Two Distinct High Throughput Screens of PAS Kinase Yield Convergent Insight to Enzyme Function

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PAS domain-linked protein kinase enzymes can be 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. Several activators discovered in this screen possessed conspicuous alkyl side chains, potentially offering clues to the properties of an endogenous ligand for the enzyme. With the exception of one compound that exerted inhibitory specificity for PASK but not PKA, CKIε and CaMK II, most inhibitors identified were shown to be nonspecific ATP competitors of the catalytic domain of PAS kinase. The results of these two HT screens provide novel insight into the regulation and biological function of PASK.

Intracellular signal transduction pathways are involved in the regulation of nearly every decision made by living cells. Reversible phosphorylation of tyrosine, serine and threonine residues on proteins is the most common mechanism controlling intracellular signaling (1). Transiently changing phosphorylation events are involved in signaling pathways that govern metabolic decisions, the assembly and disassembly of macromolecular complexes, cell growth, cell death and differentiation. It has been calculated that the mammalian "kinome" encodes 518 members (2). Many of these protein kinase enzymes are poorly understood in terms of regulation and function. PAS kinase (PASK) is one such kinase about which little is known. PASK is composed of a canonical C-terminal serine/threonine kinase domain and two N-terminal PAS domains (3), and is unique in qualifying as the only mammalian protein kinase regulated in *cis* by PAS domains. Although clear evidence has been presented indicative of the regulatory functions of the PAS A domain of PAS kinase (3,4), how this domain might connect the enzyme to intrinsic or extrinsic cues remain unknown.

Mammalian PASK has two orthologs in yeast that coordinately regulate translation and glycogen synthesis (5). Genetic and biochemical data have demonstrated that the yeast PASK enzymes phosphorylate both UDP-glucose pyrophosphorylase (Ugp1p) and glycogen synthase (Gsy2p) to negatively regulate glycogen synthesis (5). The yeast enzymes also phosphorylate three proteins

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[4]The abbreviations used are: 5'TOP, 5'-terminal oligopyrimidine tract; BSA, bovine serum albumin; CKIε, casein kinase Iε; CaMK II, calcium-calmodulin dependent kinase II; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbant assay; FL, full-length; HeLa, human cervical cancer cells; HTS, high throughput screening; IRES, internal ribosome entry sites; MALDI-TOF, matrix-assisted laser desorption ionization – time of flight; PAS, Per-Arnt-Sim; PBS, phosphate buffered saline; PKA, protein kinase A; ROS, reactive oxygen species.

involved in translation: Tiff11p (eIF1A), Caf20p and Sro9p (5). With the exception of glycogen synthase, none of the mammalian counterparts of these proteins have been found to qualify as substrates for the mammalian PASK (data not shown). Furthermore, studies of PASK knockout mice have revealed no overt developmental phenotype and few, if any, clues to the biological function of this enzyme (6). Here we report the results of two unbiased HT screens that provide new insight into pathways both upstream and downstream of mammalian PASK.

EXPERIMENTAL PROCEDURES

Reagents - [γ-^{32}P]-ATP and chromatographic reagents for protein purification were obtained from Amersham Biosciences. Unless otherwise noted all other chemicals and reagents were obtained from Sigma.

Protein Expression and Purification - All substrate proteins except AlaRS were expressed in *E. coli* strain BL21 (DE3) RIL (Stratagene). AlaRS and PASK were expressed in, and purified from, Sf-9 cells infected with a recombinant baculovirus as previously described (3). 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. RPS3a was further purified using MonoS chromatography. 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 – 0.2 mM IPTG/4 h /37°C; RPS3a, AlaRS, GAPDH, and UCK2 – 0.2 mM IPTG/ 16 h /25°C.

Kinase Substrate Screen - 150 L of HeLa-S3 cells (~150 g 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 (pH 7.4), 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%

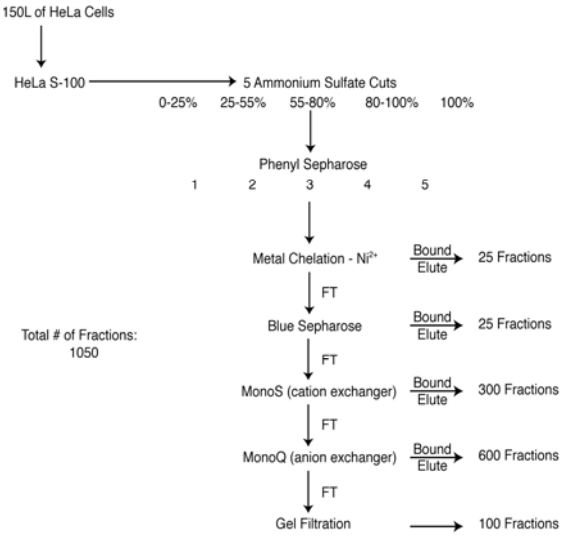


Fig. 1**. Fractionation scheme for the biochemical PASK substrate assay.** 150 liters of HeLa cells were lysed to generate a solube extract. The extract was divided into 5 ammonium sulfate cuts. Each protein precipitate was solubilized and placed over a phenyl sepharose column. Proteins were eluted in 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 and 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.

glycerol. Dialyzed fractions were then batch bound to 2 mL of Ni-NTA agarose and bound proteins eluted in 50 mM Tris (pH 8.0), 5 mM β-ME, 10 mM NaCl and 250 mM imidazole. Eluted proteins were dialyzed in 50 mM Tris (pH 8.0), 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% 1M 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 1mL 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 (3). 40 µl of each fraction was assayed with recombinant PASK 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 (7).

Phosphorylation Site Mapping and Mutagenesis - Substrate phosphorylation sites were identified by subjecting the recombinant protein to phosphorylation by PASK followed by SDS-PAGE and in-gel trypsinolysis. Labeled tryptic peptides were fractionated and identified as previously described for yPsk2 substrates (5). Phosphorylated residues were mutated to alanine via primer sowing method.

HT Chemical Screen - For this assay, the 384 Costar # 3703 white plates were coated at 4ºC overnight with 50 µl of 10 µg/ml Neutravidin (Pierce Chemical Co.) Recombinant PASK was expressed and purified as previously described (3). The assay buffer was composed of 40 mM HEPES (pH 7.0), 100 mM KCl, 6.25 mM $MgCl_2$, 2 mM DTT, 0.35 mM ATP and 1 µM biotinylated substrate peptide No. 1677, NH_2-KKERLLDDRHDSGLDSMKDEEYEQGK-biotin. 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, NH_2-KKERLLDDRHDSGLD(p)SMKDEEYEQGK-biotin. The negative control was assay buffer without added PASK. DMSO was mixed with a complete enzyme reaction in columns 2 and 23 (Fig. 5). 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 was a monoclonal antibody directed against the phosphorylated peptide No. 2491. 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 30 µl 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, 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 PASKwere cherry picked from 3 mM

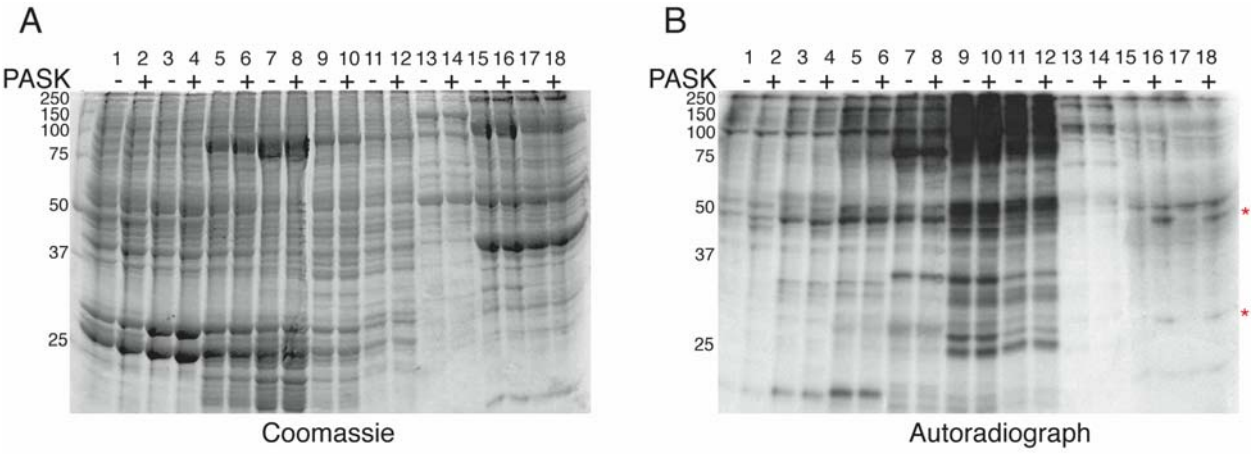


Fig. 2. **Biochemical screen for PASK substrates**. Kinase reactions containing [γ-^{32}P]ATP in the absence (-) or presence (+) of recombinant PASK were analyzed by SDS-PAGE and Coomassie Blue staining (A). Stained gels were dried and exposed to autoradiographic film (B). * Red asterisks denote PASK-dependent 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 a linear salt gradient.

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

Secondary Follow-up Inhibitor Assay – Identified inhibitors of the PASK enzyme were evaluated in a secondary gel-based assay in parallel with protein kinase A (New England Biolabs), calcium/calmodulin-dependent kinase II (New England Biolabs), and casein kinase Iε. Reaction conditions for each enzyme were as follows: PASK phosphorylation assays consisted of 15 nM PASK in 40 mM Hepes (pH7.5), 100 mM KCL, 5 mM $MgCl_2$, and 2 mM [γ-^{32}P]ATP (100-200 cpm/pmol). 5 µg of UCK2 or histone IIB was used as a PASK substrate. 1250U of CaMK II was activated according to manufacturer's protocol. Each CaMK II phosphorylation reaction contained 250U of activated CaMK II, 1X CaMK II reaction buffer, 2 mM [γ-^{32}P]ATP (100-200 cpm/pmol) and 5 µg of histone III as substrate. PKA phosphorylation assays consisted of 5U of PKA, 1X PKA reaction buffer, 2 mM [γ-^{32}P]ATP (100-200 cpm/pmol), and 5 µg of histone III as a substrate. CKIε phosphorylation assays were performed using 20 nM CKIε in 40 mM Hepes (pH 7.0), 5 mM MgCl2 and 2 mM [γ-^{32}P]ATP (100-200 cpm/pmol). 5 µg of α-casein was used as the CKIε substrate. All kinase reactions were performed in 30 µl reaction volumes, and incubated at 30°C for 30 min. Reactions were stopped by the addition of 2X-SDS loading buffer, boiled for 5 min, and loaded onto SDS-PAGE. Coomassie stained substrate bands were excised from the gel, placed in 10 ml of scintillation fluid, and radioactivity detected with an LS 6500 scintillation counter (Beckman).

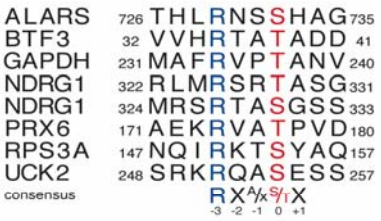
RESULTS

Biochemical HT Screen for PASK Substrates - In order to discover direct phosphorylation targets of PASK, we performed 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

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



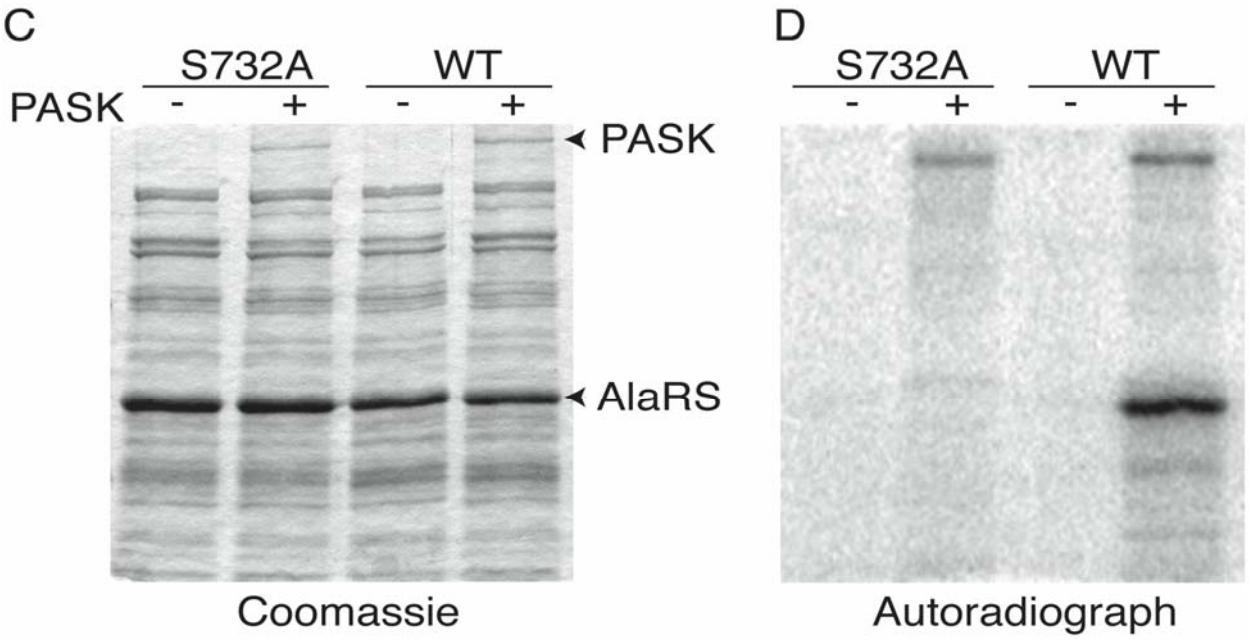


Fig. 3. Identification of PASK substrates and phosphorylation sites.
Table (A) includes those substrates identified as PASK *in vitro* substrates in which phosphorylation sites were mapped. (B) Alignment of PASK-dependent phosphorylation sites in substrates identified and the discovery of a consensus motif: 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 variant 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).

substrates in their native and often hetero-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, hetero-oligomeric state (5). 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 (Fig. 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 (8). The advantage of using ammonium sulfate precipitation as the first step is the long-term stability of proteins in this precipitant state. Thus, 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 were collected as a function

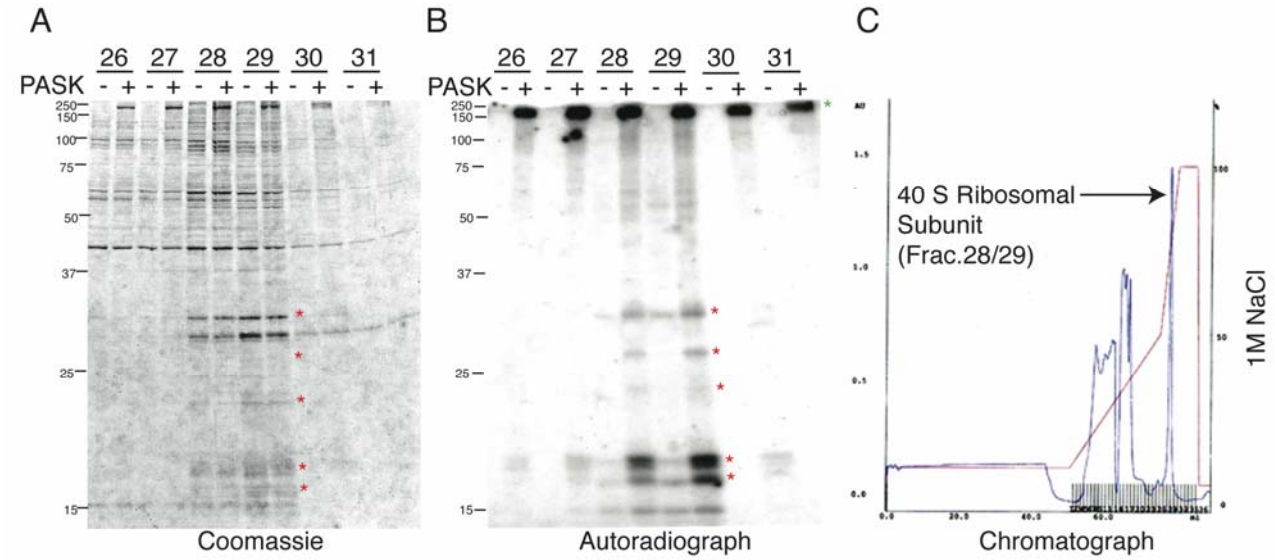


Fig. 4. PASK phosphorylates multiple subunits of the 40S ribosome. Kinase reactions containing [γ-^{32}P]ATP assayed in the absence (-) or presence (+) of recombinant PASK were analyzed by SDS-PAGE and Coomassie Blue staining (A). Stained gels were dried and exposed to autoradiographic film (B). Fractions 26-31 correspond to successive fractions eluted off 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 denotes PASK-dependent phosphorylation of individual ribosomal proteins while * green asterisks denote PASK-dependent autophosphorylaton.

of isocratic reduction in salt concentration. Each of the 5 phenyl sepharose fractions were separately placed over a series of chromatographic media as described in Fig. 1, leading to the multi-dimensional separation of HeLa proteins into roughly 1,000 fractions.

Each fraction was tested for PASK-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 Fig. 2. Although a number of proteins were phosphorylated in a PASK-independent manner (Fig. 2*B*, *lanes 9-10*), two polypeptides with apparent molecular masses of 43kD and 30kD were clearly phosphorylated only when PASK enzyme was added (Fig. 2*B*, *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 (Fig. 3*A*). 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 (supplemental data, Table I), we chose these seven polypeptides for more extensive analysis.

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 (EXPERIMENTAL PROCEDURES). 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

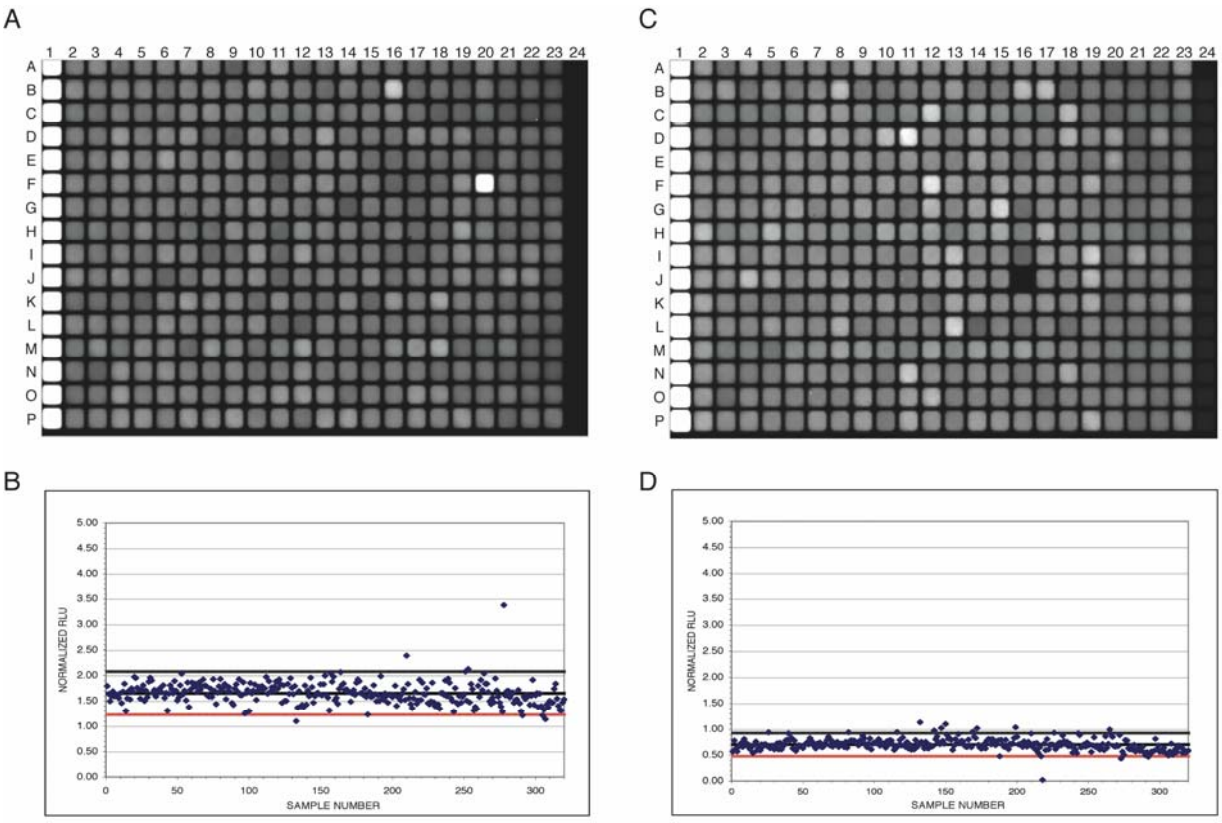


Fig. 5. High-throughput screen of PASK identifies activators and inhibitors.
Luminescence image of 384-well PASK assay plates identifying an activator, well F20 (A) and an inhibitor, well J16 (C). The plates contain a positive control in lane 1, negative control in lane 24, and internal DMSO controls 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.

phosphorylation sites. Fig. 3*C* and *D* show an example of this, in which a serine to alanine substitution of AlaRS residue 732 fully eliminated PASK-dependent phosphorylation. In all cases, with the exception of NDRG1, PASK phosphorylated each target substrate at a single site. The identification of these eight PASK phosphorylation sites revealed a consensus motif, R-X-A/x-S*/T* (Fig. 3*B*). An R-X-X-S*/T* consensus has been recognized as the preferred substrate of several other kinases, in particular calcium/calmodulin-dependent kinase II (9), the founding member of the sub-classification group of the mammalian kinome to which PASK belongs (2). Partial specification of PASK substrate recognition may be conferred by alanine at the -1 position relative to the serine or threonine modified by the enzyme.

Alanyl-tRNAsynthetase 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 C-terminal 261 residues of AlaRS. The identified phosphorylation site, S732, lies between domains thought to be important for tRNA editing and oligomerization (10,11). 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 of the C-terminal tail. This region is flexible, susceptible to proteolysis (12) and

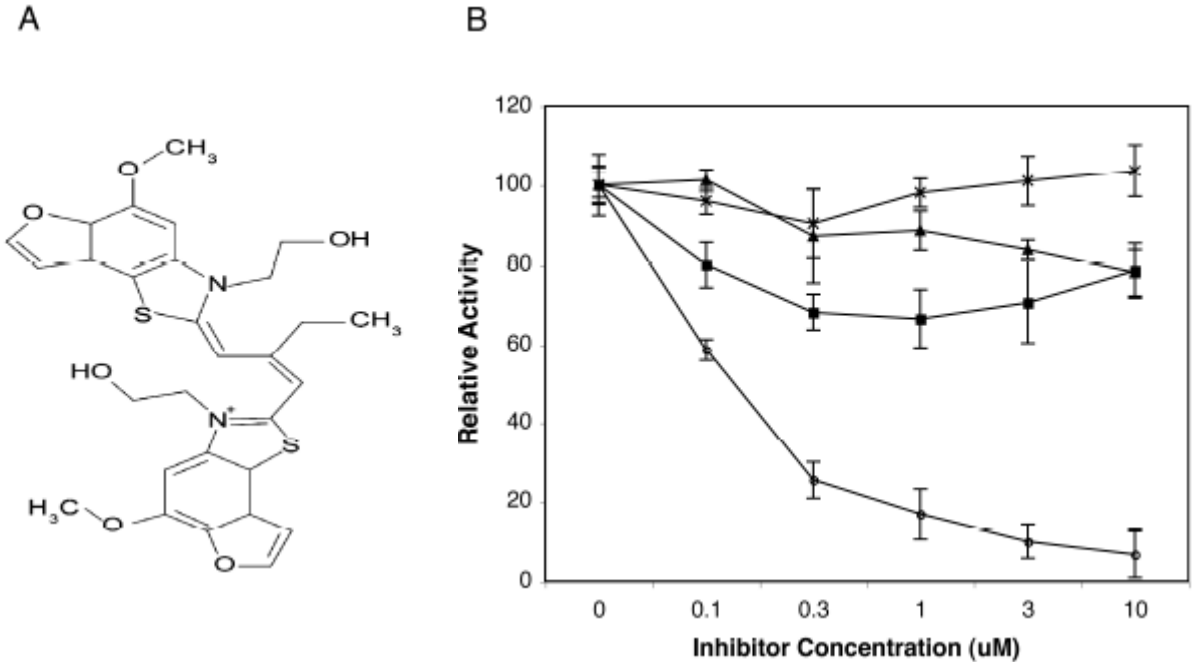


Fig. 6. An HTS identified a PASK specific inhibitor. Chemical structure (A) and inhibition curves of a PASK (○) specific inhibitor identified in the HTS that does not inhibit PKA (x) or CKIε (▲). CaMK II (■) is marginally inhibited, retaining ~75-80% activity (B). Gel-based assays were performed as described in EXPERIMENTAL PROCEDURES using 0.1-10 μM compound. Reactions were quenched at 30 min and the incorporation of ^{32}P into substrates was deteremined using liquid scintillation counting of excised phosphorylated substrates from the gel.

dispensable for enzymatic function (unpublished observations). GAPDH is classically known for its "housekeeping" role in glycolysis although other roles have emerged (13). We mapped the site of phosphorylation to threonine 237, which is conserved across all species (14) and positioned at the homotetramer interface (15). NDRG1 belongs to a family of stress-regulated proteins for which no clear function has yet to be assigned (16). We identified serine residues 328 and 330, in the C-terminal tail of NDRG1, as the sites phosphorylated by PAS kinase. These sites, Ser-328 and Ser-330, were already known to be phosphorylated *in vivo* (17). 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 threonine residue 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 (18).

40S Ribosomal Proteins are PASK Substrates - An unexpected abundance of PASK substrates were observed to be phosphorylated in two fractions, 28 and 29 (Fig. 4, *A* and *B*). These fractions consisted of a very sharp OD_{280} peak that eluted off the monoQ column at approximately 500mM NaCl (Fig. 4*C*). The sharpness of this peak, coupled with distinct absorbance at OD_{260}, indicated 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 are contained within 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

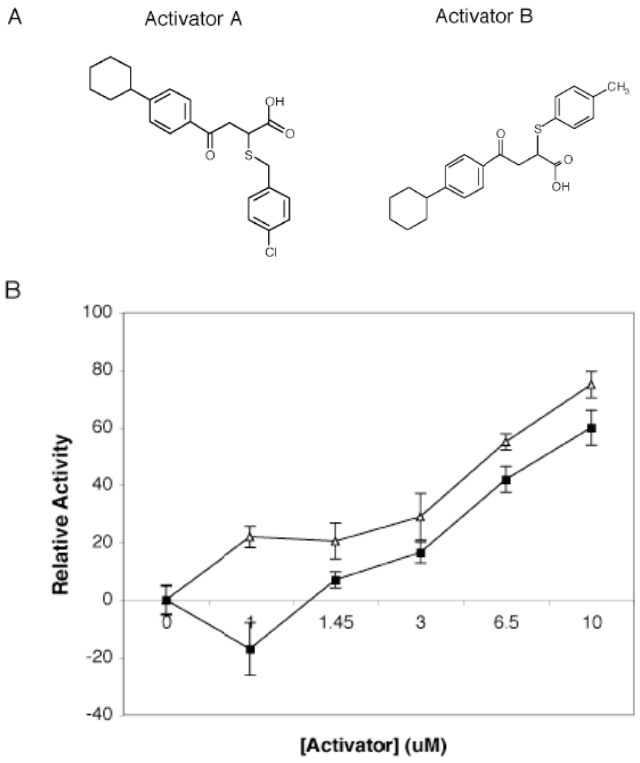


Fig. 7. An HTS identified PASK activators. Chemical structures and activation curves of 2 PASK activators, activator A (Δ) and activator B (■) that mildly activated the PASK enzyme. Assays performed as described for HTS using 1-10 μM compound

identified the precise sites of PASK-mediated phosphorylation on each polypeptide.

Chemical HT Screen for Modulators of PASK - 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 (EXPERIMENTAL PROCEDURES). The assay was performed in 384-well 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 Fig. 5 *A* 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 (Fig. 5, *B* 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 assay conditions identical to the initial screen. Out of the 739 inhibitors identified in the primary HT screen, 95 were observed in follow-up assays to inhibit PASK in a dose-dependent manner. The 95 confirmed inhibitors were 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 of inhibitory to the catalytic activity to CKIε, CaMK II and PKA as they were to PASK. One inhibitor did display modest inhibitory specificity. As shown in Fig. 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 inhibitory towards PAS kinase, yet failed to inhibit PKA, CKIε and CaMK.

Of the initially identified 665 activators, only 10 were observed to retain activity upon follow-up rescreening. Furthermore, these compounds were weak activators, modestly activating PASK between 1.6 and 2 fold levels. It is notable that the HT screen identified two highly related compounds 2-4-(4-Chloro-benzylsulfanyl)-4-(4-cyclohexyl-phenyl)-4-oxo-butyric acid (Activator A) and 4-(4-Cyclohexyl-phenyl)-4-oxo-2-p-tolylsulfanyl-butyric acid (Activator B) only differing in the carbon chain of the thiol-linker and the electron withdrawing chlorine as opposed to the electron donating methyl on the benzyl ring (Fig. 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. Additionally, three of the other confirmed activators contain conspicuous alkyl side chains characteristic of lipid-like molecules, of which such compounds were highly underrepresented in the compound library (supplemental data, Fig. 1).

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 appear to be 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 (19-22), and that purified ribosomal subunits are phosphorylated upon exposure to an exogenous kinase (23-26). Even more compelling is the phosphorylation of ribosomes by *in vivo* ^{32}P-labeling experiments in a wide variety of animal and plant tissues (19,27-32). Many of the 40S ribosomal proteins phosphorylated *in vivo* match the identities of the PASK substrates identified herein, including ribosomal proteins S2, S6, S8 and S10. We have no evidence indicative of functional significance for 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 (33). Furthermore, careful measurements calculate an average half-life of just over 20 min for the turnover of ribosomal protein phosphorylation in reticulocytes (34). 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 (34). 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) (35). Likewise, dephosphorylation of eIF4E leads to translation of specific mRNAs that have internal ribosome entry sites (IRES) (36). The observations reported herein open the possibility that PASK may regulate translational selectivity in a here-to-fore 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 (37). An emerging role of aminoacyl-tRNA synthetases, other than their classical role in tRNA charging, includes translational regulation (38). 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 (39).

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* (5). One yeast PASK substrate, Tif11p (eIF1A), mediates the transfer of Met-tRNA to the 40S ribosomal subunit generating the 40S preinitiation complex (40). 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 (41). Thus, in the course of evolution, the direct PASK substrate may have changed from Tif11p (eIF1A) in yeast to RPS3A in mammals, yet the ultimate regulatory consequences governing 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 (5). Among these are the DED1 and DBP1 genes required for translation initiation and genetically associated to both eIF4E and CAF20 (42).

An additional 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 (43). Recently, *Arabidopsis thaliana* BTF3 was shown in a yeast 2-hybrid screen to interact with eIF4E (44). 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 (44). 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 (45). Speculatively, it is possible that PAS kinase mediated phosphorylation of Caf20p in yeast is functionally analogous to the role of the mammalian enzyme in phosphorylating BTF3.

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 discovered in this study. Uridine-cytidine kinase 2 is one such metabolic enzyme discovered as a PASK 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 (46). Furthermore, an associative link exists between increased rates of uridine kinase activity, the energy requirements of the cell (47,48), and the proliferation and transformation of human cells (49).

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 translational elongation (50). PAS kinase has recently been shown to link glucose availability with the regulation of specific transcription and translation events. Rutter and colleagues have demonstrated that preproinsulin gene expression and translation are induced by glucose in a PASK-dependent manner (51). These data argue in favor of nutrient-coupled translational regulation of specific mRNAs by PAS kinase.

The switch between global and regulated translation of specific mRNAs is well documented to be induced under conditions of cellular stress. The inhibition of global translation results in an increased saving of cellular energy, preventing the synthesis of unwanted polypeptides that may interfere with stress-response pathways. Such global inhibition of translation may facilitate selective translation of proteins that are required for cell survival under stress conditions that trigger this change including 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 (52,53). Peroxiredoxins represent a new class of antioxidant enzymes dedicated to protect the cell against oxidative stress. PASK-dependent phosphorylation of peroxiredoxin 6 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 cellular stress and initiate an adaptive response that

tailors 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, the question arises as to under what conditions might PASK be stimulated to phosphorylate these substrates? Studies from yeast show that PSK1 is upregulated in response to H_2O_2 (54). Furthermore, the promoters of PSK1 and PSK2 have conserved stress response elements (STREs) known to regulate the expression of other genes in response to oxidative stress (55). Thus, studies of PASK in mammals and yeast collectively point to the sensing of nutrients and stress.

The HT screen used to identify synthetic chemicals capable of regulating PASK led to the identification of both activators and inhibitors of the enzyme. Follow-up analysis on activators identified in the primary screen gave evidence that the vast majority of initially identified compounds were false-positives. Among the handful of compounds that survived our secondary assay, two observations are note worthy. First, two of the chemical activators, designated as activator A and activator B in Fig. 7, were remarkably similar in chemical structure. These compounds, 2-4-(4-chloro-benzylsulfanyl)-4-(4-cyclohexyl-phenyl)-4-oxo-butyric acid (activator A) and 4-(4-cyclohexyl-phenyl)-4-oxo-2-p-tolylsulfanyl-butyric acid (activator B) differed only in the carbon chain of the thiol-linker and an electron withdrawing chlorine as opposed to the electron donating methyl on the benzyl ring. The fact that both of these compounds, emerging from a screen of over 200,000 synthetic organic chemicals, survived to the final list of activators, supports the conclusion that this particular chemical structure is indeed capable of modest activation of the purified PASK enzyme. Second, three of the remaining 10 confirmed activators contain a conspicuous alkyl side chain. Given that less than 0.1% of the synthetic chemicals in the 200,000 test compound library contain alkyl side chains, we speculate that this particular chemical feature will prove to be important to the ultimate mechanism of activation.

Relative to PASK activator compounds found in our HT screen, a far higher percentage of PASK inhibitors survived follow-up confirmation. Among these, all but one proved to be non-specific when cross-assayed against casein kinase Iε (CKIε), protein kinase A (PKA) or calcium/calmodulin-dependent protein kinase II (CaMK II). As shown in Fig. 6, 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 without affecting the activities of CKIε, PKA or CaMK II. Given that PASK and CaMK II are members of a small, related family within the vertebrate protein kinome, it is encouraging to think that medicinal chemistry efforts on this compound might someday lead to a potent and selective inhibitor of PASK.

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Supplemental Data – Table 1

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 qualified 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)[b]	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

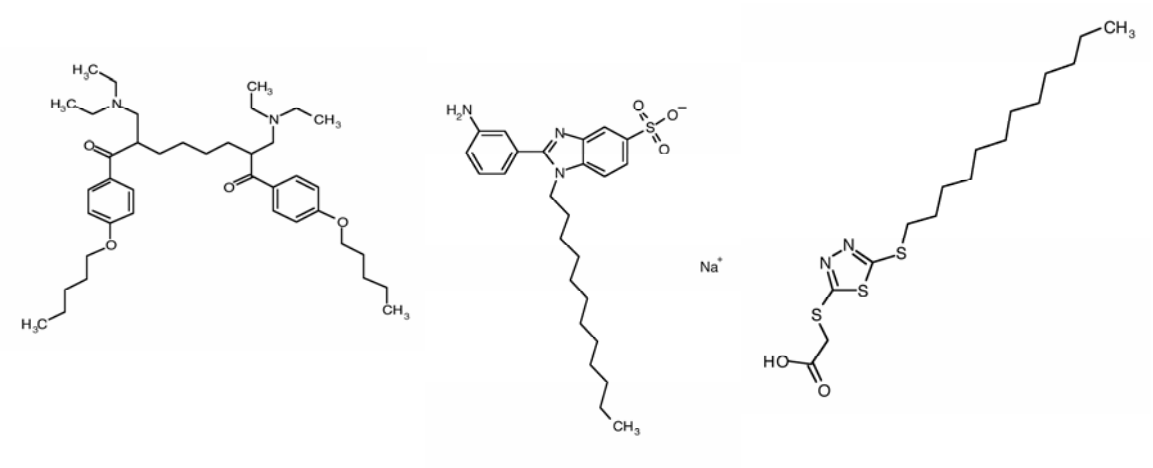
		TNF-induced protein	20556217
C4 B2 Q12	42,+	guanine aminohydrolase	11837778
		glycyl tRNA synthetase	3845409
C3 B2 S15-T	18,+++	Histone H2B	350170
		heparin binding protein HBp15	33150766
		cytoplasmic phosphotyrosyl phosphatase	179661
C3 B2 S15-B	16,+++	histone H2A.5	70686
		histone H2A.F/Z	6912616
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	
		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 beta	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-M1	25,+		
C2 B2 S35-M2	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

Supplemental Data – Figure 1



Chemical structures of three compounds which mildly activated the PASK enzyme. Note that all compounds have alkyl side chains of various lengths.