Identification and Characterization of a Novel Inositol Polyphosphate 5-Phosphatase*

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We have identified a cDNA encoding a novel inositol polyphosphate 5-phosphatase. It contains two highly conserved catalytic motifs for 5-phosphatase, has a molecular mass of 51 kDa, and is ubiquitously expressed and especially abundant in skeletal muscle, heart, and kidney. We designated this 5-phosphatase as SKIP (Skeletal muscle and Kidney enriched Inositol Phosphatase). SKIP is a simple 5-phosphatase with no other motifs. Baculovirus-expressed recombinant SKIP protein exhibited 5-phosphatase activities toward inositol 1,4,5-trisphosphate, inositol 1,3,4,5-tetakisphosphate, phosphatidylinositol (PtdIns) 4,5-bisphosphate, and PtdIns 3,4,5-trisphosphate but has 6-fold more substrate specificity for PtdIns 4,5-bisphosphate (K_m = 180 μM) than for inositol 1,4,5-trisphosphate (K_m = 1.15 mM). The ectopic expression of SKIP protein in COS-7 cells and immunostaining of neuroblastoma N1E-115 cells revealed that SKIP is expressed in cytosol and that loss of actin stress fibers occurs where the SKIP protein is concentrated. These results imply that SKIP plays a negative role in regulating the actin cytoskeleton through hydrolyzing PtdIns 4,5-bisphosphate.

Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) is known to play a role in the generation of two second messengers, inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DG), by phospholipase C. PtdIns(4,5)P_2 mobilizes Ca^{2+} stores, and DG activates protein kinase C (1, 2). In addition to its role as a lipid second messenger, PtdIns(4,5)P_2 binds to several actin-regulating proteins such as vinculin, profilin, coflin, and gelsolin (3–6) to suppress their actin-gelating activity (7). An inositol polyphosphate 5-phosphatase—IP5Pase, synaptojanin 1, previously identified with a 5-phosphatase activity towards PtdIns(4,5)P_2, bound to vinculin, -actinin, and profilin

**EXPERIMENTAL PROCEDURES**

**Materials**—Inositol 1,3,4,5-tetrakisphosphate and inositol 1,3,4,5-tetraisophosphate were purchased from Sigma. [3H]Insitol 1,3,4,5-tetraisophosphate, [3H]inositol 1,3,4,5-tetraisophosphate, [3H]phosphatidylinositol 4,5-bisphosphate, and [γ-32P]ATP were purchased from NEN Life Science Products.

**Molecular Cloning of the Human Inositol Polyphosphate 5-Phosphatase**—Partial human cDNA clone of PIPP (22) (GenBank™ accession number H14886) was obtained from Genome System, Inc. (St. Louis, MO). H14886 is a 468-bp human cDNA clone whose putative amino acid sequence contains an IP5Pase-conserved catalytic motif 2-like sequence. The human testis AZAP1 cDNA library (Stratagene) was screened using this cDNA as a probe. All DNA probes were 32P-labeled using a random primer labeling kit (Takara Shuzo) following the manufacturer’s protocol.

**Northern Blot Analysis**—A membrane containing mRNA (2 μg of poly(A) was contained in each lane) (human 12-lane multiple tissue Northern (MTN) blot) was prepared from CLONTECH. The 1.9-kb base pair human IP5Pase cDNAs including the coding region of SKIP were 32P-labeled using a random primer labeling kit (Takara Shuzo) and probed with a following the manufacturer’s protocol.

**Bacterial Expression of Recombinant SKIP**—The bacterial expression vector for human SKIP was constructed by ligating the 990-bp HindIII fragment, corresponding to amino acid residues 157–448, into the pQE-30 bacterial expression vector (Qiagen). The 1.9-kb recombinant protein was expressed and purified on Ni^{2+}-nitrilotriacetic acid-agarose beads as described by the manufacturer.

**Antibodies**—Polyclonal anti-SKIP antibodies were made by immunizing rabbits with the recombinant His_{6}-tagged human SKIP. The
Antibodies were affinity-purified from sera by conjugating the antibody proteins to CNBr beads (Amersham Pharmacia Biotech). The anti-Myc polyclonal antibody was purchased from Santa Cruz Biotechnology.

**Baculovirus Expression and Purification of Recombinant SKIP**—

The cDNA constructs, GST-tagged full-length SKIP (GST-SKIP), and GST-tagged partial human SHIP 1 cDNA (nucleotides 1,461–4,079; GenBank® accession number U57650) (GST-SHIP) were constructed by subcloning BglII-BamHI site polymerase chain reaction products encoding full-length GST amplified from pGEX-2T into the BamHI site of pFASTBAC1 (Life Technologies, Inc.) and then introducing either an EcoRI site full-length human SKIP fragment amplified by polymerase chain reaction or a BamHI site partial human SHIP 1. GST, GST-SKIP, GST-SHIP, and phosphatidylinositol 3-kinase catalytic subunit p110α proteins were expressed in Sf9 cells by infecting them with 1 ml of 1 × 10^8

**Fig. 1.** Nucleotide and predicted amino acid sequences of human SKIP. **A**, schematic representation of the genomic structures of the three different isoforms of human SKIP cDNA clones. The genome DNA sequence of human SKIP was obtained from the human genome database (Homo sapiens chromosome 17, clone hRPK.107_N_19, GenBank®/EBI Data Bank accession number AC006405). Clone 2 has a 230-bp insertion corresponding to exon II, and Clone 3 has a 937-bp deletion in the C-terminal non-coding region (exon XIII). Positions of the start methionine and stop codon of each clone are indicated by asterisks. **B**, nucleotide and predicted amino acid sequences of human SKIP cDNA Clone 1. The nucleotide and amino acid numberings for human SKIP are indicated on the right. The amino acid numbering indicates the position relative to the putative start methionine. The in-frame stop codon and the position of insertions in Clones 2 and 3 are indicated (underlined and vertical line, respectively), and conserved motifs 1 and 2 of IP5Pase are highlighted as white on black. A 937-bp deleted sequence in Clone 3, corresponding to exon XIII, is shaded. **C**, nucleotide and putative amino acid sequences of human SKIP cDNA Clone 2. The nucleotide and amino acid numberings are indicated on the right. The in-frame stop codon is underlined, and a 230-bp inserted sequence is shaded.
and resolved by TLC in chloroform/methanol/acetic acid/H2O (43:38:5:7, v/v/v/v) and then washed five times with wash buffer (40 mM Tris-HCl, pH 7.2, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) and three times with buffers for inositol phosphatase (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 μM aprotinin, and 10 μM leupeptin) and phospholipids (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 200 μM bovine serum albumin, 3 mM MgCl₂, and 2 mM cetyltrimethylammonium bromide). Inositol polyphosphate 5-phosphatase activity assay was carried out as described by Connolly et al. (26), using [3H]Ins(1,3,4,5)P₄ and [3H]Ins(1,3,4)P₃ as substrates and purified baculovirus-expressed recombinant proteins as enzymes (26). Inositol polyphosphates were separated by high pressure liquid chromatography using a Partisphere strong anion exchange (SAX) column (Whatman) and visualized by autoradiography after treating the TLC plate with ENHANCE spray (NEN Life Science Products).

The phosphatase activity for PtdIns(4,5)P₂ was measured as described previously (28). [3H]PtdIns(4,5)P₂ was sonicated with a phosphatase buffer for phospholipids (20 mM Tris-HCl, pH 7.2, 150 mM NaCl, 200 μg/ml bovine serum albumin, 3 mM MgCl₂, and 2 mM cetyltrimethylammonium bromide) and then incubated with purified recombinant protein at 37 °C for 20 min. Radiolabeled lipids were extracted and resolved by TLC in chloroform/methanol/acetic acid/H₂O (43:38:5:7, v/v/v/v) and visualized by autoradiography after treating the TLC plate with ENHANCE spray (NEN Life Science Products).

The phosphatase activity for PtdIns(3,4,5)P₃ was measured according to the method of Woscholski et al. (29). The phosphatidylinositol 3-kinase catalytic subunit p110α was used to produce [32P]PtdIns(3,4,5)P₃. p110α protein was expressed in Sf9 cells and purified as described by Funaki et al. (30). PtdIns(3,4,5)P₃/PtdIns(3,4,5)P₄ mixture was extracted with chloroform/methanol (2:1, v/v) and then dried up with nitrogen gas. Phosphatase activity assay was performed under the same conditions as those for the PtdIns(4,5)P₂ phosphatase activity assay. The lipids were extracted and separated by TLC using n-propyl alcohol/2 M acetic acid (2:1, v/v) and visualized by autoradiography.

**Human SKIP**

| motif1 | motif2 |
|--------|--------|
| DLIWFEDA | GKKRAPDTRILWRK |
| DLIWFEDA | GKKRAPDTRILWRK |
| DLIWFEDA | GKKRAPDTRILWRK |
| DLIWFEDA | GKKRAPDTRILWRK |

**Mouse SKIP**

| Type I | Type II |
|--------|---------|
| DLIWFEDA | DLIWFEDA |

**PIPP**

| Type I | Type II | Synaptotagmin | OCR | SHIP |
|--------|---------|---------------|-----|------|
| VLIWFDAGMERIE | VLIWFDAGMERIE | LVWFDAGMERIE | LVWFDAGMERIE | LVWFDAGMERIE |

**Collagen**

| Brain | Kidney | Liver | Muscles | Cartilage | Aorta | Blood leukocytes |
|-------|--------|-------|---------|-----------|-------|-----------------|
| 9.5   | 7.5    | 4.4   | 1.35    | 2.4       | 51    | 43              |

**Fig. 2.** Alignment of amino acid sequences of human SKIP and other IP5Pases. Two conserved motifs of IP5Pases are aligned. Amino acids identical to human SKIP are highlighted as white on black. Similar amino acids are shaded. Sequence identities between full-length SKIP and other IP5Pases are as follows: rat PIPP, 53.3%; human SHIP 1, 45.2%; human OCR, 45.3%; and human synaptotagmin 1, 45.5%, respectively.

**Fig. 3.** Northern blot analysis of the expression of human SKIP. Northern blot analysis of human SKIP was carried out by using 1.9-kilobase pair Clone 3 cDNA (see Fig. 1) as a probe. 2 μg of human mRNA was used in each lane. The numbers on the left are the sizes of the markers.

**Fig. 4.** Two different alternative splicing isoforms of human SKIP are expressed. A, Western blot analysis of endogenously expressed SKIP protein. Lysates (10 μg) from rat tissues and different cells were immunoblotted with anti-SKIP polyclonal antibody. B, over-expression of alternative splicing isoforms in COS-7 cells. Lysates from Sf9 mammalian expression vector alone (vector) and full-length Clone 1 and Clone 2 cDNA (subcloned in SRα vector) (Clone 1 and Clone 2, respectively) expressing COS-7 cells were immunoblotted with anti-SKIP antibody.
RESULTS AND DISCUSSION

cDNA Cloning of a Novel Inositol Polyphosphate 5-Phosphatase—In an attempt to identify novel inositol polyphosphate 5-phosphatases, we screened several cDNA libraries using human expressed sequence tag clone H14886 containing the 5-phosphatase catalytic motif 2-like sequence (see Fig. 2) as a probe (10, 14). We obtained two novel cDNA clones encoding 5-phosphatase-like sequences. One is PIPP, a recently isolated 107-kDa protein (24). The other encodes a putative 5-phosphatase cDNA clone obtained from a human testis cDNA library and 87% identical to the mouse cDNA clone U96724. Three alternative splicing isoforms were obtained (Fig. 1A). Clone 1 is a 2,786-bp cDNA with an open reading frame predicting a protein with a molecular mass of 51 kDa (Fig. 1B). Clone 2 has a 230-bp insertion, corresponding to exon II, in the N terminus of Clone 1 with a putative molecular mass of 43 kDa (Fig. 1, A and C). Clone 3 has a 937-bp deletion, corresponding to exon XIII, in the C-terminal non-coding region (Fig. 1B, shaded). In each splicing isoform, the open reading frame begins at the consensus start methionine with an upstream in-frame stop codon (Fig. 1, B and C, respectively) (31). This IP5Pase has two conserved catalytic motifs of IP5Pases (Fig. 2). To other IP5Pases, PIPP has a lot of similarity (24), whereas Type I phosphatase and Type III phosphatase SHIP have little resemblance. The full length of this IP5Pase also has greater identity with PIPP (53.3%) than do other IP5Pases (about 45%, see Fig. 2). However, no other motifs for interactions with other molecules are observed.

Northern Blot Analysis—The results of Northern blot analysis of the expression of the human novel IP5Pase are shown in Fig. 3. Two different transcripts of 2.0 kilobases and about 3.0 kilobases, respectively, are detected. The longer ones are the transcripts of Clone 1 and Clone 2, and the 2.0-kilobase transcript is that of Clone 3. This IP5Pase is expressed in all the tissues we tested, but its expression is especially high in heart, skeletal muscle, and kidney. Thus we designated this IP5Pase SKIP. Only the 3.0-kilobase transcript was detected in the brain.

Splicing Isoform Is Specifically Expressed According to the Type of Cell—To characterize the endogenously expressed SKIP protein, we generated a specific antibody against SKIP and blotted lysates from different tissues and cells (Fig. 4A). In addition to the 51-kDa form, a smaller 43-kDa band was detected. In rat brain lysate and mouse neuroblastoma N1E-115 cells, only the 51-kDa form was expressed. In contrast, in mouse C2C12 myoblast cells, C3H/10T1/2 cells, and Swiss 3T3 fibroblast cells, only the 43-kDa band was detected. Surprisingly, although SKIP cDNA was obtained from the human testis library, its expression was very weak in rat testis lysate.

To confirm that the 43-kDa band is really an alternatively spliced isoform, we subcloned the full length of Clone 1 and 2 into Sf9 expression vector, and then they were expressed in COS-7 cells. As shown in Fig. 4B, in addition to the 51-kDa form, endogenously expressed in COS-7 cells, the 43-kDa band was detected only in cells expressing Clone 2. This indicates that the 43-kDa band is indeed an alternative splicing isoform of SKIP.

Enzymatic Properties of Human SKIP—GST-conjugated SKIP (GST-SKIP) protein was expressed in Sf9 insect cells.
using a baculovirus system and purified with glutathione-Sepharose 4B beads. To characterize the substrate specificity, we examined the phosphatase activities for inositol polyphosphate and phosphatidylinositol (Fig. 5 and Fig. 6). SKIP hydrolyzed \( \text{Ins}(1,4,5)P_3 \) as well as \( \text{Ins}(1,3,4,5)P_4 \) to produce inositol bisphosphate and inositol trisphosphate, respectively (Fig. 5, A and B) (27). To further determine whether this phosphatase hydrolyzes D-4 or D-5 positions of inositol phosphates, we analyzed the activities for \( \text{Ins}(1,3,4)P_3 \). \( \text{Ins}(1,3,4)P_3 \) was generated by incubating \( \text{Ins}(1,3,4,5)P_4 \) with recombinant SHIP, a Type III 5-phosphatase, which hydrolyzes the D-5 position of \( \text{Ins}(1,3,4,5)P_4 \) to generate \( \text{Ins}(1,3,4)P_3 \). \( \text{Ins}(1,3,4)P_3 \) was further incubated with recombinant SKIP protein, but generation of inositol bisphosphate was not detected (Fig. 5C). This indicates that SKIP hydrolyzes the D-5 position of inositol polyphosphates.

The catalytic motifs of SKIP are very similar to those of Type II phosphatases, including PIPP and synaptojanin (see Fig. 2), which can hydrolyze lipid substrates such as PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 as well as inositol polyphosphates (24, 32–35). Next, we determined whether SKIP can hydrolyze PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3, GST-SKIP, but not GST alone, markedly hydrolyzed not only PtdIns(4,5)P_2 but also PtdIns(3,4,5)P_3, indicating that SKIP hydrolyzes PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. Thus, SKIP is classified as a Type II 5-phosphatase (34).

To analyze the substrate specificity of SKIP, we determined the apparent \( K_m \) values of the 5-phosphatase by Lineweaver-Burk plot analysis, using purified recombinant protein (Fig. 7). \( K_m \) values of the PtdIns(4,5)P_2 and Ins(1,4,5)P_3 5-phosphatase are 180 \( \mu \)M and 1.15 mM, respectively. This \( K_m \) value for PtdIns(4,5)P_2 is comparable with that of other Type II 5-phosphatases, such as the 75-kDa Ins(1,4,5)P_3 5-phosphatase (28). SKIP hydrolyzes PtdIns(4,5)P_2 with a 6-fold higher affinity than for Ins(1,4,5)P_3, indicating that SKIP functions as a 5-phosphatase that has high specificity for PtdIns(4,5)P_2 and probably also for PtdIns(3,4,5)P_3.

**Subcellular Localization of SKIP**—Next, Myc epitope-tagged SKIP was expressed in COS-7 cells and immunostained with...
cells were stained with anti-SKIP antibody (C) in the neurites where SKIP concentrated (arrowheads) and rhodamine-phalloidin (D). SKIP staining was concentrated in the center of neurites. Some strong spots were seen in the neurites, where actin staining was reduced (Fig. 9, C and D, arrowheads).

In conclusion, we have cloned a novel protein, SKIP, encoding inositol polyphosphate 5-phosphatase. SKIP has two isoforms of 51 and 43 kDa, which hydrolyze the D-5 position of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3 but have a 6-fold higher specificity for PtdIns(4,5)P_2 than for Ins(1,4,5)P_3. Actin filaments are reduced wherever SKIP is localized, suggesting that SKIP helps to negatively regulate the assembly of the actin cytoskeleton. The relationship between PtdIns(4,5)P_2 phosphatase activity and actin reorganization or regulation of its phosphatase activity remains to be elucidated.

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anti-SKIP

phalloidin

Fig. 8. Overexpression of human SKIP in COS-7 cells. The Myc epitope-tagged 51-kDa form of SKIP was expressed in COS-7 cells. The cells were stained with anti-Myc polyclonal antibody (left) and rhodamine-phalloidin (right).

anti-Myc

phalloidin

Fig. 9. Intracellular localization of SKIP in differentiating N1E-115 neuroblastoma cells. Rat neuroblastoma N1E-115 cells were cultured in 10% fetal calf serum-containing (undifferentiated, A and B) and serum-deprived (differentiated, C and D) media for 48 h. The cells were stained with anti-SKIP antibody (A and C) and rhodamine-phalloidin (B and D). SKIP staining was concentrated in the center of the cell but also seen in the neurites. Some actin staining disappeared in the neurites where SKIP concentrated (C and D, arrowheads).

anti-Myc polyclonal antibody (left) and rhodamine-phalloidin (right).
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