PAS kinase: An evolutionarily conserved PAS domain-regulated serine/threonine kinase

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PAS domains regulate the function of many intracellular signaling pathways in response to both extrinsic and intrinsic stimuli. PAS domain-regulated histidine kinases are common in prokaryotes and control a wide range of fundamental physiological processes. Similarly regulated kinases are rare in eukaryotes and are to date completely absent in mammals. PAS kinase (PASK) is an evolutionarily conserved gene product present in yeast, flies, and mammals. The amino acid sequence of PASK specifies two PAS domains followed by a canonical serine/threonine kinase domain, indicating that it might represent the first mammalian PAS-regulated protein kinase. We present evidence that the activity of PASK is regulated by two mechanisms. Autophosphorylation at two threonine residues located within the activation loop significantly increases catalytic activity. We further demonstrate that the N-terminal PAS domain is a cis regulator of PASK catalytic activity. When the PAS domain-containing region is removed, enzyme activity is significantly increased, and supplementation of the purified PAS-A domain in trans selectively inhibits PASK catalytic activity. These studies define a eukaryotic signaling pathway suitable for studies of PAS domains in a purified *in vitro* setting.

The PAS domain is a small regulatory module represented in all kingdoms of life. The majority of prokaryotic PAS domains function as the sensor modules of two-component systems (1). One of the best characterized of these two-component systems is the FixL/FixJ pathway of *Rhizobia*. This system couples nitrogen fixation to oxygen concentration by measuring oxygen and directing transcription of the genes whose products are required for nitrogen fixation (2–4). The mechanism relies on the ability of the single PAS domain of the FixL protein to sense oxygen levels via an attached heme moiety and couple that signal to conformational changes on the surface of the PAS domain (5). The conformation of the FixL PAS domain controls the activity of its transmitter histidine kinase, presumably by regulating PAS domain/kinase domain interactions differentially in the oxygen-bound and oxygen-free states (6). Exclusively in the oxygen-free state, the histidine kinase catalyzes a series of phosphotransfer reactions culminating in the phosphorylation and activation of the transcription factor FixJ. Phospho-FixJ is then competent to activate the transcription of a number of genes whose products are necessary for nitrogen fixation. Numerous other PAS domain-containing two-component systems have been identified in prokaryotic organisms (1, 7). Presumably, most such systems use a mechanism similar to FixL/FixJ, with PAS domain conformation, under control of a specific stimulus, regulating the activity of its cognate histidine kinase and thereby controlling specific cellular responses.

In eukaryotic organisms, histidine kinases and two-component signaling systems are rare. Phosphorylation relay systems involving serine/threonine and tyrosine kinases, however, are commonly used to link signaling pathways. Two paradigms help to define the common modes of regulation of eukaryotic protein kinases. First, phosphorylation of the protein kinase itself often serves to regulate its catalytic activity. An exposed loop near the active site of the kinase catalytic domain, termed the activation loop, is a major site on kinases for regulatory phosphorylation (8, 9). Many kinases require one or two amino acids in this loop to be phosphorylated for efficient catalytic activity. This mechanism allows interkinase regulation and forms the basis for the kinase cascades prevalent in eukaryotic cells.

A second regulatory paradigm is the use of an autoinhibitory segment attached in cis to the polypeptide specifying the protein kinase. Such regions have been discovered in a number of kinase subfamilies, with one of the best understood being the calmodulin-dependent kinases (10). Members of this family have a conserved region C-terminal to the canonical kinase domain that folds back onto the catalytic domain perturbing the ATP-binding site (11). Ca^{2+}-bound calmodulin activates the kinase by binding the regulatory region and relieving the kinase domain of this inhibitory interaction. The use of an inhibitory domain provides a mechanism for a unique apical signal to trigger a kinase cascade. This signal can then be propagated and amplified by a kinase phosphorylation cascade, resulting in the appropriate physiological response.

Here we report the discovery of an evolutionarily conserved protein kinase designated PAS kinase (PASK). The primary amino acid sequence of this polypeptide appears to specify two PAS domains as well as a canonical serine/threonine kinase domain. PASK is conserved with orthologs in humans, flies, and yeast. We present evidence that phosphorylation of the activation loop within the catalytic domain is critical for PASK activity. We also demonstrate that the enzymatic activity of PASK is subject to inhibition by the N-terminal PAS domain, designated PAS-A. Finally, evidence is presented indicating that the PAS-A domain expressed separately is able to transinactivate its cognate kinase domain *in vitro*.

Materials and Methods

Cloning and Expression of Wild-Type and Mutant Human PASK (hPASK). Phage clones from a HeLa cDNA library (λSCREEN/Novagen), which hybridized to a KIAA0135 (GenBank accession no. D50925) probe, were purified, and corresponding plasmid subclones were generated according to the manufacturers' protocols. All hPASK proteins were expressed by using the BAC-to-BAC baculovirus expression system (GIBCO/BRL). Homogenate from infected insect cells was generated by incubation in hypotonic buffer (Buffer At: 50 mM Tris, pH8.0/10 mM NaCl/5 mM 2-mercaptoethanol) for 30 min followed by dounce homogenization. Before centrifugation at $10^5 \times g$ for 1 h, NaCl was added to a final concentration of 0.3 M (generating Buffer At-300). The resultant S-100 was purified by using Ni-NTA resin (Qiagen, Chatsworth, CA). Full-length hPASK and point mutants were further purified by using an Amersham Pharmacia FPLC and a MonoQ 5/5 column.

Abbreviations: PASK, PAS kinase; hPASK, human PASK.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF387103).

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Antibody Generation, Native Molecular Weight Determination, and Western Blotting. Rabbit α-hPASK antibody (U2501) was generated by using the hPASK-derived peptide CWQGQDPAEGGQDPRIN (residues 428–440) and purified by using a peptide antigen affinity column, eluting with 0.2 M glycine (pH 2.5). The determination of native molecular weight was performed as described previously (12). HeLa cells were obtained from the National Cell Culture Center (Minneapolis, MN). HeLa cytosol (S-100) was prepared as described (13), except the dounce homogenate was centrifuged immediately at $10^5 \times g$. The resultant pellet was used to generate the nuclear fraction by incubating with 0.4 M NaCl for 30 min, followed by centrifugation at $10^5 \times g$.

Transient Transfections and Immunocytochemistry. HEK293 cells were transfected with a pcDNA3.1 V5/His (Invitrogen)-hPASK expression vector by the standard calcium-phosphate precipitation method. Transfected cells were plated onto matrigel-coated (Becton Dickinson) glass chamber slides. After an 18-h incubation, attached cells were fixed with formaldehyde and stained with α-V5 antibody (Invitrogen). All incubations and washes were carried out in PBS with 3% BSA. After washing, slides were incubated with biotinylated goat anti-mouse IgG antibody, washed, incubated with FITC-avidin, and washed extensively. FITC signal was imaged on a Nikon fluorescent microscope.

Enzyme Assays. hPASK phosphorylation reactions were performed by using 15 nM hPASK in 40 mM Hepes (pH 7.0)/100 mM KCl/5 mM $MgCl_2$/2 mM [γ-^{32}P]ATP (100–200 cpm/pmol). The substrate used was the AMARA peptide (AMARAASAAALARRR) (14). For determination of kinetic constants, peptide concentrations ranged between 0.08 and 1.0 mM. Reactions were incubated at 30°C, and 20-μl aliquots were spotted onto Whatman phosphocellulose (P81) papers. The papers were processed as described (15, 16). Data for kinetic analysis were fit to the Michaelis–Menten equation by using nonlinear regression analysis and the PRISM software package (GraphPad, San Diego). cAMP-dependent protein kinase reactions were performed as described (15). Myosin light chain kinase activity was measured as described (17). PAS domains were expressed in *Escherichia coli* and purified as described (C. Amezcua, S.M.H., J.R., and K.H.G., unpublished work).

Results

Identification of PASK. The PAS domain-containing region of hPASK was originally identified by low-stringency BLAST searches of the GenBank database for proteins similar to the *Bradyrhizobium japonicum* FixL PAS domain. A partial cDNA of hPASK had previously been sequenced by a high-throughput cDNA sequencing initiative (18) (GenBank accession no. D50925). On the basis of this sequence, a full-length cDNA clone of hPASK was obtained from a HeLa cDNA library. The complete sequence predicts a protein containing two PAS domains N-terminal to a serine/threonine kinase domain, which is followed by a 75-aa hydrophilic tail (Fig. 1*A*). The hPASK cDNA clone contained an additional 338 nucleotides of sequence 5′ to the start of the existing database entry, including 90 additional predicted amino acids. The complete hPASK cDNA sequence has been deposited in the GenBank database (accession no. AF387103).

By performing homology searches with hPASK, we identified a *Drosophila melanogaster* homolog, designated dmPASK (GenBank accession no. AAF47129), and two *Saccharomyces cerevisiae* homologs corresponding to ORFs YAL017W and YOL045W, designated PSK1 and PSK2, respectively (http://genome-www.stanford.edu). The sequence similarity among these proteins is restricted to three regions, corresponding to the two predicted PAS domains (Fig. 1 *B* and *C*) and an apparent serine/threonine kinase domain (Fig. 1*D*). When analyzed against an alignment of all known PAS domains by using a Hidden Markov model-based classification algorithm (http://smart.embl-heidelberg.de/) (19), the sequence corresponding to the N-terminal PAS domain was strongly predicted to encode a *bona fide* PAS domain (E value = 4.8×10^{-8}). The sequence of the second PAS domain was also predicted to encode a PAS domain, but with a significantly weaker E value of 7.1×10^{-2}. The kinase domain contains all of the subdomains and conserved residues typical for a catalytic serine/threonine kinase and is interpreted to constitute a new member of the Snf1/5′-AMP-dependent protein kinase subfamily (20).

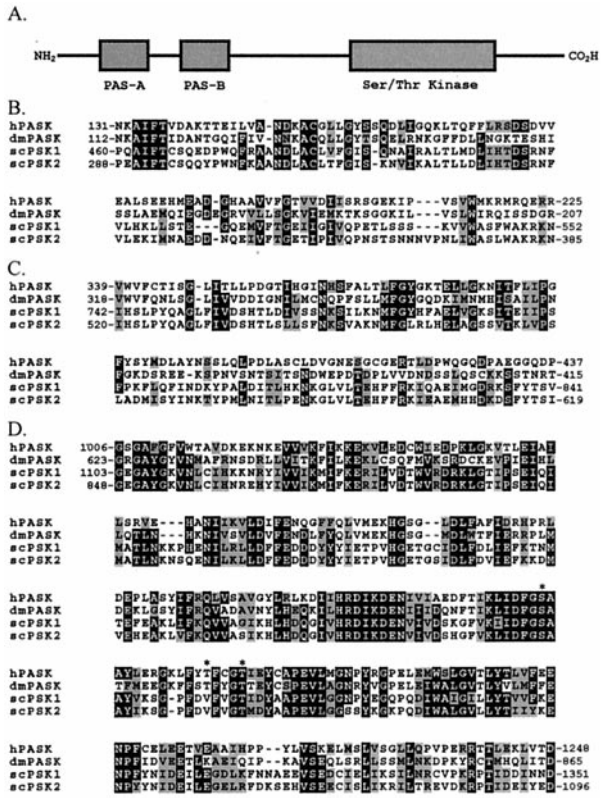

Fig. 1. Human, fly, and yeast PASK genes exhibit three regions of conservation. (*A*) Schematic diagram of the domain architecture of PASK proteins. (*B–D*) Alignment of the three conserved regions of the human, fly, and two yeast PASK proteins. Residues identical or similar in at least three of four PASK proteins are shaded in black and gray, respectively. (*B*) The N-terminal PAS domain (PAS-A). (*C*) The C-terminal PAS domain (PAS-B). (*D*) The serine/threonine kinase domain. Activation loop residues S1149, T1161, and T1165 are marked with asterisks.

Characterization of hPASK Protein. hPASK protein was purified from insect cells infected with a recombinant baculovirus encoding the full-length protein. The purified protein migrated on SDS/PAGE with an apparent molecular mass of 145 kDa, very similar to its predicted molecular mass of 142 kDa. A polyclonal antibody (U2501) directed against a peptide corresponding to residues 428–440 of hPASK was prepared in rabbits. When subjected to Western blotting with this antibody, HeLa cytosol showed one strongly reactive species that migrated identically to recombinant hPASK protein, indicating that the hPASK cDNA encodes a full-length polypeptide.

Further characterization of the hPASK protein included analysis of its homo- and/or hetero-oligomeric state. Purified recombinant hPASK and crude HeLa cytosol were subjected to a combination of glycerol gradient sedimentation and gel filtra-

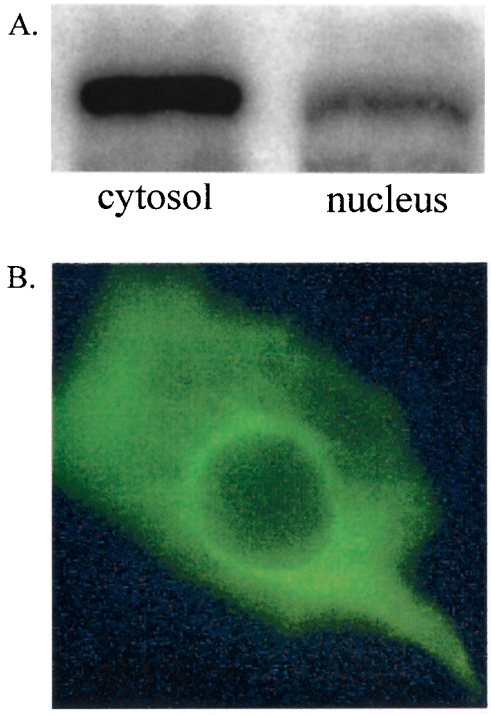

Fig. 2. hPASK protein is predominantly cytosolic. (*A*) HeLa cells were fractionated into $10^5 \times g$ soluble (cytosol) and pellet fractions. The $10^5 \times g$ pellet was extracted with 0.4 M NaCl (nucleus). Cytosolic and nuclear extracts were analyzed by Western blotting by using a polyclonal α-PASK antibody (U2501). (*B*) Immunostaining of HEK293 cells transfected with an expression vector encoding a V5 epitope-tagged version of hPASK. Cells were fixed and stained by using α-V5 monoclonal antibody and visualized by using FITC-tagged secondary antibody.

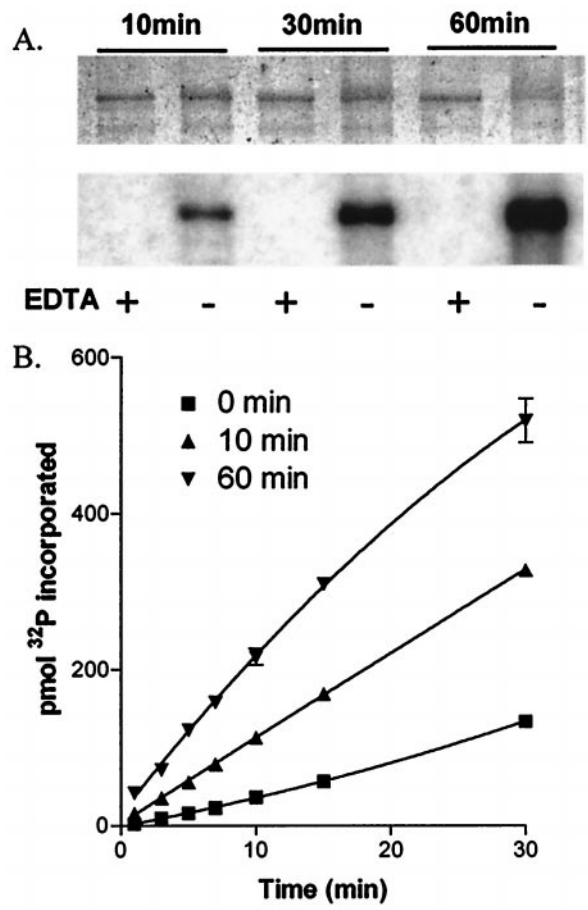

Fig. 3. hPASK is activated by phosphorylation. (*A*) Purified recombinant hPASK was incubated at 30°C in the presence of 2 mM [γ-^{32}P]ATP for the indicated time in the presence or absence of 10 mM EDTA. Samples were resolved by SDS/PAGE followed by silver staining (*Upper*). Incorporation of ^{32}P into the hPASK protein was visualized by autoradiography (*Lower*). (*B*) Untreated hPASK enzyme and the 10- and 60-min prephosphorylated samples from *A* were incubated with 0.2 mM AMARA peptide (AMARAASAAALARRR) and 2 mM [γ-^{32}P]ATP. Reactions were quenched at the times indicated, and incorporation of ^{32}P into peptide substrate was determined by liquid scintillation counting of purified peptide.

tion. These two methods facilitate experimental determination of the sedimentation coefficient and hydrodynamic radius of a specific complex, allowing calculation of a native molecular weight (21). Recombinant and HeLa-derived hPASK migrated similarly in these two assays, with calculated molecular masses of 180 and 175 kDa, respectively (data not shown). The results of these experiments indicate that the recombinant enzyme purified from insect cells likely represents a faithful model for studies of the endogenous hPASK enzyme.

Subcellular Localization of hPASK. To assess the subcellular localization of hPASK protein, HeLa cells were lysed and separated into two fractions, the cytosolic S-100 fraction and the salt-extractable P-100 fraction, chiefly comprised of nuclear proteins. Proteins present in these two fractions were subjected to Western blotting by using the U2501 α-hPASK antibody. As shown in Fig. 2*A*, hPASK is largely restricted to the S-100 fraction and presumably predominantly cytosolic. As a second confirmatory assay of subcellular localization, HEK293 cells were transiently transfected with an expression vector encoding V5-tagged, full-length hPASK. The cells were fixed and stained with α-V5 antibody and visualized with FITC-labeled secondary antibody. As shown in Fig. 2*B*, immunoreactivity was excluded from the nucleus and tended to predominate in the perinuclear region. Similar results were obtained by using untagged hPASK and the U2501 α-hPASK antibody (data not shown).

Autophosphorylating Activity of hPASK. To examine the enzymatic properties of hPASK, we incubated the purified enzyme with [γ-^{32}P]ATP, followed by SDS/PAGE and autoradiography. We detected robust Mg^{2+}-dependent incorporation of phosphate into the hPASK polypeptide (see Fig. 3*A*). The observed phosphorylation was because of hPASK kinase activity as an inactivating substitution, K1028R, destroyed the hPASK phosphorylating activity. Autophosphorylation of hPASK was strongly concentration dependent, suggesting that it results from an intermolecular interaction (data not shown).

The hPASK enzyme, as purified from insect cells, exhibited a time-dependent increase in catalytic activity over the course of a 60-min reaction (Fig. 3*B*). To directly ascertain the effect of autophosphorylation on hPASK activity, we preincubated the enzyme in the presence of ATP for 10 and 60 min before addition of AMARA peptide. This peptide substrate is phosphorylated by the Snf1 and AMPK protein kinases (14), both of which have catalytic domains highly related in amino acid sequence to the catalytic domain of hPASK. By comparison with enzyme preincubated without ATP, the autophosphorylated hPASK exhibited a time-dependent increase in initial rate (Fig. 3*B*). The time course of activation correlates with the time course over which hPASK is autophosphorylated (Fig. 3*A*). We conclude that hPASK is strongly activated by autophosphorylation.

Mapping of Autophosphorylation Sites. To obtain a better understanding of the molecular effects of phosphorylation, experi-

Table 1. Kinetic parameters of hPASK variants

	K_m, μM	k_{cat}, min^{-1}
Native hPASK	210 ± 50	60 ± 7.5
S1149A	210 ± 20	23 ± 1.5
T1161A	No detectable activity	
T1165A	No detectable activity	
S1273,77,80A	170 ± 10	66 ± 4.5
S1287,89A	200 ± 30	59 ± 7.5
S1273-89A	240 ± 40	60 ± 7.5
hPASKΔN	240 ± 30	289 ± 16

The K_m and k_{cat} values ± SEM are shown for native hPASK and hPASK mutants. The T1161A and T1165A mutant had no detectable kinase activity and were not analyzed further. Purified enzymes were assayed under conditions of varying AMARA peptide concentrations. Initial velocities were fit to the Michaelis–Menten equation using nonlinear regression to generate the K_m and k_{cat} values. Kinetic parameters were determined at least three independent times for each enzyme.

ments were conducted to determine the precise sites of autophosphorylation on the hPASK enzyme. To map these sites, we allowed the enzyme to autophosphorylate with [γ-^{32}P]ATP and then subjected it to complete trypsinolysis followed by reverse-phase HPLC. Peaks of radioactivity were analyzed by electrospray ionization–mass spectrometry. Three ions were observed to exhibit a mass corresponding to that of a predicted tryptic fragment of hPASK plus a multiple of 80, the mass of a phosphate group. Each of these putative phosphorylation sites was confirmed by phosphatase treatment, which in each case regenerated a peptide of the mass predicted from the DNA sequence. The three sites of phosphorylation corresponded to two distinct areas of the protein. The first tryptic fragment, comprising residues 1157–1179, was detected as both a singly and doubly phosphorylated peptide. This peptide corresponds to the C-terminal half of the canonical activation loop of the kinase domain and contains two threonine residues (T1161 and T1165). We were unable to detect the N-terminal half of the activation loop and thus could not assess the phosphorylation state of a third activation loop residue, S1149.

We identified two other tryptic phosphopeptides corresponding to a second region of hPASK, the C-terminal extension that consists of 75 residues following the catalytic serine/threonine kinase domain. The first of these, comprising residues 1270–1286, was detected as a singly phosphorylated species. This peptide contains three serine residues, S1273, S1277, and S1280. The second tryptic fragment, comprising residues 1287–1318, was also detected as a singly phosphorylated species. This region contains two serine residues, S1287 and S1289, as potential phosphorylation sites. These two regions, the activation loop and C-terminal extension, appear to be the major sites of phosphorylation on hPASK. The four putative phosphorylation sites identified by mass spectrometry correspond with the stoichiometry of ^{32}P incorporation observed after a 60-min reaction (3–4 mol phosphate/mol protein).

Effect of Elimination of Potential Autophosphorylation Sites. Each of the identified potential phosphorylation site residues was independently changed to alanine, an amino acid whose side chain is interpreted to mimic the dephosphorylated state. In addition to the two threonines identified above (T1161 and T1165), we also changed to alanine the third possible phosphorylation site within the putative activation loop (S1149). As shown in Table 1, the S1149A substitution had a relatively small effect on catalytic rate. The other two substitutions of activation loop residues (T1161A and T1165A), however, caused catalytic activity to be lowered below a detectable limit. The importance of phosphorylation of T1161 is consistent with Snf1 and AMPK. Phosphorylation of the corresponding residue in each of these kinases is necessary for catalytic activity, and substitution to alanine completely inactivates both enzymes (22, 23). The T1165 residue is also analogous to residues phosphorylated in members of several other kinase families, including the cyclin-dependent kinases and the mitogen-activated protein kinases (24). These data provide evidence that T1161 and T1165 represent major sites of phosphorylation on hPASK and that the phosphorylation of these residues is necessary for efficient catalytic activity.

The other two locations of autophosphorylation identified previously are localized within the polypeptide extension C-terminal to the canonical kinase domain. The first peptide observed to be phosphorylated contains three serines, S1273, S1277, and S1280. We simultaneously changed these serines to alanine to create the triple mutant S1273,77,80A. The second phosphopeptide from this region contains two serines, S1287 and S1289, which we changed to alanine to obtain the double mutant S1287,89A. In addition to these two variants, we also constructed a variant wherein all five serine residues were changed simultaneously to alanine (S1273–89A). As shown in Table 1, each of these three variants exhibited kinetic parameters indistinguishable from the native hPASK enzyme. The significance of phosphorylation in this region of the protein remains to be determined.

Effect of the PAS Domains on Kinase Activity. To assay the effect of the N terminus of the protein, including the two PAS domains (Fig. 1*A*), on kinase activity, we expressed and purified an hPASK variant missing residues 1–948 (hPASKΔN). Kinetic analysis revealed that, whereas the peptide substrate K_M values for full-length and truncated hPASK enzymes were very similar, the respective k_{cat} values were significantly different (Table 1). The catalytic rate of the hPASKΔN variant was ≈5-fold higher than full-length hPASK. These data provided preliminary evidence that the N-terminal portion of the protein contains a region that inhibits the activity of the catalytic serine/threonine kinase domain.

One possible interpretation of the aforementioned data is that one or both of the PAS domains might serve to negatively regulate the enzymatic activity of PASK. To test this hypothesis, we expressed and purified from bacteria a fragment of hPASK corresponding to the N-terminal PAS domain (PAS-A) fused to the β1 domain of Streptococcal protein G. This PAS-A domain was well folded and stable in solution, facilitating resolution of its three-dimensional structure by NMR spectroscopy (C. Amezcua and K.H.G., unpublished data). Such observations provided unequivocal evidence that the PAS-A domain of hPASK indeed represents a canonical PAS domain. Addition of increasing amounts of the purified PAS-A domain led to a dose-dependent decrease in activity of hPASKΔN, with an IC_{50} of ≈100 μM (Fig. 4*A*). The data shown in Fig. 4*A* are consistent with the prediction that the PAS-A domain of the intact enzyme serves to negatively regulate the catalytic serine/threonine kinase domain. We have been unable to identify a fragment of PASK corresponding to the second PAS domain, PAS-B, which will fold properly when expressed in bacteria. Therefore, we are currently unable to assess the role of this PAS domain in regulation of kinase activity.

Specificity of PAS-A Domain/Kinase Domain Functional Interaction. We used two approaches to assess the specificity of the inhibitory effect of PAS-A on hPASK activity. First, the catalytic activity of hPASKΔN was assayed in the presence of high levels of two heterologous PAS domains, the PAS-A domain of NPAS2 (25) and the PAS-B domain of EPAS1 (26). These two domains are also well folded and stable in solution, and preliminary NMR-based studies confirm that they both adopt a PAS domain structural fold (P. Erbel, T. C. Holdeman, and K.H.G., unpub-

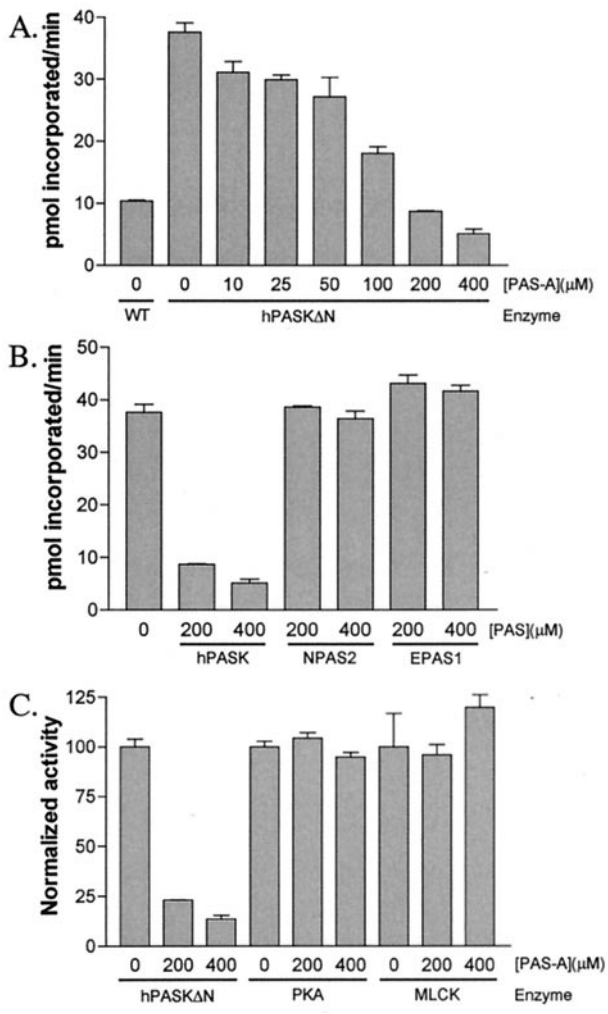

Fig. 4. PAS-A specifically inhibits enzymatic activity of hPASKΔN. (*A*) Native hPASK and hPASKΔN were incubated with AMARA peptide and [γ-^{32}P]ATP in the presence or absence of various amounts of hPASK PAS-A. Reactions were quenched after 5 min, and incorporation of ^{32}P into peptide substrate was determined by liquid scintillation counting of purified peptide. Incorporation of ^{32}P into peptide per minute is plotted with error bars indicating SD. (*B*) hPASKΔN was incubated with AMARA peptide and [γ-^{32}P]ATP in the presence of hPASK PAS-A, NPAS2 PAS-A, and EPAS1 PAS-B at the concentrations indicated. Reactions were processed and displayed as in *A*. (*C*) hPASKΔN, cAMP-dependent protein kinase (PKA), and myosin light chain kinase (MLCK) were incubated with their respective substrates and [γ-^{32}P]ATP in the presence or absence of various amounts of hPASK PAS-A. Reactions were stopped after 5 min, and incorporation of ^{32}P into substrate was determined by liquid scintillation counting. Incorporation of ^{32}P into substrate was normalized to hPASKΔN with no added PAS-A and plotted (error bars indicate SD).

lished observations). Under conditions and concentrations where the hPASK PAS-A domain caused almost complete inhibition of kinase activity, these two heterologous PAS domains showed no inhibitory activity (Fig. 4*B*).

As a second test of the specificity of inhibition, we sought to assess whether the PAS-A domain of hPASK might generally inhibit serine/threonine kinases. As control enzymes, we used cAMP-dependent protein kinase and myosin light chain kinase. In both cases, high levels of the hPASK PAS-A domain had no detectable effect on kinase activity (Fig. 4*C*). It was further demonstrated that PAS-A is not a substrate for the catalytic activity of hPASK and binds neither ATP nor peptide substrate. Gardner and colleagues have shown that the hPASK PAS-A and kinase domains directly interact in solution, suggesting that this interaction forms the basis of the inhibition observed herein (C. Amezcua, S.M.H., J.R., and K.H.G., unpublished data). We therefore propose that PASK represents a *bona fide* PAS domain-regulated serine/threonine kinase operative in yeast, flies, and mammals.

Discussion

hPASK is shown herein to represent an active serine/threonine kinase regulated in cis by one or both of two N-terminally located PAS domains. The genomes of yeast, flies, and mice also encode polypeptides homologous to hPASK, giving evidence of its evolutionary conservation. We tentatively speculate that the PASK enzyme will be involved in the regulation of a fundamental cellular pathway commonly shared among most eukaryotic cells. Northern blotting assays conducted with multiple tissue samples derived from adult mice are consistent with this interpretation, having given evidence of the ubiquitous expression of mouse PASK mRNA (J.R. and S.L.M., unpublished observations). Evidence of a cytoplasmic perinuclear pattern of intracellular localization for the enzyme is presented herein. This localization pattern has been described previously, often for proteins involved in RNA transport and function (27, 28). Various genetic approaches are currently being used in the budding yeast, *S. cerevisiae*, for the study of its two PASK enzymes. It is hoped that this genetic approach, coupled with continued biochemical and molecular biological studies, will help identify the intracellular pathway(s) regulated by PASK.

An "intelligent" enzyme must specify three critical functions: effective discrimination of substrate, efficient catalytic activity, and proper regulation of this activity. Substrate discrimination, particularly for protein kinases, is a complex task. The enzyme must find its correct intracellular location and, in some cases, associate in a stable manner with a distinct macromolecular scaffold. Biochemical studies of hPASK indicate that it is not tightly associated with a multiprotein scaffold. When assayed from crude cytosolic HeLa cell extracts, hPASK was observed to display hydrodynamic properties indistinguishable from the purified baculovirus-expressed enzyme. If hPASK does associate with other cellular macromolecules, such associations appear to be relatively weak. Preliminary studies in yeast indicate that the catalytic domain of either PSK protein expressed in the absence of its two PAS domains is sufficient to complement a vegetative growth defect caused by simultaneous deletion of the *psk1* and *psk2* genes (J.R. and S.L.M., unpublished data). Such observations provisionally indicate that substrate discrimination is primarily determined by the serine/threonine kinase domain of the enzyme. Likewise, if intracellular localization is important to substrate recognition and PASK function, it would appear that the catalytic domains of the yeast PSK enzymes are capable of finding their proper location in the absence of other protein sequences. These observations in yeast, coupled with the robust activity of the hPASKΔN enzyme (Fig. 4), clearly demonstrate that the kinase domain is also sufficient to fulfill the second critical function of an enzyme, efficient catalytic activity.

Remaining among the three aforementioned criteria characteristic of an "intelligent" enzyme is fulfillment of the requirement for proper regulation. The issue of enzyme regulation represents the primary focus of experiments carried out on hPASK to date. Comparison of the amino acid sequences of yeast, fly, and human forms of PASK reveal three conserved domains, including two PAS domains located on the N-terminal side of a serine/threonine kinase domain (Fig. 1). The last of these three conserved regions appears sufficient to facilitate proper recognition of substrate and efficient catalysis. We tentatively conclude that the third requisite function of an "intelligent" enzyme, proper regulation, may involve two and possibly all three of the conserved domains of PASK.

Evidence presented in this study indicates that hPASK utilizes two regulatory mechanisms, phosphorylation dependence and intramolecular PAS domain-mediated inhibition. Phosphorylation of threonine residues 1161 and 1165 appears necessary for efficient catalytic activity. The hPASK enzyme is able to autoactivate its catalytic activity, probably in trans, by phosphorylation of these two activation loop threonine residues. Whether hPASK is a biologically relevant hPASK kinase remains to be determined.

Although phosphorylation of the hPASK activation loop provides a clear and obvious means of enzyme regulation, our primary interest has instead focused on the intramolecular inhibitory activity specified by one or both of the N-terminally located PAS domains. A form of the enzyme lacking both PAS domains was observed to exhibit a significantly elevated k_{cat} relative to the native protein. Importantly, this elevation in catalytic activity was completely inhibited on trans addition of the purified PAS-A domain. This inhibitory activity was specific both to the catalytic domain and the PAS-A domain. The catalytic domain of hPASK was not inhibited by addition of either of two unrelated PAS domains. Likewise, the PAS-A domain of PASK did not inhibit the enzymatic activity of two unrelated serine/threonine kinases.

Our interest in the PASK enzyme came initially from the discovery that it represents the sole mammalian serine/threonine kinase associated with PAS domains. Extensive studies in microbial systems have characterized the PAS domain as a sensory module regulating the activity of its associated histidine kinase domain (1). In many cases the PAS domain incorporates a prosthetic group, such as heme or FAD, which facilitates sensory response to oxygen, redox, or other stimuli. We provisionally speculate that a similar paradigm may hold for PASK. Studies presented herein indicate that the PAS-A domain of PASK may serve in cis to negatively regulate the activity of its serine/threonine kinase domain. What remains to be determined, however, is whether the PAS-A domain might constitute a sensory device capable of altering the enzymatic activity of PASK in response to an intracellular metabolite, redox, or some other chemical signal. Were this the case, the PAS-A domain might represent the apical point wherein a specific stimulus might impinge on the regulatory pathway controlled by PASK.

The recent work of Gardner and colleagues has led to the resolution of the solution structure of the PAS-A domain of hPASK (C. Amezcua, S.M.H., J.R., and K.H.G., unpublished data). In such studies, it was possible to demonstrate the ability of the PAS-A domain to bind specific small synthetic chemicals in a region of the domain analogous to the heme-binding site of FixL. Moreover, they have demonstrated a direct physical interaction between the PAS-A and kinase domains of hPASK in a region overlapping the PAS-A ligand-binding region. These observations suggest that the kinase activity of PASK could be regulated by a specific cellular signal sensed by the PAS domain.

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