Molecular Cloning and Functional Characterization of a Novel Mammalian Sphingosine Kinase Type 2 Isoform*

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Sphingosine-1-phosphate (SPP) has diverse biological functions acting inside cells as a second messenger to regulate proliferation and survival, and extracellularly, as a ligand for G protein-coupled receptors of the endothelial differentiation gene-1 subfamily. Based on sequence homology to murine and human sphingosine kinase-1 (SPHK1), which we recently cloned (Kohama, T., Oliver, A., Edsall, L., Nagiec, M. M., Dickson, R., and Spiegel, S. (1998) J. Biol. Chem. 273, 23722–23728), we have now cloned a second type of mouse and human sphingosine kinase (mSPHK2 and hSPHK2). mSPHK2 and hSPHK2 encode proteins of 617 and 618 amino acids, respectively, both much larger than SPHK1, and though diverging considerably, both contain the conserved domains found in all SPHK1s. Northern blot analysis revealed that SPHK2 mRNA expression had a strikingly different tissue distribution from that of SPHK1 and appeared later in embryonic development. Expression of SPHK2 in HEK 293 cells resulted in elevated SPP levels. D-erythro-dihydrosphingosine was a better substrate than D-erythro-sphingosine for SPHK2. Surprisingly, D, L-threo-dihydrosphingosine was also phosphorylated by SPHK2. In contrast to the inhibitory effects on SPHK1, high salt concentrations markedly stimulated SPHK2. Triton X-100 inhibited SPHK2 and stimulated SPHK1, whereas phosphatidylserine stimulated both type 1 and type 2 SPHK. Thus, SPHK2 is another member of a growing class of sphingolipid kinases that may have novel functions.

Sphingosine-1-phosphate (SPP)† is a bioactive sphingolipid metabolite that regulates diverse biological processes, acting both inside and outside cells (reviewed in Refs. 1 and 2). SPP plays important roles as a second messenger to regulate cell growth and survival (3, 4). Many external stimuli, particularly growth and survival factors, activate sphingosine kinase (SPHK), the enzyme that forms SPP from sphingosine. This rapidly growing list includes platelet-derived growth factor (3, 5–7), nerve growth factor (8, 9), vitamin D3 (10), muscarinic acetylcholine agonists (11), tumor necrosis factor-α (12), and cross-linking of the immunoglobulin receptors FcεR1 (13) and FcgR1 (14). Intracellular SPP, in turn, mobilizes calcium from internal stores independently of inositol triphosphate (11, 15), as well as eliciting diverse signaling pathways leading to proliferation (16, 17) and suppression of apoptosis (4, 8, 17–19). Moreover, competitive inhibitors of SPHK block the formation of SPP and selectively inhibit calcium mobilization, cellular survival, and survival induced by these various stimuli (reviewed in Ref. 1). Thus, it has been suggested that the dynamic balance between levels of the sphingolipids metabolites, ceramide and SPP, and consequent regulation of opposing signaling pathways, is an important factor that determines the fate of cells (19). For example, stress stimuli increase ceramide levels leading to apoptosis, whereas survival factors stimulate SPHK leading to increased SPP levels, which suppress apoptosis (19). Moreover, the SPHK pathway, through the generation of SPP, is critically involved in mediating tumor necrosis factor-α-induced endothelial cell activation (12), and the ability of high density lipoproteins to inhibit cytokine-induced adhesion molecule expression has been correlated with its ability to reset this sphingolipid rheostat (12). This has important implications for the protective function of high density lipoproteins against the development of atherosclerosis and associated coronary heart disease. Recent data have also connected the sphingolipid rheostat to allergic responses (20).

Interest in SPP has accelerated recently with the discovery that it is a ligand of the G protein-coupled cell surface receptor EDG-1 (17, 21). This rapidly led to the identification of several other related receptors, named EDG-3, -5, -6, and -8, which are also specific SPP receptors (reviewed in Refs. 2 and 22). Sphinganine-1-phosphate, which is structurally similar to SPP and only lacks the trans double bond at the 4 position, but not lysophosphatidic acid or sphingosylphosphorylcholine, also binds to these receptors (23), demonstrating that EDG-1 belongs to a family of G protein-coupled receptors that bind SPP with high affinity and specificity (reviewed in Refs. 2 and 22). The EDG-1 family of receptors are differentially

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† The abbreviations used are: SPP, sphingosine-1-phosphate; SPHK, sphingosine kinase; EDG, endothelial differentiation gene; mSPHK, mouse SPHK; EST, expressed sequence tag; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; hSPHK, human SPHK; BSA, bovine serum albumin; kb, kilobase; DMS, N,N-dimethyl-

spingosine; three-DHS, D-L-threo-dihydrosphingosine; TLC, thin layer chromatography; MES, 4-morpholineethanesulfonic acid.
expressed, mainly in the cardiovascular and nervous systems, and are coupled to a variety of G proteins and thus can regulate diverse signal transduction pathways culminating in pleiotropic responses depending on the cell type and relative expression of EDG receptors. Although the biological functions of the EDG-1 family of G protein-coupled receptors are not completely understood, recent studies suggest that binding of SPP to EDG-1 stimulates migration and chemotaxis (24, 25) and as a consequence may regulate angiogenesis (26, 27). EDG-5 may play a role in cytoskeletal reorganization during neurite retraction, which is important for neuronal differentiation and development (23, 28).

Critical evaluation of the role of SPP requires cloning of the enzymes that regulate its metabolism. Recently, we purified rat kidney SPHK to apparent homogeneity (29) and subsequently cloned the first mammalian SPHK, designated mSPHK1 (30). Independently, two genes, termed LC54 and LC55, were also shown to code for SPHKs in Saccharomyces cerevisiae (31). Moreover, data base searches identified homologues of mSPHK1 in numerous widely disparate organisms, including worms, plants, and mammals, demonstrating that the enzyme is encoded by a member of a highly conserved gene family (30). Comparison of the predicted amino acid sequences of the known SPHK1s revealed five blocks of highly conserved amino acids (30). However, several lines of evidence indicate that there may be multiple mammalian SPHK isoforms. The finding that SPHK activity in platelets could be chromatographically fractionated into several forms with differing responses to detergents and inhibition by known SPP inhibitors suggested the presence of multiple enzyme forms in human platelets (32). Moreover, homology searches against a comprehensive nonredundant data base revealed that several of the expressed sequence tags (dbEST) at NCBI had significant homology to conserved domains of mSPHK1 (30), yet they had substantial sequence differences. Thus, we embarked on an effort to clone other SPHK isoforms. We report here the cloning, functional characterization, and tissue distribution of a second type of mammalian SPHK (SPHK2) that has distinct sequence, properties, and tissue distribution.

EXPERIMENTAL PROCEDURES

Materials—SPP, sphingosine, and N,N-dimethylsphingosine were from Biomol Research Laboratory Inc. (Plymouth Meeting, PA). All other lipids were purchased from Avanti Polar Lipids (Birmingham, AL). [γ-32P]ATP (3000 Ci/mmole) was purchased from Amersham Pharma Biotech. Poly-L-lysine and collagen were from Roche Molecular Biochemicals (Indianapolis, IN). Restriction enzymes were from New England Biolabs (Beverly, MA). Poly(A)+ RNA blots of multiple mouse adult tissues were purchased from CLONTECH (Palo Alto, CA). LipofectAMINE PLUS and LipofectAMINE were from Life Technologies, Inc. Poly(A)+ cDNA Cloning of Murine Sphingosine Kinase-2 (mSPHK2)—BLAST searches of the EST data base identified a mouse EST clone (Genbank's accession number AA839233), which had significant homology to conserved domains of mSPHK1 (30) yet had substantial sequence differences. Using this EST, a second isoform of SPHK, denoted mSPHK2, was cloned by two different PCR approaches.

In the first, we used the method of PCR cloning from a mouse cDNA library (Stratagene). Approximately 1 x 10⁶ phages were plated on twenty 150-mm plates, plaques were collected, and plasmids were isolated using standard procedures (33). An initial PCR reaction was carried out with a sequence specific primer (M-3–1, 5′-CTCTTGGTG-CACCTGCGCCGTTAGTTG) and the M13 reverse primer. The longest PCR products were gel-purified and used as the template for a second PCR, which contained a sequence specific antisense primer (M-3–3, 5′-CCAGTTCTGGGCACTGGAAGCC-3′) and the T3 primer. The final PCR products were subcloned by TOPO TA cloning (Invitrogen) and then sequenced. Platinum high fidelity DNA polymerase (Life Technologies, Inc.) was used for the PCR amplifications with the following cycling parameters: 30 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 2 min with a final primer extension at 72 °C for 5 min.

In a second approach, 5′-RACE PCR was performed with the 5′-RACE System for rapid amplification of cDNA ends according to the manufacturer's protocol (Life Technologies, Inc.). Poly(A)+ RNA was isolated from Swiss 3T3 fibroblasts using a Quick Prep mRNA purification kit (Amersham Pharmacia Biotech). First strand cDNA was synthesized at 42 °C for 50 min with 5 μg of Swiss 3T3 poly(A)+ RNA using a target antisense primer designed from the sequence of AA839233 (m-SPHK1, 5′-GCGATGGAGCTCCTTGG) and SuperScript II reverse transcriptase (Life Technologies, Inc.). Two consecutive PCR reactions using this cDNA as a template and LA Taq polymerase (Takara) were carried out as follows: first PCR, 94 °C for 2 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and primer extension at 72 °C for 5 min with 5′-RACE Abridged Anchor Primer, 5′-GCCACCGCGTCGACTAGTACG and the target-specific antisense primer m-SPHK2, 5′-GCGATGGAGCTCCTTGGCAGAGGCTGAGGT; second PCR, same conditions except that the annealing temperature was 65 °C, with Abridged Universal Amplification Primer, 5′-GCCACCGCGTCGACTAGTACG and m-SPHK2, 5′-AGTCCCAACATGACCCAGTCCTGGAGC. PCR products were cloned into pCR2.1 and sequenced, and final PCR products were subcloned into pCR3.1 and pcDNA 3 expression vectors.

cDNA Cloning of Human Sphingosine Kinase 2 (hSPHK2)—Poly(A)+ RNA from HEK293 cells was used for a 5′-RACE reaction. Target-specific antisense primers (h-SPHK1, 5′-CCACCTCCTCGG-CACCGCTG and h-SPHK2, 5′-GAGGACACCGGCGAAGATCC) were designed according to the sequence of a human EST clone (accession number AA295570). First strand cDNA was synthesized with 5 μg of HEK293 mRNA and h-SPHK1. This cDNA was used as a template in an initial PCR reaction using 5′-RACE Abridged Anchor Primer and h-SPHK2. Then, nested PCR was carried out using the Abridged Universal Amplification Primer and h-SPHK2. The resulting PCR products were cloned and sequenced as described above.

Overexpression and Measurement of Activity of SPHK2—HEK293 cells (ATCC CRL-1573) and NIH 3T3 fibroblasts (ATCC CRL-1658) were cultured as described previously (34). HEK293 cells were seeded at 6 x 10⁶/well in poly-L-lysine-coated 6-well plates. After 24 h, cells were transfected with 1 μg of vector alone or with vectors containing sphingosine kinase constructs and 6 μl of LipofectAMINE PLUS reagent plus 4 μl of LipofectAMINE reagent/well. 1–3 days after transfection, cells were harvested and lysed by freeze-thawing as described previously (30). In some experiments, cell lysates were fractionated into cytosol and membrane fractions by centrifugation at 100,000 x g for 60 min. SPP activity was determined in the presence of sphingosine, prepared as a complex with 4 mg/ml BSA and [γ-32P]dATP in kinase buffer (35) containing 200 mM KCl, unless indicated otherwise. 23P-SPP was separated by TLC and quantified with a phosphomager as described previously (30).

Lipid Extraction and Measurement of SPP—Cells were washed with phosphate-buffered saline and scraped in 1 ml of methanol containing a 2.5-μl conc. HCl. Lipids were extracted by adding 2 ml of chloroform, 1 ml of NaCl (1.1, v/v) and 100 μl of 3N NaOH, and the phases were separated. The containing SPP fraction was dried and redissolved in 1 ml of sphingosine, ceramide, and the majority of phospholipids, was transferred to a siliconized glass tube. The organic phase was re-extracted with 1 ml of methanol, 1 ml of NaCl (1.1, v/v) plus 50 μl of 3N NaOH, and the aqueous fractions were combined. Mass measurements of SPP in the aqueous phase and total phospholipids in the organic phase were carried out essentially as described (8, 36).

Northern Blotting Analysis—Poly(A)+ RNA blots containing 2 μg of poly(A)+ RNA/lane from multiple adult mouse and human tissues, and mouse embryos were purchased from CLONTECH. Blots were hybridized with the 1.2-kb PSI fragment of mouse EST AA839187 (mSPHK1 probe), the 1.5-kb EcoRI fragment of pCR3.1-mSPHK2, the 0.3-kb PouII fragment of pCR3.1-hSPHK2, or the 0.6-kb EcoRV-Sph1 fragment of human EST AA295570 (hSPHK2 probe), after gel-purification and labeling with [γ-32P]dATP. Hybridization in ExpressHyb buffer (CLONTECH) at 65 °C overnight was carried out according to the manufacturer's protocol. Blots were reprobed with β-actin as a loading control (CLONTECH). Bands were quantified using an imaging analyzer (BAS2000, Fuji film).

RESULTS AND DISCUSSION

Cloning of Type 2 Sphingosine Kinase

Blaze searches of the EST data base identified several ESTs that displayed significant homology to our recently cloned mSPHK1 sequence (30). Specific primers were designed from
the sequences of these ESTs and were used to clone a new type of mouse and human SPHK (named mSPHK2 and hSPHK2) by the approaches of PCR cloning from a mouse brain cDNA library and 5'-RACE PCR.

ClustalW alignment of the amino acid sequences of mSPHK2 and hSPHK2 is shown in Fig. 1A. The open reading frames of mSPHK2 and hSPHK2 encode polypeptides of 617 and 618 amino acids, respectively, with 83% identity and 90% similarity. Five highly conserved regions (C1–C5), identified previously in SPHK1s (30), are also present in both type 2 kinases.

**Fig. 1.** Predicted amino acid sequences of murine and human type 2 SPHK. A, ClustalW alignment of the predicted amino acid sequences of mSPHK2 and hSPHK2. Identical and conserved amino acid substitutions are shaded dark and light gray, respectively. The dashes represent gaps in sequences, and numbers on the right refer to the amino acid sequence of mSPHK2. The conserved domains (C1–C5) are indicated by lines. B, schematic representation of conserved regions of SPHK1 and SPHK2. The primary sequence of mSPHK2 is compared with that of mSPHK1.
Interestingly, the invariant GGKGK positively charged motif in the C1 domain of SPHK1 is modified to GGRGL in SPHK2, suggesting that it may not be part of the ATP binding site as previously proposed (30). A motif search also revealed that a region beginning just before the conserved C1 domains of mSPHK2 and hSPHK2 (amino acid 147–284) also has homology to the diacylglycerol kinase catalytic site. Compared with SPHK1, both SPHK2s encode much larger proteins containing 236 additional amino acids (Fig. 1B). Moreover, their sequences diverge considerably from SPHK1 in the center and at the amino termini. However, after amino acid 140 of mSPHK2, the sequences of type 1 and type 2 mSPHK have a large degree of similarity. These sequences (amino acids 9–226 for mSPHK1 and 141–360 for mSPHK2), which encompass domains C1–C4, have 47% identity and 79% similarity (Fig. 1B). In the carboxyl-terminal portion of the proteins there are also large homologous regions, which include the C5 domain, from amino acids 227–381 for mSPHK1 and 480–617 for mSPHK2, with 43% identity and 78% similarity (Fig. 1B). The overall divergence suggests that SPHK2 probably did not arise as a simple gene duplication event.

Tissue Distribution of Sphingosine Kinase Type 2

The tissue distribution of SPHK2 mRNA expression in adult mouse was compared with that of SPHK1 by Northern blotting (Fig. 2A). In most tissues, including adult liver, heart, kidney, testis, and brain, a predominant 3.1-kb SPHK2 mRNA species was detected, indicating ubiquitous expression. However, the level of expression was markedly variable and was highest in adult liver and heart and barely detectable in the skeletal muscle and spleen (Fig. 2A). In contrast, the expression pattern of mSPHK1 is quite different, with highest mRNA expression of a 2.2-kb transcript in adult lung, spleen, and liver, although expression in liver does not predominate as with mSPHK2. mSPHK1 and mSPHK2 were both temporally and differentially expressed during embryonic development. mSPHK1 was expressed highly at mouse embryonic day 7 and decreased dramatically after embryonic day 11 (Fig. 2B). In contrast, at embryonic day 7, mSPHK2 expression was much lower than mSPHK1 and gradually increased up to embryonic day 17. The hSPHK2 2.8-kb mRNA transcript was mainly expressed in adult kidney, liver, and brain, with much lower expression in other tissues (Fig. 2C). Interestingly, expression of SPHK2 in human kidney is very high and relatively much lower in the mouse kidney, whereas the opposite pattern holds for the liver.

Activity of Recombinant Sphingosine Kinase Type 2

To investigate whether mSPHK2 and hSPHK2 encode bona fide SPHKs, HEK293 cells were transiently transfected with expression vectors containing the corresponding cDNAs. Because previous studies have indicated that SPHK might be present in cells in both soluble and membrane-associated forms (3, 32, 37–39), recombinant SPHK2 activity was measured both in cytosol and in membrane fractions of transfected cells. As described previously (30), untreated or vector-transfected HEK293 cells have low levels of SPHK activity (Fig. 3A). 24 h after transfection with mSPHK2 or hSPHK2, in vitro SPHK activity was increased by 20- and 35-fold, respectively, and then decreased thereafter (Fig. 3A). In contrast, SPHK activity in cells transfected with mSPHK1 was much higher, 610-fold greater than basal levels 24 h after transfection and remained at this level for at least 3 more days (data not shown). As in HEK293 cells, transfection of NIH 3T3 fibroblasts with mSPHK1 resulted in much higher SPHK activity than with mSPHK2. We previously found that, similar to untransfected cells, the majority of SPHK activity in cells transfected with mSPHK1 was cytosolic (30). Similarly, in cells transfected with either mSPHK2 or hSPHK2, 17 and 26%, respectively, of the SPHK activity was membrane-associated (Fig. 3A), although Kyte-Doolittle hydrophathy plots did not suggest the presence of hydrophobic membrane-spanning domains.

Transfection of HEK293 cells with mSPHK2 and hSPHK2 also resulted in 2.2- and 3.3-fold increases, respectively, in...
SPP, the product formed by SPHK (Fig. 3B), in agreement with previous studies of sphingolipid metabolite levels after transfection with mSPHK1, which showed a lack of correlation of fold increases in SPP levels and in vitro SPHK enzyme activity (30, 34).

**Characteristics of Recombinant mSPHK2**

**Substrate Specificity**—Although SPHK2 is highly homologous to SPHK1, there are substantial sequence differences. Therefore, it was of interest to compare their enzymatic properties. Typical Michaelis-Menten kinetics were observed for recombinant SPHK2 (data not shown). The $K_m$ for d-erythro-sphingosine as substrate is 3.4 μM, almost identical to the $K_m$ previously found for SPHK1 (29). Although the naturally occurring d-erythro-sphingosine isomer was the best substrate for SPHK1 (30), d-erythro-dihydrosphingosine was a better substrate for SPHK2 than d-erythro-sphingosine (Fig. 4A). Moreover, although d,l-threo-dihydrosphingosine and phytosphingosine were not phosphorylated at all by SPHK1, they were significantly phosphorylated by SPHK2, albeit much less efficiently than sphingosine. Like SPHK1, other lipids including N,N-dimethylsphingosine (DMS), C2- or C16-ceramide, diacylglycerol, or phosphatidylinositol, were not phosphorylated by SPHK2 (Fig. 4A), suggesting high specificity for the sphingoid base.

DMS and threo-DHS have previously been shown to be potent competitive inhibitors of SPHK1 (40) and have been used to block increases in intracellular SPP levels resulting from various physiological stimuli (3, 4, 8, 11, 13, 14, 41). However, because threo-DHS is a substrate for SPHK2, it is not useful as a tool to investigate the role of SPHK2/SPP signaling. Thus, it was important to characterize the inhibitory potential of the nonsubstrate DMS on SPHK2. Surprisingly, we found that

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**FIG. 4. Substrate specificity of mSPHK2.** A, SPHK-dependent phosphorylation of various sphingosine analogs or other lipids (50 μM) was measured in cytosol from HEK293 cells transfected with mSPHK2. Data are expressed as percentage of phosphorylation of d-erythro-Sph. B–D, noncompetitive inhibition of recombinant SPHK2 by N,N-dimethylsphingosine. B, dose-dependent inhibition of mSPHK2 by DMS. SPHK activity in HEK293 cell lysates after transfection as in A was measured with 10 μM d-erythro-sphingosine in the presence of increasing concentrations of DMS. C, kinetic analysis of DMS inhibition. SPHK activity was measured with varying concentrations of d-erythro-sphingosine in the absence (open circles) or presence of 10 (filled squares) or 20 μM DMS (filled triangles). D, Lineweaver-Burk plots. The $K_m$ value for d-erythro-sphingosine was 3.4 μM. The $K_i$ value for DMS was 12 μM.
although DMS was also a potent inhibitor of SPHK2 (Fig. 4B), it acted in a noncompetitive manner (Fig. 4, C and D). The $K_i$ for DMS with SPHK2 was 12 $\mu$m, slightly higher than the $K_i$ of 4 $\mu$m with SPHK1, suggesting that it can be used to inhibit both types of SPHK.

mSPHK2 had highest enzymatic activity in the neutral pH range from 6.5 to 8 with optimal activity at pH 7.5 (Fig. 5A), a pH dependence similar to that of SPHK1 (data not shown). The activity decreased markedly at pH values below and above this range.

Effects of KCl and NaCl—Most of the SPHK activity in human platelets is membrane-associated and extractable with 1 M NaCl (32). Furthermore, the salt extractable SPHK from platelets has different properties than the cytosolic enzyme. It was thus of interest to determine the effect of high salt concentrations on recombinant SPHK1 and SPHK2. Interestingly, we found that high ionic strength had completely opposite effects on their activities. SPHK1 was markedly inhibited by either NaCl and KCl, with each causing 50% inhibition at a concentration of 200 mM (Fig. 5B). In contrast, SPHK2 activity was dramatically stimulated by increasing the salt concentration, with a maximal effect at a concentration of 400 mM, although KCl was much more effective than NaCl. However, above this concentration, SPHK2 activity decreased sharply although remaining elevated even at 1 M salt (Fig. 5C). Kinetic analysis of mSPHK2 in the presence and absence of high concentrations of salt indicated that the $K_m$ for sphingosine was unaltered, whereas the $V_{max}$ was increased (Fig. 5, D and E).

**FIG. 5.** pH dependence and salt effects on mSPHK2. A, cytosolic SPHK2 activity in transfected HEK293 cells was measured in kinase buffer with the pH adjusted using the following buffers: 200 mM sodium acetate (pH 4.5–5.5, open circles); 200 mM MES (pH 6–7, filled circles); 200 mM potassium phosphate (pH 6.5–8, open squares); 200 mM HEPES (pH 7–7.5, filled squares); 200 mM Tris-HCl (pH 7.5–9, open triangles); and 200 mM borate (pH 10, filled triangle). B–E, salts stimulate SPHK2 but inhibit SPHK1. B and C, SPHK activity in HEK293 cell lysates was measured 24 h after transfection with mSPHK1 (B) or mSPHK2 (C) in the absence or presence of increasing concentrations of NaCl (open squares) or KCl (filled circles). D, kinetic analysis of SPHK2 activation by KCl. mSPHK2 activity was measured with varying concentrations of D-erythro-sphingosine in the absence (open circles) or presence of 50 (open squares) or 200 mM KCl (filled circles). E, Lineweaver-Burk plots of data from D. The $K_m$ value is not affected by the presence of KCl. $V_{max}$ values were 0.1, 0.3, and 1 (nmol/min/mg) in the presence of 0, 50, and 200 mM KCl, respectively.

**FIG. 6.** Triton X-100 and BSA, but not phosphatidylserine, have differential effects on activity of SPHK1 and SPHK2. HEK293 cells were transfected with mSPHK1 (circles) or mSPHK2 (triangles) and the activities of each in cell lysates were measured after 24 h in the presence of the indicated concentrations of Triton X-100 (A), BSA (B), or phosphatidylserine (filled symbols) or phosphatidylcholine (open symbols) (C). Data are expressed as percentage of control activity measured without any additions.
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Substrate Presentation—Because sphingolipids are highly lipophilic, in *in vitro* SPHK assays, sphingosine is usually presented in micellar form with Triton X-100 or as a complex with BSA (42, 43). Furthermore, detergents such as Triton X-100 have been shown to stimulate the activity of SPHK in rat brain extracts (37) and the enzyme from rat kidney (29), and we previously found that the stability of rat kidney SPHK was increased in the presence of certain detergents (29). However, when the effect of increasing concentrations of Triton X-100 on the activities of SPHK1 and SPHK2 were compared, some unexpected results were found. Concentrations of detergent up to 0.005% had no effect, but at higher concentrations, SPHK2 activity was inhibited and SPHK1 activity was markedly stimulated (Fig. 6A). At a concentration of Triton X-100 of 0.5%, SPHK1 activity was increased by more than 4-fold, whereas SPHK2 was almost completely inhibited. Thus, Triton X-100 could be used to differentially determine SPHK1 and SPHK2 activities in tissues or cells that express both types.

Interestingly, increasing the BSA concentration from our usual SPHK assay conditions with sphingosine-BSA complex as substrate, i.e. 0.2 mg/ml BSA, caused a concentration-dependent inhibition of SPHK2 activity without affecting SPHK1 activity (Fig. 6B). Therefore, when measuring SPHK activity in cell or tissue extracts, the method of substrate presentation, whether in mixed micelles or in BSA complexes, must be carefully optimized because the differential effects of Triton X-100 and BSA on activity could yield different results depending on the relative expression of the two types of SPHK.

Effects of Phospholipids—Acidic phospholipids, particularly phosphatidylserine, phosphatidic acid, and phosphatidylinositol, and cardiolipin to a lesser extent, induced a dose-dependent increase in SPHK activity in Swiss 3T3 fibroblast lysates, whereas neutral phospholipids had no effect (42). In agreement, the enzymatic activity of recombinant SPHK1 and SPHK2 was stimulated by phosphatidylserine; the activity of both was maximally increased 4-fold at a concentration of 40 μg/ml (Fig. 6C) and inhibited by higher concentrations in a dose-dependent manner. These effects of phosphatidylserine appeared to be specific because other phospholipids, including phosphatidylcholine, had no effect on the enzyme activity. In contrast, the activities of the three major forms of SPHK in human platelets were not affected by phosphatidylserine (32).

The mechanism by which phosphatidylserine enhances the enzymatic activity of SPHK is not yet understood. One possibility is that phosphatidylserine possesses unique membrane-structuring properties, which better present the substrate, sphingosine. A second possibility is that SPHK contains determinants that specifically recognize the structure of the serine headgroup and that these determinants may only become exposed upon interaction of SPHK with membranes. In this regard, the molecular basis for the remarkable specificity of protein kinase C for phosphatidylserine is the subject of much debate. However, recent data reveal that lipid structure and not membrane structure is the major determinant in the regulation of protein kinase C by phosphatidylserine (44).

**Concluding Remarks**—The presence of multiple ESTs in the data base with significant homologies to SPHK1 as well as the identification of several genes in *S. cerevisiae* encoding different SPHKs (31) suggested that there may be several important and likely major type of SPHK that has some unique properties. Although SPHK2 has a high degree of homology to SPHK1, especially in the previously identified conserved domains identified in type 1 SPHKs (30), it is much larger (65.2 and 65.6 kDa for hSPHK2 and mSPHK2, respectively, versus 42.4 kDa for mSPHK1) and contains an additional 236 amino acids. Furthermore, its differential tissue expression, temporal developmental expression, cellular localization, and kinetic properties in response to increasing ion strength and detergents are completely different from SPHK1, suggesting that it most likely has a different function and regulates levels of SPP in a different manner than SPHK1, which is known to play a prominent role in regulating cell growth and survival. Thus, type 2 SPHK might be involved in regulation of some of the numerous biological responses attributed to SPP, such as angiogenesis and allergic responses.

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Type 2 Sphingosine Kinase
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