Identification and Characterization of a Novel Human Sphingosine-1-phosphate Phosphohydrolase, hSPP2*

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Sphingosine 1-phosphate (S1P), a bioactive lipid molecule that acts as both an extracellular signaling mediator and an intracellular second messenger. S1P is synthesized from sphingosine by sphingosine kinase and is degraded either by S1P lyase or by S1P phosphohydrolase. Recently, mammalian S1P phosphohydrolase (SPP1) was identified and shown to constitute a novel lipid phosphohydrolase family, the SPP family. In this study we have identified a second human S1P phosphohydrolase, SPP2, based on sequence homology to human SPP1. SPP2 exhibited high phosphohydrolase activity against S1P and dihydrosphingosine 1-phosphate. The dihydrosphingosine-1-phosphate phosphohydrolase activity was efficiently inhibited by excess S1P but not by lysophosphatidic acid, phosphatidic acid, or glycerol 3-phosphate, indicating that SPP2 is highly specific to sphingoid base 1-phosphates. Immunofluorescence microscopic analysis demonstrated that SPP2 is localized to the endoplasmic reticulum. Although the enzymatic properties and localization of SPP2 were similar to those of SPP1, the tissue-specific expression pattern of SPP2 was different from that of SPP1. Thus, SPP2 is another member of the SPP family that may play a role in attenuating intracellular S1P signaling.

Sphingosine 1-phosphate (S1P), a sphingolipid metabolite, regulates diverse biological processes including mitogenesis, differentiation, migration, and apoptosis both as an extracellular mediator and as an intracellular second messenger (1–3). Extracellular effects of S1P are known to be mediated via the endothelial differentiation gene (Edg) family of plasma membrane G-protein-coupled receptors, whereas its intracellular targets have yet to be determined (2, 3). S1P is synthesized by the phosphorylation of sphingosine and catalyzed by sphingosine kinase. Once formed, S1P is rapidly degraded by S1P lyase to hexadecenal and phosphoethanolamine or dephosphorylated by S1P phosphohydrolase.

The existence of a S1P-specific phosphohydrolase had been suggested by biochemical analyses using cultured skin fibroblasts and rat liver (4, 5). In 2000, murine S1P phosphohydrolase (mSPP1) was cloned as a S1P phosphohydrolase based on sequence homology to the yeast sphingoid base 1-phosphate phosphatase, Lcb3p/Lbp1p/Ysr2p (6). Recently, a human homolog of mSPP1, hSPP1, which exhibits 76% identity and 81% similarity to mSPP1, was identified (7). These mammalian SPP1s and their two yeast homologs, Lcb3p and Ysr3p, constitute the SPP family, which is distinct from another lipid phosphohydrolase family, the type 2 lipid phosphate phosphohydrolases (LPP), both in sequence and in biochemical properties.

Accordingly, the SPP family members are highly specific to sphingoid base 1-phosphates, including S1P, dihydrosphingosine 1-phosphate (dihydroy-S1P), and phytosphingosine 1-phosphate (6–9); yet the LPP family members have broad substrate specificities including S1P, phosphatidate (PA), lysophosphatidate (LPA), ceramide 1-phosphate, and diacylglycerol pyrophosphate (10–14). Proteins from both the SPP and LPP families contain the following three conserved motifs: motif 1, KXXXXXXRP; motif 2, PSGH; and motif 3, SRXXXXX-HXXXD. These motifs are found in a superfamily of phosphatases that include several lipid phosphatases, glucose-6-phosphatases, bacterial nonspecific acid phosphatases, and chloroperoxidase (15). The crystal structure of chloroperoxidase disclosed that these conserved motifs were in close proximity and that together they constituted a binding pocket for the cofactor vanadate, a compound structurally similar to phosphate (16). Thus, these motifs are believed to constitute the active site in members of this phosphatase superfamily, a conclusion supported by mutational analyses of yeast Dpp1p and mammalian LPP-1 (17, 18).

We report here that we have identified a second human S1P phosphohydrolase, termed hSPP2, based on sequence homology to hSPP1. hSPP2 exhibited high phosphohydrolase activity specifically against sphingoid base 1-phosphates. Additionally, in contrast to the ubiquitous expression of hSPP1, the expression of hSPP2 was restricted to specific tissues. Using immunofluorescence microscopy we also demonstrated that hSPP2 is localized to the endoplasmic reticulum (ER), suggesting that it functions in attenuating intracellular S1P signaling.

EXPERIMENTAL PROCEDURES

DNA Construction—pEFGP-C1 was purchased from Clontech.

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The abbreviations used are: S1P, sphingosine 1-phosphate; dihydro-S1P, dihydrosphingosine 1-phosphate; Edg, endothelial differentiation gene; PA, phosphatidate; LPA, lysophosphatidate; ER, endoplasmic reticulum; HA, hemagglutinin; ORF, open reading frame; PBS, phosphate-buffered saline; SPP, S1P phosphohydrolase; mSPP1, murine SPP1; hSPP1, human homolog of mSPP1; LPP, type 2 lipid phosphate phosphohydrolases; HEK, human embryonic kidney; hSPP2-HA, C-terminal HA-tagged hSPP2; mSPP1-HA, C-terminal HA-tagged mSPP1; EST, expressed sequence tag.
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RESULTS AND DISCUSSION

Identification of hSPP2—To identify a new S1P phosphohydrolase, the GenBank™ human EST data base was searched using the BLAST program for sequences similar to the hSPP1 sequence. We found several ESTs including BG696302, BM127455, BM830654, and AW779536 with high sequence homology to hSPP1. These ESTs contained different fragments but were derived from an identical cDNA, subsequently named human SPP2 (hSPP2). The ORF of hSPP2 encodes 399 amino acids with a molecular mass of 44.7 kDa (Fig. 1A). The hSPP2 sequence indicated 39.3% identity and 69.7% similarity to hSPP1. hSPP2 also shows significant homology to other SPP sequences. We found several ESTs including BG696302, BM127455, BM830654, and AW779536 with high sequence homology to hSPP1. These ESTs contained different fragments but were derived from an identical cDNA, subsequently named human SPP2 (hSPP2). The ORF of hSPP2 encodes 399 amino acids with a molecular mass of 44.7 kDa (Fig. 1A). The hSPP2 sequence indicated 39.3% identity and 69.7% similarity to hSPP1. hSPP2 also shows significant homology to other SPP family members, whereas it is divergent from the LPP family (Fig. 1B). Although the N-terminal hydrophilic region of hSPP2 was shorter than that of hSPP1, the overall hydropathy profile of hSPP2 was very similar to that of hSPP1 (Fig. 1C), suggesting that the transmembrane structure of hSPP2 is the same as

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that of hSPP1. The TopPredII 1.1 program (19) predicts hSPP2 to be an integral membrane protein with as many as nine membrane-spanning segments. The three conserved motifs found in the superfamily of phosphatases, including several lipid phosphatases, were also present in hSPP2 (Fig. 1A).

Characterization of Phosphohydrolase Activity of hSPP2—To investigate whether hSPP2 had S1P phosphohydrolase activity, HEK 293 cells were transiently transfected with a pcDNA3 hSPP2-HA encoding the C-terminal HA-tagged hSPP2 (hSPP2-HA). For comparison we also constructed a pcDNA3 mSPP1-HA that encodes the C-terminal HA-tagged mSPP1 (mSPP1-HA). Total cell lysates were prepared from HEK 293 cells, transfected with the respective plasmids, and separated by SDS-PAGE. This was followed by immunoblotting using anti-HA antibodies. A band with a apparent molecular mass of 35 kDa was specifically detected in hSPP2-HA-transfected cell lysates and was absent in vector-transfected cell lysates (Fig. 2, lanes 1 and 2). mSPP1-HA was detected as a band at 40 kDa (Fig. 2, lane 3). These mobilities were faster than the predicted molecular masses of hSPP2-HA (46.1 kDa) and mSPP1-HA (49.2 kDa).

Using membrane fractions and [4,5-3H]dihydro-S1P we also performed an in vitro phosphohydrolase assay. In contrast to vector-transfected HEK 293 cells, which had low levels of activity, cells overproducing hSPP2-HA and mSPP1-HA exhibited a 6.3- and 11.5-fold increase in phosphohydrolase activity, respectively (Fig. 3, A and B, lanes 1 and 2). However, because mSPP1-HA was expressed 1.6 times more abundantly than hSPP2-HA (Fig. 2), the specific activity of hSPP2 against dihydro-S1P was similar to that of mSPP1.

To investigate the substrate specificity of hSPP2, the in vitro phosphohydrolase assay was performed using [3-3H]S1P. Similar to the results obtained using [4,5-3H]dihydro-S1P (Fig. 3B, lanes 1 and 2), cells overproducing hSPP2-HA exhibited an ~6-fold higher phosphohydrolase activity against [3-3H]S1P than did vector-transfected cells (Fig. 3B, lanes 3 and 4). Next, we performed a competition assay using [4,5-3H]dihydro-S1P and various excess cold lipids, including dihydro-S1P, S1P, LPA, PA, and glycerol 3-phosphate. S1P inhibited the dihydro-S1P phosphohydrolase activity as efficiently as dihydro-S1P (Fig. 3C). However, LPA, PA, and glycerol 3-phosphate had little inhibitory effect (Fig. 3C). These results indicated that hSPP2 is a phosphohydrolase specific to sphingoid base 1-phosphates.

A previous study demonstrated that SPP1s are Mg2+-independent phosphohydrolases (7). To examine the requirement of Mg2+ for the SPP2 activity, the S1P phosphohydrolase assay was performed in the presence of the metal chelator EDTA. The activity of SPP was not affected by 10 mM EDTA (Fig. 4). Furthermore, addition of 10 mM Mg2+ did not enhance the phosphohydrolase activity (data not shown). These results indicated that SPP2 is also a Mg2+-independent phosphohydrolase.
We next examined the effects of various phosphatase inhibitors, such as NaF, sodium orthovanadate, and β-glycerol phosphate, as well as Triton X-100 on the SPP2 activity. Previous studies demonstrated that NaF, sodium orthovanadate, and Triton X-100 have strong inhibitory effects on SPP1 activity, whereas β-glycerol phosphate is not effective (6, 7). Similar to SPP1, SPP2 was significantly inhibited by 10 mM sodium orthovanadate and 0.2% Triton X-100 but not by 10 mM β-glycerol phosphate (Fig. 4). In contrast, 10 mM NaF, which efficiently inhibited the SPP1 activity, had no effect on the SPP2 activity (Fig. 4). Moreover, even 100 mM NaF did not inhibit the SPP2 activity (data not shown).

Immunofluorescent Localization of SPP2—S1P can act both extracellularly, via cell surface Edg family receptors, and intracellularly. Therefore, it is important to distinguish whether hSPP2 plays a role in attenuating intracellular or extracellular S1P signaling. For this purpose, we performed an indirect immunofluorescence microscopic analysis to determine the localization of hSPP2. HEK 293 cells transiently transfected with pcDNA3 hSPP2-HA were subjected to immunostaining with anti-HA antibody. Fig. 5A shows a reticular immunofluorescence staining pattern apparent in the perinuclear and cytosolic regions. This staining pattern was attributable to localization of hSPP2-HA in the ER, because it was superimposable upon the pattern of ER stained by an antibody against the ER retention signal, KDEL (Fig. 5A). This staining pattern of hSPP2 was similar to that of mSPP1 (Fig. 5B), which also co-localized with anti-KDEL antibody (data not shown). This result about the localization of mSPP1 is consistent with the recent report by Spiegel and co-workers (20). To examine the possibility that some fraction of hSPP2 is localized to the plasma membrane, we performed an immunofluorescence microscopic analysis using non-permeabilized cells. HEK 293 cells co-transfected with pEGFP-C1 and pcDNA3 hSPP2-HA were fixed and treated with or without Triton X-100, and hSPP2-HA was immunostained with anti-HA antibody. Permeabilized cells exhibiting green fluorescence protein fluorescence were stained with HA antibody (Fig. 5C), indicating that pEGFP-C1 and pcDNA3 hSPP2-HA were efficiently co-introduced. However, when the permeabilization step was omitted, cells exhibited only green fluorescence protein fluorescence and were not stained with HA antibody (Fig. 5D). This result indicated that permeabilization was essential for staining of hSPP2-HA.
Thus, SPP2 may function in the ER to dephosphorylate intracellular S1P.

Tissue Distribution of SPP2 mRNA Expression—To compare the tissue distribution patterns of SPP1 and SPP2 mRNAs, we first examined the expression of hSPP1 mRNA in human tissues by Northern blotting. Similar to previously reported data for mSPP1 mRNA (6), hSPP1 mRNA was found to be ubiquitously expressed (Fig. 6A). A predominant 3.4-kb hSPP1 mRNA species was detected in all tissues, but the species varied in levels. The levels of the species were highest in placenta and kidney and lowest in peripheral blood and small intestine. A 1.0-kb transcript, which may be a spliced isoform, was detected only in placenta (Fig. 6A, lane 10). In contrast, the expression of hSPP2 mRNA was rather tissue-specific (Fig. 6B). We observed two mRNAs of 5.1 and 1.7 kb in size, and the 5.1-kb mRNA was detected in brain, heart, colon, kidney, small intestine, and lung but not in skeletal muscle, thymus, spleen, liver, placenta, and peripheral blood leukocyte (Fig. 6B). The expression levels of the 5.1-kb mRNA were highest in kidney and heart. The expression pattern of the 1.7-kb mRNA was similar but weaker than that of the 5.1-kb mRNA (Fig. 6B). Thus, the expression pattern of SPP2 was quite different from that for SPP1.

In summary, we identified a second S1P phosphohydrolase that shows similar activity, substrate specificity, and localization to previously identified SPP1s. Sequence and substrate specificity of hSPP2 confirm that SPP2 belongs to the SPP family. Although the expression patterns of SPP1 and SPP2 were quite different, both were expressed abundantly in kidney. It is unclear why two such S1P phosphohydrolases with similar enzymatic properties exist in the same tissue. However, yeast Saccharomyces cerevisiae also contains two sphingoid base 1-phosphate phosphatases, Lcb3p and Ysr3p, which also share similar enzyme activity and substrate specificity (8) and which are both localized to the ER (21). In synthetic complete medium the Δlcb3Δ mutant, but not the Δysr3Δ mutant, was significantly protected from loss of viability after severe heat shock (9). However, the Δysr3Δ mutant showed a dramatic enhancement in survival when cultured in YPD medium (9). Moreover, transcription of YSR3 was up-regulated by heat shock (9). These results suggest that Lcb3p and Ysr3p are differentially regulated by growth and stress conditions. Therefore, it is possible that SPP1 and SPP2 are differentially regulated by unidentified conditions and have different roles in such conditions. Additional work will be needed to determine their specific function and regulation.

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