Molecular Cloning and Functional Characterization of Murine Sphingosine Kinase*

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Sphingosine-1-phosphate (SPP) is a novel lipid messenger that has dual function. Intracellularly it regulates proliferation and survival, and extracellularly, it is a ligand for the G protein-coupled receptor Edg-1. Based on peptide sequences obtained from purified rat kidney sphingosine kinase, the enzyme that regulates SPP levels, we report here the cloning, identification, and characterization of the first mammalian sphingosine kinases (murine SPHK1a and SPHK1b). Sequence analysis indicates that these are novel kinases, which are not similar to other known kinases, and that they are evolutionarily conserved. Comparison with Saccharomyces cerevisiae and Caenorhabditis elegans sphingosine kinase sequences shows that several blocks are highly conserved in all of these sequences. One of these blocks contains an invariant, positively charged motif, GGKGK, which may be part of the ATP binding site. From Northern blot analysis of multiple mouse tissues, we observed that expression was highest in adult lung and spleen, with barely detectable levels in skeletal muscle and liver. Human embryonic kidney cells and NIH 3T3 fibroblasts transiently transfected with either sphingosine kinase expression vectors had marked increases (more than 100-fold) in sphingosine kinase activity. The enzyme specifically phosphorylated \( \text{d-erythro-} \) sphingosine and did not catalyze the phosphorylation of phosphatidylinositol, diacylglycerol, ceramide, \( \text{d},\text{L-} \) threo-dihydrosphingosine or \( \text{N,N-} \) dimethylsphingosine. The latter two sphingolipids were competitive inhibitors of sphingosine kinase in the transfected cells as was previously found with the purified rat kidney enzyme. Transfected cells also had a marked increase in mass levels of SPP with a concomitant decrease in levels of sphingosine and, to a lesser extent, in ceramide levels. Our data suggest that sphingosine kinase is a prototypical member of a new class of lipid kinases. Cloning of sphingosine kinase is an important step in corroborating the intracellular role of SPP as a second messenger.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF068748 and AF068749.

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The abbreviations used are: SPP, sphingosine-1-phosphate; HPLC, high performance liquid chromatography.
other lipids were purchased from Avanti Polar Lipids (Birmingham, AL). (γ-32P)ATP (3000 Ci/μmol) was purchased from Amersham Pharmacia Biotech. Poly-l-lysine and collagen were from Boehringer Mannheim. Alkaline phosphatase from bovine intestinal mucosa, Type VII-NT, was from Sigma. Serum and medium were obtained from Biofluids, Inc. (Rockville, MD). Restriction enzymes were obtained 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.

**Protein Sequencing of Sphingosine Kinase—** Purified sphingosine kinase was microelectrophoresed on SDS-polyacrylamide gel electrophoresis and the Coomassie-stained 49-kDa band excised. After S-carboxyamidomethylation, this band was subjected to in-gel tryptic digestion as described (19). The resulting peptide mixture was separated by microbore high performance liquid chromatography on a Zorbax C18 1.0 × 150-mm reverse-phase column in a Hewlett-Packard 1090 HPLC with a 1040 diode array detector. Fractions were selected for sequencing based on differential UV absorbance at 205, 277, and 292 nm, and the peptide sequences were determined by automated Edman degradation (20). Complementary peptide sequence information was also obtained on 10% of the digest mixture by collisionally induced dissociation using microcapillary HPLC electrospray ionization/tandem mass spectrometry on a Finnigan TSQ7000 triple quadrupole mass spectrometer. Sequences of smaller peaks were determined by automated Edman degradation or matrix-assisted laser desorption ionization (21).

**Cell Culture—** Human embryonic kidney cells (HEK293, ATCC CRL-1573), Swiss 3T3 cells (ATCC CCL-92), and NIH 3T3 (ATCC CRL-1658) cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum or 10% calf serum.

**Transient Expression of Sphingosine Kinase—** Transfections of various cell types were carried out using LipofectAMINE PLUS reagent, essentially as described by the manufacturer. For human embryonic kidney 293 cells, cells were seeded at 6 × 10^4 per well in polylysine coated 6-cluster wells. After 24 h, cells were transfected with 1 μg of vector (pCMV-SPORT2) (Life Technologies, Inc.) alone or with vectors containing sphingosine kinase constructs (pCMV-SPORT2 sphk1a or pCMV-SPORT2 sphk1b), and 6 μg of LipofectAMINE PLUS reagent plus 4 μl of LipofectAMINE reagent per well. For Swiss 3T3 and NIH 3T3 fibroblasts, cells were seeded at 3 × 10^5 per well in collagen-coated 6-well clusters. After 24 h, cells were transfected with a mixture of LipofectAMINE PLUS (8 μl) and LipofectAMINE (6 μl) and vector along with a control or sphingosine kinase construct (2 μg). In some cases, as a measure of transfection efficiency, cells were co-transfected with 0.5 μg of pCEFLGFP (a kind gift of Dr. Silvio Gutkind), which encodes the green fluorescent protein, and were visualized with a fluorescence microscope.

**Assay of Sphingosine Kinase Activity—** Cells were harvested and lysed by freeze-thawing in buffer (20 mM Tris, pH 7.4, 20% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 1% sodium orthovanadate, 40 mM β-glycerophosphate, 15 mM NaF, 10 μg/ml leupeptin, aprotinin and soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM 4-deoxyxypyrrolidine). In some experiments, cell lysates were fractionated into cytosol and membrane fractions by centrifugation at 100,000 × g for 60 min (4°C). Sphingosine kinase activity was determined in the presence of 50 μM sphingosine, 0.25% Triton X-100, and [γ-32P]ATP (10 μCi, 1 μM) containing MgCl2 (10 mM) in buffer A as described previously (18). The labeled SPP was separated by thin layer chromatography on silica gel G60 with 1-butanol/ethanol/acetic acid/water (80:20:10:20, v/v) and visualized by autoradiography. The radioactive spots corresponding to authentic SPP were identified as described (22) and quantified with a Molecular Dynamics Storm PhosphorImager.

**Results and Discussion**

**Sequencing and Cloning of Sphingosine Kinase—** The 49-kDa sphingosine kinase polypeptide, purified from rat kidney, was excised from an SDS gel and subjected to trypsin digestion. The resulting peptides were separated by micropillary reversed-phase HPLC and sequences of 8 peptides were determined by Edman degradation or matrix-assisted laser desorption ionization mass spectrometry (Table I). Homology searches (BLAST) against a comprehensive nonredundant data base revealed no matches to known proteins. However, when the database of expressed sequence tags (dbEST) at NCBI was searched using the tBLASTn algorithm, an EST (GenBank accession number AA011725) containing sequences homologous to 3 of the 8 peptides (peptides 5, 2, and 4) was retrieved. A further search with peptides 1, 3, and 7 yielded 4 additional ESTs (GenBank accession numbers AA000819, AA107451, AA592274, and AA389543). The nucleotide sequences of mouse ESTs AA000819 and AA592274 were then used to search dbEST to obtain EST AA389187. Clones AA107451 and AA389187 were highly homologous at their 3′-ends, but were slightly divergent at their 5′-ends. Sequencing of the full-length cDNAs revealed apparent open reading frames coding for 381 and 388 amino acid polypeptides containing sequences highly homologous to 7 isolated peptides distributed throughout the protein, and these are thus designated sphk1a and sphk1b (Fig. 1). In addition,
both contained a portion of peptide 8. SPHK1a and -1b have predicted pIs of 6.68 and 6.89 and molecular weights of 42,344 and 43,254, respectively, in agreement with the molecular weight of purified rat kidney sphingosine kinase (18). Because SPHK1b only differs by a few amino acids at the N terminus, it may arise by alternative splicing. However, both sequences lacked Kozak consensus sequences, suggesting that these cDNAs may not include the actual initiation sequences.

SPHK1a has 2 overlapping calcium/calmodulin binding consensus sequences of the 1–8-14 Type B motif ((FILVW)XXX(IFILVW)XXX(IFILVW), containing net positive charges of 2–4) (24). In addition, near the C terminus, SPHK1a contains 2 overlapping calcium/calmodulin binding consensus sequences, one of Type B and one of Type A ((FILVW)XXX(IFILVW)XXX(IFILVW)) containing net positive charges of 3–6) (24). SPHK1b contains all the above calcium/calmodulin binding consensus sequences as well as an additional Type B motif at the N terminus. The existence of multiple calcium/calmodulin binding motifs supports our previous observations that purified rat kidney sphingosine kinase binds tightly to calmodulin-Sepharose in the presence of calcium (18).

Analysis of the domain structure of SPHK1a obtained by searching the protein data base (GenBank™ and Prosite) revealed several putative posttranslational phosphorylation motifs: one kinase A, two casein kinase II, and eight protein kinase C phosphorylation sites (Fig. 1). Interestingly, we previously demonstrated that inhibition of ceramide-induced apoptosis by protein kinase C activation results from stimulation of sphingosine kinase and concomitant increase in cellular SPP levels (3). Sphingosine kinase is thought to be mainly a cytosolic enzyme (18). Consistent with this, a hydropathy plot indicates that SPHK1a does not contain signal peptide or hydrophobic transmembrane sequences (data not shown).

Sphingosine kinase appears to be remarkably devoid of recognizable regulatory domains, including SH2, SH3, or pleckstrin homology domains. However, a domain reminiscent of a proline-rich SH3 binding site (25) is present at the C terminus. Furthermore, there were no obvious similarities between sphingosine kinase and phosphatidylinositol-3 kinase or phosphatidylinositol-4 kinase. Previously, we have shown that

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### Table I

Peptide sequences of rat kidney sphingosine kinase

Peptide sequences were determined by Edman sequencing or matrix-assisted laser desorption ionization mass spectrometry. Cysteine residues were alkylated with isopropylacetamide. In peptide 8, the gaps could not be unambiguously defined, although the ion series defined the indicated molecular weights for gaps. Masses represent the average isotopic mass.

| Peptide | Analysis | Sequence | Peptide mass | Theor. mass |
|---------|----------|----------|--------------|-------------|
| 1       | Edman    | VLVLLNPR |              |             |
| 2       | Edman    | IYYQGLAYLPVGK |         |             |
| 3       | Edman    | LFQSR    |              |             |
| 4       | Edman    | IFASLQAQK |              |             |
| 5       | Edman    | PTYQTFBB |              |             |
| 6       | MS-MS\(^a\) | YLV/L/W/F/MIVSFGSDDSPGR | 1643.76 | 1643.83 |
| 7       | MS-MS    | (R,V)/P(L,I)/LIDEEAEVSFK | 1515.8 | 1516.0 |
| 8       | MS-MS    | (228.1 Da/A/m/Q/k)/212.1 Da/C PR | 1217.6 | 1217.5 |

\(^a\) MS, mass spectrometry.
Sphingosine kinase is activated by acidic phospholipids including phosphatidic acid, phosphatidylinositol, phosphatidylinositol 4,5-bisphosphate, and particularly phosphatidylserine, yet bears no similarity to proteins known to bind these phospholipids, such as phosphatidic acid phosphohydrolase, phospholipase C, or protein kinase C. On the other hand, a data base search identified homologs of sphingosine kinase in numerous widely disparate organisms, demonstrating that sphingosine kinase is a member of a novel but highly conserved gene family (Fig. 2). Two of these genes, named LCB4 and LCB5, were recently shown to code for sphingosine kinases from *Saccharomyces cerevisiae* (26). Most of the other cDNA sequences were from ESTs or putative open reading frames identified from *Caenorhabditis elegans* and *Schizosaccharomyces pombe* genomic sequences. A comparison of these sequences reveals the location of several blocks of highly conserved amino acids, one or more of which might constitute critical portions of catalytic or substrate binding sites. Five regions, in particular (C1 to C5), are highly conserved in all of these sequences. C1 contains an invariant positively charged motif, GGKGK, which may be part of the ATP binding site. Moreover, the C1 and C3 subdomains of sphingosine kinase show high amino acid similarity to residues 296–315 and 378–389 of human diacylglycerol kinase with 35% and 58% identity, respectively (27, 28). These residues are present in subdomain 1 (C4-a), which is conserved in all of the known diacylglycerol kinase family members (27, 29, 30). Although subdomain 1 in diacylglycerol kinases contains a GXGXXG box, which is similar to the GXGXXG_{1-1}_K motif, known to participate in ATP binding to protein kinases, the downstream K is missing. However, the GXGXXG box (31) is not present in sphingosine kinase. Furthermore, recent studies demonstrated that this box may not be required for diacylglycerol kinase activity (32).

**Tissue Distribution of Sphingosine Kinase**—The tissue distribution of sphingosine kinase mRNA expression in adult mouse tissues was analyzed by Northern blotting (Fig. 3). In most tissues, including adult brain, heart, spleen, lung, kidney, and testis, a predominant 2.4-kilobase mRNA species was detected, indicating ubiquitous expression of sphingosine kinase. However, the level of expression was markedly variable among the different tissues. The mRNA levels were highest in adult lung and spleen and there were barely detectable levels in skeletal muscle and liver. The mSPHK1 mRNA detected by Northern blotting was slightly larger than the size of the cDNA (1.9 kilobases). Interestingly, expression of mSPHK1a mRNAs...
in various mouse tissues did not closely correlate with the relative sphingosine kinase activities in rat tissues, because we previously found that spleen and kidney have higher specific activities than liver, which has about twice the activity of brain (18).

Recombinant Sphingosine Kinase Activity—To investigate whether SPHK1a and SPHK1b encode bona fide sphingosine kinases, HEK293 cells were transiently transfected with pcMVSPORT2 expression vectors containing either SPHK1a or SPHK1b cDNAs, and sphingosine kinase activity was measured. Modest levels of endogenous sphingosine kinase activity were present in control cells (either untransfected or transfected with an empty vector) (Fig. 4). Cells transfected with SPHK1a exhibited 300-fold increased sphingosine kinase activity 24 h after transfection that remained at this level for 4 days. In contrast, cells transfected with SPHK1b showed only 120-fold increased sphingosine kinase activity after 24 h and then decreased gradually to control levels after 4 days (Fig. 4).

Transfection of either Swiss 3T3 or NIH 3T3 fibroblasts with SPHK1a or SPHK1b also resulted in marked increases in sphingosine kinase activity (Table II). As in HEK293 cells, transfection of 3T3 cells with SPHK1a led to much larger increases in sphingosine kinase activity than with SPHK1b. It should be noted that transfection efficiency was quite good and similar in all three cell lines (Table II).

Cells transfected with either SPHK1a or SPHK1b exhibited substantial increases in cytosolic (870- and 17-fold, respectively) and membrane-associated (800- and 45-fold, respectively) sphingosine kinase activity (Fig. 5). The relative amounts of sphingosine kinase activity in cytoplasmic versus membrane fractions were similar in vector-transfected and SPHK-transfected cells. Both membrane-associated and cytosolic sphingosine kinase activities have been described in mammalian tissues and cell lines (1, 23, 39–36), and it has been suggested that these activities might have distinct physiological roles and might be derived from different gene products. In contrast, we demonstrate here that sphingosine kinase expressed from a single gene product is present both in the cytoplasm and in membranes.

The substrate specificity of sphingosine kinase activity from cells transfected with either SPHK1a or SPHK1b was the same as found previously for purified rat kidney sphingosine kinase (18). The naturally occurring d-erythro-trans-isomer was the best substrate. However, d,l-erythro-dihydrosphingosine was phosphorylated to a similar extent, whereas, phytosphingosine, d,l-threo-dihydrosphingosine, ceramide, diacylglycerol, and phosphatidylinositol were not phosphorylated (Fig. 6A). Typical Michaelis-Menten kinetics were observed with d-erythro-sphingosine with $K_m$ values of 2.15 $\mu$M (data not shown), in excellent agreement with values found with sphingosine kinase purified to homogeneity from rat kidneys (18). N,N-dimethylsphingosine and d,l-threo-dihydrosphingosine have previously been used to inhibit sphingosine kinase and decrease SPP levels stimulated by various physiological stimuli (1–3). Both of these sphingolipids were potent competitive inhibitors of sphingosine kinase from transfected cells (Fig. 6, B and C).

Transfection of NIH 3T3 cells with SPHK1a also resulted in marked changes in mass levels of sphingolipid metabolites

![Graph](https://example.com/graph1.png)

**FIG. 4.** Activity of sphingosine kinase expressed in HEK293 cells. HEK293 cells were transiently transfected with the pcMVSPORT2 expression vector alone (○) or containing either the SPHK1a cDNA (pcMVSPORT2spHK1a, ▲) or the SPHK1b cDNA (pcMVSPORT2spHK1b, ■). At the indicated times, sphingosine kinase activity in cell lysates was measured as described under “Experimental Procedures.” Data are means of duplicates and are representative of three independent experiments.

**FIG. 5.** Distribution of sphingosine kinase in transfected HEK293 cells. HEK293 cells were transiently transfected with pcMVSPORT2 vector alone or containing either SPHK1a cDNA or SPHK1b cDNA. After 72 h, sphingosine kinase activity was measured in cytosol and particulate fractions as described under “Experimental Procedures.” Data are means ± S.D. A, thin layer chromatography analysis of [32P]SPP formed by cytosolic (C) or membrane (M) fractions from vector- or sphingosine kinase-transfected cells. B, sphingosine kinase activity in cytosolic (open bars) and membrane (filled bars) fractions. The data are expressed as percent of the total activity.

### Table II

| Cells    | DNA Activity | Transfection efficiency |
|----------|--------------|-------------------------|
|          | pmol/min/mg protein | fold increase |
| HEK293   | Control      | 29                      | 1.0 | 32.2 |
|          | SPHK1a       | 23,200                  | 800 | 42.0 |
|          | SPHK1b       | 657                     | 22.5| 45.5 |
| NIH 3T3  | Control      | 31                      | 1.0 | 33.7 |
|          | SPHK1a       | 12245                   | 395 | ND  |
|          | SPHK1b       | 167                     | 5.4 | 28.5 |
| Swiss 3T3| Control      | 6.2                     | 1.0 | 38.1 |
|          | SPHK1a       | 3200                    | 516 | ND* |
|          | SPHK1b       | 15.7                    | 2.5 | 42.1 |

* ND, not determined.

The relative sphingosine kinase activities in rat tissues, because we previously found that spleen and kidney have higher specific activities than liver, which has about twice the activity of brain (18).
Mass levels of SPP increased 21-fold compared with cells transfected with vector alone, with a concomitant 67% decrease in levels of sphingosine and, to a lesser extent, in ceramide levels (29%). However, it should be noted that the absolute decrease in mass of ceramide was actually much greater than the decrease in mass of sphingosine. Our results suggest that transfected SPHK1a is active in intact cells, and overexpression alters the balance of sphingolipid metabolites within cells.

In summary, we have cloned and characterized the first mammalian sphingosine kinases. These kinases have characteristics similar to sphingosine kinase purified to homogeneity from rat kidney. Sequence analysis reveals that murine sphingosine kinases belong to an evolutionarily conserved family of enzymes that is distinct from other known lipid kinases. Cloning of sphingosine kinase should help to clarify the role that regulation of sphingosine kinase plays in SPP-mediated signaling pathways initiated by growth factors, cytokines, and immunoglobulin receptors. Furthermore, it provides a useful tool to determine how the balance of sphingolipid metabolites influences cell fate.

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