We have cloned a novel inositol polyphosphate 5-phosphatase from the rat brain cDNA library. It contains two highly conserved 5-phosphatase motifs, both of which are essential for its enzymatic activity. Interestingly, the proline content of this protein is high and concentrated in its N- and C-terminal regions. One putative SH3-binding motif and six 14–3–3 binding motifs were found in the amino acid sequence. This enzyme hydrolyzes phosphate at the D-5 position of inositol 1,4,5-trisphosphate, inositol 1,3,4,5-tetrakisphosphate, and phosphatidylinositol 4,5-bisphosphate, consistent with the substrate specificity of type II 5-phosphatase, OCRL, synaptojanin and synaptojanin 2, already characterized 5-phosphatases. When the Myc-epitope-tagged enzyme was expressed in COS-7 cells and stained with anti-Myc polyclonal antibody, a signal was observed at ruffling membranes and in the cytoplasm. We prepared several deletion mutants and demonstrated that the 123 N-terminal amino acids (91–432) and a C-terminal proline-rich region containing 277 amino acids (725–1001) were essential for its localization to ruffling membranes. This enzyme might regulate the level of inositol and phosphatidylinositol polyphosphates at membrane ruffles.

Inositol and phosphatidylinositol polyphosphates play important roles in a variety of signal transduction systems. Therefore, intracellular levels of these second messenger molecules are thought to be tightly controlled and promptly changed by the enzymes in response to extracellular stimuli. Inositol polyphosphate 5-phosphatase is the enzyme that specifically hydrolyzes phosphate at the D-5 position of inositol or phosphatidylinositol polyphosphates and has been conserved from yeast to human. Seven different enzymes and numerous splice variant isoforms have been isolated in mammals (1–15). All 5-phosphatases possess two highly conserved catalytic motifs and are classified into three groups based on their substrate specificity. 1) Type I 5-phosphatase; this enzyme hydrolyzes only water-soluble substrates such as Ins(1,4,5)P_3^1^ and Ins(1,3,4,5)P_4\_2^3\_4. 2) SHIP 1 (for SH2 containing inositol polyphosphate 5-phosphatase 1) and SHIP 2; SHIP 1 selectively dephosphorylates Ins(1,3,4,5)P_4\_3\_4 and PtdIns(3,4,5)P_3\_4\_4 that contain phosphate at the D-3 position of the inositol ring (5, 7, 8). SHIP 2 hydrolyzes PtdIns(3,4,5)P_3\_4\_4 but its Ins(1,3,4,5)P_4\_4 phosphatase activity has not been confirmed (17, 18, 3) Type II 5-phosphatase, OCRL, synaptojanin and synaptojanin 2; these enzymes exhibit broad substrate specificity. They hydrolyze water-soluble substrates such as Ins(1,4,5)P_3\_3 and Ins(1,3,4,5)P_4 and lipid substrates such as PtdIns(4,5)P_2\_2 and PtdIns(3,4,5)P_3\_3 (6, 11, 12, 16, 19–22). Synaptojanin 2 is a recently discovered 5-phosphatase, and its catalytic motifs are identical to synaptojanin (11, 12). OCRL was identified as a causative gene of Lowe’s oculocerebrorenal syndrome, an X chromosome-linked developmental disorder (2). OCRL showed a strong preference for lipid substrate (16). The cells derived from the Lowe’s oculocerebrorenal syndrome patient were defective in OCRL activity and accumulated 2–3-fold more PtdIns(4,5)P_2\_2 than normal cells (20).

There is evidence that phosphatidylinositol polyphosphates such as PtdIns(4,5)P_2\_2 and PtdIns(3,4,5)P_3 play important roles in the regulation of the actin cytoskeleton (22–24). PtdIns(4,5)P_2\_2 binds to actin-binding proteins such as vinculin, α-actinin, profilin, and gelsolin, and promotes actin filament formation (25–27). Of the seven distinct 5-phosphatases, only synaptojanin has been demonstrated to hydrolyze PtdIns(4,5)P_2\_2 to actin regulatory proteins such as vinculin, α-actinin, and profilin in vitro (22). In addition, it has been shown that PtdIns(4,5)P_2\_2 and PtdIns(3,4,5)P_3 modulate the function of various proteins such as protein kinase C (28), phospholipase D (29, 30), protein kinase B/Akt (31), ATP-sensitive potassium channel (32–34), and ADP-ribosylation factor (35). Therefore, it is plausible that lipid phosphate, like OCRL and synaptojanin, hydrolyzes those phosphatidylinositol polyphosphates and plays an important role in the actin depolymerization mechanism as well as other functions.

Here, we report the cloning and characterization of a novel 5-phosphatase. This enzyme dephosphorylates the D-5 position of inositol and phosphatidylinositol polyphosphates at ruffling membranes.

**EXPERIMENTAL PROCEDURES**

**Molecular Cloning of the Rat Novel Inositol Polyphosphate 5-Phosphatase**—A partial human cDNA clone (GenBank**™**) accession number H14886) was obtained from Genome System, Inc. (St. Louis, MO). H14886 is a 468-base pair human cDNA fragment, and its deduced amino acid sequence contains a 5-phosphatase catalytic motif-like sequence. The fragments were labeled by random hexamer priming and used to screen a ZAP II rat brain cDNA library (Stratagene, La Jolla, CA). Positive clones were subcloned into pBluescript SK(–) by an in vivo excision method and sequenced. Complete sequence data was obtained from both strands using a conventional dideoxy-termination method.

**Northern Blot Analysis**—Membranes containing mRNA (2 μg of poly(A) RNA was contained in each lane) were purchased from OriGene Technology, Inc. (Rockville, MD). Total RNA was isolated from cultured cells using MagExtractor-RNA- and MagExtractor System (TOYOBO Co., Ltd., Osaka, Japan), and approximately 10 μg of the RNA was...
blotted on a nylon membrane. The sequence of rat novel 5-phosphatase specific region, nucleotides 2362–3000, was amplified by polymerase chain reaction and used as an \[a\]-\(^{32}\)P-dCTP-labeled 0.7-kb cDNA probe. Hybridization was performed following the protocol of OriGene Technologies, Inc., and the probe was hybridized at 42 °C.

Cell Culture and Expression of Recombinant Proteins in COS-7 Cells—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (Nissui, Tokyo, Japan) containing 10% fetal calf serum and 60 mg/ml kanamycin, and kept at 37 °C in a humidified atmosphere of 95% air and 5% CO\(_2\). Rat novel 5-phosphatase cDNA, its deletion mutants and partial human SHIP 1 cDNA (nucleotides 1461–4079 of human SHIP 1 cDNA; GenBank™ accession number U57650) were subcloned into the eukaryotic pCMV6-Myc expression vector. Constitutively active Rac1, RacG12V construct, was ligated into the eukaryotic pEF-BOS-FLAG expression vector. All constructs were transfected into COS-7 cells by a conventional electroporation method, and cells were harvested or fixed after 48 h (22). The expression and the size of expressed proteins were checked by immunoblotting.

Dephosphorylation of Novel Inositol Polyphosphate 5-Phosphatase—Recombinant novel 5-phosphatase expressing cells (1 \(\times\) 10^6 cells/60-mm tissue culture dish (Falcon)) were washed once with PBS(−) and harvested with 200 \(\mu\)l of the reaction buffer for alkaline phosphatase (50 mM Tris-HCl, pH 7.5, 1 mM MgCl\(_2\), 10 \(\mu\)g/ml leupeptin, and 10 \(\mu\)g/ml aprotinin). After a brief sonication, the cell lysate was incubated for 10 min at 30 °C. It was further incubated for 15 min at the same temperature. The mixture was analyzed by immunoblotting.

Enzyme Assay—Cells (7 \(\times\) 10^6 cells/150-mm tissue culture dish (Falcon)) transfected with Myc-epitope-tagged novel 5-phosphatase construct or empty vector were cultured for four days and harvested with 1 ml cold lysis buffer (40 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10 \(\mu\)g/ml leupeptin, and 10 \(\mu\)g/ml aprotinin). The cells were briefly sonicated and centrifuged at 14,000 \(\times\) g for 20 min at 4 °C. Supernatant was collected and rotated with 25 \(\mu\)l of anti-Myc monoclonal antibody (Santa Cruz Biotechnology, Inc.) for 1 h at 4 °C. Then protein A beads (Pierce) (50 \(\mu\)l) were added, and the solution was rotated for 1 h. After that, the beads were washed with lysis buffer five times and treated with SDS-PAGE sample buffer (equal volume) and boiled for 5 min. The sample was resolved on a 12% SDS-PAGE gel and transferred onto a PVDF membrane. The membrane was blocked with 5% milk powder in TBST for 1 h and incubated with primary antibodies for 2 h. The immunoblot was detected with a chemiluminescent detection kit (Thermo Fisher).
Novel Inositol Polyphosphate 5-Phosphatase

**FIG. 2. Comparison of characteristic amino acid sequences of inositol polyphosphate 5-phosphatases with that of PIPP.** Two highly conserved motifs of 5-phosphatase are aligned with PIPP. Asterisks (*) indicate the amino acids shown to be important for 5-phosphatase activity (45, 46). The enzymes are classified by specificity for substrates. Type I 5-phosphatase hydrolyzes Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄. The GenBank™ accession numbers are as follows: type I (X75094); SHIP 1 (U57650); SHIP 2 (Y14385); type II (M74161); OCRL (M88162); synaptojanin (U45479).

times and finally suspended in buffer for inositol polyphosphates or PtdIns(4,5)P₂ phosphatase assay.

**RESULTS AND DISCUSSION**

cDNA Cloning of the Novel Inositol Polyphosphate 5-Phosphatase—The predicted amino acid sequence of human EST clone H14886 is very similar to OCRL and synaptojanin, and contains the 5-phosphatase catalytic motif2-like sequence (2, 6). Therefore, we regarded H14886 as a partial cDNA fragment with the length of our cDNA clone (Fig. 4). Searches of the current GenBank™ database with BLASTN algorithm revealed that part of synaptojanin and synaptojanin 2 are homologous to the 1900–2040-base pair region of the rat putative 5-phosphatase cDNA sequence (60 and 69%, respectively), which includes the 5-phosphatase catalytic motif2.

**FIG. 3. Immunoblotting of PIPP recombinant protein.** Recombinant PIPP was expressed in COS-7 cells and detected by immunoblotting with anti-Myc polyclonal antibody (left). Cell lysate incubated without calf intestine alkaline phosphatase (CIAP) for 15 min at 30 °C (middle), and with calf intestine alkaline phosphatase (right).
acids but the dephosphorylated PIPP still seemed slightly larger in SDS-polyacrylamide gel electrophoresis (Fig. 3). This may be due to the proline-rich sequence of PIPP (40).

Our results suggest that recombinant PIPP is highly phosphorylated in the cytoplasm. But Western blot analysis revealed that anti-phosphotyrosine antibody (PY20) did not stain phosphorylated PIPP (data not shown). Therefore, PIPP is probably phosphorylated at serine/threonine residues, which might regulate the activity.

Northern Blot Analysis—The results of Northern blot analysis are shown in Fig. 4. PIPP was expressed in brain, heart, kidney, stomach, small intestine, and lung. The size of the mRNA was estimated at 3.4 kb in those tissues. In spleen, thymus, skeletal muscle, testis, and skin, no signal was observed. All cultured cells examined expressed PIPP, but Jurkat and HL-60 cells showed an especially high PIPP mRNA content. Only liver showed the 2.4-kb band. This signal might indicate the existence of a splicing isoform of the PIPP or another undiscovered 5-phosphatase in liver.

Enzymatic Activity—Myc-epitope-tagged PIPP was expressed in COS-7 cells and purified by immunoprecipitation using anti-Myc monoclonal antibody. When Ins(1,4,5)P3 was incubated with recombinant PIPP and the mixture was analyzed by high performance liquid chromatography, two peaks were observed (Fig. 5A). The retention time of these two peaks indicated that the former was inositol bisphosphate and the latter was substrate, Ins(1,4,5)P3 (37). PIPP hydrolyzed Ins(1,4,5)P3 to inositol bisphosphate and also removed one phosphate from Ins(1,3,4,5)P4 and produced inositol trisphosphate (Fig. 5B). To determine which phosphate was hydrolyzed by PIPP, Ins(1,3,4)P3 was incubated with PIPP, but no hydrolysis was observed in this case (Fig. 5C). These results indicate that PIPP specifically hydrolyzes phosphate at the D-5 position in Ins(1,4,5)P3 and Ins(1,3,4,5)P4.

The catalytic motifs of PIPP are very similar to OCRL and synaptojanin. These 5-phosphatases hydrolyze inositol polyphosphates as well as PtdIns(4,5)P2 and PtdIns(3,4,5)P3 (16, 19–22). We also checked the lipid phosphatase activity of PIPP. As shown in Fig. 6, when [3H]PtdIns(4,5)P2 was incubated with PIPP, [3H]phosphatidylinositol monophosphate was formed. When the phosphate at the D-5 position of PtdIns(4,5)P2 was labeled with 32P and incubated with PIPP, [32P]phosphatidylinositol monophosphate was not formed (data not shown), showing that PIPP has lipid phosphatase activity and can hydrolyze phosphate at the D-5 position of PtdIns(4,5)P2. Thus, according to the substrate specificity, PIPP is classified with type II 5-phosphatase, OCRL, synaptojanin, and synaptojanin 2 (21).

Overexpression of PIPP and Its Deletion Mutants in COS-7 Cells—Myc-epitope-tagged PIPP was expressed in COS-7 cells, and its cellular localization was revealed by anti-Myc monoclonal antibody. As indicated in Fig. 7A, some PIPP was clearly condensed at the cell periphery and some dispersed in the cytoplasm. The same cells were stained with rhodamine-phalloidin to visualize the actin cytoskeleton (Fig. 7B). Some PIPP was localized in cortical areas with the actin filaments locating at ruffling membranes. However, alterations in the actin cytoskeleton were not observed, in contrast to reports on overexpression of OCRL and synaptojanin in cells (22, 47).

We prepared several deletion mutants of PIPP to determine the region essential for its localization to ruffling membranes and co-expressed it with constitutively active Rac1 mutant in COS-7 cells (Fig. 8, panel I). Constitutively active Rac1 mutant co-localizes with actin filaments at ruffling membranes and...
accelerates membrane ruffling (48, 49). Apparently, PIPP co-localized with the active Rac1 mutant (Fig. 8, panel II, A and B) confirming that PIPP localizes at ruffling membranes. Mut2 and Mut3 exhibited the same localization pattern as the full-length of PIPP (data not shown). Truncated PIPP without the N-terminal proline-rich region (Mut4) located to membrane ruffles at a much lower intensity than wild type or mutants with partial proline-rich regions (Mut2 and Mut3, Fig. 8, panel II, C and D). When the C-terminal proline-rich region containing 277 amino acids (725–1001) was deleted (Mut6), the localization of the enzyme to the ruffling membranes was abolished (Fig. 8, panel II, E and F). Mut5 did not contain either the N- or C-terminal proline-rich regions and did not localize to ruffling membranes (Fig. 8, panel II, G and H). These results indicate that the C-terminal proline-rich region from residues 725 to 1001 is essential for the localization of PIPP to ruffling membranes and a part of the N-terminal proline-rich region from residues 311 to 433 contributes to the localization. PIPP contains one putative SH3-binding motif and five 14–3-3ζ-binding motifs in the proline-rich regions that are involved in its cellular distribution. The identification of PIPP-binding proteins should deepen the understanding of the function of PIPP.

Mut1 did not contain the two catalytic motifs of 5-phosphatase localized to ruffling membranes (Fig. 8, panel II, I and J). Membrane ruffling, induced by constitutively active Rac1 mutant, was not affected by the co-expression of any deletion mutants of PIPP (Fig. 8, panel II, B, D, F, H, and J). These results indicate that PIPP does not participate in the re-organization of the actin cytoskeleton but may be involved in modulation of the function of inositol and phosphatidylinositol polyphosphate-binding proteins that are present at membrane ruffles.

In summary, we have cloned a novel 5-phosphatase from a rat brain cDNA library. It is a proline-rich protein and hydrolyzed the D-5 position of phosphate in Ins(1,4,5)P3, Ins(1,3,4,5)P4, and PtdIns(4,5)P2. Therefore, we designated this novel 5-phosphatase as PIPP. PIPP is localized at membrane ruffles and may be involved in the modulation of the function of proteins that are present at membrane ruffles.
