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Pim Kinase Substrate Identification and Specificity

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The Pim family of Ser/Thr kinases has been implicated in the process of lymphomagenesis and cell survival. Known substrates of Pim kinases are few and poorly characterized. In this study we set out to identify novel Pim-2 substrates using the Kinase Substrate Tracking and Elucidation (KESTREL) approach. Two potential substrates, eukaryotic initiation factor 4B (eIF4B) and apoptosis inhibitor 5 (API-5), were identified from rat thymus extracts. Sequence comparison of the Pim-2 kinase phosphorylation sites of eIF4B and mouse BAD, the only other known Pim-2 substrate, revealed conserved amino acids preceding the phosphorylated serine residue. Stepwise replacement of the conserved residues produced a consensus sequence for Pim kinase recognition: RXRHXS. Pim-1 and Pim-2 catalyzed the phosphorylation of this recognition sequence 20-fold more efficiently than the original (K/R-K/R-R-K/R-L-S/T-a; a = small chain amino acid) Pim-1 phosphorylation site. The identification of the novel Pim kinase consensus sequence provides a more sensitive and versatile peptide based assay for screening modulators of Pim kinase activity.

Key words: Pim-2, Pim-1, eIF4B, peptide substrate, consensus sequence, KESTREL, kinase assay.

The small family of Pim protein kinases consists of three ubiquitously expressed members: Pim-1, Pim-2 and Pim-3. Pim-1 and Pim-2 are highly expressed in hematopoietic tissues and subject to regulation by a variety of cytokines and growth factors (*1*). Up-regulation of Pim-1 and Pim-2 plays an important role in cellular proliferation, survival and differentiation (*2*, *3*). Mice deficient for all Pim kinases exhibit significantly reduced body size, and the hematopoietic cells from these mice exhibit impaired differentiation and proliferation in response to a variety of growth factors (*1*).

Pim kinases are encoded by *pim* family proto oncogenes. Pim-1 and Pim-2 were originally identified as proviral integration sites in Moloney murine leukemia virus (MMLV)-induced lymphoma (*4*, *5*). Both *pim-1* and *pim-2* transgenic mice have a low rate of spontaneous tumor incidence, but are more susceptible to lymphomagenesis upon viral infection or chemical carcinogen exposure (*6*, *7*). The *myc* gene is believed to collaborate with the *pim* genes during this oncogenic process (*8*, *9*). In humans, aberrant Pim-*1* and Pim-2 expression has been associated with prostate cancer, lymphoma, leukemia and multiple myeloma (*10–12*). Pim-3 shares sequence similarity to Pim-1 and Pim-2, and it is believed to have overlapping functions with Pim-*1* and Pim-2.

While the specific proteins and mechanisms involved in Pim kinase dependent signal transduction of growth, differentiation and survival are not well understood, potential Pim kinase substrates and substrate recognition sequences have been proposed. Several proteins have been shown to be phosphorylated by Pim-1, including cdc25A (*13*), a protein phosphatase which activates Cdk*2* to promote progression of the cell cycle; PTP-U2S (*14*), a tyrosine phosphatase which can enhance differentiation and the onset of apoptosis through HP-*1*; PAP-1 (*15*), a novel protein functioning in transcription repression and splicing regulation; p*2*1 (*16*), a CDK inhibitor which regulates cell cycle; NFATc*1* (*17*), a transcription factor mediating gene expression during T cell activation; SOCS-*1* (*18*), a suppressor of cytokine signaling proteins, which functions in JAK-STAT pathways; BAD (*19*), which binds to Bcl-*2* family proteins to induce cell death. A Pim-1 peptide substrate consensus sequence, K/R-K/R-R-K/R-L-S/T-a, where a is an amino acid residue with a small side chain, has been described (*20*). Pim-2 is presumed to phosphorylate peptides of similar sequence, and has recently been shown to phosphorylate BAD (*21*, *22*), as well as a *14* mer peptide (*23*).

In this study, we set out to identify novel Pim-2 substrates using the KESTREL approach (*24*). Two potential Pim-2 substrates were identified, eIF*4*B and API-5, both of which are involved in cell cycle progression and apoptosis. Direct phosphorylation of eIF*4*B by Pim-2 at Ser406 was confirmed by *in vitro* phosphorylation of recombinant eIF4B. Moreover, a Pim kinase substrate consensus sequence, RXRHXS, was deduced by sequence comparison and analogous synthetic peptide phosphorylation analysis. We determined the importance

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of Arg and His residues in recognition by Pim kinases and showed that this consensus sequence was phosphorylated 20-fold more efficiently than the Pim-1 kinase recognition sequence.

MATERIALS AND METHODS

Expression and Purification of GST-Pim-1 and GST-Pim-2 Protein—GST-Pim-1 expression and purification have been described earlier (*25*). A clone containing the human Pim-2 kinase coding sequence (clone BG177355, Image Consortium) was amplified by PCR with the following oligonucleotide primers: 5′-CUACUACUACUA CCATGGGAATGTTGACCAAGCCTCTACAG-3′ and 5′-C AUCAUCAUCAUGCGGCCGCTTAGGGTAGCAAGGACC AGGCCAAAGG-3′. The *pim-2* gene product was digested with Nco I and Not I, and ligated into the pET41a expression vector (Novagen Corp.). The cloned *pim-2* gene was confirmed by sequencing. The GST-Pim-2/pET41a construct was transformed into *Escherichia coli* strain BL21 (Invitrogen) and GST-Pim-2 expression was induced by 0.5 mM IPTG. The GST-Pim-2 protein was purified by affinity chromatography using Glutathione Sepharose 4B beads (Pharmacia), according to the manufactures protocol, and stored in buffer containing 50 mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid-N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)) at pH 7.4, 100 mM NaCl, 5% Glycerol (v/v), 0.2 mM TCEP (Tris(2-carboxyethyl)-phosphine hydrochloride). The GST-Pim-2 appears to be 70–80% pure by Commassie-stain on SDS-PAGE.

Tissue and Cell Extract Preparation for KESTREL—Cells were lysed in 50 mM Tris (pH 7.5), 10% glycerol, 1% Triton X-100, 0.03% Brij 35, 1 mM EDTA, 1 mM EGTA, 10 μg/ml Leupeptin, 1 mM Pefabloc and the extract was cleared by centrifugation at $28000 \times g$ for 30 min. Proteins were also extracted from the sediment by incubation in 50 mM Tris (pH 7.5), 0.5 M NaCl, 10% glycerol, 1% Triton X-100, 0.03% Brij 35, 1 mM EDTA, 1 mM EGTA, 10 μg/ml Leupeptin, 1 mM Pefabloc followed by centrifugation as above. Rat tissues were minced using a household mincer until they formed a homogeneous paste. This paste was then resuspended in 5 volumes of 50 mM Tris (pH 7.5), 0.03% Brij 35, 1 mM EDTA, 1 mM EGTA, 10 μg/ml Leupeptin, and 1 mM Pefabloc, using a household blender. Protein extracts were poured through Bionet tissue to remove large debris, subsequently filtered through 0.45 μm PES (Polyethersulphone) and then desalted on Sephadex G25 fine.

Chromatography on Heparin Sepharose and Source Q—Desalted protein extract was applied to heparin sepharose HP at a flow rate of 100 cm/h. The column was developed in 30 mM MOPS (3-(N-morpholino) propanesulfonic acid-4-morpholinepropane-sulfonic acid) (pH 6.9), 5% glycerol, 0.03% Brij35, 7 mM 2-mercaptoethanol to 1.2 M NaCl. The flow-throughs were titrated to pH 8.2 with 1 M NaOH and applied to Source 15Q at a flow rate of 100 cm/h. The columns were developed in 30 mM Tris HCl (pH 8.2), 5% glycerol, 0.03% Brij35, 7 mM 2-mercaptoethanol to 1 M NaCl. For Substrate Finder, three fractions were eluted at 0.3 M, 0.6 M and 1 M NaCl, respectively (Fig. 1A). For purification purposes, non linear gradients were used and up to 40 fractions were collected (Supplemental Fig. 1).

KESTREL Kinase Assay—Desalted protein extracts or aliquots of column fractions were diluted 10-fold in 50 mM Tris (pH 7.5), 1 mM EGTA, 14 mM 2-mercaptoethanol, 10 μg/ml Leupeptin, 1 mM Pefabloc. The aliquots were incubated with or without active Pim-2 (1–10 mUnits) in the presence of 5 mM Mg-acetate and 30 kBq [γ^{32}P]ATP for 5 min. 1 Unit of Pim-2 is defined as the amount of protein (91 μg or 1.52 nmol of GST-Pim2 protein) used to incorporate 1 nmole phosphate into substrate (AKRRRLSA) in 1 min. The reactions were stopped by addition of SDS-loading buffer and heat denaturation. The samples were subjected to SDS-PAGE and subsequent electro-transfer to PVDF. Radio-labeled proteins were visualized by autoradiography.

Identification of the Substrate Proteins by Mass-spectrometry—Partially purified substrates were phosphorylated for 10 min with Pim-2 in the presence of 5 mM $MgCl_2$ and 0.1 mM [γ^{32}P]ATP. The reactions were stopped by adding SDS-loading buffer and the samples were alkylated by incubation with 50 mM iodoacetamide for 30 min. The proteins were separated by SDS-PAGE and stained with colloidal commassie. The substrates were excised from the control lane, which did not contain Pim-2, in order to avoid contamination with the kinase. The gel pieces were digested with trypsin and analyzed by MALDI-TOF-TOF mass spectrometry on an Applied Biosystems 4700 proteomics analyzer, as described previously (*26*). ^{32}P labeled phosphopeptides were enriched, using immobilized metal-affinity chromatography (IMAC) with PHOS-select resin (Sigma), as described previously (*26*).

Isolation, Cloning, Mutagenesis, Expression and Purification of eIF4B—The *eif4b* gene was isolated from adult human brain mRNA by RT-PCR using the Access RT-PCR system (Promega, Madison, WI). Primer sequences were as follows: EIF4B F (5′ primer): 5′-AAAAGGATCCGCGGCCTCAGCAAAAAAGAAGAATA AGAAGGGGAAGACTATCTC-3′; EIF4B R (3′ primer): 5′-TTTGCGGCCGCCTATTCTGCATAATCTTCTCCCTCA TTTTCATCTTCACCATCAACAGAGAGAGCAGCATACT T-3′. A 1.9 kb PCR product was extracted and cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA). Plasmids were screened by sequencing for the right insert and a validated clone was digested with BamHI and Not1 and cloned into pGEX-6P-1 (GE Healthcare, Piscataway, NJ). This clone was mutagenized to create eIF4B S406A using the QuikChange II XL Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). The mutagenic primer sequences were as follows: EIF4B S406A F: 5′-CTC GGGAGAGACACCCAGCATGGCGAAGTGAAGAAAC-3′; EIF4B S406A R: 5′-TTCCTGAGTTTCTTCACTTCG CCATGCTGGGTGTCTCTCC-3′. The constructs were validated by DNA sequencing, expressed in BL21 cells using 50 μM IPTG for 16 h at 15°C for induction. GST-eIF4B was purified over GSH-agarose and validated by SDS-PAGE and Mass-Spectrometry Fingerprinting.

Phosphorylation of Recombinant eIF4B—An aliquot of (5.2 μg) total GST-eIF4B, equivalent to 4 μg (0.042 nmol)

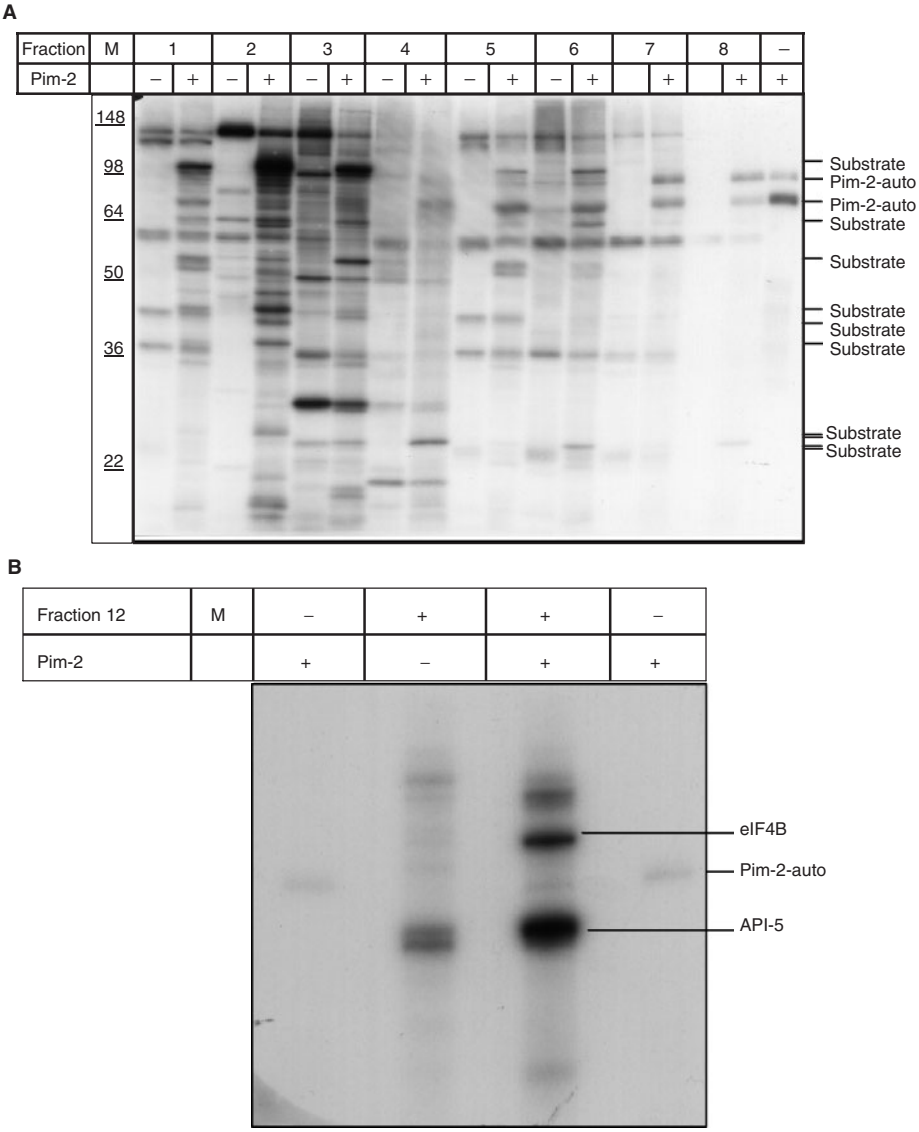

Fig. 1. (**A**) **Pim-2 phosphorylates multiple substrates in rat thymus extracts.** The following samples were incubated in the absence (–) or presence (+) of 7 mUnits Pim-2: desalted rat thymus extract (1), 0.3 M NaCl eluate from heparin sepharose (2), 0.6 M NaCl eluate from heparin sepharose (3), 1.2 M NaCl eluate from heparin sepharose (4), the heparin flowthrough (5), 0.3M NaCl eluate from Source 15Q (6), 0.6 M NaCl eluate from Source 15Q (7), and 1 M NaCl eluate from Source 15Q (8). The reactions were subjected to SDS-PAGE and transferred to PVDF, before autoradiography. Proteins that were radio-labeled only in the presence of Pim-2 were identified as potential Pim-2 substrates. (B) Phosphorylation of partially purified substrates with Pim-2. Rat thymus extracts in Heparin sepharose fractions 26 (Supplemental Fig. 1) were further purified on Source 15 S and then Source 15 Q. Fraction 12 of the 15Q column was concentrated, desalted and phosphorylated with 10 mUnits Pim-2 in the presence of 5 mM magnesium and 0.1 mM [γ^{32}P]ATP. The reactions were stopped with SDS and separated on SDS-PAGE. The radio-labeled proteins were excised and processed for identification.

in the 95 kDa band, were phosphorylated with active recombinant Pim-2, AKT1 or RSK1 in the presence of 0.1 mM [γ^{32}P]ATP and 10 mM Mg-acetate at 30°C for various periods of time. The reactions were stopped with 4% SDS and analyzed by SDS-PAGE, colloidal Coomassie staining and autoradiography. For further analysis the 95 kDa band was excised from the gel and the incorporated ^{32}P was measured in a Beckmann LS6500 Scintillation Counter. The stoichiometry and initial rates of phosphorylation were calculated, using the radioactivity of 1 nmol [γ^{32}P] ATP as a reference.

The Kinase Proteins and Substrate Peptides—Active PKCα, PKA and Akt2 proteins were purchased from Upstate Biotech (Catalog #14-484, #14-114, #14-447). AKT1 and RSK1 were provided by the Department of Signal Transduction Therapy, University of Dundee. Pim kinase substrate peptides (>95% purity) were purchased from Anaspec Inc. and dissolved in water. The following peptides, biotinylated at the amino terminus, were obtained: control (ctl), AKRRRLSA; P1, AKRRHLSA; P2, AKRRHLSY; P3, ARRRHLSY; P4, ARRRRLSY; P5, ARRKHLSY. The PKCα substrate

peptide (PLSRTLSVAAKK) and Akt substrate peptide (ARKRERTYSFGHHA) were purchased from Anaspec Inc. The PKA substrate peptide (GRTGRRNSI) was purchased from Upstate Biotech.

Steady State Kinetics Assay—Catalysis of phosphoryl transfer to peptide substrates was determined by the traditional radiometric method. First, 20 μl of 3 nM GST-Pim-1 or 15 nM GST-Pim-2 was mixed with 20 μl of 0.6 to 600 μM peptide substrate; then the kinase reaction was initiated by adding 20 μl of 300 μM ATP, containing 760 μCi/mole [γ^{33}P]ATP. The kinase buffer contained 25 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 50 mM KCl, 0.02% BSA, 0.01% CHAPs, 100 μM Na_3VO_4 and 200 μM TCEP. The 60 μl reaction mixture was incubated at room temperature for various times (2, 5, 10, 20 and 30 min) and quenched with 0.05% H_3PO_4 solution. The final concentrations of the reaction components were 1 nM GST-Pim-1 or 5 nM GST-Pim-2, 0.2 to 200 μM peptide substrate and 100 μM ATP. The phosphorylated product was captured with filter plates (Millipore #MAPHN0B10), washed four times with 0.05% H_3PO_4 solution, followed by scintillation counting. No autophosphorylation of the Pim kinases was detected under these conditions. The reaction velocity (v) was determined from plots of cpm versus reaction time, and the data were fit to the Michaelis-Menten equation, $[v = V_{max}[S]/(K_M + [S])]$, with Excel Fit.

PKLight Protein Kinase Assay—The PKLight assay reagent from Cambrex (Cat# LT07-500) was used. For Pim kinase assays, 1 nM GST-Pim-1 or GST-Pim-2, 5 μM peptide substrate and 1 μM ATP were mixed in 45 μl kinase buffer. The reactions were incubated at room temperature for times indicated in figure legend (Fig. 4A) or for 60 min. Then 45 μl of PKLight reagent A, which had been diluted 6 times with kinase buffer, was added to the reaction mixture and incubated for 15 min. Luminescence was detected with an Analyst AD reader (LJL BioSystems, Inc). The luminescence measurement reflects the amount of ATP remaining in the wells after the kinase reaction, and it is inversely related to kinase activity. Thus the kinase activity was expressed as % of ATP consumption, relative to control reaction with no peptide. For additional kinase assays, 1 nM PKCα, 0.2 nM PKA, or 10 nM Akt2 were incubated with either 1 μM PKA substrate peptide or 5 μM substrate peptides described in the figures, and 1 μM ATP at room temperature for 60 min. Lipid activator and 0.2 mM Ca^+ were supplemented in the PKCα kinase buffer.

Screening Assay Evaluation—To assess the quality of a screening assay, the Z' factor is calculated from the following equation: $Z' = 1 - 3*(SD(S) + SD(B))/(Avg(S) - Avg(B))$, where S stands for "Signal", B stands for "Background", SD stands for "Standard Deviation" and Avg stands for "Average" value. An assay with Z' value greater than 0.7 is considered an excellent assay, and Z' value between 0.5 and 0.7 is acceptable.

RESULTS

KESTREL Method Identified API-5 and eIF4B as Potential Pim-2 Substrates—In this study we employed the KESTREL approach to search for novel substrates of Pim-2. Protein extracts were prepared from rat brain, heart, lung, liver, kidney, spleen, skeletal muscle, thymus and testis, and from Hela, HEK293 and Jurkat cell lines. The extracts were fractionated and then incubated with recombinant Pim-2 in a KESTREL kinase assay. We screened eight fractions per tissue and analyzed radio-labeled protein bands on SDS gels. Potential Pim-2 substrates were found in all extracts examined, totaling well over 100 detectable bands. However, in some extracts, such as from kidney or heart, we detected only a few weak signals, whereas in other extracts, such as from thymus (Fig. 1A) or spleen and some of the cell lines, we obtained several very robust signals. Since Pim-2 was reported to regulate cell proliferation and survival mainly in hematopoietic cells, it was not surprising to find that the most abundant substrate bands identified by KESTREL were present in the thymus and spleen.

We decided to concentrate on substrates from the thymus (Fig. 1A). To this end, 1500 mg thymus extract was prepared from 40 rat organs. The extract was desalted and chromatographed on Heparin Sepharose to produce 40 fractions that were screened for the presence of Pim-2 substrates. Several major substrates eluted in fractions 19 to 27 (0.35–0.5 M NaCl) from the Heparin column (Supplemental Fig. 1A and B). Fractions 26 was desalted and further purified over Source 15S. S-fraction 10 that contained substrates was further purified over Source 15Q. Two substrate bands were detected in fraction 12 upon elution from Source 15Q and subsequent incubation with 0.1 mM [γ^{32}P] ATP, 5 mM Mg-acetate and 10 mUnits of Pim-2 (Fig. 1B).

The two substrate bands were excised and subjected to tryptic digestion, followed by Mass-Spectrometry Fingerprinting and Tandem-MS identification (Supplemental Table 1). A 57 kDa substrate protein was identified as apoptosis inhibitor 5 (API-5). A 70 kDa protein band contained a mix of eukaryotic translation initiation factor 4B (eIF4B) and the GTP-binding protein G1 to S-phase transition 1 (GST1). In order to examine which of the two proteins of the 70 kDa band was the Pim-2 substrate, we applied an aliquot of the sample to GTP-sepharose and tested both the flow-through and the GTP-eluate for substrates of Pim-2. If the GTP-binding protein was the substrate, then it would be retained on the column, whereas if eIF4B was the substrate, we would find it in the flow-through. We found that Pim-2 phosphorylated a protein in the flow-through, but not in the eluate, indicating that eIF4B was the likely substrate (Supplemental Fig. 2). Using a different approach to this end, we phosphorylated fraction 12 with Pim-2, carried out a tryptic digest and purified the phospho-peptides using immobilized metal-affinity chromatography (IMAC). Only one phospho-peptide "ERHPSWR" with a mass of 1047.4525 (peptide plus PO_4 plus H^+) was identified by Tandem MS-sequencing, corresponding to amino acids 402 to 408 of eIF4B. Hence, we concluded that the substrate in this 70 kDa band was indeed eIF4B.

To further verify that Pim-2 can phosphorylate eIF4B at S406 *in vitro*, wild type and mutant forms of recombinant GST-eIF4B (wt and S406A respectively) proteins were subjected to phosphorylation. As shown

in Fig. 2A and B, recombinant Pim-2 kinase directly phosphorylated GST-eIF4B protein. The stoichiometry of eIF4B phosphorylation was determined to be 0.5 mole of PO_4 per mole of eIF4B, suggesting that only one primary residue was phosphorylated by Pim-2. The eIF4B S406A mutant was weakly phosphorylated in comparison to wt eIF4B, indicating that Ser406 is indeed the primary Pim-2 phosphorylation site (Fig. 2C). eIF4B was observed to be phosphorylated with less efficiency by Akt1, and no evidence for phosphorylation at Ser406 was obtained: Akt1 phosphorylated wt eIF4B and the eIF4B S406A mutant with equivalent efficacy. Consistent with a recent publication (*27*), eIF*4*B was observed to be an excellent substrate for recombinant RSK1. Thus, eIF*4*B has been identified as a substrate for Pim-2 and Ser406 is the primary phosphorylation site.

Determination of Pim Kinases Consensus Sequence—Recently it was shown that Pim-2 phosphorylates murine pro-apoptotic protein BAD at Ser112 (*21*). A comparison of the sequences surrounding the Pim-2 phosphorylation sites of BAD (Ser112) and eIF4B (Ser406) suggests that Pim-2 has specific substrate recognition requirements: (1) Arg residues are present at the −5 and −3 position relative to serine; (2) His is present at the −2 position; (3) a hydrophobic residue, Tyr for BAD and Trp for eIF*4*B, is located at the *+1* position (Fig. 3). These conserved residues reveal a strong basic environment at the amino terminal side of Pim-2 kinase phosphorylation site, and are in general agreement with the Pim-*1* substrate peptide sequence described previously: K/R-K/R-R-K/R-L-S/T-a (*20*). Although higher order protein structure may have overriding influence on primary sequence, there are plenty of examples demonstrating that the amino acids surrounding the local phosphorylation site play an important role on recognition by many kinases. We set out to examine whether those conserved residues (RXRHXSϕ, where ϕ is a hydrophobic residue) near the BAD phosphorylation site (Ser*1*12) and the eIF4B phosphorylation site (Ser406) are critical for recognition by Pim-1 and Pim-2 as substrate.

Alterations to the amino acid sequences of peptide substrates have been routinely employed to define the consensus residues that are important for kinase recognition. In this study, three peptides were modeled from control peptide AKRRRLSA to further explore substrate specificity: in peptide P1 (AKRRHLSA), Arg has been replaced by His at the −2 position; in peptide P2 (AKRRHLSY), the small side chain residue Ala at position +1 was replaced by the hydrophobic Tyr residue; in peptide P3 (ARRRHLSY), Arg was substituted for Lys at position −5. Catalysis of phosphoryl transfer to each peptide was determined (Table 1).

Peptides P1 and P2, which contain His at position −2, were better Pim-2 substrates than the control peptide (Table 1). Under the conditions tested, a hydrophobic residue at the +1 position was not observed to be important for substrate recognition by Pim-2, as evidenced by the fact that P1 and P2 were both phosphorylated effectively by Pim-2. The P3 peptide was the most effective peptide substrate, exhibiting a 20-fold increase in k_{cat}^{app}/K_M^{app} compared to the control peptide. Pim-1 shares the same consensus sequence as Pim-2 (Table 1). The substitution of His for Arg at the −2 position significantly improved Pim-1 catalytic efficiency. Like Pim-2, Pim-1 showed no preference for either hydrophobic residue or small residue at the +1 position. The P3 peptide was the best substrate peptide among the three modified substrate peptides, with decreased K_M^{app} and increased k_{cat}^{app} values, resulting in ≥20-fold increase in k_{cat}^{app}/K_M^{app} compared to the control peptide. Thus, the RXRHXS sequence motif is preferred by Pim-1 and Pim-2 kinases.

Specific Residues Requirement in RXRHXS Motif—The recently solved crystal structure of the Pim-1/Pimtide complex showed that Pim-1 clearly exhibited preference towards substrate peptides with basic residues at the −3 and −5 positions (*28*). To further evaluate the importance of each specific residue in RXRHXS motif for Pim kinase phosphorylation, two more peptides were tested: P*4*, ARRRRLSY, and P*5*, ARRKHLSY. In comparison to the consensus P*3* peptide, ARRRHLSY, P*2*, P*4* and P5 peptides have one conservative substitution at the −5, −*2* and −3 positions, respectively.

The P2, P3, P4, P5 and ctl peptides were subjected to phosphorylation by Pim kinases to determine substrate specificity (Fig. 4A). The PKLight Protein Kinase Assay (Cambrix) was used to measure ATP consumption. The Arg residue at the −3 position was the most critical one to be recognized by Pim-1 and Pim-2, since conservative substitution of this residue to Lys in peptide P5 drastically reduced P5 phosphorylation. Replacement of the −2 His by Arg or the −5 Arg by Lys also significantly decreased phosphorylation efficiency of these peptides, as shown in peptides P4 and P2, respectively (Fig. 4A). Therefore, the specific residues Arg, Arg and His at the −5, −3 and −2 positions all contributed to the recognition by Pim-1 and Pim-2 kinases.

Many kinases prefer phosphorylation sites with basic residues upstream of phosphoacceptor residues. To test how specific the identified consensus sequence is to Pim kinases, the Pim kinase consensus peptide P3 and its variants were subjected to phosphorylation by PKCα, PKA and Akt2 (Fig. 4B). It has been reported that all the PKC isoforms favor peptides with a hydrophobic amino acid at the +1 position and a basic residue at −3 position. In addition, PKCα prefers peptides with basic amino acids at the +2, +3, +4 and −5 positions (*29*). We observed that PKCα phosphorylated its own substrate peptide (PLSRTLSVAAKK) very efficiently, but none of the Pim peptides were good substrates to PKCα (Fig. *4*B). The Pim consensus sequence described here is similar to the established Akt phosphorylation site, RKRXRTY*S*FG, which contains Arg at the *−5* and the −3 position (*30*). However, Akt*2* also showed clear preference to its own substrate peptide (ARKRERTYSFGHHA) over any of the Pim peptides. PKA phosphorylated its substrate peptide (GRTGRRNSI) as well as peptides ctl and P4. It appeared that PKA selected against peptides with His at −2 position, since P*2*, P*3* and P5 were poor substrates to PKA (Fig. 4B). None of the three kinases tested preferred the P3 Pim consensus peptide. Pim kinases recognized PKCα, PKA and Akt*2* peptides with less (Pim-1) or similar (Pim-2) efficiency to P2 and P4 (data not shown). These results suggested that peptides with

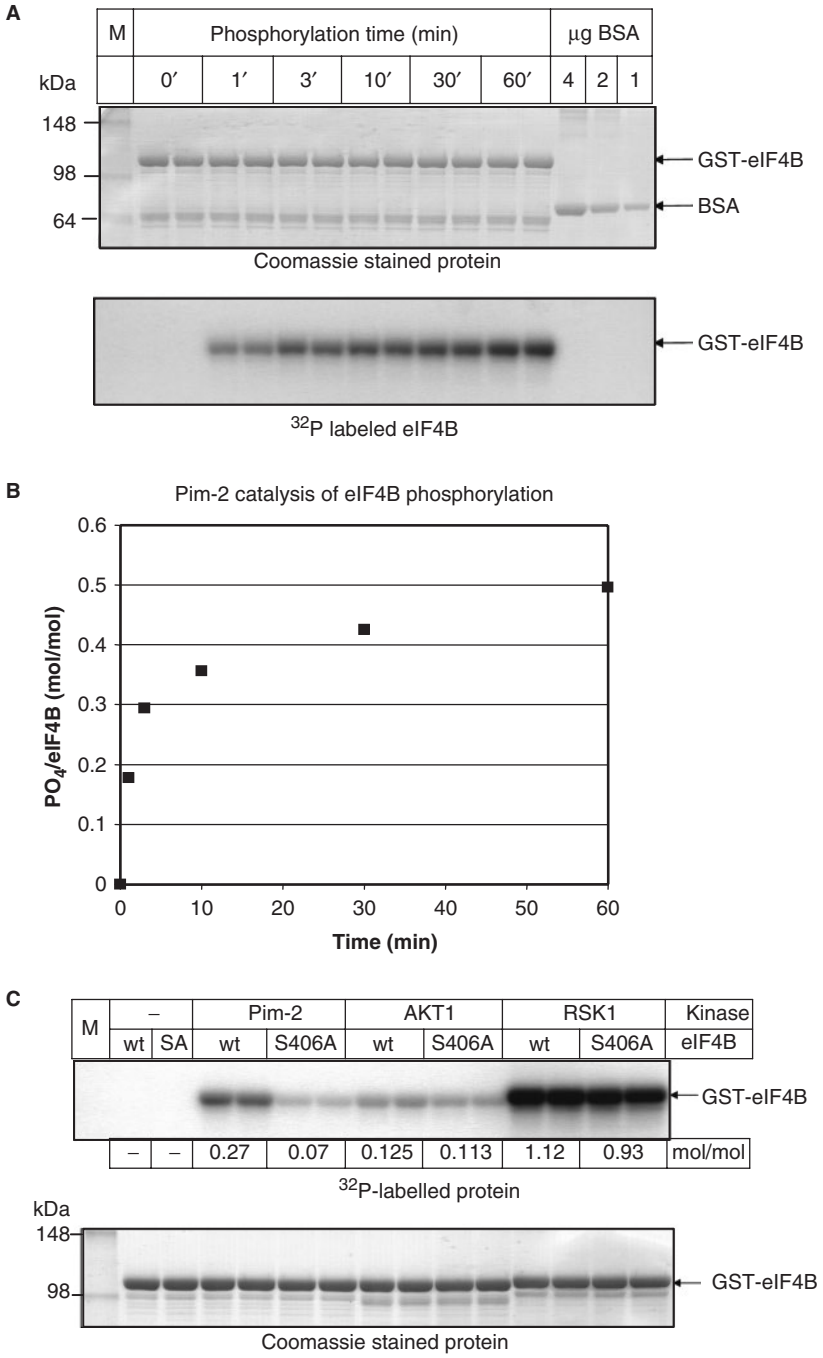

Fig. 2. **(A) Stoichiometric *in vitro* phosphorylation of eIF4B by Pim-2.** GST-eIF4B was incubated with 0.1 μg recombinant Pim-2 in the presence of 0.1 mM [γ^{32}P] ATP for the indicated periods of time. Phosphorylated proteins were separated by SDS-PAGE, and the gel was stained with colloidal coomassie and autoradiographed. (B) The eIF4B bands from Fig. 2A were excised and the incorporated ^{32}P was measured in a Scintillation Counter. The amount of GST-eIF4B per lane was estimated to be 4 μg = 0.042 nmol, using BSA as standard. The stoichiometry of phosphorylation was plotted versus time. (C) Comparison of eIF4B phosphorylation catalyzed by Pim-2, AKT1 and RSK1. GST-eIF4B (wt) or the mutant GST-eIF4B (S406A) were incubated for 10 min in the presence of 0.1 mM [γ^{32}P] ATP without (–) or with 0.1 μg Pim-2, AKT1 or RSK1 respectively. The reaction mixtures were processed as in A. The eIF4B bands were excised and the incorporated ^{32}P was measured in a Scintillation Counter. The stoichiometry of the phosphorylation is indicated.

the RXRHXS motif were specific for recognition by Pim kinases.

Use of Pim Consensus Sequence to Establish High Throughput Screen—Since Pim-1 and Pim-2 proteins promote cell survival and aberrant Pim kinase expression is linked to tumorgenesis, they may serve as suitable targets for cancer drug development. The cytokine induced expression of Pim proteins in

hematopoietic cells and impaired proliferation response in Pim deficient lymphoid cells suggest that Pim proteins might also play a role in mediating immune responses. Therefore, the Pim substrate consensus sequence can be useful to establish a homogeneous, non-radioactive high throughput screen (HTS) assay to search for modulators of Pim-1 and Pim-2 enzymatic activity. A robust kinase assay in PKLight format was developed using 5 μM P3 peptide and catalytic amounts (1 nM) of enzyme. Staurosporine was observed to be a potent Pim-1 and Pim-2 inhibitor with $IC_{50} = 3–4$ nM. This assay is a reliable HTS format, yielding a Z' of 0.85–0.90 (Fig. 5).

DISCUSSION

Two potential Pim-2 substrates, eIF4B and API-5, were identified through a preliminary KESTREL analysis. It is interesting to note that both eIF4B and API-5 are involved in cell growth and survival. Phosphorylation of eIF4B at Ser406 was discovered by mass spectrometry, and confirmed by the preferential phosphorylation of recombinant GST-eIF4B (wt) relative to GST-eIF4B S406A mutant protein (Fig 2A–C). Further biochemical and functional studies are necessary to unambiguously determine the role of Pim-2 in modulating eIF4B function. Nonetheless, the identification of eIF4B as a Pim-2 substrate may shed light on how eIF4B exerts its function during cell growth and survival. The eIF4B protein is one of the eukaryotic translation initiation factors that controls the rate of protein translation (*31*). Phosphorylation of eIF4B can be induced by mitogens and is linked to cell cycle progression. The ribosomal S6 kinase (*32*) and RSK (*27*) phosphorylated eIF4B at Ser422 and modulated eIF4B function. Akt1 also phosphorylated eIF4B weakly, but not at Ser*4*06 (Fig. 2C). Ribosomal S6 kinase is one of the down stream targets of PI3K/Akt/mTOR pathway. Interestingly, Pim-*2* has been observed to promote cell survival in an Akt pathway independent manner (*33*, *34*). Pim-2 and Akt may respond to different signals through independent pathways by phosphorylating the same substrate to bring about the same effect, as observed with BAD (*35*). eIF4B might be another common substrate of the Pim-2 and Akt pathways *in vivo*.

Control peptide (ctl):	AKRRRL*S*A
BAD S112 region:	I**R**S**RH**S*S*Y
eIF-4B S406 region:	P**R**E**RH**P*S*W
Consensus sequence:	X**R**X**RH**XSϕ

Fig. 3. **Sequence comparison.** The original Pim-1 consensus sequence (*20*) was compared to the sequence of the Pim-2 phosphorylation sites of BAD and eIF4B. The conserved residues between BAD (Ser112) and eIF4B (Ser406) were indicated by bold letters. ϕ represents hydrophobic residues.

The identification of a novel Pim kinase phosphorylation site on eIF4B Ser406 contributed to the development of a Pim kinase consensus sequence. Several potential Pim-2 substrate peptides were made based on the eIF4B phosphorylation site information obtained from the KESTREL study and the previously described BAD phosphorylation site. The peptide with the RXRHXS consensus sequence was determined to be phosphorylated most efficiently by the Pim kinases (Table 1). The substrate specificity for the newly refined Pim kinases consensus sequence was 20-fold greater than that observed for the previously described Pim-1 substrate peptide. The enhanced enzymatic activity exhibited by Pim-2 towards these modified substrate peptides appears to have resulted from enhanced affinity for Pim-2, as indicated by the reduced K_M^{app} values of the peptides (Table 1). Using a different approach, the combinatorial peptide library method, Hutti *et al*. identified the Pimtide, ARKRRRHPSGPPTA, as a substrate of Pim-2 (*23*). The larger Pimtide contains the RXRHXS motif and exhibits similar affinity for Pim-2 as measured by K_M, and slightly improved Pim-*2* specific activity (k_{cat}/K_M) for the Pimtide substrate has been reported. While we observed no preference for either hydrophobic (Tyr) or small (Ala) residues at the +1 position (Table 1, P*1* versus P2), a Gly residue at the *+1* position of the Pimtide substrate was suggested to contribute to Pim-2 recognition. The consensus sequence RXRHXS determined in this study is the minimal sequence required for Pim-1 and Pim-2 kinase recognition.

A large number of protein S/T kinases (*e.g*., the CaM kinase family, PKC family, PKA and phosphorylase kinase) prefer substrates with Arg at the −3 position (*36*). For Pim-1 and Pim-2 recognition, an Arg residue at the *−3* position is not sufficient. The control peptide AKRRRLSA, which had Arg at the *−3* position, but neither Arg at the *−5* nor His at the −2, was a poor substrate for Pim kinases (Fig. *4*A). Even conservative substitutions of any of the three basic residues in the RXRHXS motif lead to reductions in catalytic efficiency, indicating that each specific residue in the RXRHXS motif was necessary for Pim kinases recognition (Fig. *4*A). This consensus sequence is quite specific for

Table 1. **Kinetic constants for peptide substrate phosphorylation by Pim-1 and Pim-2.**

Peptide	Pim-1			Pim-2		
	k_{cat}^{app} (min^{-1})	K_M^{app} (μM)	k_{cat}^{app}/K_M^{app}	k_{cat}^{app} (min^{-1})	K_M^{app} (μM)	k_{cat}^{app}/K_M^{app}
ctl: AKRRRLSA	81.4 ± 13.0	44.0 ± 4.24	1.85	4.37 ± 0.99	22.0 ± 5.66	0.199
P1: AKRRHLSA	350 ± 84.3	25.5 ± 0.71	13.7	3.55 ± 0.62	2.25 ± 1.20	1.58
P2: AKRRHLSY	247 ± 28.2	13.3 ± 1.06	18.6	5.17 ± 1.12	3.00 ± 0.00	1.72
P3: ARRRHLSY	242 ± 35.7	5.93 ± 1.51	40.8	4.56 ± 0.59	1.05 ± 0.64	4.34

The kinetic parameters were determined using the indicated peptide as the variable substrate, and ATP concentration was fixed at 100 μM. Kinase reactions were carried out at room temperature. The mean and standard deviation of four experimental data points are reported.

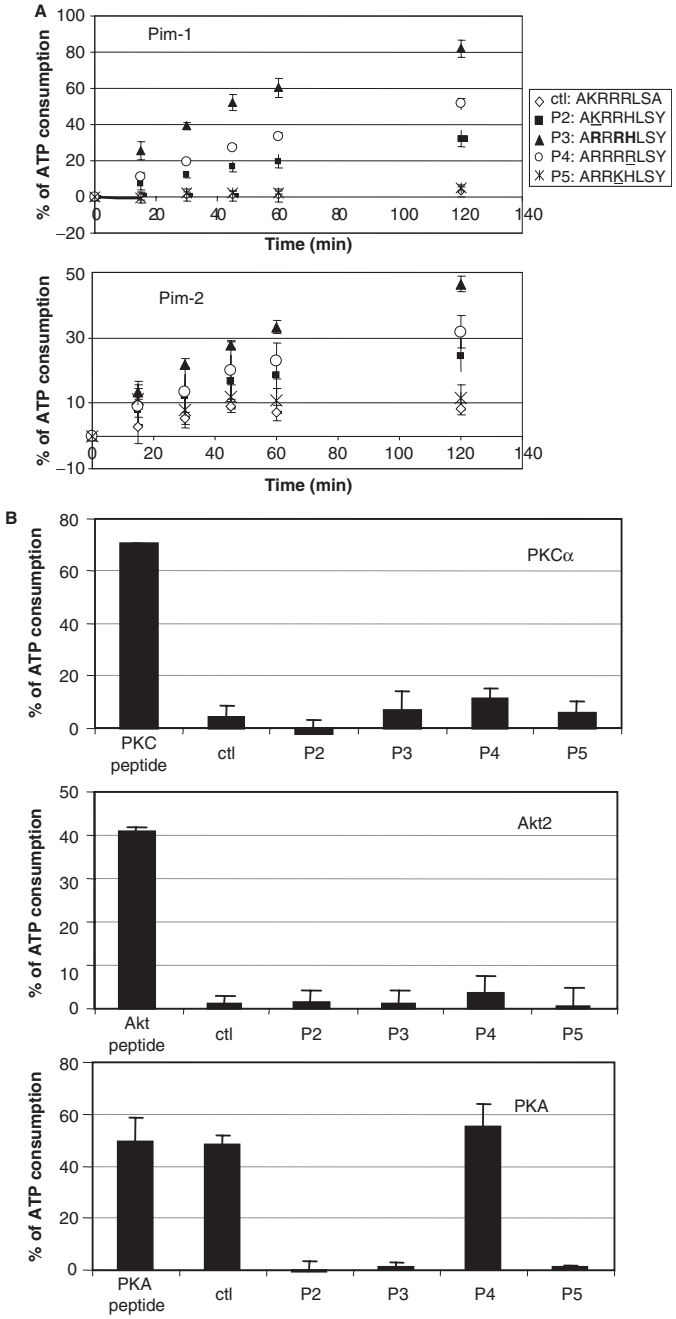

Fig. 4. **(A) The specific residues requirement by Pim-1 and Pim-2 were determined by PKLight kinase assay.** The kinase reaction was carried out by incubating 1 nM Pim kinase, 1 μM ATP and 5 μM of indicated peptide for indicated time periods at room temperature. Each assay was performed in duplicate and the mean % of ATP consumption from three independent experiments is plotted versus time. (B) The substrate specificities were determined by the PKLight kinase assay. 1 nM PKCα, 0.2 nM PKA or 10 nM Akt2 were incubated with either 1 μM PKA substrate peptide or 5 μM substrate peptides described, and 1 μM ATP at room temperature for 60 min. Each assay was performed in quadruplicate and the mean % of ATP consumption from two independent experiments is reported for each peptide substrate.

Pim kinases, since PKCα, PKA and Akt*2* all phosphorylated P3 poorly (Fig. 4B). It is particularly interesting to note that Akt2 and Pim kinases exhibited distinct substrate specificities. The two enzymes share some common substrates, such as BAD (*19*, *21*, *37*, *38*) and p21 (*16*, *39*), which are involved in cell cycle regulation and apoptosis. But Pim-*2* and Akt have been shown to promote hematopoietic cell growth and survival through overlapping but independent pathways (*40*), indicating that they may favor different substrates. Both Pim-*1* and PIM-2 have also been reported to phosphorylate BAD at Ser112 (RSRHS*S*), but not at Ser*1*36 (RGRSR*S*) (*19*, *21*), while Akt phosphorylated BAD at both sites (*37*). In this study, Akt*1* was observed to phosphorylate eIF4B at lower efficiency than Pim-2, and no evidence for Akt*1* dependent phosphorylation of eIF4B at Ser406 was

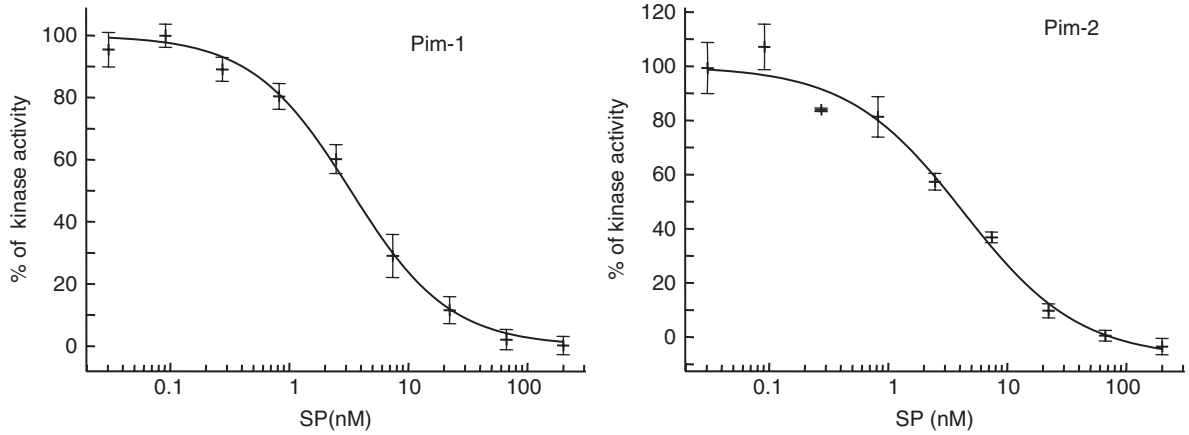

Fig. 5. **Staurosporine inhibition of Pim kinases.** The PKLight kinase assay was performed in a 60 min reaction at room temperature with 1 nM Pim kinase, 1 μM ATP, 5 μM P3 substrate peptide and various concentrations of Staurosporine. Each assay was performed in duplicate. Three independent experiments were performed and one representative result is shown.

obtained (Fig. 2C). Comparison of the Pim-1/Pimtide structure with the Akt/Aktide complex revealed that the two kinases utilized different residues to interact with their substrate peptides, especially at *−5* position (*28*). Therefore Pim kinases and Akt may possess distinct substrates specificities, depending on amino acids around consensus sequence.

In summary, the KESTREL approach has been used to identify two cell cycle regulatory proteins, eIF4B and API-5, as potential substrates of Pim-2. Through sequence comparison and stepwise amino acids substitution, we determined the Pim kinases substrate consensus sequence: RXRHXS, and showed that this consensus sequence is preferred by Pim kinases. Though it is not practical to predict putative kinase substrates by scanning protein databases with the phosphorylation motif, the stringent consensus sequence identified herein will be helpful in mapping the phosphorylation sites of physiological substrates, and evaluating the potential kinase substrates identified by *in vitro* assays. The consensus sequence is also ideal for the development of a high throughput screens for modulators of Pim kinase activity.

Supplementary data are available at JB online.

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