Protein Phosphatase 6 Subunit with Conserved Sit4-associated Protein Domain Targets IκBα*

Bjarki Stefansson and David L. Brautigan

From the Center for Cell Signaling, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Protein Ser/Thr phosphatases compose a PPP family that includes type-2 PP2A, PP4, and PP6, each with essential functions. The human PP6 gene rescues sit4, mutants of Saccharomyces cerevisiae, and Sit4 phosphatase function depends on multiple Sit4-associated protein (SAP) subunits. We report here finding a SAPs sequence domain encoded in only a single gene each in Schizosaccharomyces pombe, Caenorhabditis elegans, and Drosophila but in three distinct open reading frames in Xenopus, Mus musculus, and Homo sapiens. The SAPS proteins are more divergent in sequence than PP6. Northern hybridization showed differential distribution of the human SAPS-related mRNA in multiple human tissues, named as PP6R1, PP6R2, and PP6R3. Antibodies were generated, distribution of endogenous PP6, PP6R1, PP6R2, and PP6R3 proteins was examined by immunoblotting, and the abundance of mRNA and protein in various tissues did not match. FLAG-tagged PP6R1 and PP6R2 expressed in HEK293 cells co-precipitated endogenous PP6, but not PP2A or PP4, showing specificity for recognition of phosphatases. The SAPS domain of PP6R1 alone was sufficient for association with PP6, and this predicts that conserved sequence motifs in the SAPS domain accounts for the specificity. FLAG-PP6R1 and FLAG-PP6R2 co-precipitated HA-IκBα. Knockdown of PP6 or PP6R1 but not PP6R3 with siRNA significantly enhanced degradation of endogenous IκBα in response to tumor necrosis factor-α. The results show SAPS domain subunits recruit substrates such as IκBα as one way to determine specific functions for PP6.

Protein phosphorylation by kinases and protein dephosphorylation by phosphatases is the most common mechanism for regulating cellular processes. Protein phosphatases fall into major families based on different structures, specificity and catalytic mechanisms. The protein Ser/Thr phosphatase PPP family comprises the type-1 and type-2A phosphatases that are conserved throughout eukaryotic evolution. The PP2A, PP4, and PP6 phosphatases are sensitive to inhibition by low doses of okadaic acid that distinguishes them from the type-1 phosphatase. The regulation and function of PP2A has been studied the most (1, 2), and there are a few reports about PP4 (3), but the function of PP6 is essentially unknown in metazoans. In Saccharomyces cerevisiae genetic studies have implicated the essential Sit4 phosphatase, the homolog of PP6, in regulation of G1 to S progression (4–6). In addition, Sit4 affects a variety of processes including transcription, translation, bud formation, glycogen metabolism, monovalent ion homeostasis, H+ transport, and telomere function (7–11) (for reviews, see Refs. 12 and 13). Mammalian PP6 is a functional homolog of Sit4 because human PP6 can rescue a temperature-sensitive mutant of sit4 from arrest in G1 phase of the cell cycle, a phenotype that is rescued neither by type-1 phosphatase nor PP2A (14, 15). Sit4 associates with the protein product of the essential S. cerevisiae gene Tap42, which acts as a multicopy suppressor of the sit4 temperature-sensitive mutant (16, 17). The binding of Tap42 to Sit4 is evolutionary conserved because α4, the human homolog of Tap42, binds to PP6 (18). Unlike other known regulatory subunits, α4 binding is not specific to one type of phosphatase because it also associates with PP2A and PP4 (18–20). The α4 protein is the only subunit that has been identified for mammalian PP6 to date. In yeast, besides Tap42, Sit4 co-precipitates with three other subunits termed Sit4-associated protein 55 (SAP155), SAP185, and SAP190 (21). Deletion of multiple SAPs produced the same phenotype as deletion of Sit4, indicating that Sit4 and SAP functions depend on one another (21). Pheno- typic changes in response to deletion or overexpression of different SAP genes in S. cerevisiae suggest distinctive cellular functions for each of these proteins (21–24). Strains lacking SAP185 and SAP190 are hypersensitive to rapamycin and show constitutive phosphorylation of eukaryotic initiation factor eIF2α (23). Increased expression of SAP155 but not other SAPs conferred resistance to Kluyveromyces lactis zymocin (22). Overexpression of SAP155 decreases K+ efflux, whereas SAP155 deletion increases efflux. In contrast, overexpression of SAP185 increases K+ efflux, whereas deletion of SAP185 decreases efflux (24). Thus, specific cellular functions of PP6 are expected to be dependent on subunits related to the yeast SAP proteins.

An important biological role for PP6 in mammalian cells is indicated by its recent identification in a genome-wide screen for kinases and phosphatases that affect cell survival (25).
Knockdown of PP6 by siRNA gave one of the highest rates of apoptosis. Here, we report the identification of three human SAPS-related genes (called PP6R1, PP6R2, and PP6R3) and show the distinctive tissue distribution of the mRNA and expression of the proteins that differs from the mRNA distribution. We demonstrate that these putative regulatory subunits specifically associate with PP6 phosphatase compared with PP2A or PP4, and the SAPS domain itself is sufficient for recognition of PP6. Furthermore, we show that PP6R1 and PP6R2 co-precipitate IκBe, and using siRNA to knockdown expression of PP6 or PP6R1, we found enhanced degradation of IκBe, consistent for it being a substrate for PP6 that would oppose the action of IKK.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Chemicals**—Antibodies against PP6 were prepared against a peptide corresponding to residues 292–305 at the extreme C terminus of human PP6, affinity purified from chicken egg yolk and used at a 1:5000 dilution. Chickens were immunized with purified recombinant GST-PP6R1 (BC002799) made in bacteria, and the IgY was purified from chicken egg yolk was used at a 1:1000 dilution. Rabbits were immunized with purified recombinant GST-PP6R2 (BC000976) made in bacteria, and antibodies were purified from serum using protein A-agarose and used at a 1:500 dilution. Sheep was immunized with purified recombinant GST-PP6R3 (DQ111954) made in bacteria, and antibodies were affinity-purified from serum against MBP-PP6R3 (DQ111954) and used at a 1:4000 dilution. Rabbit antibodies against the C subunit of PP2A were made previously and were used at a 1:3000 dilution. Flag-FLAG and anti-actin antibodies were purchased from Sigma-Aldrich and used at a 1:1000 dilution. Anti-PP4 antibodies were purchased from Stratagene and used at a 1:500 dilution. Anti-glyceraldehyde-3-phosphate dehydrogenase antibodies were purchased from Chemicon International and used at a 1:1000 dilution. Mouse anti-His, antibodies and rabbit anti-IκBe (sc-7156) antibodies were purchased from Santa Cruz Biotechnology and used at a 1:1000 dilution. Chicken anti-HA antibodies were purchased from Aves Labs, Inc. (Tigard, Oregon) and used at a 1:1000 dilution. Goat anti-rabbit Alexa Fluor 680, donkey sheep Alexa Fluor 680, and goat anti-mouse Alexa Fluor 680 antibodies were purchased from Molecular Probes and Invitrogen and used at a 1:3000 dilution. Goat anti-rabbit IRDye 800 and anti-chicken IRDye 800 antibodies were purchased from Rockland Immunochemicals and used at a 1:3000 dilution. Chemicals not otherwise specified were purchased from Fisher. Tumor necrosis factor-α (TNFα) was purchased from R&D Systems, Inc. (Minneapolis, MN) and used at a concentration of 20 ng/ml.

**Sequence Alignment and Construction of Phylogenetic Tree**—Sequence alignment of PP6R1 (KIAA1115, accession number BC0002799), PP6R2 (KIAA0685, accession number BC000976), and PP6R3 (C11orf23, accession number DQ111954) was performed using ClustalW method. PostScript output from aligned sequences was generated using ESPript (26). For the phylogenetic tree Clustal alignment of SAPS-related proteins was done using ClustalX 1.83, and un-gapped versions of the alignments were created in MacClade 4.05. The Parsimony Consensus (unrooted) tree was generated using PAUP’4beta10 (the most current version of PAUP) and with a Branch and Bound Search using the degapped Clustal alignment. There were two equally maximum parsimony trees found, and the figure is of a strict consensus of those two. The bootstrap proportions are from 5000 replications, done using a pure heuristic search. The phylogram was generated by using both the Clustal degapped alignment and the PAUP consensus tree in the Phylib 3.62 PROML program (Protein Maximum Likelihood without a molecular clock constraint) and having it compute optimal branch lengths for the provided tree topology given the protein alignment. Sequence identities between metazoan and human SAPS-like proteins were calculated using DNA Star (MegAlign 5.0) after ClustalW alignment of sequences.

**Plasmids**—DNA for human PP6R1, PP6R2, and PP6R3 was amplified by PCR from clones purchased from Open Biosystems (Huntsville, AL). PP6R1, OBS#60510, GenBank™ accession number BC002799; PP6R2, OBS#875 GenBank™ accession number BC000976; PP6R3, OBS#8642489, Incyte Human cDNA. The PCR primer for amplifying OBS#60510 started at nucleotide 13 to provide an N-terminal methionine for the protein. DNA for human PP6R3 (GenBank™ accession number DQ111954) was amplified by PCR from HeLa cDNA generated by Thermoscript poly (dT) reverse transcription-PCR (Invitrogen) following the manufacturer’s protocol. The primers for PCR of PP6R3 (DQ111954) were 5’-GGA TCT TCG ATG TTA CAA ATT CAG AAC AGT ACA GAG-3’ as the forward primer and 5’-CCT GAG TCA TAC AGG GCC ATT CAC TG-3’ as the backward primer. The PP6R1, PP6R2, and PP6R3 insert were ligated in-frame with the FLAG epitope at the EcoRI/XhoI sites in the mammalian expression vector pcDNA3-FLAG and, with the exception of PP6R3 Incyte Human cDNA, in-frame with the sequence of GST at the EcoRI/Xhoh sites in the bacterial expression vector pGEX-4T-1. PP6R3 (DQ111954) was ligated in-frame with maltose-binding protein at the EcoRI/Xbal sites in the bacterial expression vector pMAL-c2X. FLAG-PP2A-Aα plasmid was generated as described before (27). FLAG-α4 plasmid was generated as described before (19). DNA for human IκBe was amplified by PCR from a clone (catalog number EHS1001–7517588, GenBank™ accession number BC063609) purchased from Open Biosystems (Huntsville, AL) and was ligated in-frame with HAα at the BamHI/EcoRI sites in the mammalian expression vector pKH3. The FLAG-PP6R1 construct was used for PCR to make fragments of PP6R1 using primers 5’-GGA ATT CAT GGT GCC CTG CTG C-3’ as forward and 5’-GTT CCA GTC ACA GGT CCA CCA TGT TCT TCT TCT-3’ as backward for PP6R1 (1–465), the same backward primer and a forward primer of 5’-GGA ATT CAT GGT GGC CCT CAT CAA CCG-3’ for PP6R1 (72–465), and 5’-GGA ATT CAT GGT GCA CCT GGT GAA CAC-3’ as forward and 5’-GCT CCA GCT ATT GGG AGC CTC G-3’ as backward for PP6R1 (462–825). Fragments were ligated in-frame with the FLAG epitope at the EcoRI/Xhol sites in the mammalian expression vector pcDNA3-FLAG.

**Cell Culture and Transfection**—HEK293 cells were grown in modified Eagle’s medium containing 2 mM l-glutamine, 10% fetal bovine serum, and antibiotic-antimycotic (Invitrogen) at...
Protein Phosphatase 6 Regulatory Subunits

37 °C in a humidified 5% CO₂ atmosphere. Cos7 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotic-antimycotic (Invitrogen) at 37 °C in humidified 5% CO₂ atmosphere. Cells were either transiently transfected using 5–10 μg of plasmid DNA with FuGENE 6 (Roche Applied Science) or calcium phosphate as the delivery method. For calcium phosphate transfections sterile deionized H₂O and 10 μg of plasmid were added to 62.5 μl of 2 M CaCl₂ to make up a solution of 500 μl. The calcium/DNA mix was added dropwise to 500 μl of 2× HEPES-buffered saline (280 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄, 12 mM dextrose, 1 M CaCl₂ to make up a solution of 500 μl of 2× HEPES-buffered saline (280 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄, 12 mM dextrose, 1 M CaCl₂) while continuously bubbling the mixture with air. After 30 min at room temperature, the mixture was added to cells at 40% confluence in 100-mm dishes. The day after transfection cells were washed with phosphate-buffered saline, 10 ml of growth medium was added, and cells were left for 24–48 h at 37 °C.

Immunoprecipitation and Immunoblotting—Hypotonic lysis of cells was performed by washing cells with phosphate-buffered saline and rinsing briefly with H₂O and then adding lysis buffer: 20 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μM Pefabloc, protease inhibitor mixture set V, EDTA-free (concentration as suggested by manufacturer, Calbiochem catalog #539137), 1 μM microcystin-LR (ALEXIS Biochemicals), 30 mM β-mercaptoethanol. Cells were left in lysis buffer for 30 min on ice and homogenized using a Dounce tissue grinder, and the homogenate was centrifuged at 12,000 × g (4 °C) for 10 min. Supernatants were used for immunoprecipitation with a mixture of anti-FLAG beads (M2; Sigma) and CL-4B-Sepharose (Sigma) beads. After rotation with supernatant at 4 °C for 2 h, beads were washed 3 times in lysis buffer and boiled in 2× SDS sample buffer. For co-immunoprecipitation of HA-IκBe cells were lysed in a buffer containing 1% Nonidet P-40 (IGEPAL CA-630) and 1 μM microcystin-LR as described before (27). SDS-PAGE was done according to Laemmli using Bio-Rad acrylamide/Bis 29:1 (catalog #161–10032). Protein Phosphatase 6 Regulatory Subunits

PCR products were used to make 32P radioactive DNA probes for Northern blot using Linear Amplification according to manufacturer’s instructions in Strip-EZ PCR Kit (Ambion, Austin, TX). NucAway Spin Columns (Ambion) were used to remove radioactive nucleotides from DNA probes. The Northern blot was probed following the Northern RNA Blot application guide Version 4.0 from Origene Technologies, Inc. Incubation of Northern blot with probes was done overnight with ULTRAhyb Ultrasensitive Hybridization buffer (Ambion). After exposure (1–3 days) using a PhosphorImager, 445SI (GE Healthcare), probes were removed from the Northern blot following the Strip-EZ PCR Kit instructions, and the blot was subsequently reprobed.

siRNA Knockdowns—siRNAs were purchased from Dharmacon (Lafayette, Colorado). siRNAs used as controls were designed against luciferase 5’-TAAGGCTATGAAAGAGATTAC-3’. PP6 siRNAs are CGACACGCCATATTTTCCCTTT as the sense sequence for PP6 siRNA 1 and TGGGCTGATCATTACACTTT as the sense sequence for PP6 siRNA 2. Sequences for PP6R1 siRNAs are TCAATTTGCTCAACAGGATT as the sense sequence for PP6R1 siRNA 1, GAAAGCACCTTTGACAAAGTT for PP6R1 siRNA 2, and CGTC-GAAGGATGAAATCATT for PP6R1 siRNA 3. PP6R1 knockdown in Fig. 7C was performed using siRNA Smart Pool catalog number M-020420-00 (Dharmacon) that contains four different siRNAs. PP6R3 knockdown was performed using siRNA Smart Pool catalog number M-014646-00 (Dharmacon) that contains 4 different siRNAs. Knockdowns were done in HeLa cells plated into either 60-mm dishes (60,000 cells) and 12-well plates (40,000 cells). 24 h later cells were transfected with siRNA (120 nM) using Oligofectamine (Invitrogen) following the manufacturer’s instructions. After overnight incubation, fresh media was added to the cells, and 24 h later cells were lysed in 2× SDS sample buffer.

RESULTS

Conservation of SAPS-related Phosphatase Subunits among Eukaryotes—Based on sequence similarity to the yeast SAP proteins that are subunits for Sit4 protein phosphatase, we identified three potential subunits for human PP6. We searched genome databases by BLAST using either yeast SAP155, SAP185, or SAP190 full-length protein sequences and identified three human open reading frames: KIAA1115, KIAA0685, and C11orf23, designated here as PP6R1, PP6R2, and PP6R3, respectively. Alignment of the sequences of human PP6R1, PP6R2, and PP6R3 (Fig. 1) showed different N- and C-terminal sequences and a central region of ~400 residues with common sequence motifs that is predicted to be mostly composed of α helices (28, 29). This is referred to as the “SAPS domain” because the sequence motifs are conserved from S. cerevisiae SAP subunits. We note that the Pfam data base (protein families data base of alignments and hidden Markov models (HMM) refers to this as the SAPS domain, different from an unrelated DNA binding domain called SAP (30). We also found three SAPS-related sequences in mouse and three in Xenopus laevis that aligned with S. cerevisiae (Fig. 2). Although the SAPS domain is conserved, there is not a one to one correspondence between the four SAPs in S. cerevisiae and three vertebrate.
SAPS-related proteins. Searches for SAPS-related sequences in the genomes of other organisms yielded only a single SAPS gene in *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Schizosaccharomyces pombe* (Fig. 2). Construction of an unrooted phylogenetic tree for eukaryotic SAPS proteins shows a separate arm for vertebrate SAPS proteins, with three branches that show nearly

![Protein Sequences of SAPS-Related Proteins and Sequence Alignment](image-url)

**FIGURE 1.** Protein sequences of SAPS-related proteins and sequence alignment. Sequences for PP6R1 (KIAA1115, accession number BC002799), PP6R2 (KIAA0685, accession number BC000976), and PP6R3 (C1orf23, accession number DQ111954) were aligned using the Clustal method (see “Experimental Procedures”), and identical residues in all three proteins are printed in reverse. The SAPS domain (according to Pfam assignment) is underlined with a thick black line. PP6R3 appears to have multiple alternatively spliced forms (Q3K3R5, Q5H9R7, Q6MB2, Q9HCL4, Q68CR3, Q9H2K5, Q9H2K6, OBS#8642489 Incyte Human cDNA from Open Biosystems). These spliced forms produce differences in the N-terminal half of the SAPS domain, whereas the C-terminal half appears the same. The schematic below the sequences shows the relative size and position of the SAPS domains in the PP6R1, PP6R2, and PP6R3 proteins. The broken line between the N terminus and the SAPS domain in PP6R3 denotes the existence of multiple spliced versions of PP6R3.

![Schematic Diagram](image-url)

**SAP6 Domains**

Protein Phosphatase 6 Regulatory Subunits

AUGUST 11, 2006 • VOLUME 281 • NUMBER 32

JOURNAL OF BIOLOGICAL CHEMISTRY

22627
equal distances between species (Fig. 2). This indicates separate lines of descent for each of the three vertebrate SAPS and shows that PP6R1 and PP6R3 diverged together on a separate arm from PP6R2. Another arm of the tree includes D. melanogaster, C. elegans, S. pombe, and S. cerevisiae. The single SAPS sequence in S. pombe (called EKC1) lies between the two SAP185/SAP190 and SAP4/SAP155 clusters in S. cerevisiae. Interestingly the S. cerevisiae SAP4/SAP155 are as closely related to C. elegans as to the Ekc1 protein in S. pombe. The S. cerevisiae SAPS have 23–25% sequence identity to EKC1 but only about 10% identity to the SAPS proteins in metazoans. This contrasts with the 61% sequence identity between Sit4 and human PP6. The third arm of the tree includes multiple SAPS proteins in plants. Based on this phylogenetic analysis we propose that multiple SAPS proteins in S. cerevisiae converged to a single SAPS protein in S. pombe, C. elegans, and D. melanogaster, which diverged into multiple SAPS proteins in plants and vertebrates. The functions of all the metazoan SAPS proteins are unknown.

**Distribution of mRNA for PP6 and Human SAPS Proteins—**
The tissue distribution of mRNA for human SAPS sequences and the PP6 catalytic subunit was analyzed by Northern hybridization with specific probes. Expression of mRNA for PP6, PP6R1, PP6R2, and PP6R3 in 12 human adult tissues was compared (Fig. 3). A predominant mRNA of 4 kilobases for PP6R1 was present at about equal levels in all the tissues, with the exception of testis, which had much higher concentration. Several tissues showed a slightly smaller mRNA including testis, which showed two additional smaller species. The relatively equal distribution of the 4-kilobase mRNA among various tissues led us to call this (KIAA1115) PP6R1. For PP6R2 (KIAA0685) there were two mRNAs of about equal amounts expressed in all the tissues with the highest levels in testis followed by liver, heart, and brain. In contrast, the mRNA for PP6R3 (C1orf23) was highly expressed in heart with barely detectable levels in other tissues, including testis. Our observation of a same-sized mRNA for each gene among different tissues indicates a uniform processing of transcripts for PP6R1 and PP6R2. Equal loading of poly(A)-mRNA was demonstrated by hybridization for β-actin mRNA (Fig. 3, bottom panel). For the PP6 catalytic subunit the major mRNA of 1.6 kilobases, consistent with a protein of 36 kDa, was most highly expressed in heart with lower levels in kidney, liver, stomach, brain, and less in skeletal muscle and small intestine (Fig. 3). In agreement with a previous report (14), we also detected a PP6 mRNA of around 4 kilobases in most tissues, with the highest levels in heart and testis (not shown).

**Distribution of PP6 Protein in Tissues, Cells, and Subcellular Fractions—**We produced and affinity-purified a specific PP6 antibody against a peptide corresponding to residues 292–305 at the extreme C terminus of the PP6 catalytic subunit. The specificity of this antibody was demonstrated by comparing equal amounts of recombinant His<sub>6</sub>PP6 and His<sub>6</sub>PP2A by Western blotting (Fig. 4A). Using this antibody PP6 was detected in all mouse tissues examined at the predicted molecular size of 36 kDa (Fig. 4B), with the highest levels in lung, liver, and spleen and relatively low levels in skeletal muscle and heart. Thus, there were striking differences in the tissue distribution of PP6 between the Northern hybridization and Western blotting, the most obvious being heart, which had the highest levels of PP6 mRNA but relatively low levels of PP6 protein. This might indicate regulation of expression at the level of translation in different tissues and raises a caution about analysis and interpretation of PP6 gene expression by DNA microarray. Comparison of PP6 expression in cultured human cell lines showed the highest levels in breast cancer (MCF7), prostate...
cancer (PC-3), cervical cancer (HeLa) cells relative to fibroblast HS-68, and retinal epithelial ARPE19 cells (not shown). Subcellular fractionation of PC-3 cells showed that PP6, like PP2A, was predominantly in the cytosol compared with detergent-soluble (membrane) and detergent-insoluble (cytoskeletal) particulate fractions that contained most of cadherin and vimentin, respectively, as markers (not shown). The same predominant cytoplasmic distribution of PP6 was observed in subcellular fractionation of HeLa and HEK293 cells. Furthermore, based on immunoblotting serial dilutions of recombinant His$_6$PP6 and His$_6$PP2A protein as standards together with extracts of tissue culture cell lines, we calculated that PP6 protein levels are from 5% (HS68) to 15% (MCF7) of the amount of PP2A. Our results show that PP6 is a widely distributed cytosolic protein phosphatase that comprises a significant fraction of type 2A phosphatase in cells.

**Tissue Distribution of SAPS Proteins**—Immunoblotting with specific antibodies was used to examine the distribution of PP6R1, PP6R2, and PP6R3 (Fig. 4B). Chicken anti-PP6R1 IgY antibody showed a protein of around 120 kDa compared to the calculated size of 98 kDa for BAA83067.3, in highest amount in mouse lung, bladder, spleen, and pancreas. Again, as with PP6 catalytic subunit, the protein distribution in various tissues did not correspond to the relative levels of the PP6R1 mRNA. Anti-PP6R2 rabbit antibody was purified using protein A-agarose, and immunoblotting extracts of mouse tissues showed a band at around 130 kDa compared to the calculated size of 105 kDa for AAH00976.2. This protein was in highest concentration in bladder, a smooth muscle, with lower concentration in heart and pancreas and with much lower but detectable levels in other tissues. The PP6R2 mRNA and protein were both found in heart; in contrast, the liver had abundant mRNA but little protein, whereas bladder had abundant levels of protein, but small intestine, another smooth muscle tissue, had low levels of mRNA. Affinity-purified sheep anti-PP6R3 antibody showed a protein of around 140 kDa relative to the calculated size of 99 kDa for Q9HCL4, which is the longest PP6R3 isoform in GenBank. The PP6R3 protein was most abundant in lung, bladder, spleen, and pancreas. The PP6R1 and PP6R3 proteins had nearly identical tissue distribution; however, these proteins were distinguished from one another by different migration in SDS-PAGE and differential staining with chicken and sheep antibodies, respectively. Antibody specificity was confirmed by siRNA knockdown of PP6R1 and PP6R3 that lowered the intensity of the 120- and the 140-kDa bands, respectively (see Figs. 6 and 7). Those tissues with highest expression of PP6R1 and PP6R3 proteins corresponded to those with the highest expression of PP6 catalytic subunit. It is interesting to note that PP6R1 and PP6R3 are on a common branch on the phylogenetic tree. The basis for the disparities between mRNA and protein for PP6 and these SAPS proteins and the biological significance of the disparities are not understood; however, the situation dictates that data on gene expression derived from DNA microarrays needs to be followed up by analysis of protein levels such as by immunoblotting. Both PP6R1 and PP6R3 were >95% cytoplasmic in subcellular fractionation of HeLa and HEK293 cells analyzed by immunoblotting. The abnormal migration of PP6R1 and PP6R3 proteins probably arises from low binding of SDS to the C-terminal regions outside the SAPS domain, because the SAPS domain (44 kDa) itself migrated where predicted, but the C-terminal domain (39 kDa) itself displayed abnormally slow migration in SDS-PAGE (see Fig. 5C).
Specific Association of SAPS-related Proteins with PP6—The SAPS domain alone and the human PP6R proteins with a SAPS domain specifically associate with PP6 (Fig. 5). FLAG-tagged PP6R1, PP6R2, and PP6R1 (1–465) (consisting mainly of the SAPS domain) were individually expressed in HEK293 cells. Endogenous PP6 but not the catalytic subunit of PP2A co-precipitated with each of these FLAG-tagged proteins (Fig. 5). The co-precipitation of PP6 with PP6R1 (1–465) is consistent with the hypothesis that the C-terminal region is not required for association with PP6. As a control to demonstrate specificity, FLAG-PP2A-Aα coprecipitated endogenous PP2A as expected, but not PP6. These results show that these SAPS-related proteins specifically recognize PP6 and not the closely related phosphatase, PP2A.

As a further test of specificity we individually expressed FLAG-tagged versions of PP6R1, PP6R2, and PP6R1 (1–465) with FLAG-α4 as the control. Endogenous PP6 co-precipitated with each of these FLAG-tagged proteins (Fig. 5B). Less PP6 was recovered with FLAG-α4 compared with the SAPS-related proteins, and although a band of PP6 is not apparent in Fig. 5B, it was obvious at higher exposure. On the other hand endogenous PP4 only co-precipitated with FLAG-α4, not with any of the FLAG tagged SAPS-related proteins even though they were all expressed at much higher levels compared with FLAG-α4 (Fig. 5B). FLAG-PP6R1 and FLAG-PP6R2 were individually co-expressed with HA-PP6, and the FLAG- and HA-tagged proteins were recovered together by FLAG immunoprecipitation. These immunoprecipitates exhibited phosphatase activity with 32P-labeled myelin basic protein as substrate that was inhibited by 50 nM okadaic acid (not shown), demonstrating that PP6 was active when bound to either PP6R1 or PP6R2. Thus, the PP6R1 and PP6R2 discriminate between the known members of the type-2A phosphatase family and specifically associate with the PP6 catalytic subunit.

Despite multiple attempts, FLAG-PP6R3 was not recovered by immunoprecipitation from extracts of transiently transfected HEK293 cells. Independent PP6R3 clones of different lengths (see “Experimental Procedures”) were tested, with similar results. Mostly FLAG-tagged fragments of PP6R3 were detected by anti-FLAG immunoblotting when transfected cells were lysed directly in SDS sample buffer (not shown). The results suggested that ectopic PP6R3 was especially susceptible to proteolytic degradation. As an alternative approach, we expressed PP6R3 in bacteria as a GST fusion protein. The GST-PP6R3 as well GST-PP6R1 and GST-PP6R2 pulled down endogenous PP6 but not PP2A from extracts of PC-3 cells (data not shown). Furthermore, we have co-immunoprecipitated endogenous PP6 complexes with chicken anti-PP6R1 and sheep anti-PP6R3 antibodies (not shown). Altogether, the results demonstrate that all three SAPS-related proteins, PP6R1, PP6R2, and PP6R3, are specific for binding PP6, and we propose that these are regulatory subunits for PP6.

The SAPS domain alone recognizes PP6. We expressed FLAG-tagged proteins encompassing the N-terminal half (1–465), the C-terminal half (462–825), or just the SAPS domain (72–465) of PP6R1 and prepared FLAG immunoprecipitates. Endogenous PP6 bound to the N-terminal half and the SAPS domain but not the C-terminal half of PP6R1 (Fig.
screen revealed that both PP6R1 (KIAA1115) and PP6R2 (KIAA0685) along with PP6 were associated with tagged IκBε but not tagged IκBα or IκBβ. Recovery of this IκBε protein complex was not affected by treating cells with TNFα, suggesting a stable association that occurred in non-stimulated cells. We co-expressed FLAG-PP6R1, FLAG-PP6R2, or FLAG-PP2A-Aα along with HA-IκBε and prepared FLAG immunoprecipitates. HA-IκBε co-precipitated with FLAG-PP6R1 and FLAG-PP6R2 but not with FLAG-PP2A-Aα (Fig. 7A). Co-precipitation of FLAG-PP6R1 or FLAG-PP6R2 with HA-IκBε was unchanged after 30 min of treatment of cells with TNFα (not shown). These results confirmed the published data. Phosphorylation of IκBε occurs in response to TNFα signaling and promotes its dissociation from NFκB and degradation via the proteasome (32, 33). If the SAPS-related proteins mediate dephosphorylation of IκBε by PP6, then one would predict increased degradation of IκBε in response to a decrease of PP6R1 or PP6 itself. Knockdown of PP6R1 with single siRNAs in HeLa cells enhanced by up to 20% compared with siRNA controls the loss of endogenous IκBε at 90 min after the addition of TNFα (Fig. 7B). Furthermore, knockdown of PP6 catalytic subunit by two different siRNAs also enhanced the degradation of endogenous IκBε relative to siRNA control in response to TNFα (not shown). We conducted another series of experiments to examine the time dependence of PP6R1 knockdown on IκBε degradation using a pool of four siRNAs against PP6R1 compared with controls with a single siRNA against luciferase. There was a statistically significant enhancement of TNFα-induced degradation of endogenous IκBε at 30, 60, and 90 min. (Fig. 7C). Knockdown of PP6R3 by a pool of four siRNAs did not enhance degradation of IκBε relative to siRNA control in response to TNFα (Fig. 7C). This showed specificity for PP6R1 vs PP6R3 in effects on IκBε. Our results are consistent with PP6R1 binding both PP6 phosphatase catalytic subunit and its putative substrate IκBε to promote the dephosphorylation and thereby oppose degradation of IκBε in response to TNFα.

DISCUSSION

In this study we identified three human SAPS-related proteins and showed the tissue distribution of their mRNA and protein. Conservation of SAPS-related protein sequences is unusual because both S. cerevisiae and vertebrates (mouse, Xenopus, and humans) contain three or more SAPS-related proteins, whereas S. pombe, C. elegans, and D. melanogaster contain only one gene each. Thus, the three vertebrate PP6 regulatory subunits do not directly correspond to the four separate SAP proteins in S. cerevisiae. We imagine that the functions of the multiple SAP proteins in S. cerevisiae were condensed into the S. pombe SAPS-related protein Ekc1 that later diverged into separate sequences found in vertebrates and another separate set in plants. We expect that each of these vertebrate SAPS-related proteins will support specific functions for the PP6 phosphatase. Even though the physiological functions of PP6 are largely unknown, our evidence indicates that PP6 has a role in the NFκB pathway.

One of the most striking outcomes of our experiments was the difference between the levels of mRNA and PP6R proteins
in various tissues. PP6R1 was the most widely distributed mRNA and was found in every adult human tissue. This uniform distribution of PP6R1 is in stark contrast with the more narrow distribution of PP6R2 and PP6R3 mRNA, which was predominantly expressed in heart. It was gratifying to discover that tissues with the highest levels of PP6 mRNA (heart and liver) also contained high levels of mRNA for one or more of the subunits (PP6R2 and PP6R3 in heart, PP6R2 in liver). However, this correspondence was overshadowed by differences between the tissue distribution of mRNA and protein. For example, immunoblotting revealed that PP6R1 and PP6R3 were especially abundant in lung and spleen and PP6R2 in bladder even though these tissues did not have corresponding high levels of mRNA. This observation calls for cautious interpretation of expression profiling of PP6 and KIAA1115, KIAA0685, and C11orf23 by DNA microarrays.

It was of interest to determine the expression level and subcellular distribution of PP6 protein in various tissue culture cell lines. Quantitation indicated that PP6 makes up to 15% of the amount of PP2A protein using recombinant proteins as immunoblotting standards. More than 90% of PP6, PP6R1, and PP6R3 in tissue culture cells was soluble, compared with the particulate fraction, consistent with it being predominantly a cytosolic enzyme. Therefore, some of the effects of okadaic acid (or other type-2A phosphatase inhibitors such as microcystin, cantharidin, or calyculin-A) previously assigned to inhibition of PP2A may instead have been due to inhibition of PP6 because these phosphatases have nearly identical sensitivity to these inhibitors.3

There is one common regulatory subunit called α4 that directly binds all three type-2A phosphatases, which is the mammalian version of the *S. cerevisiae* Tap42 protein (18). This implies that PP2A, PP4, and PP6 have a shared three-dimensional structure and common sites for interactions with α4. On the other hand, sequence differences between the three mammalian Ser/Thr phosphatases of the type-2A family are sufficient to specify association with distinctive regulatory subunits (34–36). PP2A utilizes a scaffolding subunit called the A subunit made of 15

3 T. D. Prickett and D. L. Brautigan, unpublished results.
Protein Phosphatase 6 Regulatory Subunits

HEAT repeats to recruit a member of one of four families of regulatory B subunits into ABC heterotrimers (1). On the other hand PP4 has been purified from tissues in association with two different regulatory subunits called PP4R1 and PP4R2 (34, 36). Here we demonstrated specificity of PP6 association with SAPS-related subunits by co-precipitation from cells and pull-down assays with cell extracts, and these precipitates exhibited phosphatase activity consistent with a tethering function for the SAPS domain. We observed binding of recombinant GST-PP6R1 with purified His6-PP6 (not shown) that demonstrates direct protein-protein interaction between the catalytic and regulatory subunits. However, this does not preclude that other subunits may be incorporated in complexes with SAPS domain proteins and PP6. Knockdown of PP6 reduced the levels of PP6R1 and PP6R3, whereas knockdown of either PP6R1 or PP6R3 did not reduce the levels of PP6. We interpreted these results in terms of a pool of PP6 distributed into complexes with PP6R1, PP6R2, or PP6R3 and/or other subunits. As found for protein kinase A (37, 38) and other multisubunit enzymes, holoenzymes are relatively protected from degradation compared with the separate subunits. Reduction in levels of PP6 would reduce the levels of complexes and release regulatory subunits for degradation. Alternatively, reduction of individual regulatory subunits is not reflected in reduction of PP6 catalytic subunit, which presumably redistributes into other complexes. This scheme also applies to siRNA knockdown of PP2A subunits (39, 40). Thus, depletion of a common (catalytic) subunit reduces the levels of multiple holoenzymes and the constituent regulatory subunits, whereas depletion of an individual regulatory subunit does not necessarily reduce the level of a shared catalytic subunit.

The PP6R1, PP6R2, and PP6R3 SAPS-related proteins specifically associated with PP6 compared with PP2A or PP4, which are ~50% identical in amino acid sequence to PP6. We propose that these SAPS domain proteins function as dedicated PP6 regulatory subunits. The PP6R subunits are related in sequence to the S. cerevisiae SAP proteins that were shown about 11 years ago to be required for Sit4 phosphatase to fulfill multiple essential functions in S. cerevisiae (21). Various yeast SAP genes fall into least two complementation groups, and genetic results indicate that the SAP proteins specify discrete roles for Sit4 (21). The concept is that regulatory subunits determine phosphatase specificity by simultaneously binding phosphatase catalytic subunit and substrate. PP6R1, PP6R2, and PP6 previously were found in complexes with 1xBe, suggesting that 1xBe might be a substrate for PP6. We confirmed association of PP6R1 and PP6R2 with 1xBe and showed that knockdown of PP6R1 and PP6 but not PP6R3 by siRNA promoted the degradation of 1xBe in response to TNFα stimulation. Even though PP6R1 and PP6R3 are more closely related in sequence to one another than either is related to PP6R2 (see Fig. 2), there is apparent specificity in function. Therefore, 1xBe is a likely candidate as a specific substrate for the PP6R1/PP6 holoenzyme.

Our characterization of KIAA1115, KIAA0685, and C11orf23 as specific subunits for PP6 provides important new information about these genes that already have been identified in other studies. PP6 was recently identified in a siRNA screen as the ser/thr phosphatase with the largest effects on cell survival (25). PP6 mRNA is overexpressed in pancreatic tumors relative to normal tissue, overexpressed in high grade human ductal carcinoma in situ, and is induced with androgen treatment of LNCaP prostate cancer cells (41–43). Consistent with a role for PP6 in cancer, PP6R1 mRNA is one of the top 20 genes overexpressed in hepatocellular carcinoma and metastatic liver tumors, not in normal liver (44). PP6R1 mRNA was overexpressed in pediatric acute lymphoblastic leukemia (45). PP6R2 was one of 64 genes induced by β1-integrin ligation on B cells and was strongly induced by interleukin 13 in human airway smooth muscle (46, 47). However, our results demonstrate the levels of mRNA for PP6 subunits do not correspond to the levels of their respective proteins, so these links to cancer need to be followed up by a study of the proteins. In the case of PP6R3, there is indication for a role in insulin-dependent diabetes mellitus (IDDM), because it maps as one of four genes in the IDDM4 locus at 11q13 (48). Regulatory subunits for PPP family protein phosphatases are responsible for modulating catalytic activity, restricting substrate specificity, recruiting substrates, and determining intracellular localization, based on both genetic and biochemical experiments. In these phosphatase subunits generate biochemical diversity for a limited set of PPP catalytic subunits and define relatively narrow functions for the resulting holoenzymes. Overall, there is every reason to expect that PP6 and its subunits have fundamental roles in human physiology and pathology.

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Note Added in Proof—We recently produced by reverse transcription-PCR another PP6R3 from HeLa cells that does express as a FLAG-tagged protein in 293 cells.

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