mutated, cause lethality conditionally in the background of the psk1 psk2 double mutant. This assay was undertaken in the budding yeast, *S. cerevisiae* and is based on the plasmid dependence assay developed by Kranz and Holm (Kranz and Holm, 1990). This strain, JRY82, has both *PSK* genes deleted (*psk1 psk2* genotype) as well as bearing *ade2, ade3, ura3* and *leu2* mutations. The *ade2* and *ade3* mutations will facilitate color selection for cells that maintain an indicator plasmid carrying the *PSK2* gene. When plated onto rich (YPD) medium, the *ADE3* gene on the pJR984A-11 plasmid causes cells of the JRY82 strain to accumulate an intermediate in the adenine biosynthetic pathway that gives the cells a deep red color. If cells rely on this plasmid for viability, all cells of the colony are red. If cells do not rely on this plasmid, which is the case for the parental JRY82 strain, they invariably produce sectored colonies bearing pie-shaped segments of white cells. Since the adenine biosynthetic intermediate accumulated in JRY82 cells carrying the pJR984A-11 plasmid is slightly toxic, nearly every colony contains multiple white sectors (as the cells suffer a slight selective pressure to lose the plasmid and thereby eliminate accumulation of the biosynthetic intermediate). In spite of screening over 300,00 mutant clones using this technique, we did not identify any synthetic lethal mutations for the PSK genes. Looking back from what we know now, there are several possible explanations for the failure of this approach. Probably the most important notable is the fact that glucose was used as a carbon source in this screen. We now have data, not presented in this work, that suggest PASK in yeast are both

upregulated and activated by nonfermentable carbon sources. Furthermore, analysis of the yeast PASK promoters, reveals the presence of a conserved Mig1p binding site. Mig1p binding elements are responsible for the repression of genes in response to glucose. Thus under glucose conditions, the PASK pathway, for which we were trying to identify synthetic lethal mutants, may not have been functioning.

Identification of Yeast PSK Substrates

In order to complement the identification of high-copy suppressors of the psk1 psk2 double mutant (Chapter 2), we undertook biochemical experiments to discover putative phosphorylation targets of the enzyme. Our simple, unbiased strategy entailed the generation of a soluble extract from *S. cerevisiae*, and subsequent fractionation using various chromatographic separation techniques. We applied the extract to MonoQ, MonoS, Heparin Sepharose, Bio-Gel hydroxyapatite and Ni-NTA chromatographic columns, followed by shallow gradient elution using conditions appropriate for each resin. The purpose was to 2-fold: 1. To generate partially purified pools of protein that maintained native multiprotein complexes and 2. To reduce background phosphorylation of endogenous kinases. To each of the resultant fractions, we added [γ-^{32}P]ATP of high specificity and either purified Psk2p enzyme or buffer and incubated the reaction for 30 minutes at 25°C. Samples were then subjected to SDS-PAGE and visualized by Coomassie blue staining and autoradiography. Endogenous kinase activity led to the Psk2p-independent phosphorylation of some proportion of the proteins in the various samples. Using this unbiased approach we were, however, able to identify four distinct polypeptides phosphorylated exclusively in the Psk2p-added sample.

One fraction, contained proteins eluted from a nickel column followed by a monoQ column, is shown in Figure 3-1. Despite the fact that a number of the proteins in the sample were phosphorylated in a Psk2p-independent manner (lane2), one polypeptide was observed to be phosphorylated only in the sample containing the Psk2p enzyme (lane 3). This band of ~ 55 kD was excised from the gel, and tryptic mass fingerprinting identified it as the product of the UGP1 gene, UDP-glucose pyrophosphorylase. Ugp1p catalyzes the formation of UDP-glucose from UTP and glucose-1-P. We cloned and expressed Ugp1p as a His_6-fusion in *E. coli*, and found the purified protein to be stoichiometricly phosphorylated by Psk2p *in vitro* (Figure 3-2).

To enable functional analysis of the effect of Psk2p –mediated phosphorylation, we determined the amino acid of Ugp1p phosphorylated by Psk2p. Recombinant Ugp1p was phosphorylated by Psk2p in the presence of [γ-^{32}P]ATP followed by trypsinolysis. A single phosphorylated tryptic peptide was purified using C_{18}-reverse phase HPLC and subjected to MALDI-TOF. The molecular mass was consistent with a phosphorylated peptide comprising residues 9-28 of the Ugp1p polypeptide. This peptide contained seven serine or threonine residues, which, in turn, were individually mutated to alanine via primer sowing method. Mutated variants of the Ugp1p polypeptide were expressed in *E. coli* and assayed as phosphorylation substrates for Psk2p. Six of the seven mutants were phosphorylated equally to wildtype Ugp1p, but S11A mutant was not phosphorylated (Figure 3-2). S11 is located in a predicted helix-rich region located N-terminal to the highly conserved catalytic domain of Ugp1p (Cuff et al., 1998).

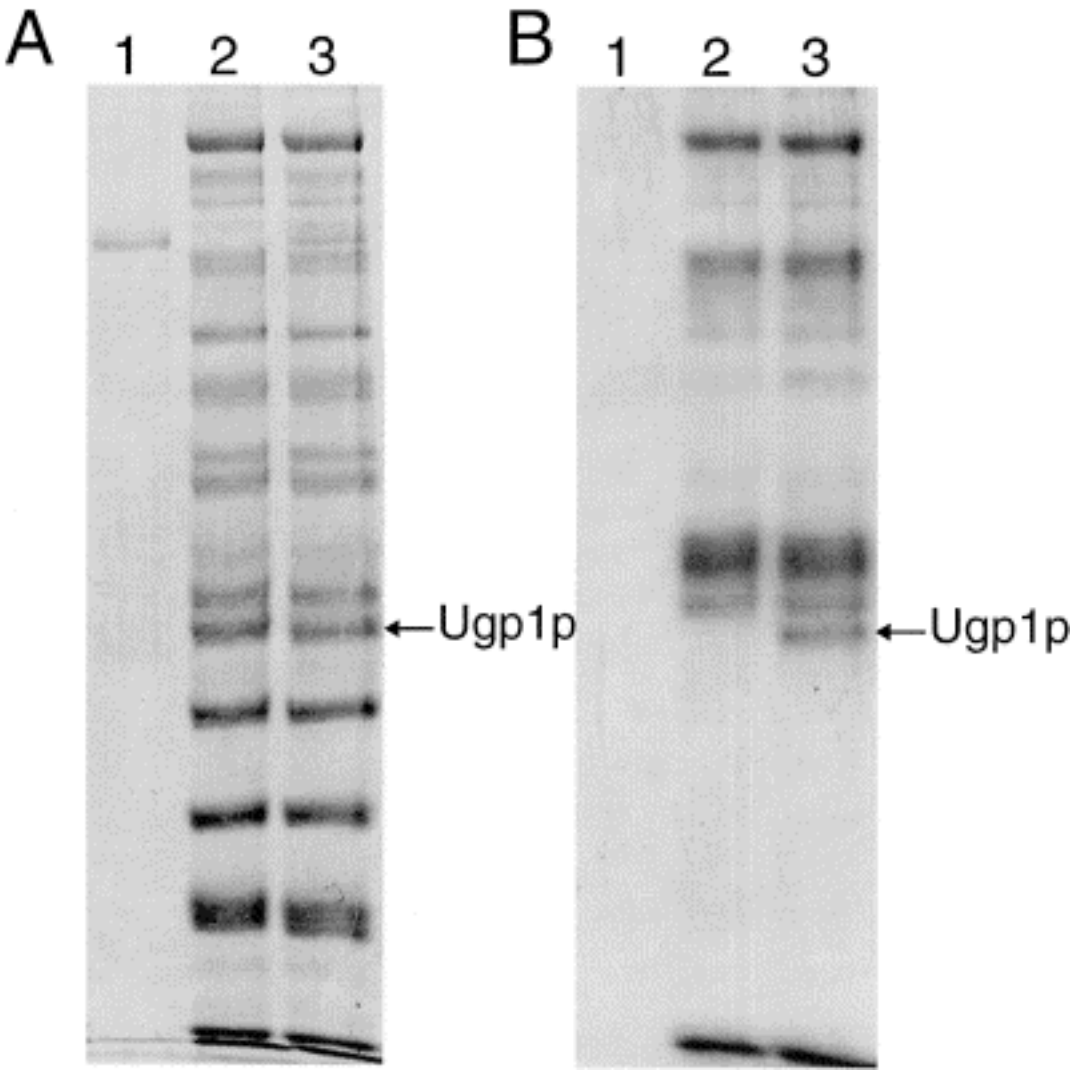


Figure 3-1 Biochemical screen for Psk2p substrates
Stained gels were dried and exposed to autoradiographic film (B). *S. cerevisiae* soluble lysate was passed through Ni^{2+}-NTA resin. Bound proteins were eluted by washing the resin with buffer containing 250mM imidazole. Lane 1: Psk2p alone; lane 2: Ni^{2+}-NTA elution alone; lane 3: Psk2p + Ni^{2+}-NTA elution. Figure from (Rutter et al., 2002).

Three additional Psk2p substrates were identified using this biochemical strategy. The second Psk2p substrate is the product of the CAF20, (Cap-associated-factor 20) gene (de la Cruz et al., 1997). Eukaryotic translation factor 4E (eIF4E) was also identified in the fraction with CAF20 and they appeared to comigrate together. These two proteins are known to constitute a stable complex upon purification to homogeneity (Altmann et al., 1989; Altmann and Trachsel, 1989). Furthermore, Caf20p can inhibit cap-dependent mRNA translation by blocking the formation of the eIF4E/eIF4G complex, which nucleates the assembly of the translation apparatus of the 5' end of mRNAs (Altmann et al., 1997). We cloned and expressed both eIF4E and Caf20p individually in *E. coli*. Surprisingly, when Caf20p was assayed individually with Psk2p and [γ-^{32}P]ATP, no significant phosphorylation was observed. In contrast, Caf20p was efficiently phosphorylated by Psk2p, when coexpressed and purified with eIF4E (Figure 3-2). This reinforces the rationale for the biochemical approach to identify kinase substrates. Other methods such as IVEC (*in vitro* expression cloning), solid phase phosphorylation and Shokat method would not have identified CAF20 as a substrate. The fact that our strategy allows for the maintenance of protein-protein interactions augments our probability to find *bona fide* PASK substrates. Phosphorylation of the recombinant Caf20p, followed by in-gel trypsinolysis and MALDI-TOF was used to identify peptides phosphorylated by PASK. Using this technique, we identified two distinct phosphorylation sites in Caf20p; one in the predominantly hydrophilic C-terminal 63 amino acids, and the second in a region including residues 58 and 59. Residues appear to be phosphorylated by Psk2p in a mutually exclusive manner. Each of the single mutants (S58A or S59A) was phosphorylated as efficiently as the native protein, while the S58A, S59A double mutant was resistant to Psk2p-mediated phosphorylation (Figure 3-2).

The third substrate identified is the product of the TIF11 gene, which encodes the eukaryotic translation initiation factor 1A (eIF1A). eIF1A is responsible for the transfer of the initiator Met-tRNA to the 40S ribosomal subunit generating the 40S preinitiation complex (Chaudhuri et al., 1997). Recombinant His_6-Tif11p expressed in *E. coli*, was phosphorylated by Psk2p (Figure 3-2). The phosphorylation site was mapped to serine 125, which is situated just C-terminal to the canonical eIF1A fold (Battiste et al., 2000). A mutant of Tif11p, S125A, was not phosphorylated by Psk2p (Figure 3-2).

The fourth Psk2p substrate identified in this biochemical screen was Sro9p. SRO9 was originally identified as a suppressor of the rho3 mutation (Matsui and Toh, 1992), and displays genetic interactions with membrane trafficking and actin cytoskeleton components (Kagami et al., 1997; Tsukada and Gallwitz, 1996). Sro9p contains a La motif, which, is involved in the binding of nacent RNA polymerase III transcripts (Rinke and Steitz, 1982). Sro9p binds RNA *in vitro*, interacts with polyribosomes, and elimination of SRO9 confers resistance to some chemical inhibitors of translation (Sobel and Wolin, 1999). Attempts to express and purify sufficient amounts of full-length Sro9p from *E. coli* were unsuccessful. We were able to express and purify an N-terminally deleted form (Sro9pΔN55). Sro9pΔN55 was found to be phosphorylated by Psk2p indistinguishable from full-length Sro9p (Figure 3-2). A single tryptic peptide was identified which contained threonine residues 101 and 103. Both single mutants (T101A and T103A) were phosphorylated similarly to wildtype Sro9p but the double mutant was immune to Psk2p-dependent phosphorylation (Figure 3-2). Similarly to Caf20p, Sro9p appears to be phosphorylated by Psk2p in a mutually exclusive manner at one of the two adjacent sites.

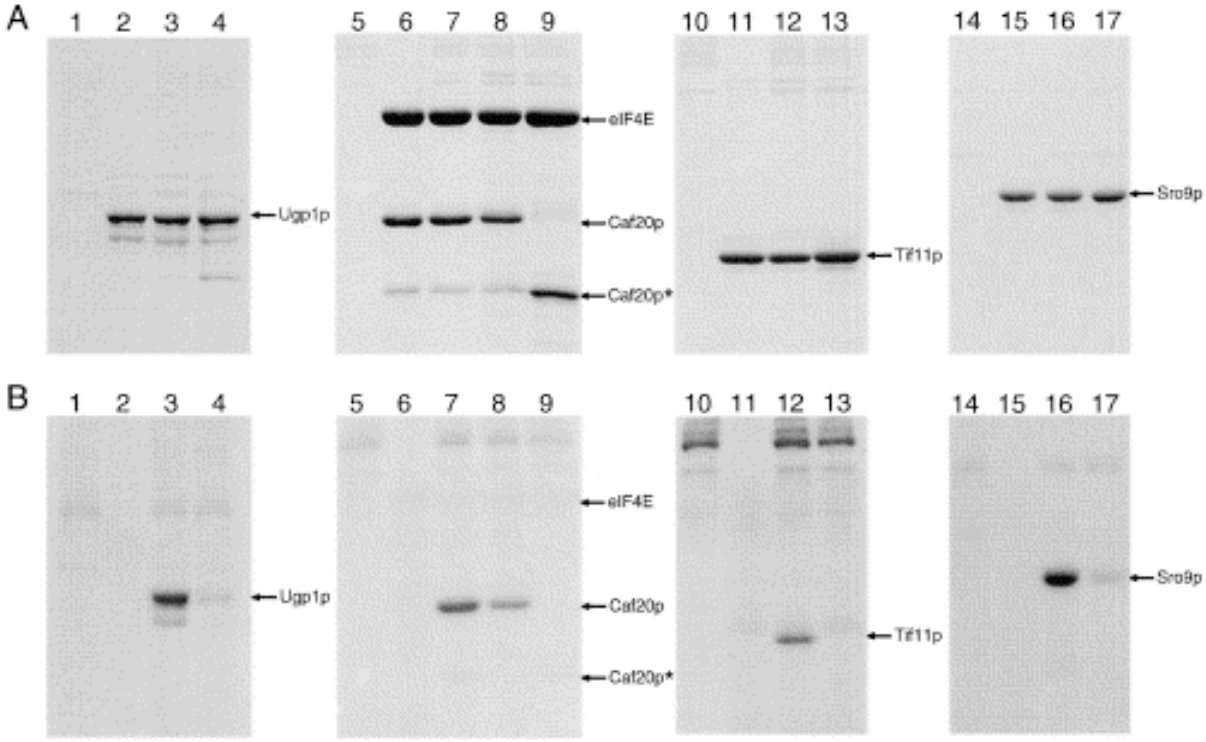


Figure 3-2 Psk2p phosphorylates Ugp1p, Caf20p, Tif11p and Sro9p
Kinase reactions containing [γ-^{32}P]ATP were analyzed by SDS-PAGE and Coomassie Blue staining (A). Stained gels were dried and exposed to autoradiographic film (B). Lanes 1, 5, 10 and 14: Psk2p alone; lanes 2,6, 11, and 15: substrate alone; lanes 3, 7, 12, and 16: Psk2p + substrate; lanes 4, 8, 9, 13, and 17: Psk2p + substrate. Substrate proteins are as follows: lanes 2 and 3: His_6-Ugp1p; lane 4: His_6-Ugp1p S11A; lanes 6 and 7: GST-eIF4E/His_6-Caf20p; lane 8: GST-eIF4E/His_6-Caf20p S58A/S59A; lane 9: GST-eIF4E/His_6-Caf20p S58A/S59A/ΔC63; lanes 11 and 12: His_6-Tif11p; lane 13: His_6-Tifllp S125A; lanes 15 and 16: His_6-Sro9pΔN55; lane 17: His_6-Sro9p T101A/T103A/ΔN55. Caf20p* signifies a naturally occurring C-terminally truncated form of Caf20p in lanes 6-8 and the Caf20pΔ63 deletion mutant in lane 9. Figure from (Rutter et al., 2002).

PSK mutants accumulate excess storage carbohydrates

The Ugp1 enzyme catalyzes an essential step in the synthesis of structural and storage carbohydrates, primarily glycogen and trehalose. As such, dysregulation of Ugp1 should lead to inappropriate accumulation of these macromolecules. Indeed, underexpression of UGP1 leads to glycogen hypoaccumulation (Daran et al., 1997) and overexpression of UGP1 leads to hyperaccumulation of glycogen (Daran et al., 1995). We hypothesized that if Psk were to phosphorylate and negatively regulate Ugp1, then deletion of both PSK genes should cause an increase in storage carbohydrate accumulation. As expected, psk1 psk2 double mutants accumulated 3-4 fold more glycogen than the wildtype strain in both log and stationary phase cultures (Figure 3-3). Single psk mutants exhibited an intermediate glycogen accumulation phenotype, with the psk2 mutant being more affected than the psk1 mutant.

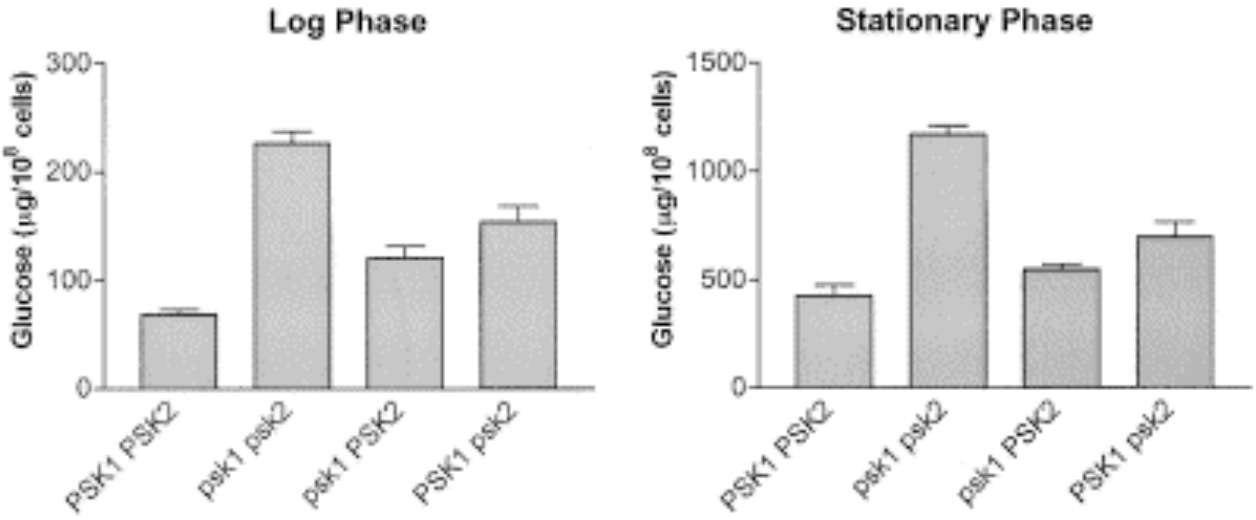


Figure 3-3 psk mutant yeast accumulate excess glycogen
Strains of the indicated genotype were grown to saturation in minimal medium with 2% glucose at 30°C. Aliquots were harvested and assayed for glycogen content (right image). An aliquot of the saturated culture was diluted in fresh medium to an OD_{600} = 0.1 and cultured for 6 hr at 30°C to an OD_{600} = 0.6. An aliquot of this culture was harvested and assayed for glycogen content (left image) which is expressed as a concentration of glucose enzymatically released from glycogen. The mean of three experiments ± S.D. is shown. Note the difference in *y* axis scale in the two figures.

Next, we wanted to demonstrate biochemically that Psk2p could indeed inhibit Ugp1 activity *in vitro* and *in vivo*. We first showed that our recombinant Ugp1 was indeed an active enzyme by its ability to generate radiolabeled UDP-glucose from [α-^{32}P]UTP and glucose 1-P. Radiolabeled UDP-glucose can be separated from free [α-^{32}P]UTP by thin layer chromatography (TLC) on polyethyleneimine cellulose. Psk2p mediated phosphorylation of Ugp1 did not inhibit its ability to generate UDP-glucose (Figure 3-4). Nor was any difference seen in catalytic rates of wildtype versus S11A Ugp1p treated with Psk2p. Furthermore, analysis of endogenous Ugp1 activity in yeast extracts from either wildtype or psk1 psk2 strains revealed no detectable difference in activity (data not shown).

Glycogen Synthase is a PSK substrate

The levels of hyperaccumulated storage carbohydrates in psk1 psk2 double mutants could not be fully explained solely on the basis of the dysregulation of the Ugp1p enzyme. First, when Ugp1p was overexpressed forty-fold higher than wildtype levels this translated in only a two-fold increase in glycogen content (Daran et al., 1995). We observed a 3-4 fold increase in glycogen content upon elimination of both PSK genes. Second, glycogen and trehalose are synthesized by the polymerization of glucose from UDP-glucose precursors. Therefore an increase in Ugp1p activity should not only cause a hyperaccumulation of glycogen but a also similar accumulation in trehalose levels. Glycogen and trehalose levels were equally affected by elimination of PSK during exponential growth, but only glycogen was increased during stationary phase. Thus additional regulatory processes must contribute to glycogen hyperaccumulation in the psk1 psk2 double mutant strain.

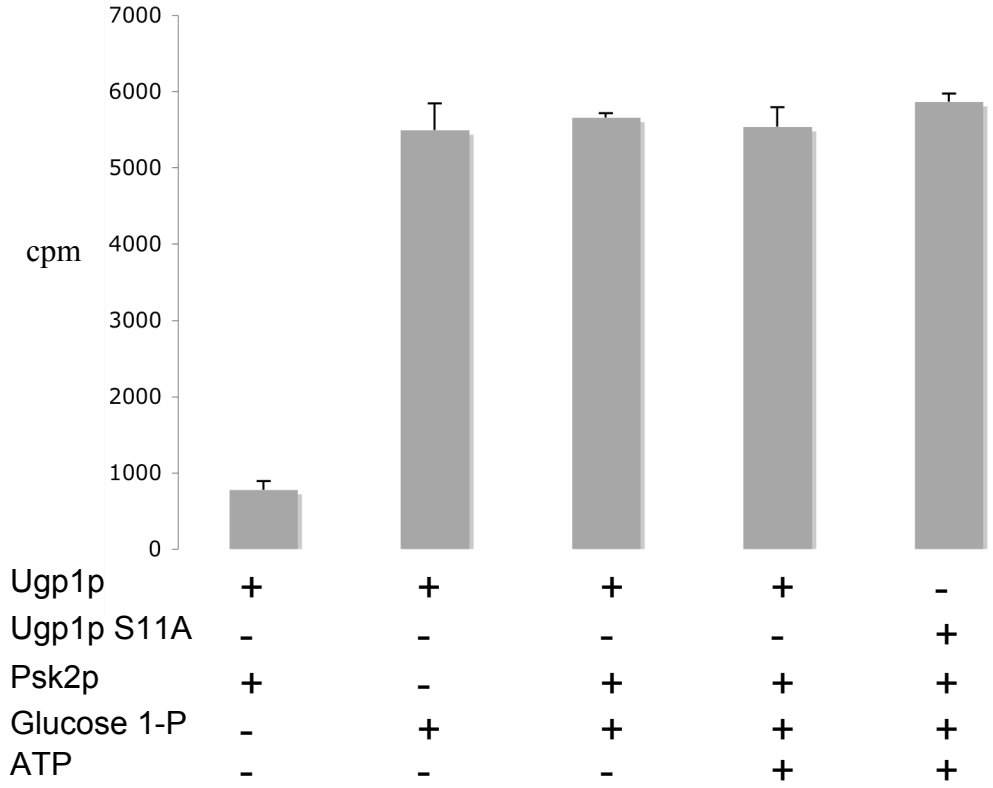


Figure 3-4 Phosphorylation of Ugp1p by Psk2p does not directly affect catalytic activity

Recombinant wildtype Ugp1p or S11A Ugp1p were incubated with recombinant Psk2p with or without ATP for 30 min at RT. Ugp1p catalytic activity was subsequently measured with the addition of [α–^{32}P]UTP and -/+ glucose 1-phosphate. Reactions were incubated for 10 min at RT, spotted on polyethyleneimine cellulose and separated by TLC. Radioactive UDP-glucose was quantitated on a phosphoimager.

Glycogen accumulation is primarily controlled by two enzymes: glycogen synthase and glycogen phosphorylase. The former is responsible for adding glucose monomers to glycogen while the latter removes glucose 1-P form glycogen. The regulation of these two enzymes in yeast is a complex network of phosphorylation, allosteric and transcriptional regulation (Francois and Parrou, 2001). In short, the two enzymes are regulated in a concerted manner by both glucose-6-P and phosphorylation. Glucose 6-P leads to an increase in glycogen synthesis, by allosterically activating glycogen synthase (Huang and Cabib, 1974a; Huang and Cabib, 1974b; Rothman-Denes and Cabib, 1971) and inhibiting glycogen phosphorylase (Fosset et al., 1971; Lin et al., 1995). Phosphorylation causes a decrease in glycogen synthesis by inhibiting glycogen synthase (Hardy and Roach, 1993; Rothman-Denes and Cabib, 1970) and activating glycogen phosphorylase (Lin et al., 1995; Lin et al., 1996). We hypothesized that the hyperaccumulation phenotype seen in the PSK double mutant strain could be due to either inhibition of glycogen synthase or activation of glycogen phosphorylase. To investigate this, we cloned, expressed and purified yeast glycogen synthase (Gsy2p) and glycogen phosphorylase (Gph1p), and assayed each enzyme for phosphorylation by Psk2p. Psk2p did not phosphorylate Gph1p (Figure 3-5), but it did efficiently phosphorylate Gsy2p (Figure 3-5). Futhermore, incubation with Psk2p and ATP caused a decrease in glycogen synthase activity when assayed in the absence of glucose 6-P (data not shown). We found the phosphorylation site to be serine 654 (Figure 3-5), which is one of the three residues already known to confer phosphorylation-mediated inhibition of glycogen synthase in yeast (Hardy and Roach, 1993).

As mentioned above, glycogen synthase is regulated allosterically by glucose 6-phosphate and by phosphorylation. These two events are interconnected, as phosphorylation-mediated inhibition can be completely reversed by saturating glucose-6-P concentrations (Hardy

and Roach, 1993; Rothman-Denes and Cabib, 1971). Glycogen synthase activity measured in the presence of glucose -6-P is indicative of total enzyme concentration independent of phosphorylation state. Therefore, the contribution of inhibition, impinged upon the glycogen synthase, by phosphorylation can be demonstrated by taking a ratio of the glycogen synthase activity in the absence to presence of glucose-6-phosphate (-/+ G-6-P).

Yeast, as opposed to mammals, begin synthesizing glycogen when nutrients become scarce. As such, there was no difference in glycogen synthase activity in extracts prepared from wildtype and PSK mutant strains in the presence and absence of excess glucose-6-P during exponentially growth (data not shown). When cells reached the transition from log to stationary phase (OD_{600}=1.5 in minimal medium), a significant increase was observed in the glycogen synthase activity ratio in the psk1 psk2 strain relative to wildtype (Figure 3-5C). This increased activity ratio in the psk1 psk2 double mutants was retained through mid-stationary phase (OD_{600}=2.5). The data shown in Figure 3-5 are representative of eight independent experiments, in each of which the psk1 psk2 mutant exhibited an increased activity ratio relative to wildtype. This increased activity ratio can only be attributed to the hypophosphorylated state of glycogen synthase in the psk1 psk2 mutant strain. This is supported by the observation that a mutation of the Psk phosphorylation site, Serine 654, to alanine is known to cause an increase in the glycogen synthase activity ratio (Hardy and Roach, 1993).

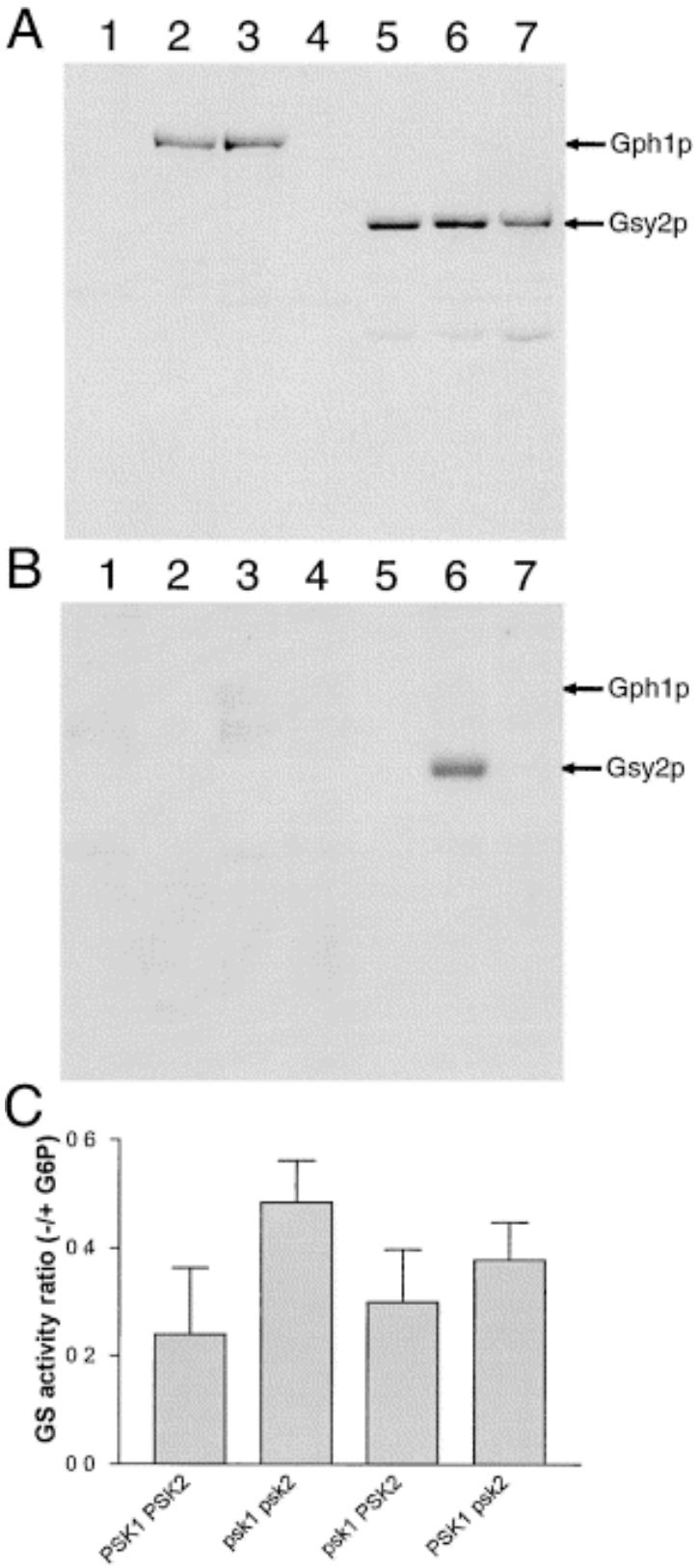

A
1 2 3 4 5 6 7
Gph1p
Gsy2p
B
1 2 3 4 5 6 7
Gph1p
Gsy2p
C
GS activity ratio (-/+ G6P)
0.6
0.4
0.2
0.0
PSK1 PSK2
psk1 psk2
psk1 PSK2
PSK1 psk2

Figure 3-5 Glycogen synthase is regulated by PAS-mediated phosphorylation
Kinase reactions containing [γ-^{32}P]ATP were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining (A). Stained gels were dried and exposed to autoradiographic film (B). Lanes 1 and 4: Psk2p alone; lane 2: Gph1p alone; lane 3: Psk2p + Gph1p; lane 5: Gsy2p alone; lane 6 Psk2p + Gsy2p; lane 7: Psk2p + Gsy2p S654A.(C) Strains of the indicated genotype were grown to an OD_{600} = 1.7 in minimal medium with 2% glucose at 30°C. Cells were harvested, extracted, and assayed for glycogen synthase activity in the presence and absence of 6.7 mM glucose-6-P. The ratio of activity in the absence of G-6-P to activity in the presence of G-6-P (−/+ G6P) is displayed. The mean of three experiments ± S.D. is shown and is representative of eight independent experiments.

Discussion

These experiments describe both genetic and biochemical techniques tailored towards understanding the biological role of PAS kinase in *S. cerevisiae*. First, a genetics approach, based on the inability of the psk1 psk2 double-mutant genotype to grow at elevated temperatures when limited to galactose as the sole carbon source, isolated enzymes in sugar flux and translation. Initially we did not believe that the gal^{ts} phenotype would necessarily be relevant to the biological role of PAS kinase. However, the data presented here argues that regulation of sugar flux may indeed represent one of the two roles of this enzyme.

Figure 3-6 illustrates the pathways used by *S. cerevisiae* when presented with galactose as the sole carbon source. This model can explain the mode of action of both high-copy suppressors and PAS kinase substrates as well as the reasoning behind the galactose phenotype. Under conditions when galactose is the sole carbon source, yeast must phosphorylate it to form galactose-1-P before it can be epimerized to glucose-1-P. This sugar must be further converted into glucose-6-P before it can enter glycolysis to produce the vital energy needs of the cell. Our data suggests that in the absence of PAS kinase, galactose falls into a shunt leading to the formation of excess glycogen. Overexpression of either phosphoglucomutase (Pgm) enzymes was able to suppress the gal^{ts} phenotype of the psk1 psk2 double mutant. This is consistent with the interpretation that the Pgm enzymes are pushing sugar flux toward glycolysis and pulling it away from the formation of storage carbohydrates.

Additionally we have shown that two enzymes relevant to the control of glycogen flux, UDP-glucose phosphorylase (Ugp1p) and glycogen synthase (Gsy2p), are direct targets of PAS kinase-mediated phosphorylation. As illustrated in Figure 3-6, Ugp1p catalyzes the conversion of glucose-1-P to UDP-glucose, which is the next to last step in the formation of both glycogen and

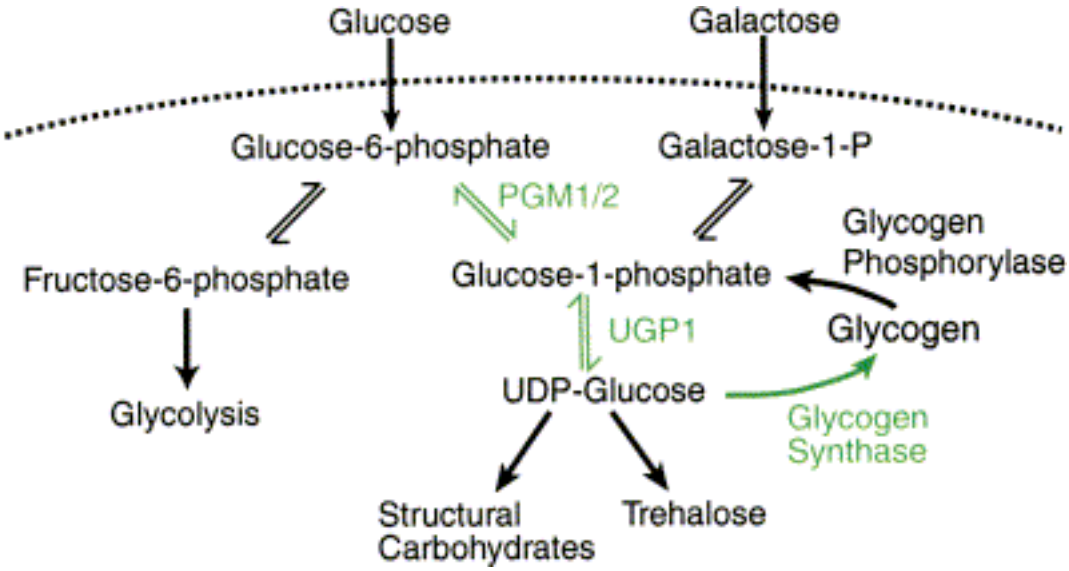


Figure 3-6 Model for PAS kinase mode of action
Upon import, galactose is phosphorylated and epimerized to glucose-1-phosphate, which can then be converted to either glucose-6-phosphate for use in glycolysis and ATP production or UDP-glucose for structural and storage carbohydrate synthesis. Similarly, when glucose is imported into the cell, it is phosphorylated and can either be isomerized to fructose-6-phosphate for glycolysis, or converted to glucose-1-phosphate. The three enzymes catalyzing sequential steps in the incorporation of glucose into glycogen (shown in green) are all related to PAS kinase, two (UGP1 and glycogen synthase) representing direct substrates of the enzyme and another (PGM) representing a high-copy suppressor the psk1 psk2 double mutant. Figure from (Rutter et al., 2002).

trehalose. Glycogen synthase then utilizes UDP-glucose directly in the polymerization of glycogen. We believe that the hyperaccumulation of glycogen in strains lacking both PAS kinase genes are a result of PSK's inability to phosphorylate and inhibit these two enzymes. They are locked into a hypophosphorylated state favoring the formation of storage carbohydrates. Directed mutagenesis of the Psk2p phosphorylation sites of both Ugp1p and Gsy2p led to phenotypes similar to those observed in the psk1 psk2 double mutant. In other words, similar defects were observed irrespective of whether phosphorylation-mediated

regulation was eliminated by removal of PAS kinase or through mutational elimination of the precise sites of phosphorylation. By negatively regulating both Ugp1p and Gsy2p, PAS kinase is interpreted to shunt the flux of galactose toward glycolysis, in part, through inhibition of glycogen synthesis.

We also clearly demonstrate through genetic and biochemical studies that PAS kinase plays a positive role in the regulation of protein synthesis. Several lines of evidence support a claim for PAS kinase in the control of protein synthesis. First, the screen for high copy suppressors of the psk1 psk2 double mutant identified a number of genes encoding translation factors and components of the translation apparatus. In particular was the identification of the uncharacterized RBS1 gene, which was the strongest suppressor identified (Rutter et al., 2002). It contains an R3H domain that has also been implicated in translational regulation (Hawkins et al., 1997), and the R3H domain was found to be essential for the activity of RBS1. Second, our unbiased biochemical screen for Psk2p substrates also led to the discovery of Tif11p, Sro9p and Caf20p, three polypeptides involved in the control of protein synthesis.

The identification of Caf20p as a Psk2p substrate is of particular interest for three additional reasons. First of all, Caf20p negatively regulates translation initiation by blocking the association of eIF4E and eIF4G (Altmann et al., 1997), which are required for the nucleation of the translational apparatus at the 5′ cap of mRNAs (Gingras et al., 1999; McKendrick et al., 1999). The DED1 and DBP1 genes, identified in the high copy suppressor screen are genetically related to both eIF4E and CAF20 (Rutter et al., 2002). These encode similar DEAD-box RNA helicases involved in mRNA translation (Chuang et al., 1997). DED1 and DBP1 suppress the temperature-sensitive growth phenotype of an eIF4E mutant, and the ded1 mutant phenotype is partially suppressed by the deletion of CAF20 (de la Cruz et al., 1997). Thus there appears to be

biochemical and genetic complementarities that exit between Caf20, DED1, DBP1 and PSK genes. Mechanistically how these are all functionally supportive remains a future avenue of research.

A second important point of Caf20p is derived from studies of translational repression in higher eukaryotes. Caf20p is thought to be the yeast analog of mammalian eIF4E binding proteins (4E-BPs). 4E-BPs compete with eIF4G for interaction with eIF4E via a short sequence that is conserved in the eIF4E binding region of Caf20p (Haghighat et al., 1995). Phosphorylation of 4E-BPs at specific sites in response to mitogenic and hormonal stimuli causes its dissociation from eIF4E, allowing functional eIF4F complexes to form on the mRNA (Gingras et al., 1999) and (Lawrence and Abraham, 1997). On the other hand, certain stress conditions can lead to inhibition of translation via dephosphorylation of 4E-BPs, thereby enhancing their affinity for eIF4E (Zanchin and McCarthy, 1995). For example, the withdrawal of glucose and amino acids from cultured mammalian cells results in a net decrease in the phosphorylation of 4E-BP1 (Hara et al., 1998). One of the effects of insulin is also the regulated phosphorylation 4E-BP1 (Gingras et al., 1999). The direct pathways responsible for regulating Caf20p in *S. cerevisiae* remain largely undefined. It is intriguing, however, that in a systematic effort to identify protein complexes in yeast, Ho and colleagues (Ho et al., 2002) discovered Caf20p to be associated with a number of proteins involved in carbohydrate metabolism.

There are still many unanswered questions that need to be addressed to understand how PAS kinase is specifically regulating translation. More than likely the two functional pathways of PASK discovered here, translation and sugar flux are coupled providing another means of the cell to regulating energy costly processes according to nutrient availability. Furthermore, in light of the plausible ligand-sensory capability of the PAS domain, we hypothesize that this system

has evolved to sense a specific metabolic product whose abundance is reflective of ambient nutritional status.

CHAPTER FOUR
Results

BIOLOGICAL ROLE OF MAMMALIAN PASK

Abstract

Extensive studies in a variety of microbial systems have shown that PAS domain-linked kinases are tuned to sense environmental cues and rapidly initiate an adaptive biological response. Two high throughput (HT) screens were employed to probe the biological role of mammalian PAS kinase. First, an unbiased biochemical screen was conducted to identify polypeptide substrates from cell extracts. This effort led to the identification of enzyme substrates involved in protein synthesis and intermediary metabolism. Phosphorylation site-mapping of these substrates identified the consensus motif, R-X-A/x-S/T, as the optimal substrate for PAS kinase. A second, independent screen interrogated a synthetic chemical library of roughly 200,000 compounds as a means of identifying small molecule activators and inhibitors of PAS kinase. All of the activators discovered in this screen proved to function independent of the PAS domain of the enzyme. Most inhibitors identified were shown to be nonspecific competitors of the catalytic domain of PAS kinase with the exception of one compound that exerted specificity for PASK over PKA, CKIε and CaMK II. The results of these two HT screens provide novel insights into the regulation and biological function of PASK.

Introduction

Mammalian PAS kinase has two orthologs in yeast that coordinately regulate translation and glycogen synthesis (Rutter et al., 2002). Genetic and biochemical data have demonstrated that the yeast PAS kinase enzymes phosphorylate both UDP-glucose pyrophosphorylase (Ugp1p)

and glycogen synthase (Gsy2p) to negatively regulate glycogen synthesis (Rutter et al., 2002). The yeast enzymes also phosphorylate three proteins involved in translation: Tiff11p (eIF1A), Caf20p and Sro9p (Rutter et al., 2002). With the exception of glycogen synthase (see Appendix 1), none of the mammalian homologs for these genes have been found to qualify as substrates for the mammalian PASK (data not shown). Furthermore, studies of PAS kinase knockout mice have revealed no overt developmental or adult phenotype (Katschinski et al., 2003), and few clues to the biological function of this enzyme. Here we report the studies of an unbiased biochemical screen that provide new insight into the biological pathways of mammalian PASK. Furthermore, we provide data on a HT synthetic chemical screen that identifies activators and inhibitors of the enzyme.

Methods

Materials

[γ-^{32}P]-ATP and chromatographic reagents for protein purification were obtained from Amersham Biosciences. All other chemicals and reagents were obtained from Sigma-Aldrich.

Protein Expression and Purification

All substrate proteins except AlaRS were expressed in *E. coli* strain BL21 (DE3) RIL (Stratagene). AlaRS and PASK was expressed in and purified from Sf-9 cells infected with a recombinant baculovirus as previously described (Rutter et al., 2001). GAPDH, NDRG1, Prx6, BTF3, UCK2, AlaRS, RPS3a were expressed as His_6-tagged proteins and purified using Ni-NTA agarose (Qiagen) according to manufacturers protocols. AlaRS, UCK2, NDRG1, and Prx6 were further purified using MonoQ chromatography (Amersham). RPS3a was further purified using

MonoS chromatography (Amersham). BTF3 was further purified using MonoQ and MonoS chromatography. GAPDH was further purified with Blue Sepharose chromatography. The induction conditions were as follows: BTF3, NDRG1, and Prx6 – 0.2mM IPTG/4 hrs/37°C; RPS3a, AlaRS, GAPDH, and UCK2 – 0.2mM IPTG/16hrs./25°C.

Kinase Substrate Screen

150 L of HeLa-S3 cells (~150g wet pellet) were obtained from the National Cell Culture Center. The cell pellet was resuspended in 5 times the volume of Buffer A (20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and protease inhibitor cocktail 1:300). The resuspended cell pellet was incubated on ice for 15 min before the cells were broken by dounce homogenization. The resulting broken cell mixture was centrifuged in three sequential steps: 1000*g*, 10,000*g*, and 100,000*g*. The 100,000*g* supernatant (S100) was considered the cytosolic fraction. The S100 was subjected to five ammonium sulfate cuts: 0-25%, 25-55%, 55-80%, 80-100%, and 100%. Protein precipitants from each cut were spun down at 10,000g and froze at -80°C until further use. Protein precipitants from each ammonium sulfate cut were resuspended in buffer A plus ammonium sulfate and fractionated over a series of successive chromatographic columns. First, the resolubilzed protein was batch bound to phenyl sepharose 6 FF and bump eluted in 5 fractions with decreasing concentrations of ammonium sulfate. Each of the 5 fractions were dialyzed in Buffer A, 100 mM NaCl, and 20% glycerol. Dialyzed fractions were then batch bound to 2 mL of Ni-NTA agarose (Qiagen) and bound proteins eluted in 50 mM Tris pH8.0, 5 mM β-ME, 10 mM NaCl and 250 mM imidazole. Eluted proteins were dialyzed in 50 mM Tris pH8.0, 5 mM β-ME, 100 mM NaCl and 20% glycerol and then assayed for PASK substrates. The flow through (FT) from the nickel column was then batch bound to 2 mL of Blue Sepharose and bound proteins were eluted in Buffer A, 1 M NaCl. Eluted proteins were dialyzed as described for nickel column and assayed for PASK substrates. The FT from the blue sepharose

column was then loaded onto a 1 mL MonoS column using an Amersham Pharmacia FPLC system. 1 mL fractions collected following a 0-100% 1 M NaCl gradient over 20 mL. Collected fractions were dialyzed as previously described and assayed for PASK substrates. The FT from the MonoS column was loaded onto a 1 mL MonoQ HR 5/5 column and again 1 mL fractions collected following a 0-100% 1 M NaCl gradient over 20 mL. Collected fractions were dialyzed as previously described and assayed for PASK substrates. The FT from the MonoQ column was loaded onto a Superdex 200 size exclusion column. 1 mL fractions collected in buffer A, 100 mM NaCl.

Kinase Assays and Protein Identification

Kinase assays were performed essentially as previously described (Rutter et al., 2001). 40 µl of each fraction was assayed with recombinant hPASK in a total reaction volume of 60 µl. After incubation for 30 min at 30°C, kinase reactions were terminated by the addition of SDS sample buffer and subjected to SDS-PAGE. Gels were stained with Coomassie Blue, dried, and analyzed by autoradiography. Proteins observed to be phosphorylated in a PASK-dependent manner were identified by mass spectrometry following in-gel trypsinolysis (Rosenfeld et al., 1992).

Phosphorylation Site Mapping and Mutagenesis

Substrate phosphorylation sites were identified by subjecting the recombinant protein to phosphorylation by hPASK followed by SDS-PAGE and in gel-trypsinolysis. Labeled tryptic peptides were fractionated and identified as previously described for yPsk2 substrates (Rutter et al., 2002). Phosphorylated residues were mutated to alanine via primer sewing method.

HTS Screen

For this assay, the 384 Costar # 3703 white plates were coated at 4ºC overnight with 50µl of 10µg/ml Neutravidin (Pierce Chemical Co.) Recombinant PASK was expressed and purified

as previously described (Rutter et al., 2001). The assay buffer was composed of 40 mM HEPES (pH 7.0), 100 mM KCl, 6.25 mM $MgCl_2$, 2 mM DTT 0.35mM ATP and 1μM biotinylated substrate peptide No. 1677, KKERLLDDRHDSGLDSMKDEEYEQGK-biotin, (synthesized at Tularik) substrate. A Biomek FX (Beckman Instruments) with a 384 well multichannel pod was used to dispense liquids into the assay plates. Volumes of 0.4 μl compound (0.3 mM) were mixed in 40 μl assay buffer. The reaction was initiated with the addition of 10 μl PASK (2 μg/ml) into 384 plates. The PASK and compounds were incubated for 60 min at room temperature. Total reaction volume was 50 μl per well. Positive and negative controls were in columns 1 and 24, respectively. The positive control was 10 nM biotinylated-phosphopeptide No. 2491, KKERLLDDRHDSGLD(p)SMKDEEYEQGK-biotin, (synthesized at Tularik). The negative control was assay buffer without added PASK. DMSO was mixed with a complete enzyme reaction in columns 2 and 23. Following the 60 min kinase reaction, the phosphorylated peptide was quantitated by a subsequent ELISA reaction. The assay plates were washed 4 times with 60 μl phosphate buffered saline per wash, using a Tecan Power Washer 384 (Tecan US).

The primary antibody, against the phosphorylated peptide, was a monoclonal antibody prepared at Tularik. The antibody buffer was composed of 2% BSA (Sigma A-2934, globulin-free) in phosphate buffered saline, primary antibody (1:10,000) and secondary antibody horseradish peroxidase-anti-mouse IgG (1:4,000). Volumes of 30ul antibody solution were dispensed into the assay plate wells and incubated for 1 hr at room temperature. The plates were washed 4 times with PBS as previously described. The luminol substrate, Pierce Super Signal Pico West (Pierce Chemical Co.), was diluted 1:10 in PBS and 30 μl dispensed per well. Luminescence for each plate was read for 25 sec using the CLIPR (Molecular Devices). For screen validation, compounds that were determined to activate or inhibit PAS kinase were cherry

picked from 3 mM compound stock library plates. The cherry picking of compounds was done with the use of a Biomek FX (Beckman Instruments) with a Span 8 pod.

Results

Biochemical screen for PASK substrates

In order to discover direct phosphorylation targets of PASK, we developed an unbiased, large-scale chromatographic fractionation scheme to separate cellular polypeptides based on their biochemical properties. The utility of this approach favored three objectives. First, extensive fractionation led to dilution of endogenous protein kinase enzymes whose activities would otherwise obscure the test activity of the PAS kinase added to each fraction. Second, fractionation led to the extensive purification of substrates such that, in all cases, we were able to identify substrates by mass spectrometry. Finally, fractionation of native cellular materials assisted in maintaining substrates in their native and often oligomeric state. The latter attribute may be particularly valuable owing to previous observations indicating that yeast PAS kinase is only capable of recognizing certain substrates in their native, oligomeric state (Rutter et al., 2002).

To maximize the probability of identifying low abundance substrates, we generated a soluble extract from 150 liters of HeLa cells. Our fractionation scheme included ammonium sulfate precipitation, hydrophobic interaction chromatography, metal chelation chromatography, cibacron blue sepharose chromatography, two forms of ion exchange chromatography, and size exclusion chromatography steps (Figure 4-1). The soluble HeLa cell extract was first fractionated into five ammonium sulfate cuts, based on typical ammonium sulfate precipitation ranges for proteins from a crude extract (King, 1972). The advantage of using ammonium sulfate precipitation as first step is the long-term stability of proteins in this precipitant state. Thus,

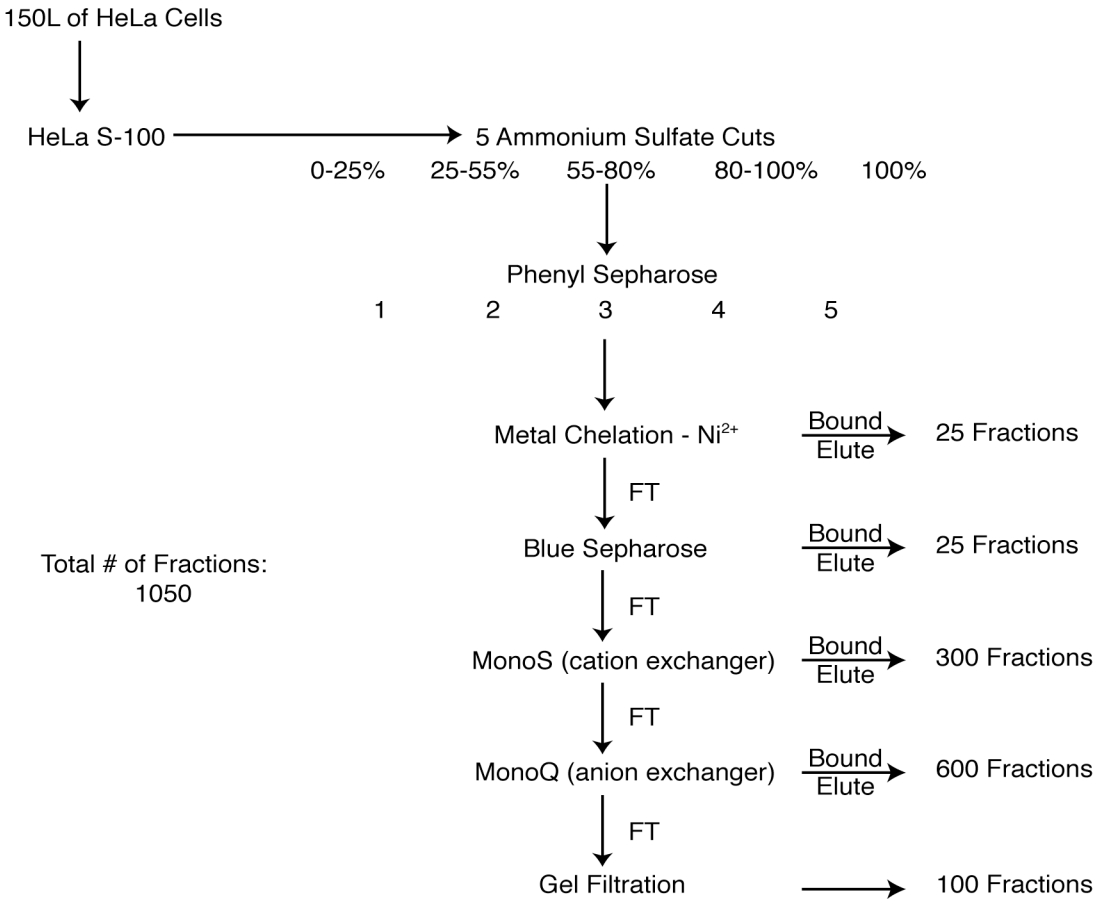


Figure 4-1 Fractionation scheme for the biochemical PASK substrate assay
150 liters of HeLa cells were lysed to generate a soluble extract. The extract was divided into 5 ammonium sulfate cuts. Each protein precipitate was solubilized and placed over a phenyl sepharose column. Proteins were eluted in 5 bump fractions. Each fraction from the phenyl sepharose column was then placed over a series of chromatographic resins. Those that bound to the various successive columns were eluted, saved and used in kinase reactions while the flow-through (FT) was placed over the next successive column beginning with Ni-NTA ending with gel filtration. The total # of fractions collected from each column is listed to the right while the sum total of all fractions generated was 1050.

protein precipitates from each cut could be frozen and stored for serial, exhaustive fractionation. Resolubilized proteins from ammonium sulfate precipitates were applied to phenyl sepharose resin and 5 subsequent fractions collected as a function of a reverse reduction in salt concentration. Each of the 5 phenyl sepharose fractions were separately placed over a series of chromatographic media as described in Figure 4-1, leading to the multi-dimensional separation of HeLa proteins into roughly 1000 fractions.

Each fraction was tested for PAS kinase-dependent protein phosphorylation using [γ-^{32}P]ATP. One such example, containing fractions that eluted off the monoQ column from the 25-55% ammonium sulfate cut, is shown in Figure 4-2. Although a number of proteins were phosphorylated in a PASK-independent manner (Figure 4-2B, lanes 9-10), two polypeptides with apparent molecular masses of 43kD and 30kD were clearly phosphorylated only when PASK enzyme was added (Figure 4-2B, lanes 16 and 18). These bands were excised from the gel, and tryptic mass spectrometric fingerprinting identified them as N-myc down regulated gene 1 (NDRG1) and uridine cytidine kinase 2 (UCK2).

Using this technique we found a number of distinct polypeptides phosphorylated only in the presence of PASK (Figure 4-3A). These included alanyl-tRNA synthetase (AlaRS), basic transcription factor 3 (BTF3), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), N-myc downregulated gene 1 (NDRG1), peroxiredoxin 6 (Prx6), ribosomal protein S3A (RPS3A), and uridine-cytidine kinase 2 (UCK2). Although additional putative substrates were identified in the screen, we choose these seven polypeptides for more extensive analysis.

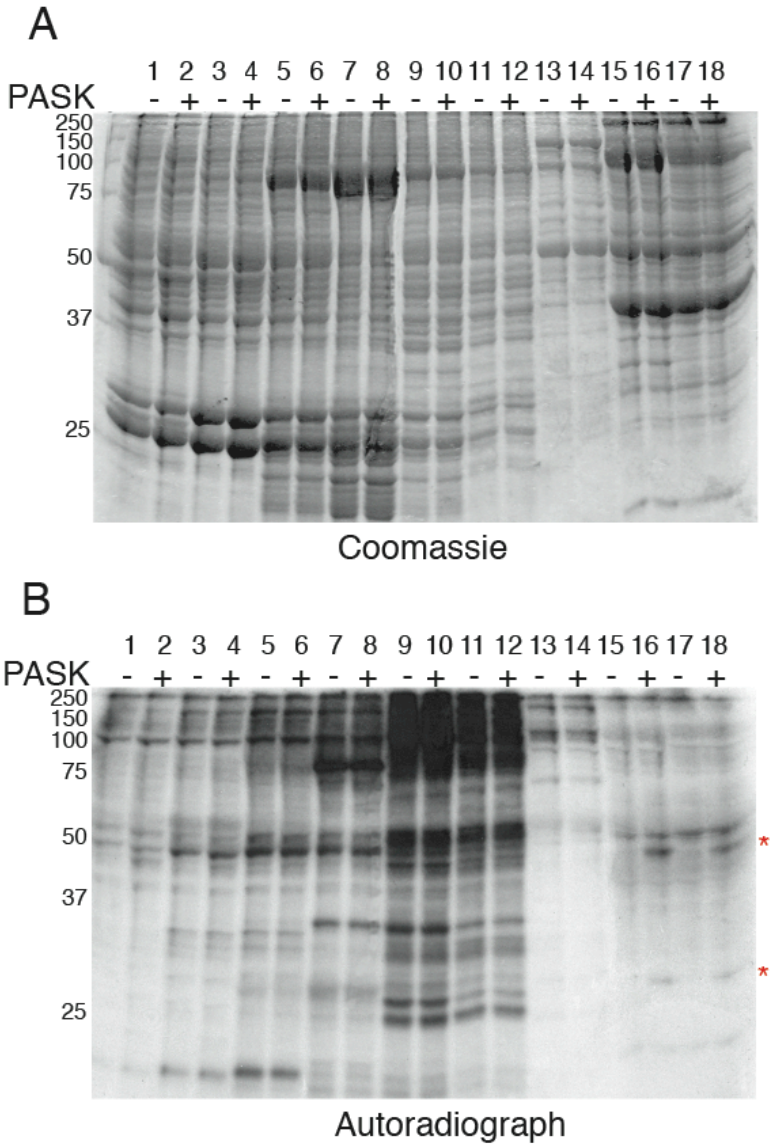


Figure 4-2 Biochemical screen for PASK substrates
Kinase reactions containing [γ-^{32}P]ATP in the absence (-) or presence (+) of recombinant PASK were analyzed by SDS-PAGE and Coomassie Blue staining (A). Stained gels were dried and exposed to autoradiographic film (B). * Red asterisks denote PASK-dependent phosphorylation events. Fractions analyzed in this gel were proteins from the HeLa soluble extract that precipitated between 25-55% ammonium sulfate, eluted in fraction 2 from phenyl sepharose column, flowed through Ni^{2+}, blue sepharose and MonoS. Proteins were bound to MonoQ and eluted with linear salt gradient.

Identification of PAS kinase phosphorylation sites

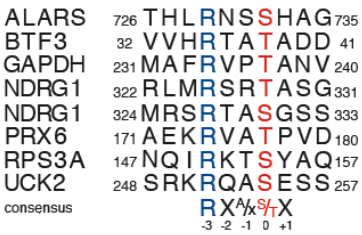
To investigate whether observed PASK substrates and the proteins identified by mass spectrometry were indeed one in the same, we cloned and expressed each substrate as His_6-fusions in either *E. coli* or Sf-9 insect cells (materials and methods). PASK efficiently phosphorylated each of the purified recombinant proteins. By phosphorylating each recombinant substrate with PAS kinase, followed by in-gel trypsinolysis and MALDI-TOF, we were able to identify each PASK-dependent phosphorylation site. Site-directed mutagenesis confirmed the relevant phosphorylation sites. Figures 4-3C and D show an example of this, in which a serine to alanine substitution of AlaRS residue 732 fully eliminated PAS kinase-dependent phosphorylation. In all cases, with the exception of NDRG1, PAS kinase phosphorylated each target substrate at a single site. The identification of these eight PAS kinase phosphorylation sites revealed a consensus motif, R-X-A/x-S*/T* (Figure 4-3B). An R-X-X-S*/T* consensus has been recognized by several other kinases, in particular calcium/calmodulin-dependent kinase II (Kennelly and Krebs, 1991), the founding member of the sub-classification group of the mammalian kinome to which PAS kinase belongs (Manning et al., 2002). Partial specification of PAS kinase substrate recognition may be conferred by alanine at the -1 position relative to the serine or threonine modified by the enzyme.

Alanyl-tRNA synthetase is a 107 kD protein that ligates alanine to the corresponding $tRNA^{ALA}$. The 28 kD polypeptide identified in our screen, as an *in vitro* substrate of PASK, corresponds to the last 261 residues of C-terminal tail of AlaRS. The identified phosphorylation site, S732, lies in a well-conserved region of the polypeptide thought to be important for oligomerization.

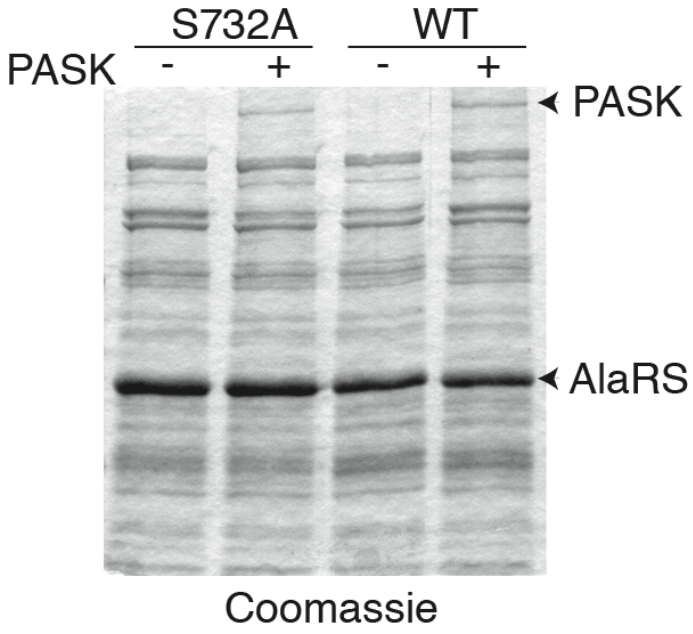
A

Substrates Identified	Size (kD)
Alanyl tRNA Synthetase (c-term.)	28
Basic Transcription Factor 3	17.6
Glyceraldehyde 3-P Dehydrogenase	36
N-myc Downregulated Gene 1	42.8
Peroxiredoxin 6	25
Ribsomal Protein S3A	29.9
Uridine Cytidine Kinase 2	29.3

B



C



D

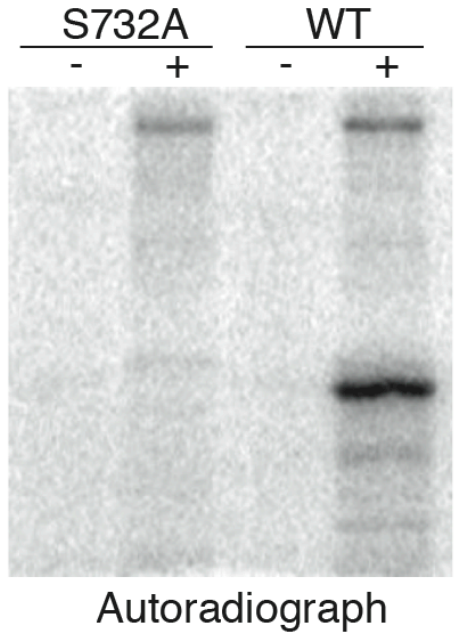


Figure 4-3 Identification of PASK substrates and phosphorylation sites
Table (A) includes those substrates identified as PASK *in vitro* substrates in which phosphorylation sites were mapped. The MW (kD) described corresponds to the size of protein identified as determined by SDS-PAGE and molecular weight standards. (B) Alignment of PASK-dependent phosphorylation sites in substrates identified and the discovery of a consensus: R-X-A/X-S*/T*. Red residues refer to phosphorylation sites whereas blue residues indicate an invariant Arg in the -3 position. Kinase reactions containing [γ-^{32}P]ATP of wildtype (WT) and S732A of c-term. AlaRS assayed in absence (-) or presence (+) of recombinant PASK were analyzed by SDS-PAGE and Coomassie Blue staining (C). Stained gels were dried and exposed to autoradiographic film (D).

Uridine cytidine kinase 2 catalyzes the rate-limiting step in the pyrimidine salvage pathway in which pyrimidine nucleosides are recycled for RNA and DNA synthesis. Phosphorylation of UCK2 by PASK occurs on residue 254 on the C-terminal tail. GAPDH is classically known for its "house keeping" role in glycolysis although other roles have emerged (Sirover, 1999). We mapped the site of phosphorylation to threonine 237, which is conserved across all species, and is positioned at the homotetramer interface.

NDRG1 belongs to a family of stress-regulated proteins for which a function has not been assigned (Shaw et al., 2002). We identified serine 328 and serine 330, in the C-terminal tail of NDRG1, as the residues phosphorylated by PAS kinase. These sites, Ser-328 and Ser-330, are already known to be phosphorylated *in vivo* (Murray et al., 2004).

Peroxiredoxin 6 (Prx6) is also upregulated in response to stress and acts a cellular defense against reactive oxygen species (ROS), especially lipid peroxides. Phosphorylation of Prx6 by PAS kinase was found to occur on residue threonine 177. Analysis of the Prx6 crystal structure demonstrates Thr-177 lies near the active site along the dimer interface suggesting that phosphorylation at this residue could affect catalytic activity.

40S ribosomal proteins are PASK substrates

Surprisingly, an abundance of PAS kinase substrates were observed to be phosphorylated in two fractions, 28 and 29 (Figures 4-4A and B). These fractions consisted of a very sharp OD_{280} peak that eluted off the monoQ column at approximately 500mM NaCl (Figure 4-4C). The sharpness of this peak, coupled with distinct absorbance at OD_{260}, suggests that many of the proteins in this fraction might be part of a large nucleoprotein complex. Indeed, size exclusion chromatography of these fractions demonstrated a very large complex eluting in the void volume

(data not shown). Mass spectrometry of PASK-dependent phosphoproteins in these fractions revealed that all were ribosomal proteins that compose the small 40S subunit of the ribosome. These observations indicate that multiple polypeptide components of the 40S ribosome are substrates for PASK in the context of the intact complex. Although having confirmed these substrates as ribosomal proteins S2, S6, S8, S10 and S14 we have not yet identified the precise sites of PAS kinase mediated phosphorylation on each polypeptide.

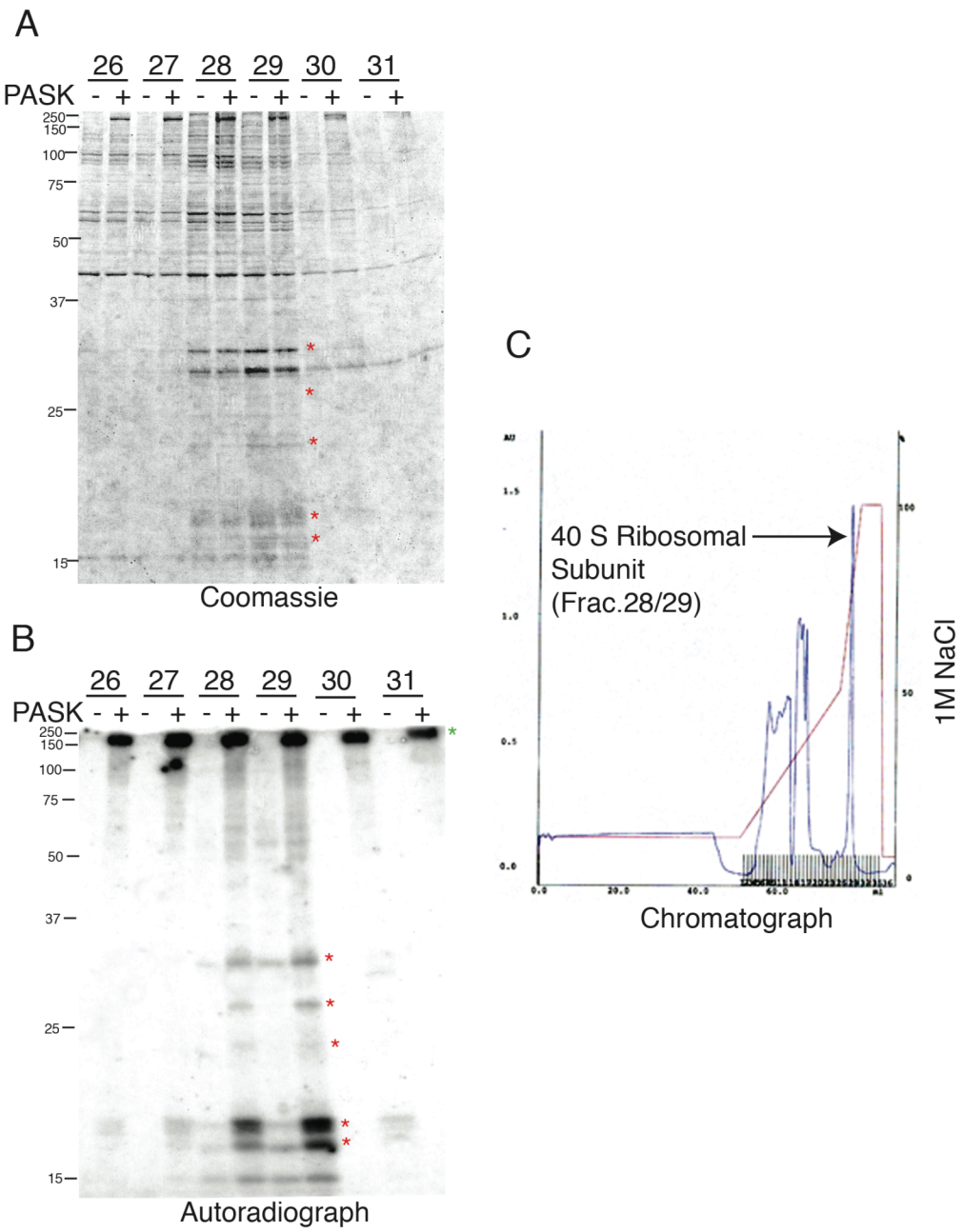


Figure 4-4 PASK phosphorylates multiple subunits of the 40S ribosome
Kinase reactions containing [γ-^{32}P]ATP assayed in the absence (-) or presence (+) of recombinant PASK were analyzed by SDS-PAGE and Coomassie Blue staining (A). Stained gels were dried and exposed to autoradiographic film (B). Fractions 26-31 correspond to successive fractions eluted off a MonoQ column during the biochemical screen. Fractions 28 and 29 correspond to a very sharp OD_{280} protein peak that was identified as the 40S small subunit of the ribosome. * Red asterisks denote PASK-dependent phosphorylation of individual ribosomal proteins while * green asterisks denote PASK-dependent autophosphorylaton.

Chemical HT screen for modulators of PAS kinase

A robotic HT screen was performed to identify potential activators or inhibitors of PASK that might either provide clues to the identity of the endogenous ligand or qualify as tools for probing PASK function. An ELISA-based assay using recombinant PAS kinase and a biotinylated peptide substrate was used to screen a library of 201,280 synthetic organic compounds (Materials and Methods). The assay was performed in 384 plates coated with Neutravidin, initiated with 10 μl PASK (2 μg/ml), and incubated for 60 minutes at room temperature. Luminescence images of two PASK assay plates in which an activator (well F20) and an inhibitor (well J16) were identified are shown in Figures 4-5A and C. Activator and inhibitor ”hits” were designated as compounds that increased or decreased PASK activity greater or less, respectively, than 2 standard deviations from the mean DMSO control (Figures 4-5B and D).

Roughly equal numbers of inhibitors (739) and activators (665) were identified from the initial screen. These 1404 compounds were individually recovered from the 3 mM stock compound library plates and retested in follow-up assays under “follow-up” assay conditions identical to the initial screen. For the followup assay, both full-length PAS kinase and ΔN PAS kinase were tested in parallel. The latter variant is known to display enhanced catalytic activity due to removal of the inhibitory PAS domain of the intact enzyme (Rutter et al., 2001). Comparison between the two enzyme samples allowed for the discrimination of compounds whose activity might be selectively dependent upon the PAS domain. Out of the 739 inhibitors identified in the primary HT screen, 95 were observed in follow-up assays to inhibit both forms of PASK. No compounds were found to selectively require the PAS domain in order to inhibit

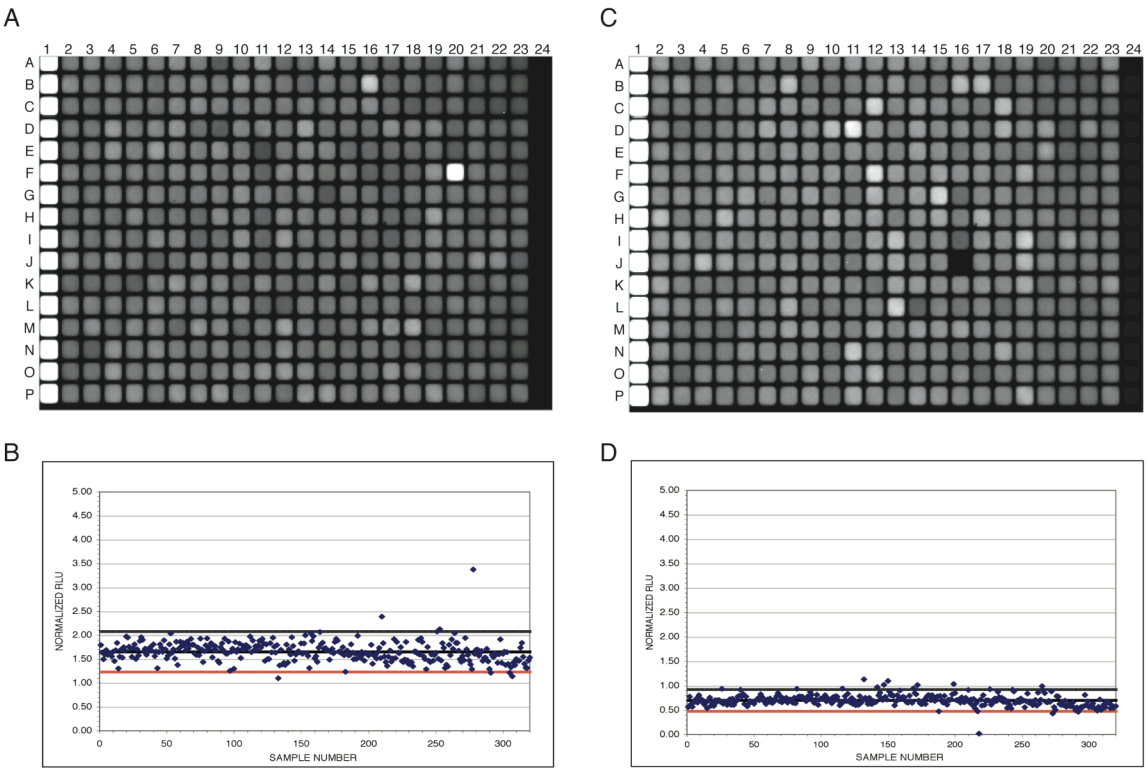


Figure 4-5 High-throughput screen of PASK identifies activators and inhibitors. Luminescence image of 384-well PASK assay plates identifying an activator, well F20 (A) and an inhibitor, well J16 (C). The plates contain a positive control in lane 1, negative control in lane 24, and internal DMSO controls in columns 2 and 23. (B) and (D) correspond to normalized relative light units (RLU) from assays plates, (A) and (C) respectively, plotted as a function of sample number. Compound "hits" were designated as compounds that increased or decreased PASK activity greater (black line) or less (red line) than 2 standard deviations from the mean DMSO control.

the catalytic activity of PASK. The 95 confirmed inhibitors were of further tested on casein kinase Iε (CKIε), Ca^{2+}-calmodulin-dependent kinase II (CaMK II) and protein kinase A (PKA) to determine specificity for PASK. The vast majority of chemical inhibitors discovered in the HT screen appear to be non-specific kinase inhibitors as they were equally toxic to CKIε, CaMK II and PKA as they were to PASK. Moreover, all inhibitors were observed to be equally toxic to both the full-length enzyme and the truncated kinase domain only fragment of PASK. One inhibitor did display modest inhibitory specificity. As shown in Figure 4-6, the symmetric 2-(2-{2-[3-2-Hydroxyl-ethyl)-5-methoxy-2,3,7,8-tetrahydrofuro[3',2':3,4]benzo[1,2-d]thiazol-2-ylmethlene]-butylidene}-5-methoxyfuro[3',2':3,4]benzo[1,2-d]thiazol-3-yl)-ethanol was equivalently inhibitory to both full length PAS kinase and the catalytic domain-only variant, yet failed to inhibit PKA, CKIε and CaMK II.

Of the initially identified 665 activators, only 10 were observed to retain activity upon follow-up rescreening. None of these 10 compounds were found to selectively activate only the full-length enzyme, suggesting that these activators do not bind or mechanistically function in a PAS-dependent manner. Furthermore, these compounds were weak activators, modestly activating PASK 1.6-2-fold. It is also notable that the HT screen identified two highly related compounds 4-(4-Cyclohexyl-phenyl)-4-oxo-2-p-tolylsulfanyl-butyric acid and 2-4-(4-Chloro-benzylsulfanyl)-4-(4-cyclohexyl-phenyl)-4-oxo-butyric acid only differing in the carbon chain of the thiol-linker and the electron withdrawing chloro as opposed to the electron donating methyl on the benzyl ring (Figure 4-7). The fact that two of these highly related compounds found their way to the final list of the confirmed activators favors the conclusion that they represent *bona fide* chemical activators of PASK.

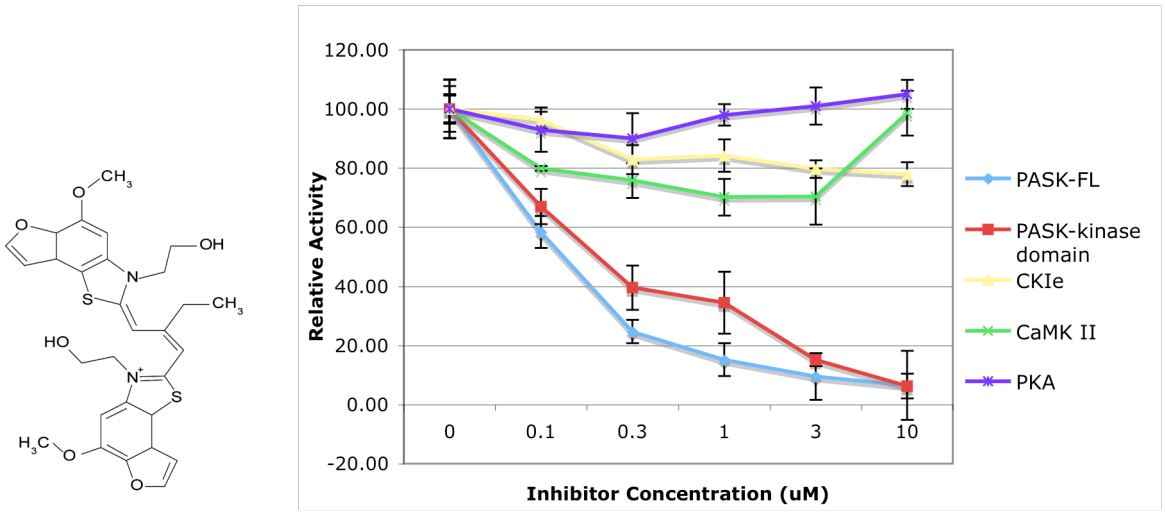


Figure 4-6 A PASK specific inhibitor identified in the HTS
Chemical structure and inhibition curves of a PASK specific inhibitor identified in the HTS that does not inhibit PKA, CKIε or CaMK II. Assays performed as described for HTS using 0.1-10 μM compound.

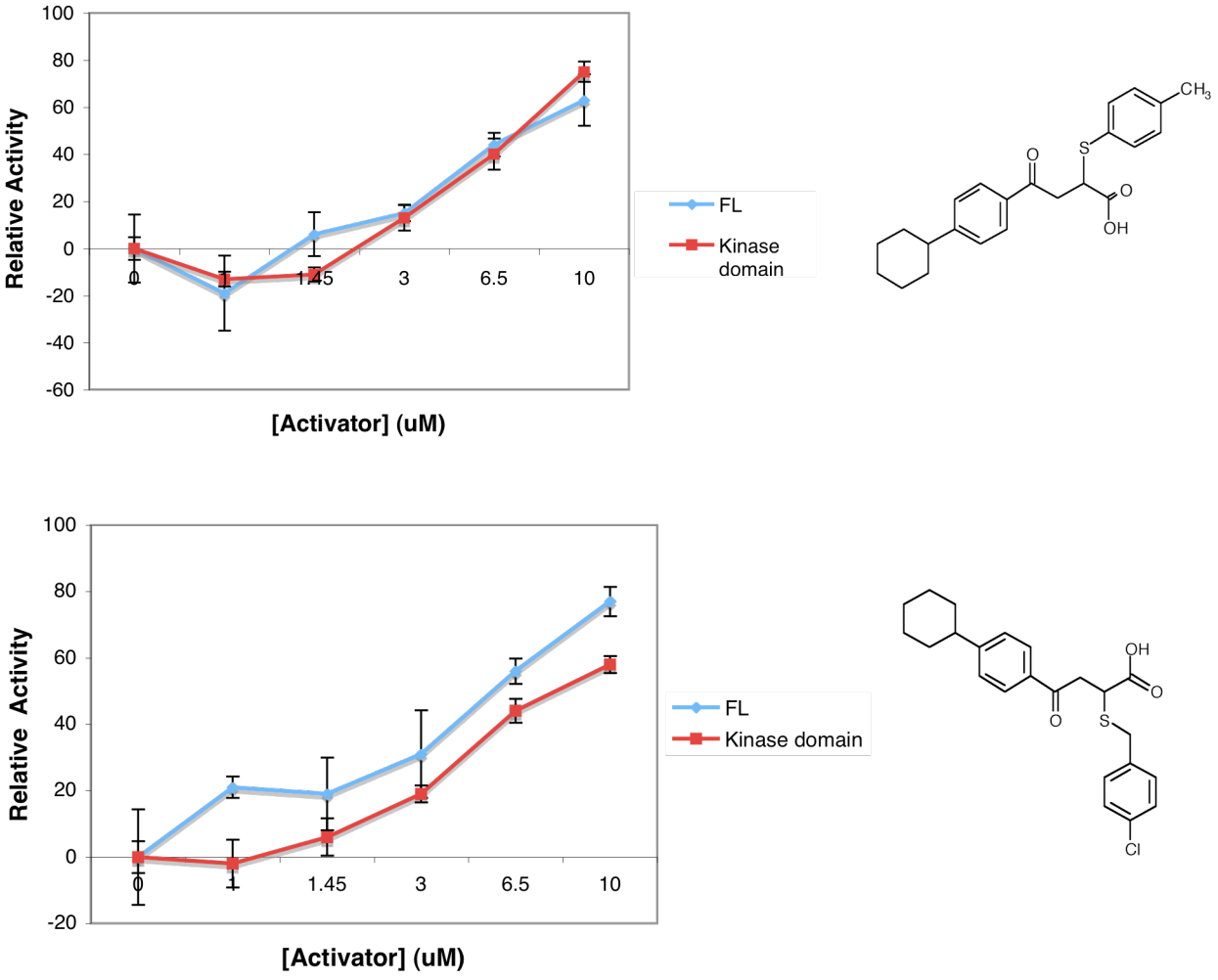


Figure 4-7 Two HT PASK activators
Chemical structures and activation curves of 2 PASK activators that weakly activated both full length and kinase domain only PASK. Kinase reactions containing both full-length and kinase domain PASK were performed in the presence of 0, 1, 1.45, 3, 6.5 and 10 µM with the above compounds as previously described. Note the commonality between the two chemical structures.

Discussion

The identification of a number of PASK substrates provides the first cues to the direct biochemical role of PASK in mammalian cells. The fact that several ribosomal proteins are phosphorylated by PAS kinase within the context of the intact 40S ribosomal complex gives tantalizing evidence indicative of a role of PASK in the regulation of translation. It is also notable that our blind screen identified ribosomal protein S3A and alanyl-tRNA synthetase as apparent PAS kinase substrates. Numerous studies have shown that ribosomal proteins can be phosphorylated in purified ribosomes incubated with [γ-^{32}P]ATP (Barden and Labrie, 1973; Jergil, 1972; Walton and Gill, 1973) (Delaunay et al., 1973), and that purified ribosomal subunits are phosphorylated upon exposure to an exogenous kinase (Ventimiglia and Wool, 1974) (Stahl et al., 1972; Traugh and Traut, 1973) (Eil and Wool, 1971). Even more compelling is the phosphorylation of ribosomes by *in vivo* ^{32}P-labeling experiments in a wide variety of animal and plant tissues (Francis and Roberts, 1982) (Gressner and Wool, 1974; Loeb and Blat, 1970; Pierre et al., 1974; Prestayko et al., 1974) (Barden and Labrie, 1973; Cawthon et al., 1974). Many of the 40S ribosomal proteins phosphorylated *in vivo* match the identities of the PASK substrates identified therein, including ribosomal proteins S2, S6, S8 and S10. We have no evidence indicative of functional significance for the PASK-mediated phosphorylation of ribosomal proteins. Similarly, others have reported that phosphorylated ribosomal subunits exhibit negligible differences in initiation rates, elongation rates and aminoacyl-tRNA binding when compared to unphosphorylated controls (Eil and Wool, 1973). Furthermore, careful measurements calculate an average half-life of just over 20 min for the turnover of ribosomal protein phosphorylation in reticulocytes (Kabat, 1972). This turnover rate is significantly slower than the time required to synthesize an average polypeptide chain, which is typically 0.25 to 0.4

min (Kabat, 1972). Thus, it appears that the phosphorylation/dephosphorylation of ribosomal proteins may not be expected to influence translation in reticulocyte lysate assays. A more plausible explanation for phosphorylation of ribosomal proteins is at the level of mRNA selection. Selective translation of specific mRNAs is best understood in the phosphorylation of ribosomal protein S6 and elongation factor 4E. Under a variety of hormonal and nutrient signals ribosomal protein S6 is phosphorylated to regulate the translation of a group of mRNAs that contain a 5'-terminal oligopyrimidine tract (5'TOP) (Meyhaus and Hornstein, 2000). Likewise, dephosphorylation of eIF4E leads to translation of specific mRNAs that have internal ribosome entry sites (IRES) (Hellen and Sarnow, 2001). The observations reported herein open the possibility that PASK may regulate translational selectivity in a heretofore unanticipated manner.

Consistent with this theme, alanyl-tRNA synthetase and GAPDH, both identified as substrates in our unbiased screen for PASK substrates could serve a similar purpose to regulate translational selectivity. GAPDH has been reported to associate with polysomes and bind *cis* acting elements important for the stability and translation of mRNA (Nagy and Rigby, 1995). An emerging role of aminoacyl-tRNA synthetases, other than their classical role in tRNA charging, includes translational regulation (Martinis et al., 1999a; Martinis et al., 1999b). In response to IFN-γ, glutamyl-prolyl-tRNA synthetase is phosphorylated and released from a multisynthetase complex, allowing it to bind to the 3' untranslated region of the ceruloplasmin mRNA and thereby inhibiting its translation (Sampath et al., 2004).

Our studies of HeLa cell substrates of PASK are generally consistent with previous biochemical and genetic studies of the two PASK enzymes of *S. cerevisiae* (Rutter et al., 2002). One yeast PASK substrate Tif11p (eIF1A) mediates the transfer of Met-tRNA to the 40S ribosomal subunit generating the 40S preinitiation complex (Chaudhuri et al., 1997). Although

mammalian eIF1A does not appear to be an *in vitro* substrate for PASK (data not shown), ribosomal protein S3A was found to be. RPS3A plays an important role in binding iMet-tRNA within the ribosome (Westermann et al., 1981). Thus, in the course of evolution, the direct PASK substrate may have changed from Tif11p (eIF1A) to RPS3A, yet the ultimate regulation of translation may remain unchanged. It is also notable that a high copy suppressor screen for genes that facilitate the growth of yeast lacking both PASK genes predominantly yielded genes encoding translation factors (Rutter et al., 2002). Among these are the DED1 and DBP1 genes required for translation initiation and genetically related to both eIF4E and CAF20 (de la Cruz et al., 1997).

Yet another parallel can be drawn between the yeast PASK substrate Caf20p and the mammalian PASK substrate basic transcription factor 3. Caf20p is known as an eIF4E binding protein that competes with eIF4F for binding to eIF4E. This event inhibits cap-dependent mRNA translation by blocking the formation of the eIF4E/eIF4G complex, which nucleates the assembly of the translation apparatus at the 5' end of mRNAs (Altmann et al., 1997). Recently, *Arabidopsis thaliana* BTF3 was shown in a yeast 2-hybrid screen to interact with eIF4E. The *A. thaliana* BTF3 polypeptide contains a sequence that is found in all eIF4E binding proteins, including Caf20p, which is responsible for competing with eIF4F (Freire, 2005). eIF4E binding proteins (4E-BPs) are critical regulators of translation in response to nutrient and other hormonal signals. Cell signaling cascades that lead to phosphorylation of 4E-BPs are known to prompt 4E-BP release from eIF4E (Gingras et al., 1999).

The phosphorylation state of ribosomal proteins and/or other proteins involved in the regulation of protein synthesis are closely coupled with nutrient availability. This may be of special significance when considering the metabolic enzymes representative of the other primary

category of PAS kinase substrates. Uridine-cytidine kinase 2 is one such metabolic enzyme discovered as a PAS kinase substrate that is essential for the generation of pyrimidine nucleoside pools. Sufficient pools of UTP and CTP are required for stability of *in vitro* translations systems Furthermore, an associative link exists between increased rates of uridine kinase activity, the energy requirements of the cell (Huang and Graves, 2003; Wharton and Pledger, 1981) and the proliferation and transformation of human cells (Shen et al., 1998).

A second metabolic PASK substrate, GAPDH, is a glycolytic enzyme responsible for converting glyceraldehyde 3-phosphate to 1:3-bis-phosphoglycerate. In glycolyis, glucose is converted to pyruvate for the generation of the cell's primary energy source, ATP. Reduced levels of intracellular ATP are known to increase phosphorylation of eukaryotic elongation factor 2 (eEF-2), resulting in a global blockage of global translational elongation (Horman et al., 2002). PAS kinase has recently been shown to link glucose availability with the regulation of specific transcription and translation events. Rutter et al. have demonstrated that preproinsulin gene expression and translation are induced by glucose in a PASK-dependent manner (da Silva Xavier et al., 2004). These data argue in favor of nutrient-coupled translational regulation of specific mRNAs by PAS kinase.

The switch between global translation and regulated translation of specific mRNAs is well documented to be induced under conditions of cellular stress (Harding et al., 2000; Holcik et al., 2000). The inhibition of global translation results in an increased saving of cellular energy, prevents the synthesis of unwanted transcripts that may interfere with stress-response pathways, and facilitates selective translation of proteins that are required for cell survival under stress conditions that trigger this change include, nutrient limitation, hypoxia, ultraviolet radiation and oxidative stress. The latter condition, oxidative stress, may be particularly

important with regard to peroxiredoxin 6 as it was identified independently in our biochemical screen as a putative PASK substrate. Oxidative stress occurs when the rate of reactive oxygen species (ROS) generation exceeds the detoxification capacity of the cell. Numerous studies have shown that various translation factors, ribosomal proteins and metabolic enzymes, particularly those in glycolysis, are exceptionally susceptible to oxidation (Cumming et al., 2004; Shenton and Grant, 2003). Peroxiredoxins represent a new class of antioxidant enzymes dedicated to protect the cell against oxidative stress. PASK-dependent phosphorylation of peroxiredoxin may directly regulate its activity. Our phosphorylation site-mapping studies revealed a PASK-mediated phosphorylation site near the catalytic site of the peroxiredoxin homodimer interface. As speculated below, the sensing ability of the PAS domain might allow PASK to sense direct cellular stress and initiate an adaptive response that couples protein synthesis to the metabolic status of the cell.

Having found that PASK phosphorylates specific polypeptide targets that are well suited to regulate both translation and intermediary metabolism, what conditions might PASK be stimulated to phosphorylate these substrates? Studies from yeast show that PSK1 is upregulated in response to H_2O_2 (Gasch et al., 2000). Furthermore, the promoters of PSK1 and PSK2 have conserved Stress Response Elements (STREs) have been shown to regulate the expression of other genes in response to oxidative stress (Ruis and Schuller, 1995). Thus, collective studies of PASK in mammals and yeast globally point to the sensing of nutrients and stress.

The HT screen used to identify chemicals capable of regulating PASK generated both activators and inhibitors of the enzyme. 10 confirmed chemical compounds were identified in the HTS to mildly activate the PASK enzyme. None of these compounds were selective, however, for the PAS domain. It is notable that three of the activators identified in the HT screen contained long alkyl chains. Might this suggest that an endogenous ligand for PASK, provided one exists,

possesses lipid-like properties? Perhaps, but even so, this would not be consistent with the hypothesis that the PAS domain is the ligand binding domain. These compounds, as well as the other activators, likely represent structure-stabilizing ligands that promote higher catalytic efficiency by stabilizing the active enzyme.

Most PASK inhibitors identified were generic kinase inhibitors which are most likely interfering with ATP binding or hydrolysis. However, one inhibitor, 2-(2-{2-[3-2-Hydroxyl-ethyl)-5-methoxy-2,3,7,8-tetrahydrofuro[3',2':3,4]benzo[1,2-d]thiazol-2-ylmethlene]-butylidene}-5-methoxyfuro[3',2':3,4]benzo[1,2-d]thiazol-3-yl)-ethanol, selectively inhibited PASK while not affecting Casein Kinase I, PKA or CaMK II. The latter, CaMK II is especially interesting as PASK is a member of the CaMK family. While this compound lacks sufficient potency to be used in cellular assays, it provides hope that further screening of related analogs would generate chemical compounds that can serve as useful biochemical tools. The results from these two HT screens collectively demonstrate novel insights into the regulation and biological role of mammalian PAS kinase.

CHAPTER FIVE
PASK and Other Observations

Abstract

PAS kinase represents the only mammalian serine/threonine kinase that is regulated by a PAS domain. Despite its demonstrated functions in controlling glycogen synthesis in yeast and glucose-responsive transcription in pancreatic β-cells, very little is known about its intracellular signaling pathways and modes of regulation. In budding yeast, PASK was observed to interact with scaffolding proteins 14-3-3 (Gavin et al., 2002). In efforts to characterize regulatory mechanisms for PASK in mammals, including 14-3-3 interactions, the following results were obtained. PASK bound to 14-3-3γ *in vitro* and *in vivo,* as demonstrated by glutathione *S*-transferase (GST)-14-3-3 fusion protein pull-down assays and co-immunoprecipitation. Phosphorylation of PASK by an upstream kinase was shown to be required for this interaction. GST-14-3-3 pull-down assays using either site-directed mutants in the PAS domain or in known phosphobinding residues of 14-3-3 demonstrated the requirements for binding. PASK bound to 14-3-3 basally, and the interaction was greatly abrogated when cells were treated with hydrogen peroxide. Furthermore, PASK was phosphorylated by MAP kinase kinase 1 *in vitro* and this phosphorylation event served two purposes. It was inhibitory for PASK catalytic activity and it generated a binding site for 14-3-3γ. Binding of 14-3-3γ to this site ameliorated the inhibition in a dose-dependent manner. Finally, to demonstrate physiological relevance, immunoprecipitation of PASK could effectively pull down endogenous MEKK1. These observations provide novel modes of regulation impinged upon PAS kinase, thereby suggesting a role for PASK in oxidative stress pathways.

Materials and Methods

Reagents

Recombinant MEKK1 was a generous gift from Dr. Melanie Cobb (UTSWMC). Wildtype, kinase-dead (K1028R), PAS A disruption mutant (LGY155-7EDI), PAS B disruption mutant (LGY369-71EDI), PAS A (I203F) and PAS A (C228F) PASK expression constructs in pcDNA3.1 V5/His (Invitrogen) were obtained from Dr. Jared Rutter (Univ. of Utah).

Cloning and Protein Expression

Human 14-3-3 isoforms beta, epsilon, gamma and zeta cDNA was amplified from a cDNA library from HBL-100 cells by PCR and cloned into the bacterial expression vector pGST-Parallel. All isoforms were expressed in *E. coli* Bl-21 (DE3) RIL (Stratagene) and purified as GST-fusions using glutathione sepharose (GE Healthcare) and further purified using MonoQ chromatography (GE Healthcare). The induction conditions were 0.2M IPTG/4 hrs/37ºC. Recombinant PASK was expressed and purified as previously described (Rutter et al., 2001).

Kinase Reactions

Kinase reactions for PASK and MEKK1 were identical to those previously described for PASK in Chapter 4 (Materials and Methods).

14-3-3 Pull-down Assays

50 µl of glutathione sepharose (GE) was washed 2X with 1ml lysis buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 20 mM NaF, 1:300 protease inhibitor cocktail (Sigma) and 1:100 phosphatase inhibitor cocktail (Sigma)). Beads were incubated with 4 µg of GST-14-3-3 in 500 µl of lysis buffer at 4°C for 1 hr. Beads were washed 2X with 1 ml lysis buffer and then incubated the appropriate cell lysate for 1 hr at 4ºC rocking. Again beads were washed 2X with

1 ml lysis buffer, eluted with 30 µl of 2X SDS-sample buffer, boiled for 5 min and 10 µl was loaded on 10% SDS-PAGE. Western blots were performed using primary antibodies: anti-PASK (U2501, 1:10,000); anti-GST (1:5,000); anti-14-3-3γ (Santa Cruz Biotech, 1:2,000). Cells were lysed upon addition of 1 ml of lysis buffer and pipetting up and down ~10 times.

PASK Stable Cell Lines and Cell Culture

Wildtype, kinase-dead (K1028R), PAS A disruption mutant (LGY155-7EDI), PAS B disruption mutant (LGY369-71EDI), PAS A (I203F), and PAS A (C228F) PASK stable expressing cell lines were generated by linearizing 2.5 µg of the constructs with Apa L1 and transfected via Lipofectamine 2000 (Invitrogen) into HEK 293 cells. Clonal populations that acquired resistance to G418 (Invitrogen) were selected, passaged and western blots performed to confirm the levels of PASK overexpression. Cells were maintained in DMEF 12:1, 10% FBS, P/S and G418.

Immunoprecipitations

Cells were lysed as described above in the 14-3-3 assays. Lysates were incubated with 1.5 µl of anti-Myc or anti-V5 antibody (Invitrogen) for 1 hr rocking at 4°C. 50 µl of prewashed (in lysis buffer) of protein G- agarose (Invitrogen) was added and again incubated for 1 hr, rocking at 4°C. Beads were subsequently washed 3X with 1 ml lysis buffer and resuspended in 50 µl 2X SDS-sample buffer. Boiled for 5 min and loaded an appropriate amount on SDS-PAGE and western blots performed using anti-Myc antibody (Invitrogen, 1:5,000) or anti-MEKK1 (Santa Cruz Biotech, 1:1,000). Secondary antibodies were HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (BioRad). Chemiluminescence substrate was Supersignal western pico (Pierce) and signal detected using autoradiographic film.

Introduction

PAS kinase (PASK) is a serine/threonine kinase that is unique in representing the only mammalian kinase with a PAS domain. The PAS domain serves as an autoinhibitory domain by binding in *cis* to the catalytic kinase domain (Rutter et al., 2001). The PAS domain also possesses a hydrophobic pocket capable of binding small organic compounds that weakly activate the catalytic activity of the kinase (Amezcua et al., 2002). It has been hypothesized that the PAS domain may act as a ligand-gated switch capable of regulating PASK in response to a distinct stimulus (Rutter et al., 2001) (Amezcua et al., 2002).

Yeast orthologs of PASK act to positively regulate translation and negatively regulate glycogen synthesis (Rutter et al., 2002). Mammalian PASK is ubiquitously expressed in all cell and tissue types. Recent studies give evidence that PASK may have a beta cell specific function in regulating the expression of the preproinsulin and pancreatic homeobox duedonum genes in response to high glucose (da Silva Xavier et al., 2004). PASK null mice have no overt phenotype and are indistinguishable from their wildtype littermates, providing little insight into the biological function of mammalian PASK (Katschinski et al., 2003).

In efforts to characterize regulatory pathways of PASK, it was noted that a yeast proteome screen characterizing protein-protein interactions demonstrated a binding event between Psk1p, one of the yeast PASK orthologs, and Bmh1/2p (Gavin et al., 2002). Bmh1/2p are 14-3-3 proteins which belong to a family of highly conserved, ubiquitously expressed proteins that generally serve as phosphoserine/threonine binding proteins (Yaffe, 2004). There are seven known 14-3-3 isoforms in mammals that typically form stable homo- and heterodimers. 14-3-3 dimers mediate the affects of many protein kinases and substrates through their ability to bind specific peptide motifs phosphorylated on serine or threonine residues

(Yaffe, 2002). Often 14-3-3 proteins function as allosteric regulators that stabilize their binding partner in a particular conformation thus, regulating activity, localization and binding interactions.

In the present study, 14-3-3 proteins were interrogated as allosteric binding partners of PASK. Here, it was discovered that a small pool of PASK in cultured cells is phosphorylated by an upstream kinase, under basal conditions, and this phosphorylation event is sufficient for specific interaction with 14-3-3γ. MAP kinase kinase 1 (MEKK1) was found to phosphorylate PASK *in vitro,* generating a 14-3-3γ binding site. Furthermore, phosphorylation of PASK by MEKK1 was found to be inhibitory for PASK autophosphorylation, and binding of 14-3-3γ to this site relieved PASK inhibition.

Results

Four of the seven 14-3-3 isoforms were cloned and expressed in *E. coli* as N-terminal GST fusion proteins. All four 14-3-3 isoforms (beta, gamma, epsilon and zeta) abundantly expressed soluble GST-fusion proteins. To assess whether PASK could directly interact with 14-3-3 proteins, GST-14-3-3 pull down assays were performed with total cell lysates generated from confluent HEK 293 cells stably transfected with human PASK. Western blots were then performed using an antibody to either GST or PASK (Materials and Methods). Out of the four 14-3-3 isoforms tested, only 14-3-3γ was able to efficiently pull down PAS kinase (Figure 5-1; data shown for beta and gamma isoforms only). Additionally, the western blot for PAS kinase demonstrated two distinct bands being pulled down by GST-14-3-3γ (Figure 5-1, P and NP). The lower band is also present in cell extracts incubated with beads alone suggesting that a small amount of PASK is nonspecifically binding the beads. The intensity of the upper band is much

stronger than the lower band and is specifically pulled down with GST-14-3-3γ. This upper band was hypothesized to be a super shifted, hyperphosphorylated (P) form of PAS kinase while the lower is a hypo- or nonphosphorylated (NP).

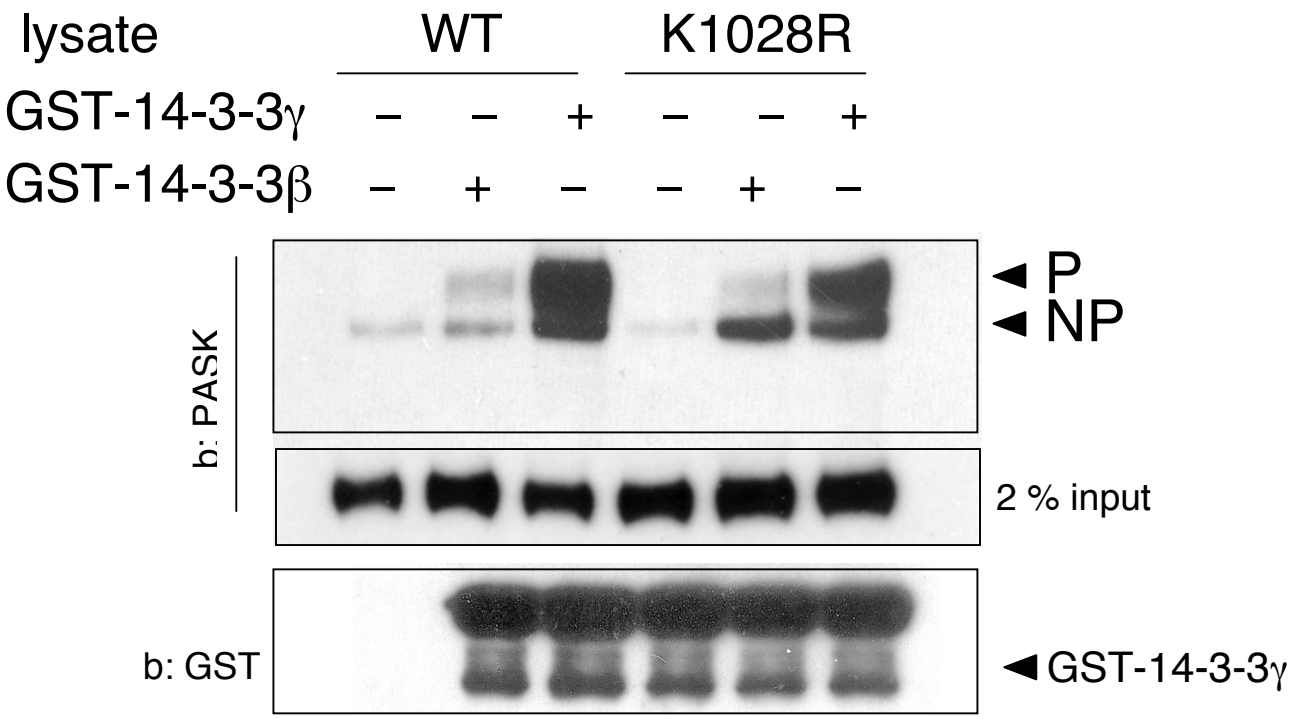


Figure 5-1 PASK specifically interacts with 14-3-3γ
GST-14-3-3γ and β were incubated with cellular lysates from cells overexpressing wildtype or kinase dead (K1028R) PASK for 1 hr. Bound complexes were pulled down with glutathione sepharose and western blots against PASK were performed. Glutathione beads incubated with lysates served as a negative control. P – Phosphorylated PASK. NP – Nonphosphorylated PASK.

Phosphorylation is required for the interaction between PASK and 14-3-3γ

It is well understood that 14-3-3 proteins generally require phosphorylation on serine and/or threonine residues in order to interact with target proteins (Yaffe, 2004). To investigate whether phosphorylation is required for PASK interaction, lysates from 293 cells, stably transfected with PASK, were treated with alkaline phosphatase for 1 hr at 37°C prior to

incubation with GST-14-3-3γ. Incubation with alkaline phosphatase completely eliminated the upper band suggesting that the upper band is a phosphorylated form of PASK and that 14-3-3γ specifically interacts with this phosphorylated form of PASK (data not shown).

PASK is phosphorylated in vivo by an upstream kinase

PASK possesses autophosphorylating activity *in vitro* that allows for the *trans* autophosphorylation of a threonine residue in the active site and two serine residues in the C-terminal tail (Rutter et al., 2001). Elimination of either or both of the two serine residues in the C-terminal tail did not affect catalytic activity (Rutter et al., 2001), and it is presumed that these phosphorylation events, if physiologically relevant, might provide a necessary docking domain for interaction with additional proteins. An antibody that recognizes phosphorylated PASK at one of the C-terminal sites did not recognize the phosphorylated state of PASK pulled down by 14-3-3γ, suggesting that this site is not responsible for 14-3-3 binding (data not shown). It was also serendipitously discovered that 14-3-3γ could effectively pull down a "kinase-dead" variant of PAS kinase, K1028R, at levels indistinguishable from wildtype PASK (Figure 5-1). This observation suggests that PASK can be phosphorylated *in vivo* by another kinase, and that this phosphorylation event is responsible for 14-3-3γ binding.

Domains responsible for the PASK/14-3-3γ interaction

To understand the domains and requirements responsible for interaction between PASK and 14-3-3γ, 14-3-3γ point mutants were generated in residues that have been shown to be critical for binding of the phosphoresidue (Yaffe, 2002). These three variants, K50L, R56L and R132L/Y138F, were expressed as described for wildtype GST-14-3-3γ and GST pull downs

were performed. 14-3-3γ variants K50L (lane 4) and R132/Y138F (lane 6) completely eliminated interaction and the ability to pull down PASK, while variant R56L (lane 5) was able to pull down PAS kinase in a manner analogous wild type (lane 3) 14-3-3γ (Figure 5-2).

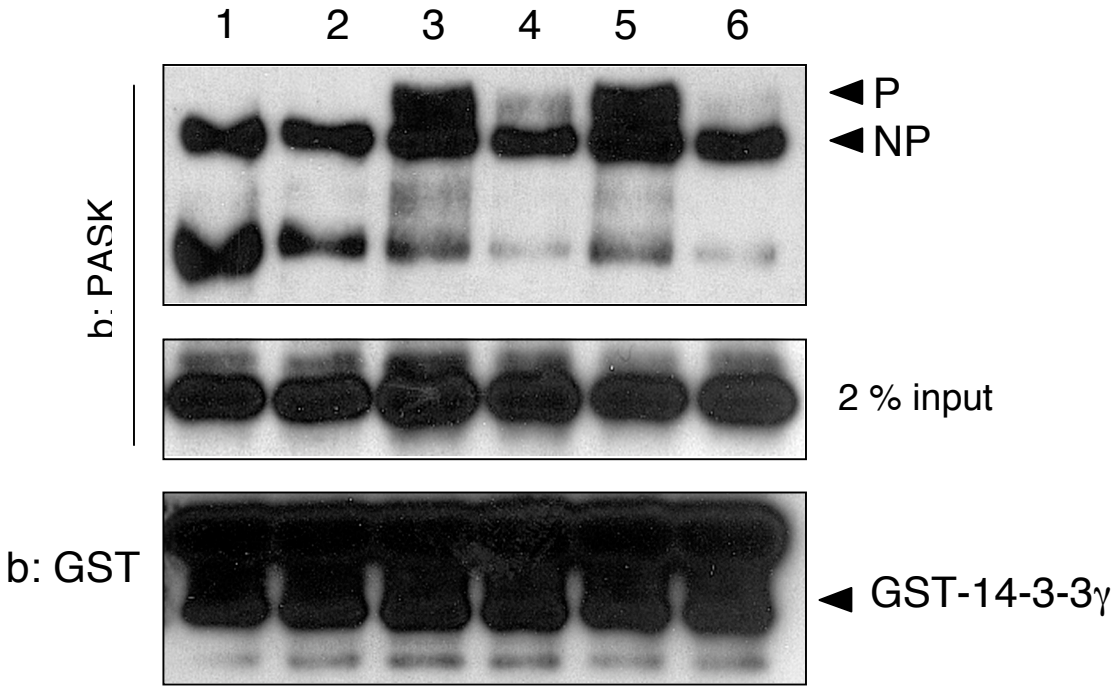


Figure 5-2 14-3-3 mutants inhibit interaction with phosphorylated PASK
GST-14-3-3γ pull down assays were performed using glutathione beads alone (lanes 1 and 2), wildtype 14-3-3γ (lane 3), 14-3-3γ K50L (lane 4), 14-3-3γ R56L (lane 5) and 14-3-3γ R132L/Y133F (lane 6) and soluble cell lysates from PASK stable 293 cell lines. Washed beads were subjected to SDS-PAGE and western blotting using anti-PASK (U2501) antibody or anti-GST antibody.

In order to determine whether either of the two PASK PAS domains might be necessary for the interaction of PASK with 14-3-3γ, stable cell lines were generated that expressed various PASK mutants (Materials and Methods). Two variants, I203F and C228F, are single amino acid substitutions within the PAS A domain and have been shown to release the inhibition that the PAS domain exerts on the catalytic domain of the enzyme (Amezcua et al., 2002). The other two

mutants are variants, containing three consecutive amino acid substitutions, in either the PAS A or PAS B domain. These triple mutants cause the PAS domains to unfold (unpublished observation – Gardner lab). PAS A mutants, I203F and C228F, interacted with 14-3-3γ indistinguishable from wildtype PASK (Figure 5-3, lanes 5-6). Conversely, the triple mutant variants of either PAS domain resulted in a lack of PASK being pulled down by 14-3-3γ (Figure 5-3, lanes 3-4). These observations indicate that both PAS A and B domains might be required for interaction with 14-3-3. Yet, other explanations may account for these observations. First, the PAS domains might structurally orient a distinct region of PASK responsible for 14-3-3γ interaction. Secondly, unfolding either PAS domain may induce conformational constraints, providing inaccessibility to interact with 14-3-3γ. Finally, the PAS domains might be required for the phosphorylation event preceding 14-3-3 binding.

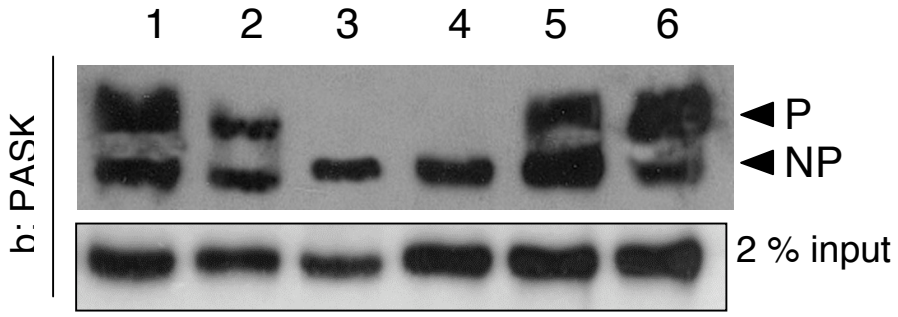


Figure 5-3 PAS domains of PASK are necessary for 14-3-3 binding
GST -14-3-3γ prebound to glutathione sepharose was incubated with cellular extracts from the following PASK stable cell lines: wildtype PASK (lane 1), K1028R kinase dead PASK (lane 2), PAS A unfolding mutant (lane 3), PAS B unfolding mutant (lane 4), PAS A I203F PASK (lane 5) and PAS A C228F PASK (lane 6). Pulled down PASK was detected by SDS-PAGE followed by western blotting with anti-PASK (U2501) antibody. P – Phosphorylated PASK; NP – Nonphosphorylated PASK.

PASK is dephosphorylated in response to H_2O_2

To identify cellular conditions that might alter the phosphorylation state of PAS kinase and/or its ability to bind 14-3-3γ, PASK stable cell lines were cultured under various conditions (growth factors and stress agents). Soluble lysates were then prepared for use in GST-14-3-3γ pull down assays. PASK stable cells treated with increasing concentrations of H_2O_2 for 30 min resulted in a dose-dependent decrease in PASK being pulled down by 14-3-3γ (Figure 5-4). This observation suggested that PASK was being either actively dephosphorylated, degraded or inhibited from interacting with 14-3-3γ in response to H_2O_2. To investigate whether PASK is actively dephosphorylated in response to H_2O_2, PASK-expressing cells were pretreated for 10 min with DMSO (control), 25 mM NaF (a serine/threonine phosphatase inhibitor), 200 μM Na_3VO_4 (a tyrosine phosphatase inhibitor), or 100 μg of catalase (H_2O_2 scavenger). Cells were subsequently treated for 30 min with 500 μM H_2O_2, lysed and the soluble lysate was utilized in GST-14-3-3γ pull down assays. As expected, cells pretreated with catalase completely blocked the ability of H_2O_2 to inhibit binding to 14-3-3γ (Figure 5-5). Similar results were obtained with cells pretreated with NaF, while those treated with Na_3VO_4 had no protective effective from the change induced by H_2O_2. These experiments demonstrate that the phosphorylation event on PASK, required for 14-3-3 binding, occurs on a serine/threonine residue and that PASK is actively dephosphorylated in response to oxidative stress.

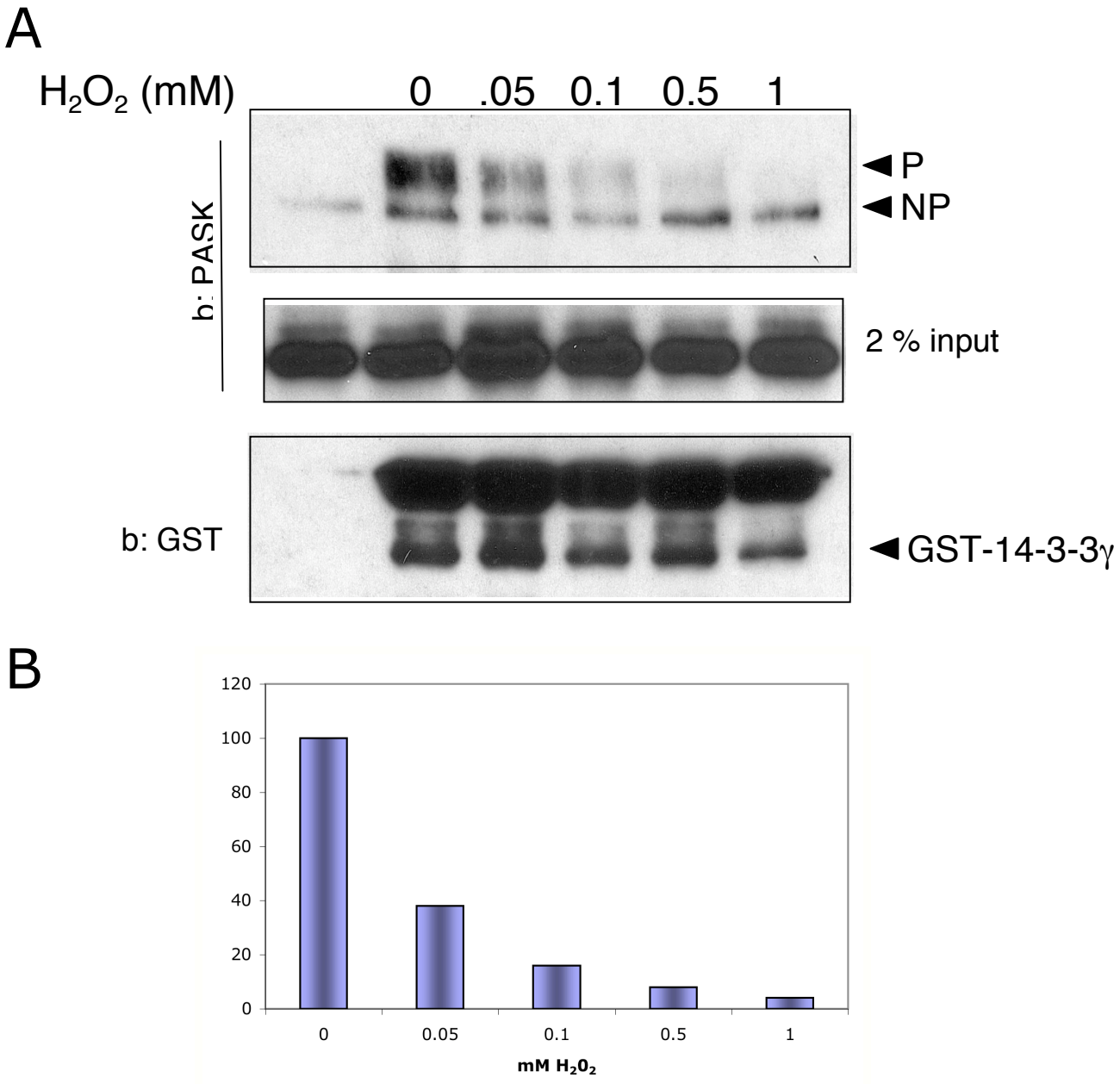


Figure 5-4 H_2O_2 leads to loss of PASK/14-3-3γ interaction
PASK stable 293 cells were treated with the indicated doses of H_2O_2 for 30 min, lysed and subjected to GST-14-3-3γ pull down assays as previously described in Figure 5-1. Western blots were performed using either anti-PASK antibody or anti-GST antibody. P – Phosphorylated PASK; NP- Non-phosphorylated PASK. Fold decrease in P-PASK being pulled down by 14-3-3γ in a dose-dependent manner upon treatment of cells H_2O_2 is demonstrated in (B). Intensity of bands from P-PASK on blot (A) was quantitatively determined using a densitometer.

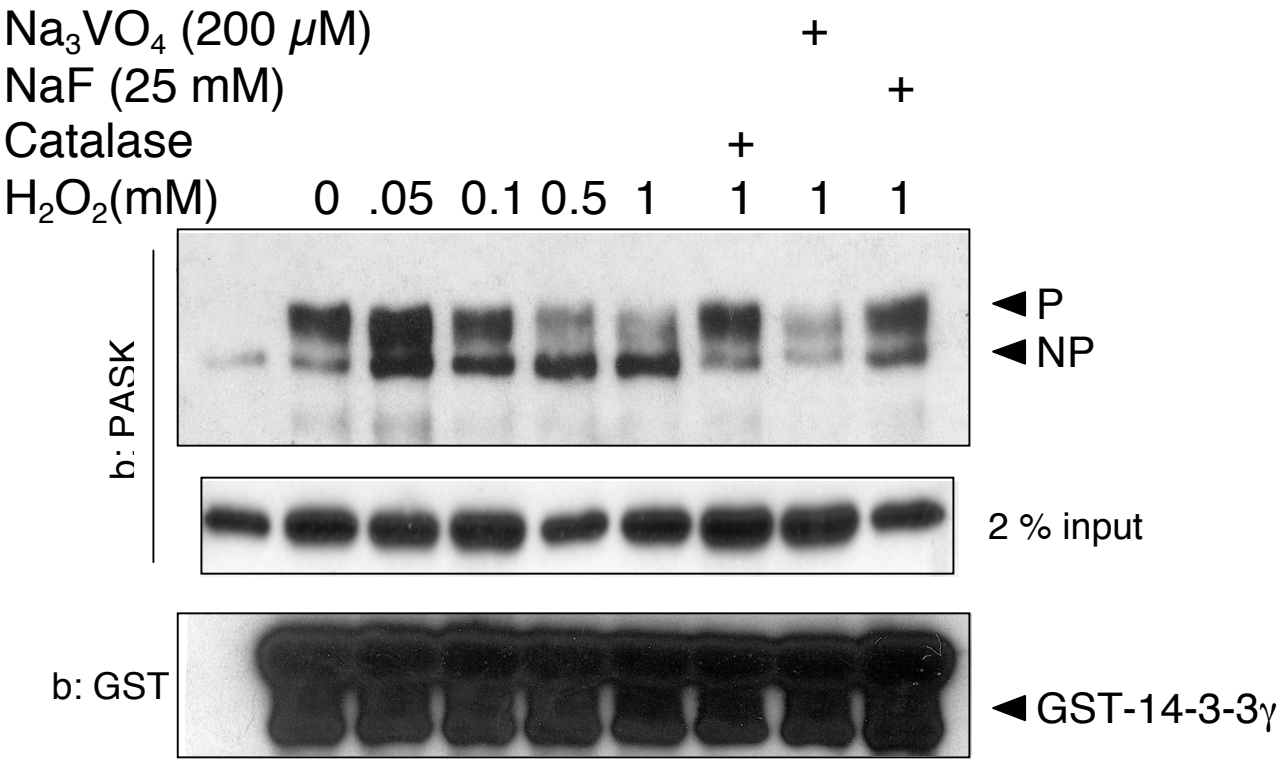


Figure 5-5 PASK is actively dephosphorylated in response to H_2O_2
PASK stable 293 cells were pretreated for 10 min with DMSO (negative control), 10 ug/ml catalase, 25 mM NaF or 200 μM Na_3VO_4 and subsequently treated with the indicated concentrations of H_2O_2 for 30 min. Cells were lysed and lysates subjected to 14-3-3γ pull down assays as previously described. Bound complexes were eluted in SDS-sample loading buffer, subjected to SDS-PAGE and western blots with anti-PASK and anti-GST antibodies.

PASK is phosphorylated by MAP kinase kinase 1 in vitro

Given that the interaction between PASK and 14-3-3γ is regulated by artificially induced oxidative stress and that peroxiredoxin, an antioxidant enzyme, was discovered as a PASK substrate (unpublished observation – BLP), it was hypothesized that the kinase responsible for PASK phosphorylation might also be regulated by oxidative stress. As such, mitogen activated protein kinase kinase 1 (MEKK1) was tested as a possible PAS kinase kinase. MEKK1 is a kinase in the initial stages of MAPK signaling cascades that influence cellular processes such as differentiation, proliferation and cell death. Furthermore, MEKK1 is inactivated by oxidation of Cys-1238 in the glycine rich loop known to be critical for ATP binding (Cross and Templeton, 2004). Oxidation of MEKK1 at Cys-1238 occurs at physiological concentrations when the cell encounters oxidative stress (Cross and Templeton, 2004). Using the "kinase-dead" variant of PASK as a substrate, to eliminate PASK autophosphorylation, MEKK1 was able to efficiently phosphorylate PASK but not casein kinase I, phosphoinositide-dependent kinase 1 or Akt (data not shown).

Phosphorylation of PASK by MEKK1 creates a 14-3-3γ binding motif

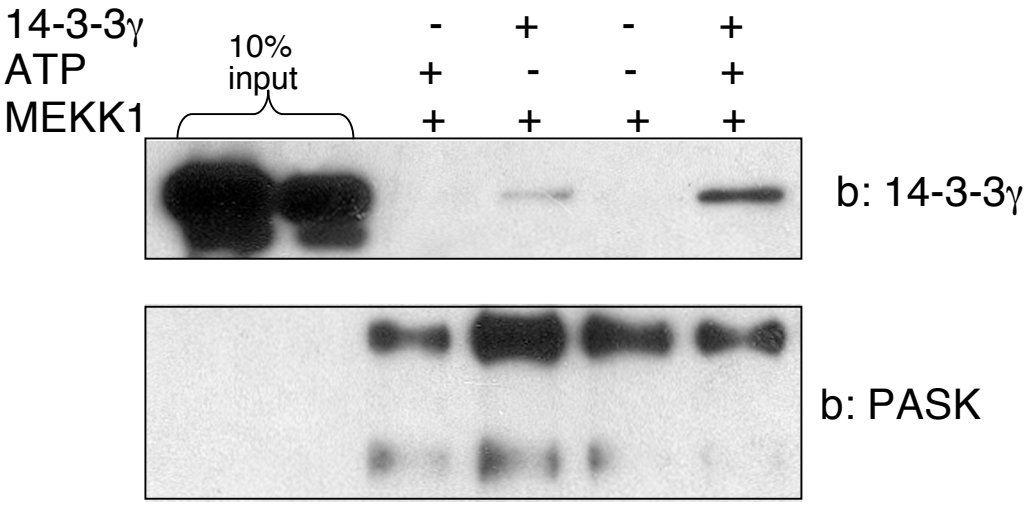
Next, it was imperative to determine whether the MEKK1-dependent phosphorylation of PASK was sufficient for 14-3-3γ binding. This experiment was performed with the aid of a C-terminal V5-tag on mammalian PASK in our stable transfected cell lines. First, PASK was immunoprecipitated from cell lysates using an anti-V5 antibody. After sufficient washing of the beads, the immunoprecipitated PASK was subjected to kinase reactions with recombinant MEKK1 in the presence or absence (negative control) of cold ATP. Following the kinase reaction, beads were washed to eliminate any MEKK1 and then incubated at 4°C with or without

14-3-3γ. Beads were washed again and western blots were performed using anti-14-3-3γ antibodies. PASK that was subjected to phosphorylation by MEKK1 was able to co-immunoprecipitate 14-3-3γ significantly more than PAS kinase treated with MEKK1 but no ATP (Figure 5-6A). This suggests that phosphorylation of PAS kinase by MEKK1 is creating a binding site for 14-3-3γ.

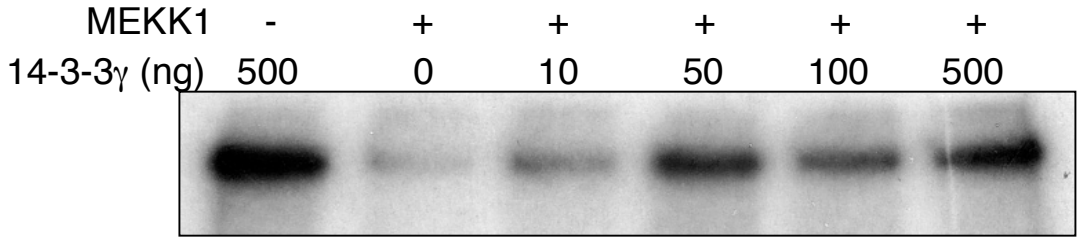
MEKK1-dependent phosphorylation of PASK is inhibitory and binding of 14-3-3γ to this site relieves the inhibition.

Binding of 14-3-3 proteins to other kinases have been reported to regulate the catalytic activity of the enzyme. To address whether the phosphorylation of PASK by MEKK1 and/or 14-3-3 binding had any affect on the catalytic activity of PASK the following experiment was performed. Immunoprecipitated PASK was again subjected to a kinase reaction in the presence of MEKK1 with or without cold ATP. After sufficient incubation, beads were washed and incubated with or without increasing concentrations of recombinant 14-3-3. Beads were washed again and immunoprecipitated PASK was subjected to a second kinase reaction in the presence of γ-^{32}P[ATP] to allow for PASK autophosphorylation. Reactions were then subjected to SDS-PAGE and autoradiography. Surprisingly, phosphorylation of PASK by MEKK1 completely inhibited PAS kinase catalytic activity (Figure 5-6B). Furthermore, titration of 14-3-3 relieved this inhibition in a dose-dependent manner (Figure 5-6 B and C). These results demonstrate that the MEKK1-dependent phosphorylation of PASK results in inhibition of PASK enzyme activity but also creates a binding site for 14-3-3γ. Binding of 14-3-3γ to this phosphorylation site ameliorates the inhibitory effect.

A


14-3-3γ
ATP
MEKK1
10%
input
- + - +
+ - - +
+ + + +
b: 14-3-3γ
b: PASK

B


MEKK1 - + + + + +
14-3-3γ (ng) 500 0 10 50 100 500

C

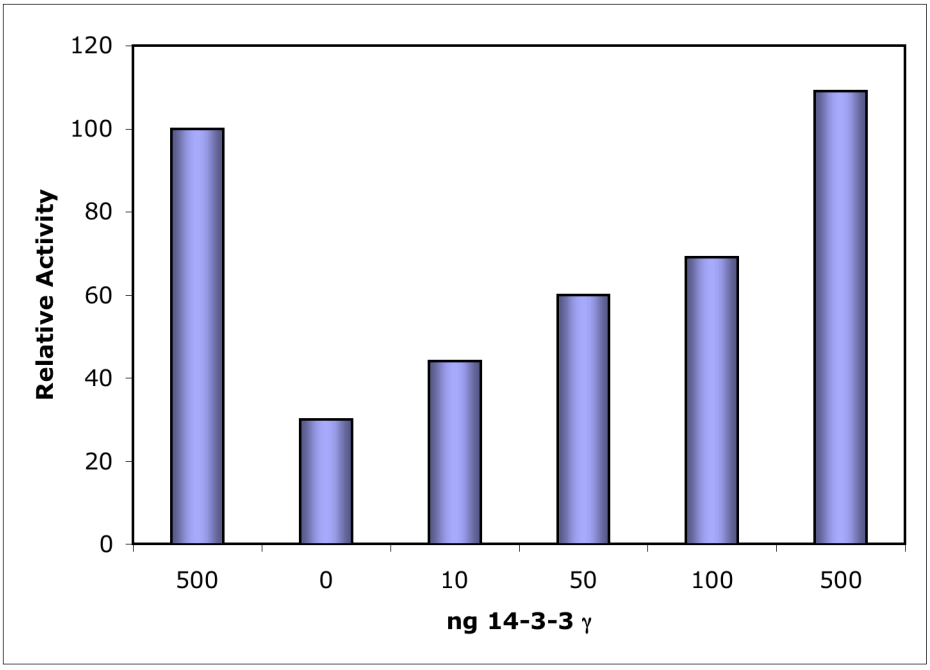

120
100
80
60
40
20
0
Relative Activity
500
0
10
50
100
500
ng 14-3-3 γ

Figure 5-6 MEKK1-dependent phosphorylation of PASK inhibits PASK catalytic activity and creates a binding domain for 14-3-3γ

PASK was immunoprecipitated from PASK overexpressing cells using an anti-V5 antibody and subjected to kinase assays in the presence of MEKK1 with or without cold ATP for 1 hr at RT. Immunobound PASK was subsequently washed, incubated with the indicated concentrations of 14-3-3γ at 4ºC for 1 hr and analyzed by SDS-PAGE and western blotting for GST (A) or subjected to kinase reactions containing [γ-^{32}P]ATP and then analyzed by SDS-PAGE and Coomassie Blue staining (B). Intensity of PASK autophosphorylation was quantitated using a densitometer (C).

Is MEKK1 the kinase responsible for phosphorylation of PAS kinase *in vivo*? To demonstrate a physiological association between PASK and MEKK1, co-immunoprecipitation experiments were performed. PAS kinase immunoprecipitated from stably transfected cells was able to efficiently co-immunoprecipitate endogenous MEKK1 whereas, MEKK1 was not co-immunoprecipitated with a LacZ control (Figure 5-7). The observation presented herein argues favorably that PASK and MEKK1 directly interact in cells.

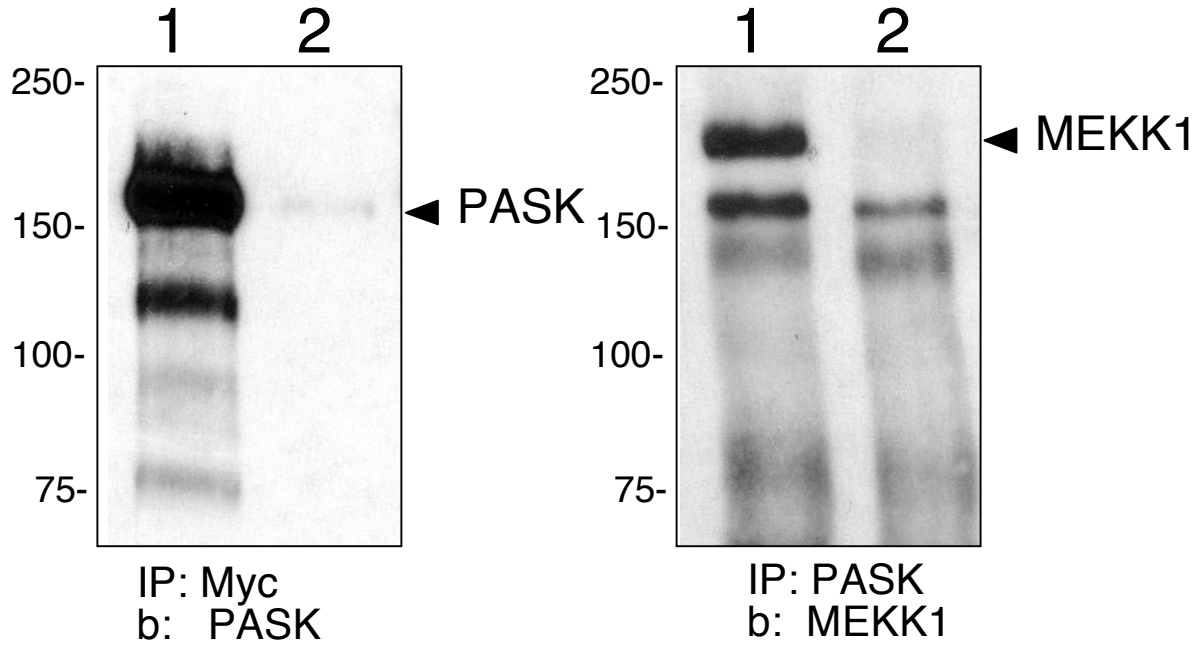


Figure 5-7 PASK Interacts with MEKK1 *in vivo*
PASK (lane 1) and LacZ (lane 2) were transiently transfected into 293 cells. 48 hr post-transfection cells were lysed, PASK and LacZ immunoprecipitated with an anti-myc antibody and analyzed by SDS-PAGE and western blot with anti-PASK antibody or anti-MEKK1 antibody.

Conclusions:

The major finding of this study is the identification of new cellular mechanisms for the regulation of PASK activity that includes phosphorylation by MEKK1 and specific interaction with 14-3-3γ. The observations reported herein demonstrate that PASK binds 14-3-3γ *in vitro* and *in vivo*. These data are consistent with previously reported observations describing an interaction between one of the yeast orthologs of PASK and 14-3-3 proteins. Despite the fact that only ~2% of the PASK in the cell was found to associate with 14-3-3γ, this same 2% appears to comprise a distinct pool of phosphorylated PASK. Why is such a small amount of PASK phosphorylated? First, PASK, albeit ubiquitous, is expressed at low levels in the cell. In efforts to study PASK function, stable cell lines overexpressing the enzyme were generated and subsequently used in this study. Thus, the overexpression of PASK, above endogenous levels, could effectively overwhelm endogenous regulatory mechanisms. Alternatively, the protein kinase responsible for *in vivo* phosphorylation of PASK may be rate-limiting, either in activity or expression levels, under basal conditions. Indeed MEKK1, which has been shown in this study to phosphorylate PASK, is reported to have low activity under resting conditions in 293 cells (Gallagher et al., 2002). Furthermore, the interaction between PASK and 14-3-3γ appears to be significant as most reports indicate that 14-3-3 isoforms are interchangeable with respect to protein binding (Fu et al., 2000), whereas the interaction with PASK was highly specific for 14-3-3γ. Consistent with this selectivity, CaMKK, a close family member of PASK, has been reported to preferentially associate with 14-3-3γ (Davare et al., 2004). Moreover, evidence presented herein suggests that the PAS domains might be important for the interaction between 14-3-3γ and PASK. Either of two triple amino acid substitution mutants, either in the PAS A or

PAS B domain, fully eliminated 14-3-3γ interactions. It is possible, though untested, that these triple mutants also impede MEKK1 phosphorylation of PASK.

Whereas no prototypic 14-3-3γ binding sites were discovered for PASK, binding of 14-3-3γ to PASK requires prior phosphorylation at an unknown serine/threonine residue. Autophosphorylation of PASK was not sufficient for the 14-3-3 interaction, demonstrating that PASK can be phosphorylated by an upstream kinase. This claim is substantiated by the fact that a "kinase-dead" version of PASK was pulled down by 14-3-3γ in cells at levels analogous to wildtype. Furthermore, this phosphorylation event appears to be regulated by cellular redox status as treatment of cells with hydrogen peroxide leads to active dephosphorylation of PASK.

MEKK1 is a likely candidate for the protein kinase responsible for *in vivo* PASK phosphorylation. PASK and MEKK1 interact *in vivo* by co-immunoprecipitation experiments. MEKK1 can phosphorylate PASK *in vitro* and this phosphorylation event is inhibitory for the catalytic activity of PASK. The MEKK1-dependent phosphorylation of PASK is interesting as the only known substrates of MEKK1 are a handful of MAPKs that are phosphorylated at both a tyrosine and threonine residue in their active sites. The site of MEKK1-dependent phosphorylation is not within the active site of PASK (data not shown). Where this phosphorylation event occurs and how it inhibits the catalytic activity of PASK remains an exciting avenue of future research. Furthermore, MEKK1-dependent phosphorylation of PASK is sufficient for the creation of a suitable binding site for 14-3-3γ.

Various groups have proposed roles for MEKK1 in the ERK1/2, JNK and p38 MAPK pathways, including those responsive to oxidative stress (Anselmo and Cobb, 2004). Recently, MEKK1 was demonstrated to be oxidized or glutathionylated in response to oxidative stress (Cross and Templeton, 2004). Glutathionylation of MEKK1 results in reversible loss of catalytic

activity. Thus, a shift in the redox balance of the cell in favor of oxidation can provide a model for MEKK1 inactivation with the concomitant activation of PASK. This model could speculatively explain a previously reported role of PASK in the pancreatic beta cell. Da Silva Xavier and Rutter have reported that PASK activity is increased in response to elevated levels of glucose in the pancreatic β-cell (da Silva Xavier et al., 2004). High glucose levels have been associated with increased mitochondrial respiration and production of reactive oxygen species (ROS) (Evans et al., 2002; Singh et al., 2004). It is possible that the increase in PASK activity observed by elevated glucose concentrations is due to the glutathionlyation/inactivation of MEKK1 and the concomitant dephosphorylation/activation of PASK. Moreover, the regulation of the preproinsulin gene by PASK in response to high glucose concentrations in the β-cell may infer that altered PASK activity can contribute to some forms of type 2 diabetes. Indeed, a majority of the pathophysiology of diabetes stems from increased mitochondrial dysfunction and reactive oxygen species (Rhodes, 2000) (Droge, 2002). Whilst unclear as to whether ROS is a causative agent, or simply a secondary effect of diabetes, the observations reported herein provide new avenues of future research. Understanding these novel modes of regulation for PASK, in both, oxidative stress pathways coupled with glucose metabolism, will provide greater insight into PASK function and its possible role in diabetes.

CHAPTER SIX
Conclusions and Future Directions

This study provides evidence for the regulation and biological function of PAS kinase in both yeast and mammalian systems. In yeast, we have identified five *bona fide* PASK substrates that regulate two fundamental, interconnected processes: glycogen synthesis and translation. Although we present genetic evidence that supports a role of PASK in positively regulating protein synthesis, mechanistically how PASK-dependent phosphorylation of these translation factors affects their activity, or under what conditions they become phosphorylated remain questions for future studies. Further support for a role of the PASK enzyme in the regulation of protein synthesis stems from an unbiased, high-throughput (HT) biochemical screen for mammalian PASK substrates. Several mammalian ribosomal proteins were phosphorylated by PAS kinase within the context of the intact 40S ribosomal complex. It is also notable that our blind screen identified ribosomal protein S3A and alanyl-tRNA synthetase as apparent PAS kinase substrates. Unfortunately, the functional roles of PASK-dependent phosphorylation of these substrates have again eluded us. As discussed in chapter 5, specific rather than global translation events are likely to be regulated by PASK in response to a defined nutrient or redox state of the cell. Future studies, such as, comparative analysis of the translation of specific mRNAs between cells under varied stress or nutrient conditions, will be needed to validate this claim.

The hypothesis that PASK might regulate the translation of specific mRNAs is particularly appealing with regard to the level and location of PASK expression in the testes. PASK expression in the testes is approximately 90-fold higher than the next highest expressing

tissue, thymus, and is confined to distinct developmental stages of the spermatocyte (Katschinski et al., 2003). Spermatogenesis is a unique and complex process of cellular differentiation. The extensive differentiation of germ cells into mature gametes requires ongoing protein synthesis and the selective translation of mRNAs encoding polypeptides required for distinct differentiation steps (Herbert and Hecht, 1999). A number of mRNAs have been reported to be synthesized early in spermatogenesis, and stored in the cytoplasm as translationally silent ribonucleoprotein complexes (Gold and Hecht, 1981; Gold et al., 1983). At the appropriate stage of spermatogenesis, these translationally silent mRNAs become translationally competent (Herbert and Hecht, 1999). A similar situation is observed during oogenesis in *Xenopus*. A fraction of RNAs are synthesized early in development and sequestered as ribonucleoprotein complexes. The temporal activation of these silent RNAs guarantees proper differentiation from oocyte to egg (Herbert and Hecht, 1999).

What proteins govern these translationally silent ribonculeoprotein complexes? The identity of polypeptides responsible for translational silencing or "masking" in mammals is poorly understood. Several lines of evidence suggest that two Y-box transcription factors, FRGY2 and mRNP3, are involved in translational masking in *Xenopus* (Deschamps et al., 1992; Murray et al., 1991; Murray et al., 1992; Tafuri and Wolffe, 1990). These proteins inhibit translation of bound RNAs both *in vitro* and *in vivo*. Other *Xenopus* proteins have been reported to associate with these complexes including Xp54, a DEAD-box helicase, believed to assist in unwinding RNA secondary structure, and influencing translation initiation (Ladomery et al., 1997; Meric et al., 1997). Interestingly, homologs of Xp54 include the *S. cerevisiae* helicases DED1 and DBP1 and the human DBY helicase. Mutations in the human DEAD-box helicase, DBY, are a frequent cause of male infertility (Van Landuyt et al., 2001). The *S. cerevisiae*

helicases DED1 and DBP1 are DEAD-box RNA helicases, required for translation initiation of yeast mRNAs (Berthelot et al., 2004; Iost et al., 1999), and qualified as high copy suppressors of the growth deficiency of a strain lacking both PASK genes (Rutter et al., 2002). Furthermore, phosphorylation is considered to be a major player in the assembly and disassembly of ribonucleoprotein complexes (Dearsly et al., 1985; Kick et al., 1987; Murray et al., 1991).

It is well documented that the energy requirements of the spermatocyte change as a consequence of maturation and differentiation (Bajpai et al., 1998). In early spermatogenesis, germ cells rely almost exclusively on lactate as the preferred energy substrate (Grootegoed et al., 1984b). Sertoli cells are responsible for providing the maturing germ cells with this substrate. Glucose on the other hand is a poor energy substrate for early spermatogenic cells (Grootegoed et al., 1984a). As germ cells mature from spermatogonia to spermatocytes, to spermatids and then spermatozoa, they gradually switch preferred energy substrates from lactate to glucose (Bajpai et al., 1998; Risley, 1990). The inability to use glucose in early spermatogenesis is universal among diverse species, and is a result of ineffective glycolytic pathways (Nakamura et al., 1984). When the activity of each glycolytic enzyme was measured in rat spermatocytes, it was reported that GAPDH and pyruvate kinase had the lowest activity (Nakamura et al., 1984). GAPDH and pyruvate kinase were both identified as *in vitro* substrates for PASK in our biochemical screen. It is possible that PASK might regulate the activity of these glycolytic enzymes in early spermatogenesis. Furthermore, the translational regulation of specific mRNAs during spermatogenesis might be regulated by PASK as a function of the changing metabolic state of the maturing germ cell. Perhaps some of the PASK substrates identified in our screen, including ribosomal proteins, might serve as translational regulators in the spermatocyte under the control of PASK-dependent phosphorylation.

As previously mentioned, translational regulation through ribosomal protein S6 and elongation factor 4E are directly coupled to nutrient, stress and/or hormonal signals. PAS kinase may function in an analogous manner. Our data, as well as that of others, suggest that PASK may qualify as an important metabolic sensor under nutrient-sensitive conditions (da Silva Xavier et al., 2004; Rutter et al., 2002). In yeast, elimination of the PASK genes resulted in a distinguishable phenotype only after cells were required to utilize galactose as a sole carbon source, at elevated temperatures (Rutter et al., 2002). Additionally, the other major group of PASK substrates identified both in yeast and mammals were metabolic enzymes. In yeast, we demonstrate that PASK phosphorylates and negatively regulates UDP-glucose pyrophosphorylase and glycogen synthase, the last two enzymes in the synthesis of glycogen (Rutter et al., 2002). Phosphorylation of glycogen synthase directly inhibits catalytic activity, whereas phosphorylation of Ugp1p alters its oligomeric state, allowing the UDP-glucose to be channeled for use in structural rather storage carbohydrates. Ongoing studies are focused on an understanding of the biochemical composition of the Ugp1p complexes and how PASK-mediated phosphorylation might change the complex.

We have also found that glycogen synthase in mammals is a physiological substrate for PASK, and that phosphorylation negatively regulates the catalytic activity of the enzyme through phosphorylation at Ser-640 (site 3a). Ser-640 is one of nine phospho-regulatory sites on glycogen synthase, yet is considered the main regulatory site in the C-terminus of the enzyme (Roach, 1990; Skurat et al., 2000; Skurat and Roach, 2000). Phosphorylation at these sites are catalyzed by several kinases including phosphorylase kinase, casein kinase I and GSK-3. *In vitro* GSK-3 can sequentially phosphorylate sites 4, 3c, 3b and 3a on glycogen synthase (DePaoli-Roach et al., 1983; Picton et al., 1982; Zhang et al., 1993), and for many years has been

heralded as “the glycogen synthase kinase”, but recent evidence challenges this assertion. Prior phosphorylation of site 5, on glycogen synthase by casein kinase, is required before GSK-3 can catalyze the phosphorylation of site 4, 3c, 3b and 3a (Picton et al., 1982). Recent reports demonstrate that a glycogen synthase variant lacking the GSK-3 recognition sequence, expressed in either COS cells or Rat-1 fibroblasts, did not prevent glycogen synthase phosphorylation at site 3a (Ser-640) and/or inactivation of the enzyme (Skurat et al., 2000; Skurat et al., 1994). These experiments provide evidence that GSK-3 may not be the relevant *in vivo* glycogen synthase kinase.

Additionally, in the first genome scan for Type II diabetes (T2D), a region on chromosome 2q37.3 showed the strongest suggestive linkage (Hanis et al., 1996). This region encompassed three candidate genes. Analysis of intragenic and intergenic SNPs caused investigators to taut that Calpain-10 was most likely the candidate for the disease susceptibility locus (Horikawa et al., 2000). In the original report, an intronic SNP, designated as UCSNP-43, was attributed to account for the associate risk of T2D in Mexican Americans (Horikawa et al., 2000). No experimental studies have provided conclusive evidence that UCSNP-43, or other implicated SNPs, affect splicing, expression levels, or protein function. Now a decade later, more than 20 genome scans among various ethnic groups, have been performed to extensively study the molecular genetics of Type II diabetes (Cassell and Hitman, 2003). The result: some studies have confirmed and others have refuted the original data, thus continuing widespread controversy as to whether calpain-10 is indeed a susceptibility gene for T2D. Recent efforts have focused on how calpain-10 can contribute to the pathogenesis of T2D. Some functional studies of calpain-10 suggest a role in insulin-stimulated GLUT4 translocation and glucose uptake (Otani et al., 2004; Sreenan et al., 2001). However, careful observers note, “...given the

notoriously promiscuous nature of the calpain family any experiments performed outside of the tightly controlled intracellular physiological environment must be viewed with extreme caution" (Turner et al., 2005). No direct physical proof has demonstrated a role for calpain-10 as a causative or predisposition factor in T2D. What relevance is this to PASK? The PASK gene is also located at 2q37.3, adjacent to calpain-10. Emerging evidence for the role of PASK in the regulation of glycogen synthase, and glucose induced expression of the preproinsulin gene in pancreatic β-cells (da Silva Xavier et al., 2004), justifies further investigation as to whether PAS kinase, and not calpain-10, might be a disease susceptibility gene for Type II diabetes.

Other metabolic, mammalian PASK substrates identified to date include glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase, peroxiredoxin 6 and uridine-cytidine kinase 2. Exhaustive studies examining the effect of PASK-dependent phosphorylation on the functional properties of these enzymes have, disappointingly yielded very little. It is possible that phosphorylation regulates subcellular localization or other regulatory phenomena not modeled in test tube reactions.

Perhaps the most intriguing aspect of this study is the understanding, or lack thereof, of the complexity that exists in the regulation of the PAS kinase enzyme. The defining feature of PAS kinase is its two, N-terminal PAS domains. Biochemical and structural studies have previously demonstrated that the PAS A domain can directly bind and inhibit the catalytic domain of the kinase (Amezcua et al., 2002; Rutter et al., 2001). Furthermore, an NMR-based screen of organic compounds showed that the PAS A domain can specifically bind small molecules within its hydrophobic core (Amezcua et al., 2002). This is consistent with many prior studies showing that PAS domains serve as small modular domains capable of sensing environmental signals. Might PASK directly bind and sense an endogenous ligand?

Experimental evidence suggests a ligand exists, but so far its identity remains a mystery. We have performed a HTS with a library of over 200,000 small molecules designed to identify potential activators and inhibitors of the enzyme. Unfortunately none of the activators identified in the HTS proved to be PAS-dependent. These chemical activators likely represent structure-stabilizing compounds that provide additional stability of the "on state". Additional screening of chemical compounds or organic extracts of cells or tissues for PAS-dependent activators may provide additional clues. Our chemical HT screen also identified a fairly specific inhibitor of the PASK enzyme. This compound inhibited PASK with an apparent IC_{50} of 200 nM, while little inhibition was observed against PKA, CKIε, and CaMKII. Further chemical synthesis of related compounds may provide added specificity and potency for the use in biochemical and cell-based assays.

The versatility of PAS domains allows them to accommodate small molecules in their hydrophobic cores and also serve as protein-protein interaction domains. As discussed, the PAS domain can directly bind to the catalytic domain of PASK (Amezcua et al., 2002). In Chapter 5, we propose that PAS domains are important for MEKK1-dependent phosphorylation of PASK. In Appendix A, we show that the PAS A domain of PASK is inhibitory towards binding to both glycogen synthase and UCK2. If the PAS domain is either deleted or disrupted, PASK associates more stably with glycogen synthase or UCK2. As discussed, how the PAS domain either directly or indirectly affects these associations is not known. Does the PAS domain bind substrate and its own kinase domain competitively? Does the PAS domain mask the putative substrate-docking domain described in Appendix A? There exist numerous possibilities, and only a careful in-depth analysis of these interactions will provide the answers to these questions.

In the process of identifying PASK substrates we observed several key regulatory events that determine substrate specificity by PAS kinase. First, by mapping the phosphorylation sites of substrates phosphorylated by PAS kinase we discovered a primary consensus motif, R-X-A/x-*S/*T. In all cases, an arginine residue was required in the -3 position and 5 of the 9 phosphorylation sites showed a preference for alanine at the -1 position. As previously mentioned, primary sequence alone is not sufficient to confer substrate specificity for most protein kinases, including PASK, for several reasons. First, many protein kinases have been observed to phosphorylate R-X-X-S/T motifs (Kennelly and Krebs, 1991; Pearson and Kemp, 1991). Second, our biochemical screen for PASK substrates entailed the incubation of thousands of polypeptides, bearing the motif R-X-X-S/T, with active, recombinant PASK, yet only a handful of polypeptides were observed to be phosphorylated in a PASK-dependent manner. Third, we observed that the efficiency of substrate phosphorylation by PASK varied between the full length and kinase domain only enzymes. In many studies, the kinase domain only enzyme was used because it lacked the inhibitory effect of the PAS domains, and hence proved more active against peptide substrates, histones and myelin basic protein. Interestingly, we found substrates such as peroxiredoxin 6, uridine-cytidine kinase 2 and glycogen synthase were better substrates for the full length enzyme than the kinase domain alone enzyme. In Appendix A, we demonstrate that a region between the PAS domains and kinase domain is important for efficient phosphorylation of UCK2 and glycogen synthase. Further studies are required to pinpoint the exact sequence(s) or structural elements that compose this putative substrate-docking domain. A further layer of regulation in the association between PASK and glycogen synthase was uncovered in the course of our studies. Glycogen directly, negatively regulates the PASK-glycogen synthase interaction but has no affect on the PASK-UCK2 interaction. Where and how

glycogen polymers exert their effect on the PASK-glycogen synthase interaction in a substrate-dependent manner remain questions for future studies. These studies demonstrate a unique characteristic of substrate specificity for the PASK enzyme. While many protein kinases require scaffolding proteins, substrate docking-associated proteins, or other extrinsic factors in order to interact with substrates, PASK appears to have many of these features "in-house". Only future studies will determine whether PASK might also rely on additional proteins for further specificity.

Activation of PASK can also occur through *trans* autophosphorylation of a key threonine residue in its active site *in vitro* (Rutter et al., 2001). Conversely, evidence is presented that PASK is phosphorylated *in vivo* by an upstream kinase. MEKK1 can physically interact with PASK *in vivo* and phosphorylate PASK on at least one serine or threonine residue that corresponds to a loss of PASK catalytic activity. Even more surprising, we found that the phospho-dependent inhibitory state of PASK could be overcome in cells via two distinct pathways. First, treatment of cells with hydrogen peroxide led to an active dephosphorylation of PASK in a dose-dependent manner that could be blocked by pretreating cells with either a serine/threonine phosphatase inhibitor or catalase. Second, we discovered that PASK could specifically associate with 14-3-3γ both *in vivo* and *in vitro* in a phospho-dependent manner. The 14-3-3 phospho-binding site on PASK could be generated by MEKK1 and binding of 14-3-3γ to this site relieves the inhibition exerted by the phosphorylation event.

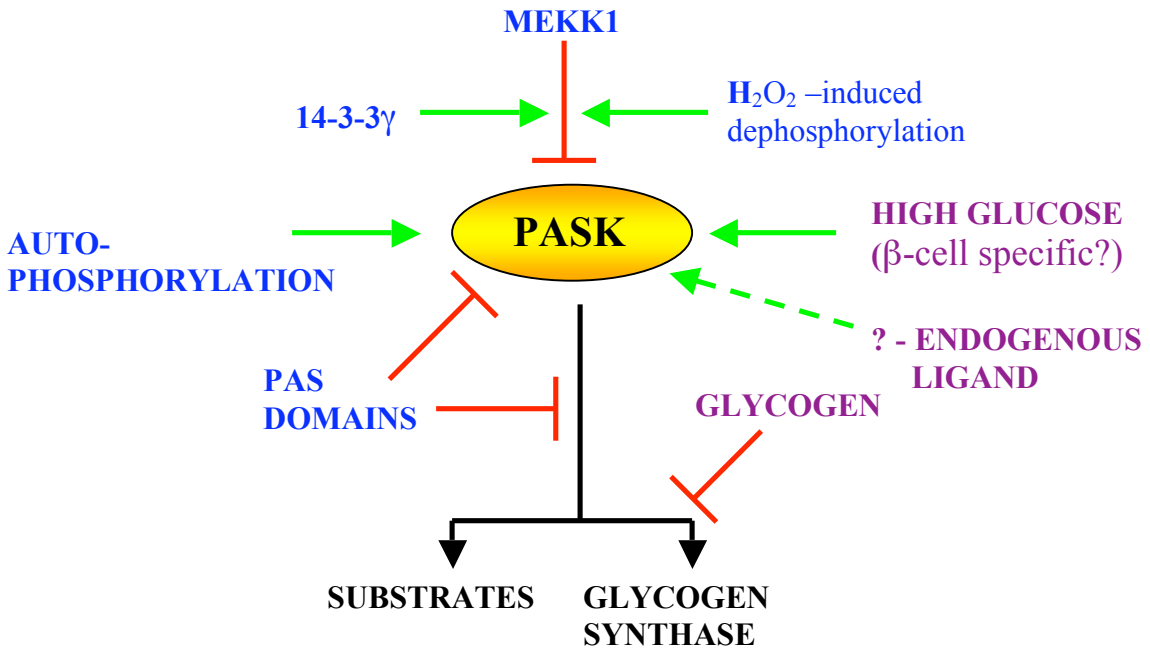


Figure 6-1 PASK inputs and regulation
PASK activation events are emphasized in green, while inhibitory events are in red. Black lines indicate direct phosphorylation of substrates. Direct interactions with PASK are shown in blue while indirect or unknown mechanisms are displayed in purple. Dashed lines indicate potential interactions.

The *in vivo* relevance of the 14-3-3 interaction with PASK can be substantiated by the following observations. First, yeast 14-3-3's and one of the yeast PASK orthologs were observed to associate in co-immunoprecipitation experiments *in vivo* (Gavin et al., 2002). Second, elimination of both 14-3-3 genes in yeast resulted in a hyperaccumulation of glycogen (Wilson et al., 2002), a phenotype remarkably similar to a strain lacking both PASK genes (Rutter et al., 2002). While 14-3-3's play a broad regulatory role in the cell, of which many factors might contribute to this phenotype, the correlation is striking, and prompts further investigation. Third, it is also notable that comparative 2-D gel analysis of proteins from brain

tissue, of wildtype and 14-3-3γ knockout mice, demonstrated a differential expression of only six polypeptides (Steinacker et al., 2005). One such protein was peroxiredoxin 6, an antioxidant enzyme, identified as an *in vitro* substrate for PASK in our biochemical screen. The mobility shift of peroxiredoxin 6 in the 14-3-3γ knockout mice compared to analogous tissue from wildtype mice was attributed to overoxidation of the peroxiredoxin 6 enzyme (Steinacker et al., 2005). What does this mean? We don't fully comprehend the significance of these correlations but it does provide credibility that studies reported herein are meaningful, and give greater insight into PASK function.

The current known modes of PASK regulation are summarized in Figure 6-1. Future studies will need to address how these regulatory processes work separately and/or concertedly to provide specificity to discrete phosphorylation events. The apparent complexity of regulation that is built into the PASK pathway is reminiscent of that seen for other nutrient sensitive kinases such as TOR and AMPK (Lindsley and Rutter, 2004). Decisions concerning growth, proliferation, development, and the corresponding specific functional responses must take into account the prevailing redox and nutrient state. The data presented in this dissertation provides evidence that PASK might coordinately regulate protein synthesis and metabolism, thereby reflecting the energy requirements of the cell.

APPENDIX A
Control of Mammalian Glycogen Synthase by PASK

Introduction

Glycogen is considered the principal storage form of glucose, and acts as a reserve supply of carbon and energy in most organisms. It consists of a large branched polymer of glucose and is stored in several locations in mammals, most importantly muscle and liver. These stores of glycogen are critical in helping mammals maintain a buffered blood glucose level. Glycogen synthesis, catalyzed by glycogen synthase, is highly regulated both allosterically by glucose 6-phosphate as well as covalent modification at multiple phosphorylation sites (Friedman and Larner, 1963; Larner, 1990; Roach, 1990). These phosphorylation events are primarily localized to two specific regions of the polypeptide, located in the N- and C-termini. Phosphorylation of these sites are known to be catalyzed by a number of different kinases including casein kinase I, phosphorylase kinase, glycogen synthase kinase 3 and more recently DYRK1A (dual specificity tyrosine phosphorylated and regulated kinase). Phosphorylase kinase and casein kinase I are known to phosphorylate the N-terminal sites Ser-7 (site 2) and Ser-10 (site 2a) respectively (Flotow and Roach, 1989; Nakielny et al., 1991). GSK-3 phosphorylates multiple sites in the C-terminus including the main regulatory site Ser-640 (Fiol et al., 1987; Picton et al., 1982). As previously mentioned, GSK-3 requires a priming event, thought to be initiated by casein kinase I, that allows for the generation of a GSK-3 consensus site. Skurat and colleagues have recently shown that DYRK1A is capable of phosphorylating Ser-640 both *in vitro* and *in vivo* (Skurat and Dietrich, 2004).

We have previously shown that glycogen synthesis in yeast is controlled in part by at least one of the PSK orthologs (Rutter et al., 2002). Deletion of both PSK1 PSK2 genes results

in a greater than 4-fold increase in glycogen accumulation compared to wildtype (Rutter et al., 2002). As described in chapter 3, we demonstrated that glycogen synthase and Ugp1p (UDP-glucose pyrophosphorylase) are both physiological targets of yeast PSK. Furthermore, genetic and biochemical data demonstrate that these phosphorylation events are important for negatively regulating the activities of these two enzymes (Rutter et al., 2002).

Here we show that mammalian glycogen synthase is also phosphorylated by PASK and that this phosphorylation event is inhibitory. Furthermore, the interaction between PASK and glycogen synthase is controlled by glycogen, the PASK PAS domain and a unique positive substrate-docking domain.

Summary of Results

Glycogen synthase was purified from rabbit muscle in both dephosphorylated (I-form) and phosphorylated (D-form) forms (DePaoli-Roach et al., 2003). As mentioned in the introduction, glycogen synthase can be activated allosterically by glucose 6-phosphate levels (Friedman and Larner, 1963). This allosteric regulation can completely activate glycogen synthase independent of its phosphorylation state. It does not, however, increase basal glycogen synthase activity in the unphosphorylated state (Larner, 1990).

PASK was able to phosphorylate the I-form of glycogen synthase but not the D-form suggesting that the relevant PASK phosphorylation sites were the same as physiological regulatory sites (Figure A-1A). Furthermore, phosphorylation of glycogen synthase by PASK completely inhibited its activity, similar to that seen for yeast Psk2p (Figure A1-B). Tryptic

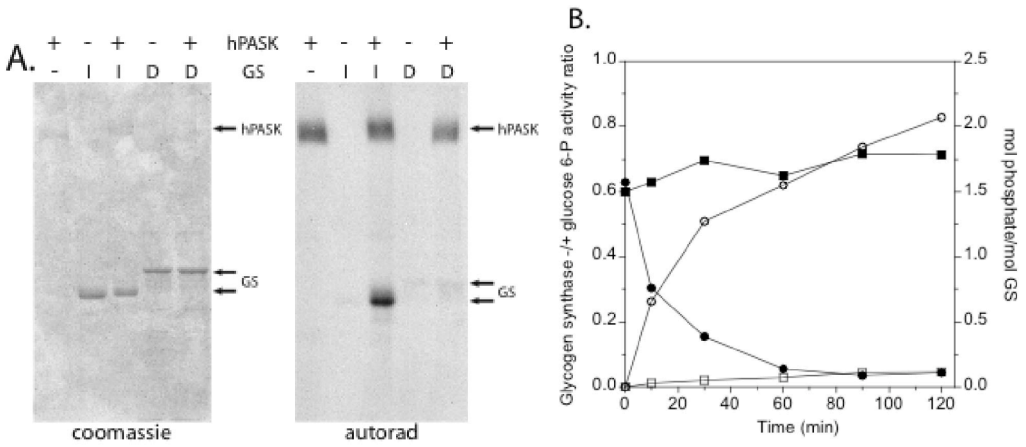


Figure A-1 PASK phosphorylates mammalian glycogen synthase
(A) Purified glycogen synthase (I and D forms) were purified from rabbit muscle and incubated with [γ-^{32}P]ATP and either PASK or buffer. Reactions subjected to SDS-PAGE, coomassie staining and autoradiography. (B) I-form of glycogen synthase subjected to phosphorylation by PASK (circles) or buffer (squares) in the presence of ATP. At indicated times, samples were subjected to SDS-PAGE for phosphorylation stoichiometry (open symbols) and glycogen synthase activity assay (black symbols). Figure from J. Rutter – submitted to PNAS.

mass fingerprinting and mutagenesis identified the relevant PASK phosphorylation site as Ser-640.

To investigate more fully the direct interaction of PASK with glycogen synthase, the following PASK deletion mutants were constructed: Δ955 (lacks residues 1-955; only the kinase domain is left) and Δ444 (lacks residues 1-444; removes the PAS domain but leaves the remaining N-terminus plus the kinase domain) (Figure A-2). The PAS A domain has inhibitory activity toward the kinase and hence Δ955 is catalytically more active (Amezcua et al., 2002; Rutter et al., 2001). This is seen in Figure A-3 where a substantial increase in activity is observed for Δ955 against histone, a nonphysiological substrate. In contrast, glycogen synthase was a worse substrate for Δ955 than for the wildtype kinase. A similar effect was seen when UCK2, a PASK substrate identified in a biochemical screen (Chapter 4), was tested.

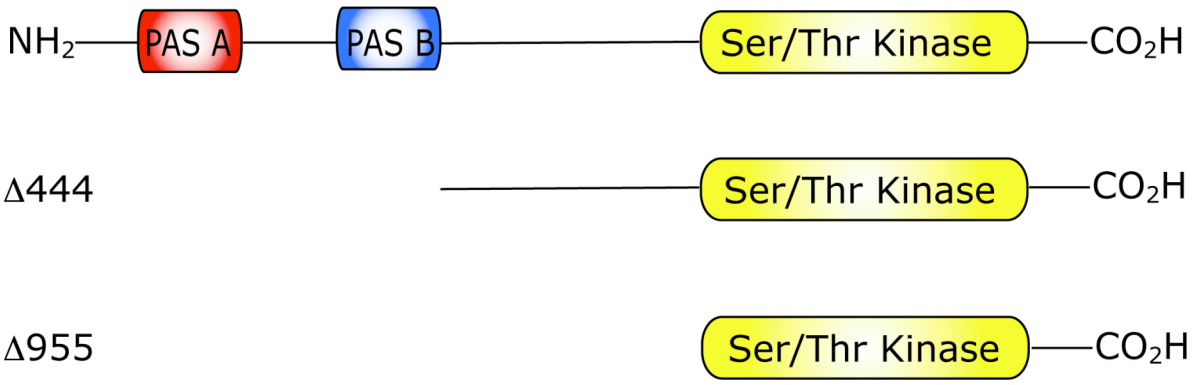


Figure A-2 Diagram of PASK deletion constructs
Two N-terminal deletions were made: Δ444 which lacks the residues 1-444 and Δ955 which lacks the entire N-terminus, residues 1-955.

Δ444 on the other hand exhibited surprisingly more activity toward both glycogen synthase and UCK2 suggesting that some region between the kinase domain and the PAS domain is important for substrate binding. This regulated binding event was further demonstrated by cotransfection-immunoprecipitation experiments with the various PASK deletion constructs and either glycogen synthase or lacZ (negative control). PASK substantially co-immunoprecipitated glycogen synthase over lacZ. Δ444, which lacks the PAS domain, caused an even higher level of glycogen synthase to be co-immunoprecipated as compared to wildtype. This reinforces the kinase assay data in Figure A-3 and demonstrates that the PAS domain is indeed inhibitory.

Perplexingly, we noticed that while purified rabbit muscle glycogen synthase (I-form) was a good substrate for PASK, recombinant bacterial-expressed muscle glycogen synthase was not. Recombinant *E. coli* expressed glycogen synthase purifies with substantial glycogen bound to it whereas rabbit muscle does not.

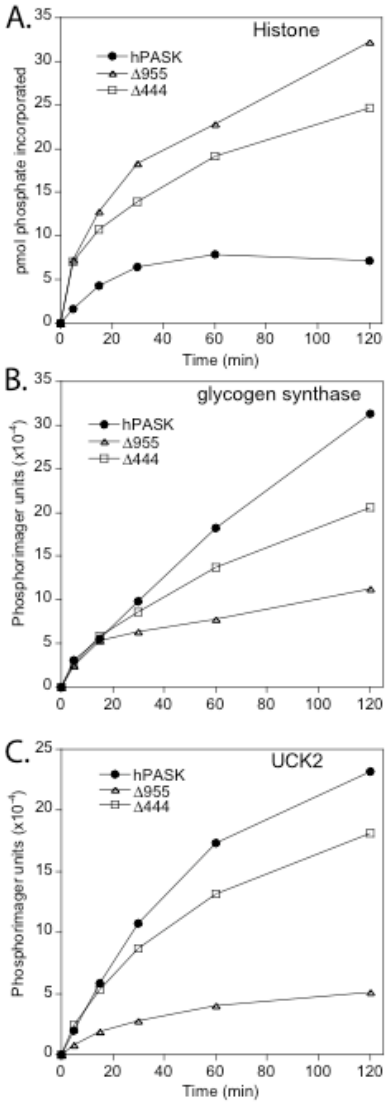


Figure A-3 Glycogen synthase and UCK2-dependent PASK phosphorylation requires a substrate docking domain (A) Histone, (B) I-form of glycogen synthase or (C) UCK2 was subjected to phosphorylation in the presence of [γ-^{32}P]ATP by wildtype PASK, Δ955 or Δ444. After incubation for the indicated time, SDS-PAGE and autoradiography were used to determine stoichiometry. Figure/data from J. Rutter (2005) – submitted to PNAS.

A simple titration experiment of glycogen into kinase reactions with PASK and purified rabbit muscle glycogen synthase showed a dose-dependent inhibition of PASK ability to phosphorylate glycogen synthase. This inhibition was half-maximal at ~35 μg/ml glycogen and reached and inhibition plateau of ~65%. Glycogen did not inhibit the ability of PASK to phosphorylate UCK2 nor the ability of DYRK to phosphorylate glycogen synthase. Since PASK and DYRK both phosphorylate the same site, Ser-640, on glycogen synthase this excludes the possibility that glycogen is occluding the phosphorylation site. It also demonstrates that the glycogen-dependent inhibition of PASK is specific for both PASK and glycogen synthase.

Conclusion

As described in the introduction (Chapter 1), several kinases have substrate docking domains that are responsible for efficient phosphorylation of their given protein substrates. PASK has now been demonstrated to have a functionally similar substrate docking domain located N-terminal to the kinase but C-terminal to the PAS domain. This domain is important for efficient phosphorylation of glycogen synthase and UCK2. Since this region is close to 600 amino acids additional experiments will need to be performed to narrow down the exact sequence(s) or structural requirements necessary for these docking interactions. Furthermore, the mechanism of how glycogen is specifically inhibiting PASK-dependent phosphorylation of glycogen synthase is unknown. How and when this inhibition occurs in cells coupled with the regulatory mechanisms exerted by the PAS domain provides many intriguing questions for future research. The fact that PAS domains, in most contexts, act as sensors and the emerging roles of PASK in regulating cellular metabolism, it is highly probable that PASK is an important nutrient sentinel.

Appendix B
Table of proteins identified in PASK substrate screen

This table compiles the mass spectrometry data that was obtained in the course of our unbiased HT biochemical screen for PASK substrates. [a]Refers to the chromatographic fraction where a given protein band(s) appeared to be phosphorylated by PASK. [b]Refers to the apparent molecular weight of the proposed substrate and the number of "+ " symbols determine the robustness of the phosphorylated band (on a scale of 1-5 with + being a weak band and +++++ being a very intense band). [c]Protein name of the polypeptide(s) identified by mass spectrometry in the excised phosphorylated gel band. Most bands contained multiple polypeptides, which are listed in descending order of the most abundant. [d]Gene accession number of the given identified polypeptide. Proteins that were confirmed as *in vitro* PASK substrates are shown in red for full length PASK and green for kinase domain only PASK. Those in blue are proteins that were tested for PASK substrates but were not phosphorylated *in vitro* while those in black have not been confirmed.

fraction[a]	size (kD)	protein[c]	gi ID[d]
E2 B1-2	35, ++++	glyceraldehyde-3-phosphate dehydrogenase	31645
		cytosolic malate dehydrogenase	5174539
D2 A-1st	37,+	glyceraldehyde-3-phosphate dehydrogenase	31645
		template activating factor I, spice form alpha	213258
C4 B1	15,++	TCP4, RNA polyII transcriptional coactivator p15	1709514
		ribosomal protein S14	5033051
		nucleoside-dephosphate kinase 2 (NM23)	4505409
		ubiquitin-conjugating enzyme E2N	4507793
		60S ribosomal protein L13	4506633
		Histone H2B	15030224
C5 B2 Q1-12	29,+	Horf6, peroxiredoxin 6	3318841
		14-3-3 beta	4507949
		tumor protein D52-like	4507643
		alanyl-tRNA synthetase	4501841
		Ran-binding protein	938026
C4 B2 Q15	28,++	tumorprotein D52-like, hD54	4507643
		alanyl-tRNA synthetase	4501841
		spermine synthetase	1082797
		Horf6, peroxiredoxin 6	3318841
		Ran-specific GTPase-activating protein	542991
C3 B2 S11	20,+++	basic transcription factor 3	20070130
		Rnase H1 small subunit	19421812
C3 B2 S14	23,++	peroxiredoxin 1	4505591
		basic transcription factor 3a	107909
		HSPC150 protein	7661808
		60S ribosomal protein L22	4506613
C4 B2 S24	20,++	basic transcription factor 3	20070130
		TNF-induced protein	20556217
C4 B2 Q12	42,+	guanine aminohydrolase	11837778
		glycyl tRNA synthetase	3845409
C3 B2 S15-T	18,+++	Histone H2B	350170
		heparin binding protein HBp15	33150766
		cytoplasmic phosphotyrosyl phosphatase	179661
C3 B2 S15-B	16,+++	histone H2A.5	70686
		histone H2A.F/Z	6912616
C3 B2 Q12-T	40,++	enolase 2; neuron specific gamma enolase	693933
		hypothetical protein; NUDC	12052969
		guanine deaminase	4758426
C3 B2 Q12-M	27,+	Ran-binding protein	938026
		hyppthetical protein	27478045
		Rho GDP dissociation inhibitor alpha	4757768
C3 B2 Q12-B	23,+	human F-actin capping protein	13124696
		tumor protein, translationally controlled	4507669
		HSP70	462325
		guanine aminohydrolase	4758426

		Ran-binding protein	938026
C3 B2 Q19-T	58,++	pyruvate kinase	478822
		FLJ22028 protein	18203811
		apoptosis inhibitor fibroblast growth factor 2	5729730
		14-3-3 beta	4507949
C3 B2 Q19-M	22,++	basic transcription factor 3	16159637
		TPM4 ALK fusion oncoprotein 1	10441388
		hypothetical protein FLJ22028	18203811
C3 B2 Q19-B	18,+	mago-nashi homolog	4505087
		6-pyruvoyltetrahydropterin synthase	17390048
		ribosomal protein L23	27498549
		14-3-3	3387922
C5 B2 gf11	48,++	eukaryotic tranlation initiation factor 4A	4503529
		PAS kinase	20521830
		fumarate hydratase	19743875
		pyruvate kinase	20178296
C2 B1-T	18,++	TCP4, RNA polyII transcriptional coactivator p15	1709514
		ribosomal L31	4506633
		Chain B, Mammalian Srp	11513833
		nucleoside-dephosphate kinase 2 (NM23)	1421609
C2 B1-B	16,++	GABA receptor associated protein-like 2	6005768
		Chain B, Mammalian Srp	11513833
		histone H2A.F/Z	6912616
C2 B2 S20-T	54, +	moesin	4505257
		PAS kinase	14583077
		flap structure-specific endonuclease I	4758356
C2 B2 S20-M	40,++	flap structure-specific endonuclease I	4758356
		APEX nuclease	18375501
C2 B2 S20-B	30,++	60S acidic ribosomal protein PO	5815233
		tropomyosin 4	4507651
		flap structure-specific endonuclease I	4758356
C2 B2 S22-T	30,++	PDGFA associated protein 1	7657441
		APEX nuclease	6980832
C2 B2 S22-M	27,+	basic transcription factor 3a	107909
		APEX nuclease	6980804
C2 B2 S22-B	22,++	H1 histone, member X	5174449
		p64 CLCP	895845
		heparin binding protein HBp15	33150766
C2 B2 S35-T	29,+	peroxiredoxin 1	4505591
		heparin binding protein HBp15	33150766
C2 B2 S35-M	24,++	ribosomal protein L22	4506613
C2 B2 S35-B	16,++	histone H2A	25092737
C2 B2 S36-T	18,+++	histone H2B	4504259
C2 B2 S36-B	16, +++	histone H2A.F/Z	6912616
C2 B2 Q13	23, +	dUTPase	3041664
		PNG gene	2208307A
C2 B2 gf11	40, +	enolase 1	45033571
		6-phosphogluconate dehydrogenase	20981679
		PTD004 protein	15530273

C1 B1-T	18, +	TCP4, RNA polyII transcriptional coactivator p15	1709514
		nucleoside-dephosphate kinase 2 (NM23)	4505409
		ribosomal protein L31	1655596
C1 B1-B	16, +	selenoprotein H	25014109
		ribosomal protein L30	4506631
C1 B2-S15-T	18, +++	Histone H2B	11036646
C1 B2-S15-B	16, +++	histone H2A.5	70686
		histone H2A.F/Z	20357599
		histone H2A, family member R	25092737
C1 B2-T1	25, +++	adenylate kinase	4502011
		peroxiredoxin 1	4505591
		ribosomal protein S3A	14755682
		uridine kinase	15928999
C1 B2-T2	24, +++	glyoxalase I	15030212
		uridine kinase	7706497
C1 B2-B	17, +++	TCP4, RNA polyII transcriptional coactivator p15	1709514
		ribosomal protein L22	4506613
		unnamed protein product	7020527
		nucleoside-dephosphate kinase 2 (NM23)	4505409
		ribosomal protein L31	4506633
		ribosomal protein S14	5032051
B3 B2 S14-T	27, ++	ribosomal protein S8	4506743
		pyridoxine 5'-phosphate oxidase	8922498
		calpain, small subunit	18314496
		6-phosphogluconate dehydrogenase	6912586
		Ran-binding protein	4506407
B3 B2 S14-M	22, ++	ribosomal protein S9	17318569
		KRT10 protein	21961605
		DNA-binding protein	189299
		ribosomal protein L18	4506607
		Y box binding protein 1	340419
		eIF-3, subunit K	10801345
B3 B2 S14-B1	17, +	ribosomal protein S25	4506707
		ribosomal protein S18	11968182
		ribosomal protein S13	4506685
		smooth muscle myosin light chain	17986264
C6 B1-T	23, ++	uridine kinase	1264408
		glyoxalase I	15030212
		peroxiredoxin 1	4505591
		splicing factor, serine/arginine rich 3	4506901
C6 B1-M	20, ++	cyclophilin B	4758950
		ribosomal protein L23	27485613
		dUTPase	3041664
		nucleoside-dephosphate kinase 2 (NM23)	35068
C6 B1-B	16, +++	TCP4, RNA polyII transcriptional coactivator p15	1709514
		ribosomal protein L22	4506613
		nucleoside-dephosphate kinase 2 (NM23)	4505409
		ribosomal L31	1655596
		histone H2B, family member F	10800140

C6 B2 S16	18, +	ribosomal protein L23	306549
		ribosomal protein L11	15431290
		eIF-4C	1082871
		Histone H2A, family member Z	29735974
C6 B2 S15-T	17, ++	ribosomal protein L22	4506613
		histone H2B	15030224
		ribosomal protein L31	4506633
C6 B2 S15-B	15, ++	Histone H2A, family member E	10800144
		Histone H2A, family member Z	4504255
B4 B2 S15-T	26,+++	RPSX4 protein	13938353
		exonuclease RRp41	9506689
		ribosomal protein S8	4506743
		14-3-3 zeta	23110942
		Ran-binding protein	4506407
		ribosomal protein S3A	20543864
B4 B2 S15-B	18, ++	RPSX4 protein	4506725
		exonuclease RRp41	9506689
		14-3-3 zeta	23110942
		ACTB protein	15277503
B6 B1	23, +++	basic transcription factor 3	1082633
		hyppthetical protein BC013949	19923969
		uridine kinase	12644008
		splicing factor, serine/arginine rich 3	4506901
		14-3-3 zeta	88168
A1 B2 Q8	45, +++	n-myc downregulated gene	14165266
		alpha 1 actin	4501881
		HSP70	24234686
A1 B2 S16	23, +	Ras-related protein Rab-7	1709999
		ribosomal protein L18	18204442
		unknown protein	13177700
B3 B2 Q8-T	45, +++	eIF-4A	4503529
		guanin deaminase	4758426
		CDC37 homolog	5901922
		alanyl-tRNA synthetase	15079238
		n-myc downregulated gene	5174657
B3 B2 Q8-B	27, ++	tumorprotein D52-like, hD54	4507643
		14-3-3 zeta	23110942
		alanyl-tRNA synthetase	15079238
		peroxiredoxin 6	4758638
		Ran-binding protein	4506407
B2 B2 Q9-T	46, ++++	eIF-4A	4503529
		alanyl-tRNA synthetase	15079238
		CDC37 homolog	5901922
		pyruvate kinase	3146989
		PASK	1710186
B2 B2 Q9-B	27, +++	14-3-3 zeta	23110942
		Ran-binding protein	4506407
		alanyl-tRNA synthetase	15079238
		peroxiredoxin 6	4758638

BIBLIOGRAPHY

Altmann, M., Krieger, M., and Trachsel, H. (1989). Nucleotide sequence of the gene encoding a 20 kDa protein associated with the cap binding protein eIF-4E from Saccharomyces cerevisiae. Nucleic Acids Res *17*, 7520.

Altmann, M., Schmitz, N., Berset, C., and Trachsel, H. (1997). A novel inhibitor of cap-dependent translation initiation in yeast: p20 competes with eIF4G for binding to eIF4E. Embo J *16*, 1114-1121.

Altmann, M., and Trachsel, H. (1989). Altered mRNA cap recognition activity of initiation factor 4E in the yeast cell cycle division mutant cdc33. Nucleic Acids Res *17*, 5923-5931.

Amezcua, C. A., Harper, S. M., Rutter, J., and Gardner, K. H. (2002). Structure and interactions of PAS kinase N-terminal PAS domain: model for intramolecular kinase regulation. Structure (Camb) *10*, 1349-1361.

Anselmo, A. N., and Cobb, M. H. (2004). Protein kinase function and glutathionylation. Biochem J *381*, e1-2.

Bajpai, M., Gupta, G., and Setty, B. S. (1998). Changes in carbohydrate metabolism of testicular germ cells during meiosis in the rat. Eur J Endocrinol *138*, 322-327.

Barden, N., and Labrie, F. (1973). Cyclic adenosine 3',5'-monophosphate dependent phosphorylation of ribosomal proteins from bovine anterior pituitary gland. Biochemistry *12*, 3096-3102.

Battiste, J. L., Pestova, T. V., Hellen, C. U., and Wagner, G. (2000). The eIF1A solution structure reveals a large RNA-binding surface important for scanning function. Mol Cell *5*, 109-119.

Berthelot, K., Muldoon, M., Rajkowitsch, L., Hughes, J., and McCarthy, J. E. (2004). Dynamics and processivity of 40S ribosome scanning on mRNA in yeast. Mol Microbiol *51*, 987-1001.

Bibikov, S. I., Barnes, L. A., Gitin, Y., and Parkinson, J. S. (2000). Domain organization and flavin adenine dinucleotide-binding determinants in the aerotaxis signal transducer Aer of Escherichia coli. Proc Natl Acad Sci U S A *97*, 5830-5835.

Blomberg, N., Baraldi, E., Nilges, M., and Saraste, M. (1999). The PH superfold: a structural scaffold for multiple functions. Trends Biochem Sci *24*, 441-445.

Boles, E., Liebetrau, W., Hofmann, M., and Zimmermann, F. K. (1994). A family of hexosephosphate mutases in Saccharomyces cerevisiae. Eur J Biochem *220*, 83-96.

Borgstahl, G. E., Williams, D. R., and Getzoff, E. D. (1995). 1.4 A structure of photoactive yellow protein, a cytosolic photoreceptor: unusual fold, active site, and chromophore. Biochemistry *34*, 6278-6287.

Bossemeyer, D. (1995). Protein kinases--structure and function. FEBS Lett *369*, 57-61.

Buzan, J. M., and Frieden, C. (1996). Yeast actin: polymerization kinetic studies of wild type and a poorly polymerizing mutant. Proc Natl Acad Sci U S A *93*, 91-95.

Cartwright, J. L., and McLennan, A. G. (1999). The Saccharomyces cerevisiae YOR163w gene encodes a diadenosine 5', 5'"-P1,P6-hexaphosphate (Ap6A) hydrolase member of the MutT motif (Nudix hydrolase) family. J Biol Chem *274*, 8604-8610.

Cassell, P. G., and Hitman, G. A. (2003). Genetics of type 2 diabetes mellitus: current progress and future directions. (London: Martin Dunitz).

Cawthon, M. L., Bitte, L. F., Krystosek, A., and Kabat, D. (1974). Effect of cyclic adenosine 3':5'-monophosphate on ribosomal protein phosphorylation in reticulocytes. J Biol Chem *249*, 275-278.

Chan, T. O., Rittenhouse, S. E., and Tsichlis, P. N. (1999). AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. Annu Rev Biochem *68*, 965-1014.

Chaudhuri, J., Si, K., and Maitra, U. (1997). Function of eukaryotic translation initiation factor 1A (eIF1A) (formerly called eIF-4C) in initiation of protein synthesis. J Biol Chem *272*, 7883-7891.

Christie, J. M., Reymond, P., Powell, G. K., Bernasconi, P., Raibekas, A. A., Liscum, E., and Briggs, W. R. (1998). Arabidopsis NPH1: a flavoprotein with the properties of a photoreceptor for phototropism. Science *282*, 1698-1701.

Chuang, R. Y., Weaver, P. L., Liu, Z., and Chang, T. H. (1997). Requirement of the DEAD-Box protein ded1p for messenger RNA translation. Science *275*, 1468-1471.

Chun, J., Hyun, S., Kwon, T., Lee, E. J., Hong, S. K., and Kang, S. S. (2005). The subcellular localization control of integrin linked kinase 1 through its protein-protein interaction with caveolin-1. Cell Signal *17*, 751-760.

Clauser, K. R., Baker, P., and Burlingame, A. L. (1999). Role of accurate mass measurement (+/- 10 ppm) in protein identification strategies employing MS or MS/MS and database searching. Anal Chem *71*, 2871-2882.

Cross, J. V., and Templeton, D. J. (2004). Oxidative stress inhibits MEKK1 by site-specific glutathionylation in the ATP-binding domain. Biochem J *381*, 675-683.

Cuff, J. A., Clamp, M. E., Siddiqui, A. S., Finlay, M., and Barton, G. J. (1998). JPred: a consensus secondary structure prediction server. Bioinformatics *14*, 892-893.

Cumming, R. C., Andon, N. L., Haynes, P. A., Park, M., Fischer, W. H., and Schubert, D. (2004). Protein disulfide bond formation in the cytoplasm during oxidative stress. J Biol Chem *279*, 21749-21758.

da Silva Xavier, G., Rutter, J., and Rutter, G. A. (2004). Involvement of Per-Arnt-Sim (PAS) kinase in the stimulation of preproinsulin and pancreatic duodenum homeobox 1 gene expression by glucose. Proc Natl Acad Sci U S A *101*, 8319-8324.

Dajani, R., Fraser, E., Roe, S. M., Young, N., Good, V., Dale, T. C., and Pearl, L. H. (2001). Crystal structure of glycogen synthase kinase 3 beta: structural basis for phosphate-primed substrate specificity and autoinhibition. Cell *105*, 721-732.

Daran, J. M., Bell, W., and Francois, J. (1997). Physiological and morphological effects of genetic alterations leading to a reduced synthesis of UDP-glucose in Saccharomyces cerevisiae. FEMS Microbiol Lett *153*, 89-96.

Daran, J. M., Dallies, N., Thines-Sempoux, D., Paquet, V., and Francois, J. (1995). Genetic and biochemical characterization of the UGP1 gene encoding the UDP-glucose pyrophosphorylase from Saccharomyces cerevisiae. Eur J Biochem *233*, 520-530.

Davare, M. A., Saneyoshi, T., Guire, E. S., Nygaard, S. C., and Soderling, T. R. (2004). Inhibition of calcium/calmodulin-dependent protein kinase kinase by protein 14-3-3. J Biol Chem *279*, 52191-52199.

Davis, R. J. (2000). Signal transduction by the JNK group of MAP kinases. Cell *103*, 239-252.
de la Cruz, J., Iost, I., Kressler, D., and Linder, P. (1997). The p20 and Ded1 proteins have antagonistic roles in eIF4E-dependent translation in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A *94*, 5201-5206.

Dearsly, A. L., Johnson, R. M., Barrett, P., and Sommerville, J. (1985). Identification of a 60-kDa phosphoprotein that binds stored messenger RNA of Xenopus oocytes. Eur J Biochem *150*, 95-103.

Delaunay, J., Loeb, J. E., Pierre, M., and Schapira, G. (1973). Mammalian ribosomal proteins: studies on the in vitro phosphorylation patterns of ribosomal proteins from rabbit liver and reticulocytes. Biochim Biophys Acta *312*, 147-151.

DePaoli-Roach, A. A., Ahmad, Z., Camici, M., Lawrence, J. C., Jr., and Roach, P. J. (1983). Multiple phosphorylation of rabbit skeletal muscle glycogen synthase. Evidence for interactions among phosphorylation sites and the resolution of electrophoretically distinct forms of the subunit. J Biol Chem *258*, 10702-10709.

DePaoli-Roach, A. A., Vilardo, P. G., Kim, J. H., Mavila, N., Vemuri, B., and Roach, P. J. (2003). Determination of mammalian glycogen synthase phosphatase activity. Methods Enzymol *366*, 17-34.

Deschamps, S., Viel, A., Garrigos, M., Denis, H., and le Maire, M. (1992). mRNP4, a major mRNA-binding protein from Xenopus oocytes is identical to transcription factor FRG Y2. J Biol Chem *267*, 13799-13802.

Droge, W. (2002). Free radicals in the physiological control of cell function. Physiol Rev *82*, 47-95.

Dunckley, T., and Parker, R. (1999). The DCP2 protein is required for mRNA decapping in Saccharomyces cerevisiae and contains a functional MutT motif. Embo J *18*, 5411-5422.

Eil, C., and Wool, I. G. (1971). Phosphorylation of rat liver ribosomal subunits: partial purification of two cyclic AMP activated protein kinases. Biochem Biophys Res Commun *43*, 1001-1009.

Eil, C., and Wool, I. G. (1973). Function of phosphorylated ribosomes. The activity of ribosomal subunits phosphorylated in vitro by protein kinase. J Biol Chem *248*, 5130-5136.

Elion, E. A. (2000). Pheromone response, mating and cell biology. Curr Opin Microbiol *3*, 573-581.

Evans, J. L., Goldfine, I. D., Maddux, B. A., and Grodsky, G. M. (2002). Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. Endocr Rev *23*, 599-622.

Favelyukis, S., Till, J. H., Hubbard, S. R., and Miller, W. T. (2001). Structure and autoregulation of the insulin-like growth factor 1 receptor kinase. Nat Struct Biol *8*, 1058-1063.

Fiol, C. J., Mahrenholz, A. M., Wang, Y., Roeske, R. W., and Roach, P. J. (1987). Formation of protein kinase recognition sites by covalent modification of the substrate. Molecular mechanism for the synergistic action of casein kinase II and glycogen synthase kinase 3. J Biol Chem *262*, 14042-14048.

Flotow, H., and Roach, P. J. (1989). Synergistic phosphorylation of rabbit muscle glycogen synthase by cyclic AMP-dependent protein kinase and casein kinase I. Implications for hormonal regulation of glycogen synthase. J Biol Chem *264*, 9126-9128.

Fosset, M., Muir, L. W., Nielsen, L. D., and Fischer, E. H. (1971). Purification and properties of yeast glycogen phosphorylase a and b. Biochemistry *10*, 4105-4113.

Frame, S., Cohen, P., and Biondi, R. M. (2001). A common phosphate binding site explains the unique substrate specificity of GSK3 and its inactivation by phosphorylation. Mol Cell *7*, 1321-1327.

Francis, T. A., and Roberts, S. (1982). Influence of the state of ribosome association on the phosphorylation of ribosomal proteins in isolated ribosome--protein kinase systems from rat cerebral cortex. Biochem J *208*, 289-300.

Francois, J., and Parrou, J. L. (2001). Reserve carbohydrates metabolism in the yeast Saccharomyces cerevisiae. FEMS Microbiol Rev *25*, 125-145.

Freire, M. A. (2005). Translation initiation factor (iso) 4E interacts with BTF3, the beta subunit of the nascent polypeptide-associated complex. Gene *345*, 271-277.

Friedman, D. L., and Larner, J. (1963). Studies On Udpg-Alpha-Glucan Transglucosylase. Iii. Interconversion Of Two Forms Of Muscle Udpg-Alpha-Glucan Transglucosylase By A Phosphorylation-Dephosphorylation Reaction Sequence. Biochemistry *128*, 669-675.

Fu, H., Subramanian, R. R., and Masters, S. C. (2000). 14-3-3 proteins: structure, function, and regulation. Annu Rev Pharmacol Toxicol *40*, 617-647.

Fu, W., Jack, R. F., Morgan, T. V., Dean, D. R., and Johnson, M. K. (1994). nifU gene product from Azotobacter vinelandii is a homodimer that contains two identical [2Fe-2S] clusters. Biochemistry *33*, 13455-13463.

Gallagher, E. D., Xu, S., Moomaw, C., Slaughter, C. A., and Cobb, M. H. (2002). Binding of JNK/SAPK to MEKK1 is regulated by phosphorylation. J Biol Chem *277*, 45785-45792.

Gasch, A. P., Spellman, P. T., Kao, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., Botstein, D., and Brown, P. O. (2000). Genomic expression programs in the response of yeast cells to environmental changes. Mol Biol Cell *11*, 4241-4257.

Gavin, A. C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J. M., Michon, A. M., Cruciat, C. M*., et al.* (2002). Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature *415*, 141-147.

Gilles-Gonzalez, M. A. (2001). Oxygen signal transduction. IUBMB Life *51*, 165-173.

Gingras, A. C., Raught, B., and Sonenberg, N. (1999). eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu Rev Biochem *68*, 913-963.

Gold, B., and Hecht, N. B. (1981). Differential compartmentalization of messenger ribonucleic acid in murine testis. Biochemistry *20*, 4871-4877.

Gold, B., Stern, L., Bradley, F. M., and Hecht, N. B. (1983). Gene expression during mammalian spermatogenesis. II. Evidence for stage-specific differences in mRNA populations. J Exp Zool *225*, 123-134.

Goldberg, J., Nairn, A. C., and Kuriyan, J. (1996). Structural basis for the autoinhibition of calcium/calmodulin-dependent protein kinase I. Cell *84*, 875-887.

Gressner, A. M., and Wool, I. G. (1974). The phosphorylation of liver ribosomal proteins in vivo. Evidence that only a single small subunit protein (S6) is phosphorylated. J Biol Chem *249*, 6917-6925.

Grishin, N. V. (1998). The R3H motif: a domain that binds single-stranded nucleic acids. Trends Biochem Sci *23*, 329-330.

Grootegoed, J. A., Jansen, R., and Van der Molen, H. J. (1984a). The role of glucose, pyruvate and lactate in ATP production by rat spermatocytes and spermatids. Biochim Biophys Acta *767*, 248-256.

Grootegoed, J. A., Jansen, R., and van der Molen, H. J. (1984b). Spermatogenic cells in the germinal epithelium utilize alpha-ketoisocaproate and lactate, produced by Sertoli cells from leucine and glucose. Ann N Y Acad Sci *438*, 557-560.

Haghighat, A., Mader, S., Pause, A., and Sonenberg, N. (1995). Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E. Embo J *14*, 5701-5709.

Hampsey, M. (1997). A review of phenotypes in Saccharomyces cerevisiae. Yeast *13*, 1099-1133.

Hanis, C. L., Boerwinkle, E., Chakraborty, R., Ellsworth, D. L., Concannon, P., Stirling, B., Morrison, V. A., Wapelhorst, B., Spielman, R. S., Gogolin-Ewens, K. J.*, et al.* (1996). A genome-wide search for human non-insulin-dependent (type 2) diabetes genes reveals a major susceptibility locus on chromosome 2. Nat Genet *13*, 161-166.

Hanks, S. K., and Hunter, T. (1995). Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. Faseb J *9*, 576-596.

Hanks, S. K., Quinn, A. M., and Hunter, T. (1988). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science *241*, 42-52.

Hara, K., Yonezawa, K., Weng, Q. P., Kozlowski, M. T., Belham, C., and Avruch, J. (1998). Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. J Biol Chem *273*, 14484-14494.

Harding, H. P., Zhang, Y., Bertolotti, A., Zeng, H., and Ron, D. (2000). Perk is essential for translational regulation and cell survival during the unfolded protein response. Mol Cell *5*, 897-904.

Hardy, T. A., and Roach, P. J. (1993). Control of yeast glycogen synthase-2 by COOH-terminal phosphorylation. J Biol Chem *268*, 23799-23805.

Hawkins, N. C., Van Buskirk, C., Grossniklaus, U., and Schupbach, T. (1997). Post-transcriptional regulation of gurken by encore is required for axis determination in Drosophila. Development *124*, 4801-4810.

Heist, E. K., Srinivasan, M., and Schulman, H. (1998). Phosphorylation at the nuclear localization signal of Ca2+/calmodulin-dependent protein kinase II blocks its nuclear targeting. J Biol Chem *273*, 19763-19771.

Hellen, C. U., and Sarnow, P. (2001). Internal ribosome entry sites in eukaryotic mRNA molecules. Genes Dev *15*, 1593-1612.

Hellman, U., Wernstedt, C., Gonez, J., and Heldin, C. H. (1995). Improvement of an "In-Gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. Anal Biochem *224*, 451-455.

Herbert, T. P., and Hecht, N. B. (1999). The mouse Y-box protein, MSY2, is associated with a kinase on non-polysomal mouse testicular mRNAs. Nucleic Acids Res *27*, 1747-1753.

Ho, Y., Gruhler, A., Heilbut, A., Bader, G. D., Moore, L., Adams, S. L., Millar, A., Taylor, P., Bennett, K., Boutilier, K.*, et al.* (2002). Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry. Nature *415*, 180-183.

Hofer, T., Spielmann, P., Stengel, P., Stier, B., Katschinski, D. M., Desbaillets, I., Gassmann, M., and Wenger, R. H. (2001). Mammalian PASKIN, a PAS-serine/threonine kinase related to bacterial oxygen sensors. Biochem Biophys Res Commun *288*, 757-764.

Holcik, M., Yeh, C., Korneluk, R. G., and Chow, T. (2000). Translational upregulation of X-linked inhibitor of apoptosis (XIAP) increases resistance to radiation induced cell death. Oncogene *19*, 4174-4177.

Horikawa, Y., Oda, N., Cox, N. J., Li, X., Orho-Melander, M., Hara, M., Hinokio, Y., Lindner, T. H., Mashima, H., Schwarz, P. E.*, et al.* (2000). Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus. Nat Genet *26*, 163-175.

Horman, S., Browne, G., Krause, U., Patel, J., Vertommen, D., Bertrand, L., Lavoinne, A., Hue, L., Proud, C., and Rider, M. (2002). Activation of AMP-activated protein kinase leads to the phosphorylation of elongation factor 2 and an inhibition of protein synthesis. Curr Biol *12*, 1419-1423.

Huang, K. P., and Cabib, E. (1974a). Yeast glycogen synthetase in the glucose 6-phosphate-dependent form. I. Purification and properties. J Biol Chem *249*, 3851-3857.

Huang, K. P., and Cabib, E. (1974b). Yeast glycogen synthetase in the glucose 6-phosphate-dependent form. II. The effect of proteolysis. J Biol Chem *249*, 3858-3861.

Huang, M., and Graves, L. M. (2003). De novo synthesis of pyrimidine nucleotides; emerging interfaces with signal transduction pathways. Cell Mol Life Sci *60*, 321-336.

Humphries, K. M., Deal, M. S., and Taylor, S. S. (2005). Enhanced dephosphorylation of cAMP-dependent protein kinase by oxidation and thiol modification. J Biol Chem *280*, 2750-2758.

Huse, M., and Kuriyan, J. (2002). The conformational plasticity of protein kinases. Cell *109*, 275-282.

Iost, I., Dreyfus, M., and Linder, P. (1999). Ded1p, a DEAD-box protein required for translation initiation in Saccharomyces cerevisiae, is an RNA helicase. J Biol Chem *274*, 17677-17683.

Ivashkiv, L. B., and Hu, X. (2004). Signaling by STATs. Arthritis Res Ther *6*, 159-168.

Jamieson, D. J., and Beggs, J. D. (1991). A suppressor of yeast spp81/ded1 mutations encodes a very similar putative ATP-dependent RNA helicase. Mol Microbiol *5*, 805-812.

Jamieson, D. J., Rahe, B., Pringle, J., and Beggs, J. D. (1991). A suppressor of a yeast splicing mutation (prp8-1) encodes a putative ATP-dependent RNA helicase. Nature *349*, 715-717.

Jeffrey, P. D., Russo, A. A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J., and Pavletich, N. P. (1995). Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex. Nature *376*, 313-320.

Jergil, B. (1972). Protein kinase from rainbow-trout-testis ribosomes. Partial purification and characterization. Eur J Biochem *28*, 546-554.

Johnson, S. A., and Hunter, T. (2005). Kinomics: methods for deciphering the kinome. Nat Methods *2*, 17-25.

Kabat, D. (1972). Turnover of phosphoryl groups in reticulocyte ribosomal phosphoproteins. J Biol Chem *247*, 5338-5344.

Kagami, M., Toh-e, A., and Matsui, Y. (1997). SRO9, a multicopy suppressor of the bud growth defect in the Saccharomyces cerevisiae rho3-deficient cells, shows strong genetic interactions with tropomyosin genes, suggesting its role in organization of the actin cytoskeleton. Genetics *147*, 1003-1016.

Kallunki, T., Deng, T., Hibi, M., and Karin, M. (1996). c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. Cell *87*, 929-939.

Kallunki, T., Su, B., Tsigelny, I., Sluss, H. K., Derijard, B., Moore, G., Davis, R., and Karin, M. (1994). JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation. Genes Dev *8*, 2996-3007.

Katschinski, D. M., Marti, H. H., Wagner, K. F., Shibata, J., Eckhardt, K., Martin, F., Depping, R., Paasch, U., Gassmann, M., Ledermann, B.*, et al.* (2003). Targeted disruption of the mouse PAS domain serine/threonine kinase PASKIN. Mol Cell Biol *23*, 6780-6789.

Kemp, B. E., Bylund, D. B., Huang, T. S., and Krebs, E. G. (1975). Substrate specificity of the cyclic AMP-dependent protein kinase. Proc Natl Acad Sci U S A *72*, 3448-3452.

Kennelly, P. J., and Krebs, E. G. (1991). Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. J Biol Chem *266*, 15555-15558.

Kick, D., Barrett, P., Cummings, A., and Sommerville, J. (1987). Phosphorylation of a 60 kDa polypeptide from Xenopus oocytes blocks messenger RNA translation. Nucleic Acids Res *15*, 4099-4109.

King, T. P. (1972). Separation of proteins by ammonium sulfate gradient solubilization. Biochemistry *11*, 367-371.

Klauck, T. M., Faux, M. C., Labudda, K., Langeberg, L. K., Jaken, S., and Scott, J. D. (1996). Coordination of three signaling enzymes by AKAP79, a mammalian scaffold protein. Science *271*, 1589-1592.

Knighton, D. R., Zheng, J. H., Ten Eyck, L. F., Ashford, V. A., Xuong, N. H., Taylor, S. S., and Sowadski, J. M. (1991). Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. Science *253*, 407-414.

Kranz, J. E., and Holm, C. (1990). Cloning by function: an alternative approach for identifying yeast homologs of genes from other organisms. Proc Natl Acad Sci U S A *87*, 6629-6633.

Ladomery, M., Wade, E., and Sommerville, J. (1997). Xp54, the Xenopus homologue of human RNA helicase p54, is an integral component of stored mRNP particles in oocytes. Nucleic Acids Res *25*, 965-973.

Larner, J. (1990). Insulin and the stimulation of glycogen synthesis. The road from glycogen structure to glycogen synthase to cyclic AMP-dependent protein kinase to insulin mediators. Adv Enzymol Relat Areas Mol Biol *63*, 173-231.

Lawrence, J. C., Jr., and Abraham, R. T. (1997). PHAS/4E-BPs as regulators of mRNA translation and cell proliferation. Trends Biochem Sci *22*, 345-349.

Lee, J. Y., Rohlman, C. E., Molony, L. A., and Engelke, D. R. (1991). Characterization of RPR1, an essential gene encoding the RNA component of Saccharomyces cerevisiae nuclear RNase P. Mol Cell Biol *11*, 721-730.

Lin, K., Hwang, P. K., and Fletterick, R. J. (1995). Mechanism of regulation in yeast glycogen phosphorylase. J Biol Chem *270*, 26833-26839.

Lin, K., Rath, V. L., Dai, S. C., Fletterick, R. J., and Hwang, P. K. (1996). A protein phosphorylation switch at the conserved allosteric site in GP. Science *273*, 1539-1542.

Lindsley, J. E., and Rutter, J. (2004). Nutrient sensing and metabolic decisions. Comp Biochem Physiol B Biochem Mol Biol *139*, 543-559.

Loeb, J. E., and Blat, C. (1970). Phosphorylation of some rat liver ribosomal proteins and its activation by cyclic AMP. FEBS Lett *10*, 105-108.

Lu, Z., Xu, S., Joazeiro, C., Cobb, M. H., and Hunter, T. (2002). The PHD domain of MEKK1 acts as an E3 ubiquitin ligase and mediates ubiquitination and degradation of ERK1/2. Mol Cell *9*, 945-956.

Malaisse, W. J. (1983). Insulin release: the fuel concept. Diabete Metab *9*, 313-320.

Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002). The protein kinase complement of the human genome. Science *298*, 1912-1934.

Marin, O., Meggio, F., Marchiori, F., Borin, G., and Pinna, L. A. (1986). Site specificity of casein kinase-2 (TS) from rat liver cytosol. A study with model peptide substrates. Eur J Biochem *160*, 239-244.

Martinis, S. A., Plateau, P., Cavarelli, J., and Florentz, C. (1999a). Aminoacyl-tRNA synthetases: a family of expanding functions. Mittelwihr, France, October 10-15, 1999. Embo J *18*, 4591-4596.

Martinis, S. A., Plateau, P., Cavarelli, J., and Florentz, C. (1999b). Aminoacyl-tRNA synthetases: a new image for a classical family. Biochimie *81*, 683-700.

Matsui, Y., and Toh, E. A. (1992). Yeast RHO3 and RHO4 ras superfamily genes are necessary for bud growth, and their defect is suppressed by a high dose of bud formation genes CDC42 and BEM1. Mol Cell Biol *12*, 5690-5699.

Matthews, M. B., Sonenberg, N., and Hershey, J. W. B. (2000). Origins and principles of translational control. (Cold Spring Harbor, New York
: Cold Spring Harbor Laboratory Press).

McKendrick, L., Pain, V. M., and Morley, S. J. (1999). Translation initiation factor 4E. Int J Biochem Cell Biol *31*, 31-35.

Meggio, F., Marchiori, F., Borin, G., Chessa, G., and Pinna, L. A. (1984). Synthetic peptides including acidic clusters as substrates and inhibitors of rat liver casein kinase TS (type-2). J Biol Chem *259*, 14576-14579.

Meggio, F., and Pinna, L. A. (2003). One-thousand-and-one substrates of protein kinase CK2? Faseb J *17*, 349-368.

Meric, F., Matsumoto, K., and Wolffe, A. P. (1997). Regulated unmasking of in vivo synthesized maternal mRNA at oocyte maturation. A role for the chaperone nucleoplasmin. J Biol Chem *272*, 12840-12846.

Meyhaus, O., and Hornstein, E. (2000). Translational control of top mRNAs (Cold Spring Harbor: Cold Spring Harbor Press).

Murray, J. T., Campbell, D. G., Morrice, N., Auld, G. C., Shpiro, N., Marquez, R., Peggie, M., Bain, J., Bloomberg, G. B., Grahammer, F.*, et al.* (2004). Exploitation of KESTREL to identify NDRG family members as physiological substrates for SGK1 and GSK3. Biochem J *384*, 477-488.

Murray, M. T., Krohne, G., and Franke, W. W. (1991). Different forms of soluble cytoplasmic mRNA binding proteins and particles in Xenopus laevis oocytes and embryos. J Cell Biol *112*, 1-11.

Murray, M. T., Schiller, D. L., and Franke, W. W. (1992). Sequence analysis of cytoplasmic mRNA-binding proteins of Xenopus oocytes identifies a family of RNA-binding proteins. Proc Natl Acad Sci U S A *89*, 11-15.

Mylin, L. M., Bushman, V. L., Long, R. M., Yu, X., Lebo, C. M., Blank, T. E., and Hopper, J. E. (1994). SIP1 is a catabolite repression-specific negative regulator of GAL gene expression. Genetics *137*, 689-700.

Nagy, E., and Rigby, W. F. (1995). Glyceraldehyde-3-phosphate dehydrogenase selectively binds AU-rich RNA in the NAD(+)-binding region (Rossmann fold). J Biol Chem *270*, 2755-2763.

Nakamura, M., Okinaga, S., and Arai, K. (1984). Metabolism of pachytene primary spermatocytes from rat testes: pyruvate maintenance of adenosine triphosphate level. Biol Reprod *30*, 1187-1197.

Nakielny, S., Campbell, D. G., and Cohen, P. (1991). The molecular mechanism by which adrenalin inhibits glycogen synthesis. Eur J Biochem *199*, 713-722.

Ogawa, A., Takayama, Y., Sakai, H., Chong, K. T., Takeuchi, S., Nakagawa, A., Nada, S., Okada, M., and Tsukihara, T. (2002). Structure of the carboxyl-terminal Src kinase, Csk. J Biol Chem *277*, 14351-14354.

Otani, K., Han, D. H., Ford, E. L., Garcia-Roves, P. M., Ye, H., Horikawa, Y., Bell, G. I., Holloszy, J. O., and Polonsky, K. S. (2004). Calpain system regulates muscle mass and glucose transporter GLUT4 turnover. J Biol Chem *279*, 20915-20920.

Owen, D. J., Papageorgiou, A. C., Garman, E. F., Noble, M. E., and Johnson, L. N. (1995). Expression, purification and crystallisation of phosphorylase kinase catalytic domain. J Mol Biol *246*, 374-381.

Parrou, J. L., and Francois, J. (1997). A simplified procedure for a rapid and reliable assay of both glycogen and trehalose in whole yeast cells. Anal Biochem *248*, 186-188.

Pearson, R. B., and Kemp, B. E. (1991). Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. Methods Enzymol *200*, 62-81.

Picton, C., Woodgett, J., Hemmings, B., and Cohen, P. (1982). Multisite phosphorylation of glycogen synthase from rabbit skeletal muscle. Phosphorylation of site 5 by glycogen synthase kinase-5 (casein kinase-II) is a prerequisite for phosphorylation of sites 3 by glycogen synthase kinase-3. FEBS Lett *150*, 191-196.

Pierre, M., Creuzet, C., and Loeb, J. E. (1974). Localization of phosphoproteins in ribosomal subunits and in free and bound polysomes of rat liver. FEBS Lett *45*, 88-91.

Prestayko, A. W., Klomp, G. R., Schmoll, D. J., and Busch, H. (1974). Comparison of proteins of ribosomal subunits and nucleolar preribosomal particles from Novikoff hepatoma ascites cells by two-dimensional polyacrylamide gel electrophoresis. Biochemistry *13*, 1945-1951.

Prowse, C. N., Deal, M. S., and Lew, J. (2001). The complete pathway for catalytic activation of the mitogen-activated protein kinase, ERK2. J Biol Chem *276*, 40817-40823.

Rhodes, C. (2000). Diabetes Mellitus: A Fundamental and Clinical Text, 2nd edn (Philadelphia: Lippincott-Raven Publishers).

Rinke, J., and Steitz, J. A. (1982). Precursor molecules of both human 5S ribosomal RNA and transfer RNAs are bound by a cellular protein reactive with anti-La lupus antibodies. Cell *29*, 149-159.

Risley, M. S. (1990). Support of Xenopus laevis spermatogenesis in vitro by different energy substrates. Biol Reprod *42*, 511-522.

Roach, P. J. (1990). Control of glycogen synthase by hierarchal protein phosphorylation. Faseb J *4*, 2961-2968.

Rosenfeld, J., Capdevielle, J., Guillemot, J. C., and Ferrara, P. (1992). In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. Anal Biochem *203*, 173-179.

Rosenmund, C., Carr, D. W., Bergeson, S. E., Nilaver, G., Scott, J. D., and Westbrook, G. L. (1994). Anchoring of protein kinase A is required for modulation of AMPA/kainate receptors on hippocampal neurons. Nature *368*, 853-856.

Rothman-Denes, L. B., and Cabib, E. (1970). Two forms of yeast glycogen synthetase and their role in glycogen accumulation. Proc Natl Acad Sci U S A *66*, 967-974.

Rothman-Denes, L. B., and Cabib, E. (1971). Glucose 6-phosphate dependent and independent forms of yeast glycogen synthetase. Their properties and interconversions. Biochemistry *10*, 1236-1242.

Ruis, H., and Schuller, C. (1995). Stress signaling in yeast. Bioessays *17*, 959-965.

Rutter, J., Michnoff, C. H., Harper, S. M., Gardner, K. H., and McKnight, S. L. (2001). PAS kinase: an evolutionarily conserved PAS domain-regulated serine/threonine kinase. Proc Natl Acad Sci U S A *98*, 8991-8996.

Rutter, J., Probst, B. L., and McKnight, S. L. (2002). Coordinate regulation of sugar flux and translation by PAS kinase. Cell *111*, 17-28.

Sampath, P., Mazumder, B., Seshadri, V., Gerber, C. A., Chavatte, L., Kinter, M., Ting, S. M., Dignam, J. D., Kim, S., Driscoll, D. M., and Fox, P. L. (2004). Noncanonical function of glutamyl-prolyl-tRNA synthetase: gene-specific silencing of translation. Cell *119*, 195-208.
Schulman, H. (2004). Activity-dependent regulation of calcium/calmodulin-dependent protein kinase II localization. J Neurosci *24*, 8399-8403.

Sharrocks, A. D., Yang, S. H., and Galanis, A. (2000). Docking domains and substrate-specificity determination for MAP kinases. Trends Biochem Sci *25*, 448-453.

Shaw, E., McCue, L. A., Lawrence, C. E., and Dordick, J. S. (2002). Identification of a novel class in the alpha/beta hydrolase fold superfamily: the N-myc differentiation-related proteins. Proteins *47*, 163-168.

Shen, F., Look, K. Y., Yeh, Y. A., and Weber, G. (1998). Increased uridine kinase (ATP: uridine 5'-phosphotransferase; EC 2.7.1.48) activity in human and rat tumors. Cancer Biochem Biophys *16*, 1-15.

Shenton, D., and Grant, C. M. (2003). Protein S-thiolation targets glycolysis and protein synthesis in response to oxidative stress in the yeast Saccharomyces cerevisiae. Biochem J *374*, 513-519.

Shih, M., Lin, F., Scott, J. D., Wang, H. Y., and Malbon, C. C. (1999). Dynamic complexes of beta2-adrenergic receptors with protein kinases and phosphatases and the role of gravin. J Biol Chem *274*, 1588-1595.

Singh, P., Jain, A., and Kaur, G. (2004). Impact of hypoglycemia and diabetes on CNS: correlation of mitochondrial oxidative stress with DNA damage. Mol Cell Biochem *260*, 153-159.

Sirover, M. A. (1999). New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. Biochim Biophys Acta *1432*, 159-184.

Skurat, A. V., and Dietrich, A. D. (2004). Phosphorylation of Ser640 in muscle glycogen synthase by DYRK family protein kinases. J Biol Chem *279*, 2490-2498.

Skurat, A. V., Dietrich, A. D., and Roach, P. J. (2000). Glycogen synthase sensitivity to insulin and glucose-6-phosphate is mediated by both NH2- and COOH-terminal phosphorylation sites. Diabetes *49*, 1096-1100.

Skurat, A. V., and Roach, P. J. (2000). 2nd edn (Philadelphia: Lippicont-Raven).

Skurat, A. V., Wang, Y., and Roach, P. J. (1994). Rabbit skeletal muscle glycogen synthase expressed in COS cells. Identification of regulatory phosphorylation sites. J Biol Chem *269*, 25534-25542.

Sobel, S. G., and Wolin, S. L. (1999). Two yeast La motif-containing proteins are RNA-binding proteins that associate with polyribosomes. Mol Biol Cell *10*, 3849-3862.

Sreenan, S. K., Zhou, Y. P., Otani, K., Hansen, P. A., Currie, K. P., Pan, C. Y., Lee, J. P., Ostrega, D. M., Pugh, W., Horikawa, Y.*, et al.* (2001). Calpains play a role in insulin secretion and action. Diabetes *50*, 2013-2020.

Stahl, J., Welfle, H., and Bielka, H. (1972). Studies on proteins of animal ribosomes. XIV. Analysis of phosphorylated rat liver ribosomal proteins by two-dimensional polyacrylamide gel electrophoresis. FEBS Lett *26*, 233-236.

Stein, S. C., Woods, A., Jones, N. A., Davison, M. D., and Carling, D. (2000). The regulation of AMP-activated protein kinase by phosphorylation. Biochem J *345 Pt 3*, 437-443.

Steinacker, P., Schwarz, P., Reim, K., Brechlin, P., Jahn, O., Kratzin, H., Aitken, A., Wiltfang, J., Aguzzi, A., Bahn, E.*, et al.* (2005). Unchanged survival rates of 14-3-3gamma knockout mice after inoculation with pathological prion protein. Mol Cell Biol *25*, 1339-1346.

Tafuri, S. R., and Wolffe, A. P. (1990). Xenopus Y-box transcription factors: molecular cloning, functional analysis and developmental regulation. Proc Natl Acad Sci U S A *87*, 9028-9032.

Tasken, K., and Aandahl, E. M. (2004). Localized effects of cAMP mediated by distinct routes of protein kinase A. Physiol Rev *84*, 137-167.

Taylor, B. L., and Zhulin, I. B. (1999). PAS domains: internal sensors of oxygen, redox potential, and light. Microbiol Mol Biol Rev *63*, 479-506.

ter Haar, E., Coll, J. T., Austen, D. A., Hsiao, H. M., Swenson, L., and Jain, J. (2001). Structure of GSK3beta reveals a primed phosphorylation mechanism. Nat Struct Biol *8*, 593-596.

Thomas, J. A., Schlender, K. K., and Larner, J. (1968). A rapid filter paper assay for UDPglucose-glycogen glucosyltransferase, including an improved biosynthesis of UDP-14C-glucose. Anal Biochem *25*, 486-499.

Traugh, J. A., and Traut, R. R. (1973). Recent advances in the preparation of mammalian ribosomes and analysis of their protein composition. Methods Cell Biol *7*, 67-103.

Tsukada, M., and Gallwitz, D. (1996). Isolation and characterization of SYS genes from yeast, multicopy suppressors of the functional loss of the transport GTPase Ypt6p. J Cell Sci *109 (Pt 10)*, 2471-2481.

Turner, M. D., Cassell, P. G., and Hitman, G. A. (2005). Calpain-10: from genome search to function. Diabetes Metab Res Rev.

Van Landuyt, L., Lissens, W., Stouffs, K., Tournaye, H., Van Steirteghem, A., Liebaers, I., Blagosklonova, O., and Bresson, J. L. (2001). The role of USP9Y and DBY in infertile patients with severely impaired spermatogenesis. Mol Hum Reprod *7*, 691-693.

Vassilev, A., Kaneko, K. J., Shu, H., Zhao, Y., and DePamphilis, M. L. (2001). TEAD/TEF transcription factors utilize the activation domain of YAP65, a Src/Yes-associated protein localized in the cytoplasm. Genes Dev *15*, 1229-1241.

Venema, J., and Tollervey, D. (1999). Ribosome synthesis in Saccharomyces cerevisiae. Annu Rev Genet *33*, 261-311.

Ventimiglia, F. A., and Wool, I. G. (1974). A kinase that transfers the gamma-phosphoryl group of GTP to proteins of eukaryotic 40S ribosomal subunits. Proc Natl Acad Sci U S A *71*, 350-354.

Walton, G. M., and Gill, G. N. (1973). Adenosine 3',5'-monophosphate and protein kinase dependent phosphorylation of ribosomal protein. Biochemistry *12*, 2604-2611.

Wenger, R. H., and Katschinski, D. M. (2005). The hypoxic testis and post-meiotic expression of PAS domain proteins. Semin Cell Dev Biol.

Westermann, P., Nygard, O., and Bielka, H. (1981). Cross-linking of Met-tRNAf to eIF-2 beta and to the ribosomal proteins S3a and S6 within the eukaryotic inhibition complex, eIF-2.GMPPCP.Met-tRNAf.small ribosomal subunit. Nucleic Acids Res *9*, 2387-2396.

Wharton, W., and Pledger, W. J. (1981). Regulation of uridine kinase activity in BALB/C-3T3 cells by serum components. In Vitro *17*, 706-712.

Wilson, W. A., Wang, Z., and Roach, P. J. (2002). Systematic identification of the genes affecting glycogen storage in the yeast Saccharomyces cerevisiae: implication of the vacuole as a determinant of glycogen level. Mol Cell Proteomics *1*, 232-242.

Wong, W., and Scott, J. D. (2004). AKAP signalling complexes: focal points in space and time. Nat Rev Mol Cell Biol *5*, 959-970.

Yaffe, M. B. (2002). How do 14-3-3 proteins work?-- Gatekeeper phosphorylation and the molecular anvil hypothesis. FEBS Lett *513*, 53-57.

Yaffe, M. B. (2004). Master of all things phosphorylated. Biochem J *379*, e1-2.

Yang, S. H., Whitmarsh, A. J., Davis, R. J., and Sharrocks, A. D. (1998). Differential targeting of MAP kinases to the ETS-domain transcription factor Elk-1. Embo J *17*, 1740-1749.

Zanchin, N. I., and McCarthy, J. E. (1995). Characterization of the in vivo phosphorylation sites of the mRNA.cap-binding complex proteins eukaryotic initiation factor-4E and p20 in Saccharomyces cerevisiae. J Biol Chem *270*, 26505-26510.

Zetterqvist, O., Ragnarsson, U., Humble, E., Berglund, L., and Engstrom, L. (1976). The minimum substrate of cyclic AMP-stimulated protein kinase, as studied by synthetic peptides representing the phosphorylatable site of pyruvate kinase (type L) of rat liver. Biochem Biophys Res Commun *70*, 696-703.

Zhang, F., Strand, A., Robbins, D., Cobb, M. H., and Goldsmith, E. J. (1994). Atomic structure of the MAP kinase ERK2 at 2.3 A resolution. Nature *367*, 704-711.

Zhang, W., DePaoli-Roach, A. A., and Roach, P. J. (1993). Mechanisms of multisite phosphorylation and inactivation of rabbit muscle glycogen synthase. Arch Biochem Biophys *304*, 219-225.

VITA

Brandon Linn Probst was born in Burley, Idaho, on July 9, 1976, the son of Steve and Beth Probst. After graduating from Yreka High School, Yreka, California in 1994, he enrolled at Brigham Young University in Provo, Utah. There he received the degree of Bachelor of Science with a major in microbiology and minors in chemistry and molecular biology in 2000. In May 2000 he entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas. He was awarded the degree of Doctor of Philosophy in September 2005. After graduation, he will be a research scientist at Joyant Pharmaceuticals Inc., Dallas, TX. In 1998, he married Stephanie Naseath of Carlsbad, CA. He has three sons: Taylor, Tanner and Hunter.

Permanent Address: 1009 Ann Dr.
Wylie, TX 75098