Identification and Characterization of a Sac Domain-containing Phosphoinositide 5-Phosphatase*  

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We have characterized a novel Sac domain-containing inositol phosphatase, hSac2. It was ubiquitously expressed but especially abundant in the brain, heart, skeletal muscle, and kidney. Unlike other Sac domain-containing proteins, hSac2 protein exhibited 5-phosphatase activity specific for phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. This is the first time that the Sac domain has been reported to possess 5-phosphatase activity. Its 5-phosphatase activity for phosphatidylinositol 4,5-bisphosphate ($K_m = 14.3 \mu M$) was comparable with those of Type II 5-phosphatases. These results imply that hSac2 functions as an inositol polyphosphate 5-phosphatase.

Phosphatidylinositol (PI)$^1$ phosphates, trace amounts of phospholipids in eukaryotic cells, exist as seven different molecules that are produced as a result of the phosphorylation of single or multiple sites of the inositol head group of phosphatidylinositol, including PI(4)P, PI(3)P, PI(5)P, PI(4,5)P$_2$, PI(3,4)P$_2$, PI(3,5)P$_2$, and PI(3,4,5)P$_3$. These lipids are now widely recognized as important regulators in a variety of cellular functions such as membrane trafficking, cytoskeletal reorganization, cell survival and cell proliferation (1–4).

A variety of phosphoinositides are hydrolyzed by phosphatases with different substrate specificities. They are classified into four groups according to the position of the phosphate that they hydrolyze, 1-, 3-, 4-, or 5-phosphatase. Among many phosphoinositide phosphatases, 5-phosphatase forms a fairly large family. According to the substrate specificity, it is classified into four types. Type I 5-phosphatases hydrolyze only water-soluble substrates such as Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ (5).

Type II enzymes hydrolyze not only the water-soluble inositol phosphates but also lipid substrates such as PI(4,5)P$_2$ and PI(3,4,5)P$_3$. Type II 5-phosphatases are further divided into four subgroups. All type II 5-phosphatases have two conserved motifs, WXGDXNR and KXRPAW/(T/C)DR(IV)LW/R/K. The first subgroup includes a 75-kDa protein isolated from human platelets and is now called 5-phosphatase II or GIP (GAP domain-containing inositol 5-phosphatase) (6). This subgroup also includes Lowe’s oculocerebrorenal syndrome (OCRL) protein. This protein hydrolyzes the phospholipid substrates 10–30-fold more than type I 5-phosphatase (7). In the kidney proximal tubule cells from a Lowe’s syndrome patient, PI(4,5)P$_2$ was found to accumulate, even though other inositol polyphosphate 5-phosphatases were present, suggesting that OCRL protein regulates the PI(4,5)P$_2$ levels in the cells (8). Synaptojanin 1 and 2 are also subjected to group II enzymes that participate in synaptic vesicle trafficking. Synaptojanins form complexes with dynamin, amphiophysin, and Grb2 to promote synaptic vesicle recycling (9, 10).

Synaptotagmins contain two phosphatase domains. One is a phosphatase domain at the C terminus, which is common to all type II 5-phosphatases. This domain can hydrolyze PI(4,5)P$_2$ to PI(4)P but cannot use PI(4,5)P$_3$ or PI(3,5)P$_3$ as a substrate (11). In addition to this 5-phosphatase, synaptojanins contain Sac domains in the N terminus, which are homologous to yeast Sac1. Recently, the synaptojanin Sac domain and yeast Sac1 protein were found to dephosphorylate PI(4,5)P$_3$ but not PI(4,5)P$_2$ (12). Thus synaptojanins have both a PI(4,5)P$_2$ 5-phosphatase activity and a phosphoinositide phosphatase activity.

Proline-rich inositol polyphosphate 5-phosphatase (PIPP) is a recently identified type II 5-phosphatase that localizes at membrane ruffling areas (13). This phosphatase hydrolyzes phosphatidylinositol 5-phosphate at the 5-position of Ins(1,4,5)P$_3$, Ins(1,3,4,5)P$_4$, and PI(4,5)P$_2$. 5-Phosphatase that induces arborization (Pharbin) and skeletal muscle- and kidney-enriched inositol phosphatase (SKIP) also seem to be members of the type II 5-phosphatases (14, 15). Pharbin has a CaaX motif at the C terminus and localizes at membranes. It hydrolyzes Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ more effectively than PI(4,5)P$_2$. On the other hand, SKIP preferentially hydrolyzes phosphoinositides, such as PI(4,5)P$_2$ and PI(3,4,5)P$_3$ to water-soluble inositol polyphosphates.

Type III enzymes hydrolyze phosphate at the 5-position of phosphoinositide and inositol polyphosphate, which have a 3-position phosphate group. There are two such enzymes designated as SHIP 1 and 2 SH$_3$-containing inositol phosphatase (16, 17). SHIPs contain an SH$_2$ domain at the N terminus and form complexes with intracellular signaling molecules such as Grb2 and Shc (18). Because these enzymes hydrolyze PI 3-kinase products such as PI(3,5)P$_2$ and PI(3,4,5)P$_3$, SHIPs are thought to have a negative function and to terminate signals from PI 3-kinase. Type IV 5-phosphatase was originally reported to hydrolyze only PI(3,5)P$_2$ and PI(3,4,5)P$_3$, but it has now been found that it also hydrolyzes PI(4,5)P$_2$ (19). However, this group of 5-phosphatases does not use water-soluble inositol phosphates as substrates.

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‡ The abbreviations used are: PI, phosphatidylinositol; PI(3)P, phosphatidylinositol (3)-monophosphate; PI(4)P, phosphatidylinositol (4)-monophosphate; PI(5)P, phosphatidylinositol (5)-monophosphate; PI(4,5)P$_2$, phosphatidylinositol (4,5)-bisphosphate; PI(3,4,5)P$_3$, phosphatidylinositol (3,4,5)-trisphosphate; Ins(1,4,5)P$_3$, inositol (1,4,5)-trisphosphate; Ins(1,3,4,5)P$_4$, inositol (1,3,4,5)-tetrakisphosphate; GST, glutathione $S$-transferase; hSac2, human Sac2; OCRL, Lowe’s oculoencephalorenal syndrome; SHIP, SH$_3$-containing inositol phosphatase; GIP, GAP domain containing inositol 5-phosphatase; pharbin, 5-phosphatase that induces arborization; PIPP, proline-rich inositol polyphosphate 5-phosphatase; SKIP, skeletal muscle- and kidney-enriched inositol phosphatase.
The Sac domain was originally found in yeast Sac1p (20, 21). The SAC1 mutation suppressed the phenotypes seen in Sec14 mutant yeast. Sec14p is the yeast PI/phosphatidylcholine transfer protein. Several of the Sac domain-containing proteins have been identified in yeast. Fig4p protein, which was composed only of the Sac domain, was identified from the yeast mutant that causes mating defects (22). Inp51p, Inp52p, and Inp53p are 5-phosphatases that contain the Sac domain in the N terminus-like synaptojanin. Recently, Guo et al. (12) found that these Sac domains, except Inp51p, possess phosphoinositide phosphatase activity. The Sac domain is 400 amino acids in length and consists of seven conserved motifs that appear to define the catalytic regions of enzyme activity (see Fig. 3). The sequence RXXXNCLDCLDRTN within the sixth motif is the most particular. The CXXR(T/S) motif found within this sequence appears to define the catalytic region of the phosphatase and is also seen in a variety of metal-independent protein phosphatases (12). In addition to synaptojanins, three additional Sac domain-containing proteins (KIAA0851, KIAA0966, and KIAA0274) exist in humans. Among these proteins, only the rat homolog of KIAA0851 (rSac1), which is the most closely related to yeast Sac1p, was characterized (23). This enzyme showed the same spectrum for the substrate specificity of yeast Sac1p and hydrolyzed PI(3)P, PI(4)P, PI(3,5)P2.

![Fig. 1. Nucleotide and corresponding amino acid sequence of hSac2 cDNA. The amino acid sequence of the Sac domain is underlined.](source: http://www.jbc.org/)

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However, KIAA0966 (hSac2) and KIAA0274 (hSac3) still have not been characterized. We studied the characterization of hSac2 and determined the lipid specificity. This enzyme has a different substrate specificity than rSac1, in that it can dephosphorylate the 5-position of phosphoinositide, PI(4,5)P\(_2\), and PI(3,4,5)P\(_3\). It hydrolyzes PI(4,5)P\(_2\) most effectively, and its substrate specificity for PI(4,5)P\(_2\) is comparable with those of Type II phosphatases.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phosphoinositides (PI(3)P, PI(4)P, PI(3,4)P\(_2\), PI(3,5)P\(_2\), PI(4,5)P\(_2\), and PI(3,4,5)P\(_3\)) and phosphatidylinositol (Ins(1,4,5)P\(_3\) and Ins(1,3,4,5)P\(_4\)) were purchased from Cell Signals, Inc. \(^\text{[g]-32P}\)ATP was purchased from PerkinElmer Life Science Products. Tetramethylrhodamine B isothiocyanate-conjugated wheat germ agglutinin and rhodamine-labeled phalloidin were each purchased from Sigma and Molecular Probes. Polyclonal antibody against c-Myc was purchased from Santa Cruz Biotechnology.

**FIG. 2.** Comparison of the domain structures of the various Sac domain-containing proteins. hSac2, yeast Sac1p, rat Sac1p, Inp52p, and Inp53p share the homology sequence at the C terminus of the Sac domain (white box). Inp51p, Inp52p, and Inp53p have a type II 5-phosphatase catalytic domain and a proline-rich region at the C terminus of the protein. However, hSac2 has no putative domain besides the Sac domain.

**FIG. 3.** Comparison of highly conserved sequences of the various Sac domains with that of hSac2. The Sac domains of hSac2, Sac1p, rat Sac1p, Inp52p, and Inp53p contain the conserved amino acids that encode the putative catalytic sequence (C\(_{\text{x5}}\)R) in motif 6. However, those of Inp51p are replaced with other amino acids, which are the reason for the lack of phosphatase activity seen in this protein. Highly conserved residues are highlighted as white on black.

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**Materials**—Phosphoinositides (PI(3)P, PI(4)P, PI(3,4)P\(_2\), PI(3,5)P\(_2\), PI(4,5)P\(_2\), and PI(3,4,5)P\(_3\)) and phosphatidylinositol (Ins(1,4,5)P\(_3\) and Ins(1,3,4,5)P\(_4\)) were purchased from Cell Signals, Inc. \(^{\text{[g]-32P}}\)ATP was purchased from PerkinElmer Life Science Products. Tetramethylrhodamine B isothiocyanate-conjugated wheat germ agglutinin and rhodamine-labeled phalloidin were each purchased from Sigma and Molecular Probes. Polyclonal antibody against c-Myc was purchased from Santa Cruz Biotechnology.

**FIG. 4.** Northern blot analysis of expression of hSac2. Northern blot analysis of human hSac2 was carried out using N-terminal 666-base pair hSac2 cDNA as a probe. 2 \(\mu\)g of human mRNA was used in each lane. The numbers on the left are the sizes of the markers. Lane 1, brain; lane 2, heart; lane 3, skeletal muscle; lane 4, colon (no mucosa); lane 5, thymus; lane 6, spleen; lane 7, kidney; lane 8, liver; lane 9, small intestine; lane 10, placenta; lane 11, lung; lane 12, peripheral blood leukocyte. kbp, kilobase pair.

**FIG. 5.** Enzymatic properties of the recombinant hSac2 protein. **a**, phosphatase activity analysis of the recombinant GST-hSac2 protein for phosphoinositide and inositol phosphate substrates in vitro. 50 \(\mu\)M of various phosphoinositide and inositol phosphate substrates were incubated with 25 ng of GST-hSac2 at 37 °C for 15 min. The reaction product was measured by the malachite green method. **b**, kinetic properties of hSac2. PI(4,5)P\(_2\) (5-100 \(\mu\)M) was incubated with 25 ng of GST-hSac2 at 37 °C for 15 min. The reaction product was measured by the malachite green method. \(K_m\) values were calculated by Lineweaver-Burk analysis.
Molecular Cloning of hSac2—Full-length human cDNA clone of human Sac2 (GenBank accession number HJ06369) was obtained from Kazusa DNA Research Institute. hSac2 is a 4924-bp cDNA with 17-bp poly(A)+ stretch, which was inserted at the SalI-NotI site of pBlue-script II SK+ vector.

Northern Blot Analysis—A membrane containing mRNA (2 μg of poly(A) was contained in each lane) (human 12-lane multiple tissue Northern blot) was purchased from CLONTECH. The N-terminal 666-base pair cDNA were 32P-labeled using a random primer labeling kit (Takara Shuzo) following the manufacturer's protocol.

Baculovirus Expression of Recombinant hSac2—The expression constructs of glutathione S-transferase (GST) or GST-tagged full-length hSac2 were constructed as follows. GST construct was produced by ligating BglII/BamHI site polymerase chain reaction product encoding full-length GST amplified from pGEX-2T bacterial expression vector into the BamHI site of pFASTBAC1 (Life Technologies, Inc.). Then BamHI-NotI site full-length hSac2 fragments amplified by polymerase chain reaction were introduced into the BamHI site of the GST construct (GSThSac2). The GST and GSThSac2 proteins were expressed in Sf9 cells by infecting them with 1 ml of 1 × 10^9 units/ml recombinant virus and cultured in SF900 medium containing 5% fetal calf serum at 28 °C for 48 h.

Phosphatase Activity Assay—Baculovirus-expressing Sf9 cells were centrifuged and lysed in a cold lysis buffer (40 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The cells were briefly sonicated and centrifuged at 10,000 × g at 4 °C for 30 min. The supernatant was applied to 400 μl of glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech), then washed five times with a wash buffer (40 mM Tris-HCl, pH 7.5, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100) and three times with assay buffers for phosphoinositide phosphatase (50 mM Tris maleate, pH 6.0, 5 mM MgCl 2, 25 mM KCl, 0.25% β-octyl glucoside, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The recombinant proteins were eluted with three bed volumes of elution buffer (20 mM glutathione in 50 mM Tris maleate, pH 6.0). Phosphoinositide phosphatase and inositol 5-phosphatase activity assays were carried out as described by Maehama et al. (24) using 50 μM PI(3)P, PI(4)P, PI(5)P, PI(3,4)P 2, PI(3,5)P 2, PI(4,5)P 2, PI(3,4,5)P 3, Ins(1,4,5)P 3, and Ins(1,3,4,5)P 4 as substrates and purified baculovirus-expressed recombinant proteins as enzymes. Recombinant hSac2 protein was added, the mixture was further incubated at 37 °C for 15 min, and the phosphate release was quantified using a malachite green-based colorimetric assay for inorganic phosphate. (24).

RESULTS AND DISCUSSION

Identification of Novel Sac Domain-containing Protein—The Sac domain is a recently identified novel inositol polyphosphate phosphatase, and several family proteins have been identified in yeast. Nemoto et al. (23) identified a rat homologue of yeast Saclp, which was a prototypic member of the Sac domain-containing protein. Sac domain-containing proteins possess conserved amino acid motifs that are essential for inositol polyphosphate phosphatase activities. In an attempt to identify another member of the Sac domain-containing proteins, we searched the cDNA data base based on the amino acid sequence of these motifs and identified two novel clones. One is KIAA0274, (GenBank accession number NM104845) which is homologous to yeast Fig4p protein (22). The other is KIAA0966 (GenBank accession number XM005971), a 4923-bp clone, with an estimated open reading frame of 3396-bp that encodes a protein with a molecular mass of 120 kDa (Fig. 1). Unlike other Sac domain-containing proteins, KIAA0966 has no yeast counterparts (Fig. 2). We named this hSac2 (human Sac2). hSac2 shares 34.2% amino acid identity with Saclp and possesses seven motifs conserved in the Sac domain-containing proteins. As shown in Fig. 3, an active site motif CXXR(T/S) is found within the sixth motif, which also exists in metal-dependent protein phosphatases and inositol polyphosphate phosphatases. Some dissimilar amino acids in...
the fifth motif (Val^{117} to Asp^{227}) were also observed (Fig. 3).

Tissue Distribution of hSac2—The expression of hSac2 was analyzed by Northern blot analysis. The 4.9-kilobase transcript was detected in every tissue tested but was especially high in the brain, heart, skeletal muscle, kidney, and placenta (Fig. 4).

Enzymatic Properties of hSac2—The Sac-containing proteins studied so far exhibited intrinsic inositol phosphatase activities. Yeast Sac1 and rat Sac1 exhibited phosphatase activities for PI(3)P, PI(4)P, and PI(3,5)P_2 but not PI(4,5)P_2 (22). We expressed GST-conjugated full-length hSac2-2 protein in insect cells and purified the protein. The intrinsic phosphatase activities of the purified recombinant protein for the phosphoinositides were analyzed. Unexpectedly, hSac2 only hydrolyzed PI(5)P_2 and PI(3,4,5)P_3, as synaptojanin 1 or SKIP did (Fig. 5a). It did not hydrolyze PI(3)P, PI(4)P or PI(3,5)P_2, as rat Sac1 did. We also analyzed its phosphatase activities for inositol phosphates, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. Neither of them was hydrolyzed, and this revealed that hSac2 is a phosphoinositide-specific 5-phosphatase (Fig. 5a). The mutant in which asparagine at amino acid 460 was replaced with alanine lost its substrate specificity.

In other parts of the Sac domain are present, which may contribute to the substrate specificity. In mammalians, at least three Sac-containing proteins (Sac1, Sac2, and Sac3) are present in addition to synaptotagmins. Thus it remains to be determined whether these Sac proteins have different substrate specificities and functions.

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