A Novel Transmembrane Ser/Thr Kinase Complexes with Protein Phosphatase-1 and Inhibitor-2*

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Protein kinases and protein phosphatases exert coordinated control over many essential cellular processes. Here, we describe the cloning and characterization of a novel human transmembrane protein KPI-2 (Kinase/Phosphatase/Inhibitor-2) that was identified by yeast two-hybrid using protein phosphatase inhibitor-2 (Inh2) as bait. KPI-2 mRNA was predominantly expressed in skeletal muscle. KPI-2 is a 1503-residue protein with two predicted transmembrane helices at the N terminus, a kinase domain, followed by a C-terminal domain. The transmembrane helices were sufficient for targeting proteins to the membrane. KPI-2 kinase domain has about 60% identity with its closest relative, a tyrosine kinase. However, it only exhibited serine/threonine kinase activity in autophosphorylation reactions or with added substrates. KPI-2 kinase domain phosphorylated protein phosphatase-1 (PP1C) at Thr320, which attenuated PP1C activity. KPI-2 C-terminal domain directly associated with PP1C, and this required a YTF motif. Inh2 associated with KPI-2 C-terminal domain with and without PP1C. Thus, KPI-2 is a kinase with sites to associate with PP1C and Inh2 to form a regulatory complex that is localized to membranes.

Reversible phosphorylation of proteins is the major mechanism for control of myriad functions in eukaryotic cells. Kinases and phosphatases catalyze the opposing reactions, and we have been interested in how these activities are reciprocally regulated to produce abrupt and transient changes in the phosphorylation state of target proteins.

Protein phosphatase-1 (PP1) is a predominant serine/threonine protein phosphatase that is extraordinarily conserved from yeast to mammalian cells. In the yeast Saccharomyces cerevisiae, PP1C is encoded by GLC7 (DIS2) and has over 80% sequence identity with mammalian PP1C (1). Yeast genetics and mammalian cell biochemistry has shown PP1C is essential for cell survival and multiple cellular functions, including glycogen metabolism, muscle contraction, cell cycle progression, chromosome segregation, and neuronal signaling (2–11). These different processes are regulated by distinct PP1 holoenzymes in which the same catalytic subunit interacts with different regulatory subunits (R subunit) (6, 12, 13). R subunits directly restrict activity of PP1C and target the enzyme to a specific subcellular location or substrate. Well known R subunits include the Gα/Gi subunit, which targets PP1C to glycogen particles (14–16), and the MYPT1 subunit, which targets PP1C to myosin and moesin/ERM proteins of the actin cytoskeleton (17, 18).

Control of PP1 by R subunits is supplemented by a series of small inhibitor phosphoproteins such as inhibitor-1 (19), its homologue dopamine and cAMP-regulated phosphoprotein (DARPP-32) (20, 21), PHI-1 (23), and inhibitor-2 (Inh2) (24, 25). Inh2 is common partner of PP1C and is conserved from yeast to human. In the yeast, GLC8 encodes a protein resembling mammalian Inh2, although there is only 28% identity in primary sequence (8, 26). Intracellular localization and isolation of Inh2 and PP1 (27–29) raised questions as to whether Inh2 is bound to PP1 or might associate with other partners besides PP1. To address these questions, we performed the yeast two-hybrid study using Inh2 as bait.

KPI-2 is one of the proteins identified, cloned, expressed, and characterized. Here we describe the biochemical properties of this novel transmembrane protein, which has a kinase domain and a C-terminal domain that associates with PP1C. KPI-2 is a serine/threonine kinase that can autoprophosphorylate and also phosphorylate PP1C at Thr320 and inactivate its phosphatase activity. KPI-2 interacts with Inh2 in conjunction with PP1, and this forms a complex where PP1C is associated with two different regulators.

EXPERIMENTAL PROCEDURES

Materials—Calf thymus Histone H1 and Microcystin-LR were purchased from Calbiochem-Novabiochem. [32P]ATP (30 Ci/mmol) was purchased from Amersham Biosciences. Anti-FLAG M2 antibody and anti-FLAG M2-agarose affinity gel were obtained from Sigma. Mouse anti-PP1 monoclonal antibody was purchased from Transduction Laboratories. Phospho-Thr-Pro monoclonal antibody and phospho-PP1C-Thr320 were from Cell Signaling Technology. Phospho-Ser antibody was from BIOMOL Research Laboratories, Inc. FuGene 6 transfection reagent was from Roche Molecular Biochemicals. S9 cells were obtained from Invitrogen. Restriction enzymes were purchased from New England BioLabs.

Yeast Two-hybrid System—The library screen was performed by using human Inh2 as bait. Residues 1–197 of Inh2 were inserted into a pGBT10 vector, a derivative of pGBT9 that contains the Gal4 DNA-binding domain. The library was a 9-day embryonic mouse cDNA clone.
Transmembrane Kinase-Phosphatase-Inhibitor Complex

into pVP16 vector, which contains the Gal4 activation domain (created by Dr. S. Hollenberg, Fred Hutchinson Cancer Center, Seattle, WA). The screening was done by using the large-scale, sequential transformation method (30). 2 × 10⁶ colonies were screened. Positive clones were tested first for expression of the HIS3 gene (His⁺) by growth of the clones on the plates lacking histidine (SD/Tryp-/Leu-/-His). The positives of those were tested for expression of the reporter gene, lacZ, using an assay for β-galactosidase activity. Clones were rescued by electroporation into Escherichia coli HB101 and grown on M9 plates lacking leucine, which allowed for analysis of positives by transformation tests and DNA sequencing.

For protein-protein interaction, we used an alternative yeast two-hybrid system. Inh2 or PP1C gene was fused to the Gal4 DNA-binding domain (full-length vector, and KPI-2 C-terminal wild-type (residues 1099–1503), AA-mutant, or IB-4 (KPI-2 fragment from two-hybrid screen) was fused to the Gal4-activation domain in a pVP16 vector. Both bait and prey plasmids were cotransformed into HF7c cells. Protein-protein interaction was determined by checking the growth of clones on the plate lacking histidine (SD/Tryp-/Leu-/-His).

Cloning and Construction of Different Expression Vectors—mRNA was isolated from HeLa cells with a QuickPrep™ Micro mRNA purification kit (Amersham Biosciences). N- and C-terminal portions of KPI-2 cDNA were synthesized separately using a One-Step RT-PCR kit (Qiagen). Primers were designed according to the sequence of KIAA1079 cDNA, and the following primers were used (Fig. 1A): for N-terminal, forward primer containing BamHI site 5’ - TAT AAT GGA TCC ACC ATG CCC CCG CCG TT-3’; reverse primer containing HindIII site 5’ - TCT GGT GTT TCT TGC TGA ATG AAG CTT TTA GTA AGT-3’; for C-terminal, forward primer containing HindIII site 5’ - ACT TAC TAA AAG CTT CAT TGT CCA GGA AAG AAC ACA-3’, reverse primer containing XhoI site 5’- CAC TCG AGG TCC TTT TCT CCG TCT CCG CTG CTT CC-3’. The amplified C terminus was subcloned into a pCMV pcDNA vector with an HindIII/XhoI site. The full-length KPI-2 was created by inserting the N-terminal portion into the plasmid containing the C-terminal portion at the BamHI-HindIII site. The construct of KPI-2 full-length was named pCMV-KPI-2-full-length. The HindIII site was repaired to original sequence by using a Stratagene dIII site repair kit according to the manufacturer instructions (Clontech). The membrane was probed following kinase reaction and autoradiography, the band corresponding to the KPI-2 kinase and MBP substrate phosphorylation used 0.25 mg/ml myelin basic protein (MBP), and protein and measuring the radioactivity with a scintillation counter. The samples were sonicated to lyse all the cells. After centrifugation, the supernatants were pooled and then centrifuged at 40000 × g for 10 min. The supernatants were transferred to new tubes, and concentrated by using Centricon Centrifugal Filter Devices (Millipore). The membrane pellets were solubilized with lysis buffer containing 1% Nonidet P-40. The protein concentration was measured, and an equal amount of protein was subjected to SDS-PAGE and immunoblotted with anti-myc antibody.

Expression and Purification of Recombinant Kinase Protein—The dominant pFastBac-KPI-2 kinase (94–600) was transformed into DH10Bac-competent cells for transformation into Bacmid. The recombinant Bacmid DNA was identified by PCR and transfected into S9 cells and incubated for 5 days at 27°C. The recombinant baculoviruses were harvested to obtain the P1 viruses and then amplified with P1 viruses to produce high titer P2, P3 viruses. The virus titer was measured by the plaque reduction assay of the cell culture center of the University of Virginia.

For protein expression, S9 cells were infected with recombinant baculoviruses, and cells were collected 60 h later by centrifugation. The cell pellet was resuspended in lysis buffer (20 mM imidazole–HCl, pH 7.0, 20 mM potassium phosphate, 150 mM NaCl, 1% Nonidet P-40, 0.1% β-mercaptoethanol, 1 mM diethiothreitol, and protease inhibitors as above) and the cells were sonicated in liquid nitrogen and the lysate was sonicated for 10 min. The samples were sonicated to lyse all the cells. After centrifugation, the supernatants were incubated with nickel-nitrioltriacetic acid resin for 1 h at 4°C and transferred to a column. The column was washed with buffer containing 20 mM imidazole–HCl, pH 7.0, 20 mM potassium phosphate, 300 mM NaCl, 10% glycerol, 0.1% β-mercaptoethanol, 1 mM diethiothreitol, and proteinase inhibitors as above and eluate was collected. The samples were concentrated by using Centricon Centrifugal Filter Devices (Millipore). The material was then dialyzed against 20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 50 mM NaF, 1% Nonidet P-40, 20 mM β-glycerophosphate, 1 mM NaVO₄, 1 mM dithiothreitol, 0.1% β-mercaptoethanol, and 1 mM Pefabloc-sc, 10 μg/ml leupeptin, and 10 μg/ml pepstatin for 30 min on ice. The lysates were centrifuged by centrifugation at 10,000 × g for 10 min. For Western blotting, equal amount of proteins were subjected to SDS-PAGE and immunoblotted with specific antibodies. For immunoprecipitation, the lysates were incubated with anti-FLAG M2-agarose affinity gel for 1 h at 4°C. The beads were washed three times with the lysis buffer and then subjected to SDS-PAGE and immunoblotted with anti-FLAG, anti-PP1C, or anti-Inh2 antibody.

Preparation of Cell Membranes—COS7 cells transfected with pCMV-KPI-2-N-terminal for 24 h were washed once with phosphate-buffered saline, and scraped in ice-cold buffer containing 10 mM Tris/HCl, pH 8.0, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM Pefabloc-sc, 10 μg/ml leupeptin and 10 μg/ml pepstatin. The cells were disrupted by homogenation in lysis buffer, were clarified by centrifugation at 1000 × g for 10 min at 4°C. The pellet was homogenized again, and the supernatants were pooled and then centrifuged at 4000 × g for 10 min. The supernatants were mixed with an equal volume of the same buffer containing 0.25% s croose and centrifuged at 100,000 × g for 1 h. The supernatants were transferred to new tubes, and concentrated by using Centricon Centrifugal Filter Devices (Millipore). The membrane pellets were solubilized with lysis buffer containing 1% Nonidet P-40. The protein concentration was measured, and an equal amount of protein was subjected to SDS-PAGE and immunoblotted with anti-myc antibody.

Kinase Assay—Auto phosphorylation was performed by incubating purified recombinant KPI-2 kinase at 30°C for 20 min in 20 μl of reaction buffer containing 20 μM Hepes, pH 7.4, 1 mM MnCl₂, 10 mM MgCl₂, 5 mM β-glycerophosphate, 0.1 mM Na₃VO₄, 2 mM dithiothreitol, 0.4 mM Pefabloc-sc, and 100 μM [γ³²P]ATP (10 μCi). The reaction was terminated by the addition of 6X SDS sample buffer and boiling for 5 min. The samples were resolved by SDS-PAGE, and the gel was stained with Coomassie Blue. The phosphorylation was detected by autoradiography and quantified by excising the band corresponding to the protein and measuring the radioactivity with a scintillation counter.

Histone H1—Histone H1 recombinant His₄–Histh, or poly(Glu/Tyr) (4:1) under the same conditions. The eluant from S9 cells transfected with wild type baculovirus was processed in parallel.

Cell Culture, Transfection, Immunoprecipitation, and Western Blotting—COS7, HeLa, and HEK293T cells were cultured in Dulbecco’s modified Eagle’s Medium supplemented with 10% heat-inactivated newborn calf serum (Invitrogen). Cells were grown in 10-cm plates in a humidified incubator at 37°C and 5% CO₂ and subcultured every 2–3 days. Cells were transfected by using calcium phosphate method according to the manufacturer’s instructions. After 24 h of transfection, the cells were harvested and lysed with lysis buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 50 mM NaF, 1% Nonidet P-40, 20 mM β-glycerophosphate, 1 mM NaVO₄, 1 mM dithiothreitol, 0.1% β-mercaptoethanol, 1 mM Pefabloc-sc, 10 μg/ml leupeptin, and 10 μg/ml pepstatin) for 30 min on ice.
washed extensively, and PP1C binding was detected by immunoblotting with anti-PP1C antibody. The content of the GST fusion protein was determined by immunoblotting with anti-GST antibody.

Phosphorylase Phosphatase Assay—PP1C activity was assayed by the release of \(^{32}\)P phosphate from \(^{32}\)P-labeled phosphorylase A as described in Shenolikar and Ingebritsen (33). Activity of PP1C purified from rabbit skeletal muscle was determined in a reaction mixture (40 \(\mu\)l) containing 20 mM MOPS, pH 7.4, 2 mM MgCl\(_2\), 1 mg/ml bovine serum albumin, and 15 \(\mu\)M \(^{32}\)P-labeled phosphorylase A at 30 °C for 15 min. Acid-soluble \(^{32}\)P was analyzed by liquid scintillation counting.

RESULTS

Identification of Novel Kinase KPI-2—To identify binding proteins for Inh2, we employed yeast two-hybrid analysis with human Inh2 (residues 1–197) as bait. One of six independent clones was a fragment of an uncharacterized protein encoded by the human open reading frame of KIAA1079 (NM-011575). The fragment identified in the Inh2 screen, called IB-4, was the region of residues 1344–1450, including the PP1C binding motif VTF (Fig. 1A).

We cloned the cDNA from HeLa cells by using RT-PCR and named it KPI-2 (Kinase/Phosphatase/Inhibitor-2). Our KPI-2 cDNA clone was 4512 nucleotides in length encoding a 1503-amino acid residue protein (GenBank\textsuperscript{TM} accession number AY130988) and matched with human chromosome 7q21.3-q22.1. The KIAA1079 sequence differs from KPI-2 by missing 94 nucleotides that were thought to be an intron with a mismatched splicing junction.

The domain structure of KPI-2 protein is shown in Fig. 1A. There are two predicted transmembrane helices (available at www.enzim.hu/hmmtop/) that extend between amino acid residue ranges 11–29 and 46–63 near the N terminus. There is one kinase domain (residues 137–407) (www.kinase.com), which contains an ATP binding motif (residues 143–168) and has about 60% sequence identity with mouse apoptosis-associated tyrosine kinase (AATYK) (34–37). In the C-terminal region, there is a predicted PP1C binding motif KKA\textsuperscript{VTFFD}D that contains key Val and Phe at amino acid residue 1355 and 1357. This site was recovered in the two-hybrid clone IB-4.

Identification of KPI-2—Northern blot of RNA from eight human tissues. The membrane (Clontech) was probed with \(^{32}\)P-labeled cDNA corresponding to the kinase domain (280–1800 nt) of KPI-2 and was exposed to x-ray film with intensifying screen for 72 h at −70 °C.
their sequences (Fig. 2A). Because of the predicted transmembrane helices in the N terminus, we expected the KPI-2 protein to be membrane-bound. To test this, we expressed KPI-2 N-terminal-(1–703) in COS7 cells, and prepared soluble and membrane fractions from these cells. The myc-tagged KPI-2-(1–703) protein was recovered entirely in the membrane fraction solubilized by 1% Nonidet P-40 and did not appear in the soluble fraction (Fig. 2B). Phospholemman was used as a plasma membrane protein marker, and protein phosphatase 2A catalytic subunit (PP2Ac) (cytosolic protein marker) antibodies.

Expression, Purification, and Autophosphorylation of Recombinant KPI-2 Kinase—We expressed the KPI-2 kinase domain (residues 94–600) as a His6-tagged fusion protein in Sf9 cells. Following metal-ion affinity chromatography, one major protein of ~75 kDa was detected in the Coomassie Blue-stained gel (Fig. 3A, left panel), and this protein also reacted with anti-His antibody (Fig. 3A, right panel). The 75-kDa KPI-2 kinase from Sf9 cells was purified by Mono Q anion-exchange chromatography (not shown), and an autophosphorylation assay was performed. The purified recombinant kinase was incubated with [32P]ATP, and autophosphorylation was observed (Fig. 3B, left panel). We also performed a kinase assay using different substrates. Myelin basic protein (MBP) was phosphorylated efficiently, but Histone H1 and His6-Inh2 were relatively poor substrates under the same conditions (Fig. 3B, right panel). The tyrosine kinase substrate poly(Glu:Tyr) (4:1) was not phosphorylated at all, even with prolonged incubation (data not shown).
was located at the Thr320 site in PP1C. PP1C was inhibited by or phosphorylated PP1C are presented as the mean ± H11006 labeled phosphorylase A. Phosphorylase phosphatase activity of PP1C/H9262 was performed immediately with 15–PP1C, purified from rabbit skeletal muscle, by KPI-2 kinase-(94 plus/minus KPI-2 kinase. Western blotting using anti-phospho-antibody (Fig. 3threonine protein kinase. phosphorylated KPI-2. This evidence shows that KPI-2 is a serine/threonine protein kinase requires the VTF motif. Phosphorylation of PP1C by KPI-2 Reduces PP1C Activity—KPI-2 is a serine/threonine protein kinase with a PP1 binding motif in the C-terminal domain that would bring the kinase and phosphatase together. We checked whether KPI-2 could phosphorylate PP1C and alter its activity. Purified PP1C was incubated with or without purified KPI-2 kinase-(94–600). Reactions contained 100 μM ATP8S, which was used instead of ATP to retard dephosphorylation of PP1C during the reaction. Phosphatase assays were performed using [32P]phosphorylase α as substrate. PP1C activity was reduced 70% by reaction with KPI-2 kinase compared with incubation without kinase (Fig. 4A). PP1C is known to be activated by phosphorylation at Thr320 (38–40), so we tested if the phosphorylation by KPI-2 was located at the Thr320 site in PP1C. PP1C was inhibited by incubation with 1 μM microcystin-LR and incubated with ATP plus/minus KPI-2 kinase. Western blotting using anti-phospho-PP1Cα(Thr320) antibody showed that PP1C was phosphorylated at Thr320 by KPI-2 kinase (Fig. 4B). Thio phosphorylated PP1C did not react with the phospho-specific antibody. Thus, KPI-2 phosphorylated Thr320 in PP1C, which reduces phosphatase activity.

KPI-2 Binding to PP1C Requires a C-terminal VXF Motif—There is a consensus PP1 binding motif VXF in the C-terminal domain of KPI-2. To check PP1C binding, we prepared GST-KPI-2-(1099–1503) and used HeLa cell lysates as a source of PP1C in a pull-down assay. Wild type (wt) GST-KPI-2-(1099–1503) bound PP1C, but GST alone as a control did not pull down PP1C from these same lysates. Substitution of Ala for Val1335 and Phe1337 in GST-KPI-2-(1099–1503) to give an AA-mutant protein eliminated PP1C binding (Fig. 5, upper panel). Equivalent amounts of GST and GST fusion proteins were present in the assays, shown by immunoblotting with anti-GST antibody (Fig. 5, lower panel). Similar results were obtained with GST-KPI-2-wt plus purified PP1C (not shown), showing that PP1C bound directly and did not require any other proteins for association. These results showed that a GST-KPI-2 fusion protein binds PP1C and that this binding requires the VXF motif.

Binding of VXF Motif Subunits Reduces Phosphorylase Phosphatase Activity of PP1C—GST-KPI-2-wt-(1099–1503) and the AA-mut fusion protein or GST alone were preincubated with purified PP1C, then phosphorylase phosphatase activity was measured. As shown in Fig. 6A, GST-KPI-2-wt potently reduced PP1C activity in a dose-dependent manner over the nanomolar concentration range. The AA-mutant protein that did not bind PP1C in a pull-down assay (Fig. 5) produced no reduction in phosphorylase phosphatase activity of PP1C, like GST alone, up to 100 nM (Fig. 6A).

We compared the effects of binding different regulatory subunits to PP1C. Various amounts of GST-KPI-2-(1099–1503), GST-G3M(1–240), or GST-M130(1–498) fusion proteins were incubated with purified PP1C and assayed for phosphorylase phosphatase activity of PP1C. As shown in Fig. 6B, GST-KPI-2-(1099–1503) and GST-M130(1–498) gave identical reduction of PP1C activity, but GST-G3M(1–240), which binds PP1C in a pull-down assay over this range of concentrations (41), produced less of an effect. The results showed that different regulatory subunits could use a VXF motif to bind PP1C with about the same apparent affinity but reduce PP1C activity with phosphorylase as substrate to different levels.

Inhibitor 2 Binding to KPI-2—Does KPI-2 bind to PP1C in living cells? Does Inh2 bind to KPI-2 directly or only bind to PP1C, producing an indirect association with KPI-2? We overexpressed FLAG-KPI-2-wt-(1099–1503), the KPI-2-AA-mut or

Fig. 4. Phosphorylation of PP1C by KPI-2. A, phosphorylation of PP1C, purified from rabbit skeletal muscle, by KPI-2 kinase-(94–600) was carried out by incubation with 0.1 mM ATP8S for 60 min at 30 °C. Phosphatase assay was performed immediately with 15 μM of 32P-labeled phosphorylase A. Phosphorylase phosphatase activity of PP1C or phosphorylated PP1C are presented as the mean ± S.E. for two separate experiments. B, PP1C was inactivated by incubation with 1 μM microcystin-LR, then incubated with 0.1 mM ATP plus/minus KPI-2 kinase for 60 min. PP1C phosphorylation was analyzed by Western blotting using anti-phospho-PP1Cα(Thr320) antibody (upper panel). The protein levels of PP1C in each sample were detected using anti-PP1C antibody (lower panel).

Fig. 5. KPI-2 binds to PP1C in vitro. GST-KPI-2-wt-(1099–1503) or GST-KPI-2-AA-mut or GST protein was bound to glutathione-Sepharose beads and incubated with HeLa cell lysates at 4 °C for 1 h. PP1C binding was detected by immunoblotting with anti-PP1C antibody (upper panel). GST and GST fusion proteins were detected by immunoblotting with anti-GST antibody (lower panel).
Both bait-vector (PP1C) and prey-vector (KPI-2) were cotransformed into HF7c yeast strain, and protein-protein interaction was determined by yeast two-hybrid system. pGBT10-Inh2 or PP1C were used as bait vector. pVP16-KPI-2-wt-(1099–1503), the AA-mut, and IB-4 were prey vectors. Both vectors were transformed into yeast HIS3 strain, and transformations were checked by growth on double dropout plate (SD/-Trp/-Leu) (left panel, IP). The interaction between KPI-2 and Inh2 was checked using alternative yeast two-hybrid system. pGAL10-Inh2 or PP1C was used as bait vector. pVP16-KPI-2-wt-(1099–1503), the AA-mut, and IB-4 were prey vectors. Both vectors were transformed into yeast HIS3 strain, and transformations were checked by growth on double dropout plate (SD/-Trp/-Leu) (right panel). Protein-protein interaction was determined by growth on a triple dropout plate (SD/-Trp/-Leu/-His) (top panel). PP1C was preincubated with increasing concentrations of GST-KPI-2-wt-(1099–1503) (*), GST-M130-(1–450) (●), or GST-Gm-(1–240) (○) for 10 min on ice, then PP1C activity was assayed with 32P-labeled phosphorylase A. The data are representative of three independent experiments. B, PP1C was preincubated with increasing concentrations of GST-KPI-2-wt-(1099–1503) (●), GST-M130-(1–450) (●), or GST-Gm-(1–240) (○) for 10 min on ice, then PP1C activity was assayed with 32P-labeled phosphorylase A. The data are representative of three independent experiments.

empty vector, and HA3-tagged Inh2 in COS7 cells and prepared cell extracts and immunoprecipitated them with anti-FLAG M2 agarose. PP1C bound to FLAG-KPI-2-wt but did not bind to FLAG-KPI-2-AA-mut (Fig. 7, top panel, IP). This result was completely consistent with the GST fusion protein pull-down assay (see Fig. 5). However, Inh2 bound to both FLAG-KPI-2-wt and FLAG-KPI-2-AA-mut but not to the anti-FLAG M2-agarose used as a negative control (Fig. 7, bottom panel, IP). This indicated that PP1C was not required for Inh2 binding to the KPI-2 C-terminal domain. Immunoblot analysis of the cell extracts and the immunoprecipitates revealed that FLAG-KPI-2 proteins were expressed to the same level and precipitated identically (Fig. 7, Inh2 was also expressed at the same level in each sample. These results showed: 1) FLAG-KPI-2-wt-(1099–1503) protein binds to PP1C in living cells; 2) Val1355 and Phe1357 in KPI-2 are necessary for this association; and 3) Inh2 binds to KPI-2-(1099–1503) with or without PP1C.}

**Inhibitor 2 Binding to KPI-2 Requires PP1C in Yeast—**We checked Inh2 and PP1C binding to KPI-2 in yeast by performing protein-protein interaction assay based on yeast 2-hybrid. Both bait-vector (PP1Cα or Inh2) and prey-vector (KPI-2-wt or AA-mut) were cotransformed into HIEC yeast strain, and protein-protein interaction was determined by growth of clones on the medium lacking histidine. KPI-2-wt-(1099–1503) and IB-4 interacted with both PP1Cα and Inh2. In contrast, the KPI-2-AA-mut did not bind either PP1Cα or Inh2 (Fig. 8, −His). PP1 was the positive control for binding to Inh2, and the PP1 glycoprotein targeting subunit Gα(1–240) was the positive control for binding to PP1Cα. All the strains grew well in double dropout medium as a control (Fig. 8, +His). The results confirm that KPI-2 binding to PP1C requires the C-terminal VXV motif. However, Inh2 did not associate with KPI-2-AA-mut, so in yeast Inh2 binding to KPI-2 required PP1C.

**DISCUSSION**

We identified KPI-2 as a unique human protein with transmembrane helices, a Ser/Thr kinase domain and a C terminus region that binds PP1 and Inh2. By sequence analysis the nearest relative of KPI-2 is a tyrosine kinase (AATYK), which is induced during apoptosis or terminal differentiation of 32Dcl3 cells and is otherwise expressed predominantly in brain (34–37). KPI-2 has about 60% sequence identity with AATYK within the kinase domain and shows no sequence similarity outside the kinase domain. Based on sequence alignments, Hanks and Hunter (42) proposed that protein kinase subdomains VI–VIII predict serine/threonine versus tyrosine kinase specificity. The catalytic loop in these catalytic domains is highly conserved among each of these groups of enzymes. In serine/threonine kinases, a Lys residue is...
found at the n+2 position in the subdomain VIIb relative to the essential Asp residue, and most are DLK. In contrast, the protein-tyrosine kinases have an Arg residue instead at either the n+2 position or n+4 position, like DLR or DLAXR (42, 43). There is a common DLAXR motif in KPI-2 (residues 265–269, DLALR) and its closest related kinases (AATYK and trkB) raising the expectation that KPI-2 would be a tyrosine kinase. However, our results showed that KPI-2 is a Ser/Thr kinase and does not exhibit tyrosine kinase activity, based on multiple lines of evidence. First, phosphoamino acid analysis of autophosphorylated KPI-2 showed only phospho-Ser and phospho-Thr, no phospho-Tyr. Second, immunoblotting with anti-phospho-Thr-Pro and anti-phospho-Ser antibody both showed reactivity with KPI-2. Third, KPI-2 did not associate with PP1C and with Inh2, but mutation of the VTF motif eliminated both interactions. This suggested that Inh2 bound to KPI-2 indirectly, by interacting with PP1C that was bound to the VTF of KPI-2. We have uncovered recently other examples where PP1C binds to Inh2 and a VXF-containing subunit at the same time, through separate sites (47, 48). However, in COS7 cells Inh2 bound to the wild type KPI-2 C-terminal domain as well as the AA mutant that did not interact with PP1C. We concluded that neither PP1C nor the VXF motif were required for Inh2 interaction with KPI-2. Curr-ently, KPI-2 appears to bind Inh2 in COS7 cells but not in yeast. One possibility to account for this difference is that KPI-2 phosphorylation. In mammalian cells Inh2 is fully phosphorylated at Ser36, Ser120, and Ser121 (49), but it is not known whether these CK-II sites are phosphorylated in yeast. Another possibility is that an unknown scaffold protein in mammalian cells, not in yeast, binds Inh2 to KPI-2. Regardless, our evidence shows that KPI-2 is a novel example of a PP1 regulator that brings together a PP1 kinase and PP1 inhibitor for regulation of this phosphatase at the membrane surface. The physiological function of this multienzyme complex remains an unsolved mystery for further investigations.

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