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# Distribution of sphingosine kinase activity in mouse tissues: contribution of SPHK1

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**Abstract**

Sphingosine kinase (SPHK) phosphorylates sphingosine to form a bioactive lipid mediator, sphingosine 1-phosphate (S1P). S1P mediates such diverse biological processes as regulation of cell differentiation, motility, and apoptosis both extracellularly, via S1P (Edg) family receptors, and intracellularly, through unidentified targets. In cells S1P is short-lived, so the synthetic process catalyzed by sphingosine kinase may be important in maintaining the cellular levels of the compound. Thus far, two sphingosine kinases have been reported, with SPHK1 exhibiting the higher activity. However, several studies suggest the existence of unidentified sphingosine kinases. Therefore, to further elucidate the role of SPHK1 in the formation of S1P, we investigated its contribution to the total sphingosine kinase activity in mouse tissues. We found that SPHK1 is a major sphingosine kinase in many tissues, especially in brain, heart, and colon. However, some tissues such as spleen, small intestine, and lung contained sphingosine kinase activity that was not attributable to SPHK1 or SPHK2, as determined by immunodepletion assays. Furthermore, the presence of other sphingosine kinases with different properties, i.e., higher activity toward phytosphingosine and a different subcellular distribution, is suggested.


*Keywords:* Sphingosine kinase; Sphingosine 1-phosphate; Phytosphingosine; Platelet

Sphingosine 1-phosphate (S1P) is a bioactive lipid mediator generated by sphingosine kinase (SPHK) phosphorylating sphingosine (Sph) [1]. Recent studies have shown that S1P acts extracellularly by binding to the S1P/Edg family of G protein-coupled receptors and thereby regulating multiple cellular events including cell motility, mitogenesis, and calcium mobilization [2,3]. Important roles for S1P have also been reported in angiogenesis and vascular maturation [4,5].

Like other sphingolipid metabolites, such as ceramide and sphingosine, S1P has also been implicated as a signaling molecule within the cell [1]. Although the intracellular targets of S1P have yet to be determined, intracellular S1P has been implicated in regulating such biological processes as cell differentiation [6], calcium mobilization from intracellular stores [7], and apoptosis [8].

In many cell types the normal intracellular amount of S1P is very low, due to degradation by Sphingosine phosphate lyase (SPL) and dephosphorylation by S1P phosphohydrolases. In contrast, in platelets, which lack SPL, S1P accumulates and is released upon activation [9]. Therefore, the action of sphingosine kinases in generating S1P may be a key step in regulating the concentration of S1P. To date, two sphingosine kinases, SPHK1 and SPHK2, have been identified [10,11]. For mouse SPHK1 there are two splicing isoforms, SPHK1a and SPHK1b. SPHK2 exhibits very weak activity compared to SPHK1, approximately 100-fold less. A number of external stimuli, including PDGF, TNF-α, phorbol ester, and the crosslinking of FcεRI, are known to activate SPHK1 in several cell types, however many of their mechanisms remain undetermined (reviewed in [12]).

In spite of the strong kinase activity of SPHK1, several lines of evidence suggest the presence of other sphingosine kinases in platelets and other tissues[13,14].

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Therefore, the contribution of SPHK1 to the total sphingosine activity in tissues remains unclear. To determine the significance of SPHK1 in the formation of S1P, we investigated the distribution pattern of SPHK activity in mouse tissues, particularly the contribution of SPHK1 to the total activity. Our results suggest the presence of sphingosine kinases, other than SPHK1 and SPHK2, and the possibility that sphingosine kinase isozymes may differ among tissues.

## Materials and methods

*Preparations of tissue homogenates.* Male C57BL mice were briefly anesthetized with ether and killed by decapitation. Various tissues were isolated, rinsed with phosphate-buffered saline (PBS), and stored at −80 °C. Tissues were homogenized in lysis buffer (50 mM Hepes–NaOH (pH 7.5), 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 1× protease inhibitor cocktail (Complete, Roche Molecular Biochemicals), and 10% glycerol) using a Teflon homogenizer (five strokes) and sonication (3 × 15 s). Solutions were then centrifuged at 1000*g* for 3 min at 4 °C to remove debris. Aliquots of homogenates were stored at −80 °C prior to assaying.

*Cell culture.* Chinese hamster ovary (CHO) cells were cultured in Ham's F-12 medium (Sigma, St. Louis, MO) supplemented with 10% FBS (Iwaki, Japan), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma) at 37 °C in a humidified atmosphere with 5% $CO_2$. CHO cells stably expressing FLAG-SPHK1a or pcDNA3-FLAG1 were selected in growth medium containing 0.6 g/L geneticin (G-418 sulfate, Sigma). Preparation of the plasmids pcDNA3-FLAG1 and pcDNA3-FLAG1-SPHK1a has been previously described [15].

*SPHK activity measurement.* In vitro sphingosine kinase assays were performed as described previously [16]. In brief, 100 μg protein from each tissue homogenate was incubated with 2 μCi [$\gamma$-$^{32}$P]ATP (0.2 nmol, Perkin–Elmer Life Sciences, Boston, MA), 0.5 mM cold ATP, and 50 μM D-*e*-sphingosine (Sigma) or phytosphingosine (PHS, BIOMOL, Plymouth Meeting, PA) at 37 °C for 30 min in assay buffer (20 mM Tris–HCl (pH 7.5), 12 mM β-glycerophosphate, 0.25 mM EDTA, 5% glycerol, 1 mM sodium pyrophosphate, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1× protease inhibitor cocktail, and 0.5 mM 4-deoxypyridoxine). Lipids were extracted by adding 800 μL chloroform/methanol/HCl (100:200:1), vortexing for 1 min, and centrifuging. Next, 250 μL of chloroform and 250 μL of 1 M KCl were added, and the mixture was vortexed vigorously for 5 min. Samples were centrifuged for 2 min at 4000*g* and the upper phase was removed. The organic phase was dried and suspended in chloroform/methanol (2:1). Lipids were separated by TLC on Silica Gel 60 high performance TLC plates (Merck, Germany) with 1-butanol/acetic acid/water (3:1:1). Radioactive bands were visualized and quantified using a Fujix Bio-Imaging Analyzer, BAS2000 (Fuji Photo Film, Japan).

*Immunoprecipitation.* One hundred microgram of protein from each tissue homogenate was diluted to an equal volume with lysis buffer containing Triton X-100 (final concentration 1%), incubated for 1 h at 4 °C, followed by centrifugation at 100,000*g* for 30 min at 4 °C. CHO cells were washed twice with PBS and lysed in lysis buffer. Cell suspensions were sonicated, centrifuged at 1000*g* for 5 min, and then the lysates were prepared as described above. The supernatants from these tissue or cell lysates were further subjected to a sphingosine kinase assay, immunoblotting or immunoprecipitation.

For immunoprecipitation, each solubilized lysate was incubated with an anti-mouse SPHK1 (mSPHK1) antibody (which had been raised against full-length MBP-fused mSPHK1a recombinant protein) at a 1:100 dilution for 1 h at 4 °C, followed by incubation with protein A–Sepharose 4 Fast Flow (Amersham Pharmacia Biotech, Liscataway, NJ), with continuous rotation, for 2 h at 4 °C. After centrifugation at 10,000*g* for 10 s at 4 °C, the resulting supernatant was subjected to a sphingosine kinase assay or immunoblotting. Immunoprecipitates were washed three times with wash buffer (50 mM Hepes–NaOH (pH 7.5), 150 mM NaCl, and 0.5% Triton X-100) and then they were subjected to a sphingosine kinase assay or immunoblotting. Proteins from whole cell extracts and supernatants were precipitated with 5% trichloroacetic acid prior to immunoblotting.

*Immunoblotting.* Proteins were separated by SDS–PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA.). Blocking was performed with 5% skimmed milk in TBS-T (137 mM NaCl, 20 mM Tris–HCl (pH 7.5), and 0.05% Tween 20), followed by incubation with 1 μg/mL anti-FLAG, M2 (Sigma) or a 1:1000 dilution of an anti-mSPHK1 antibody. The blot was washed with TBS-T and then incubated with a 1:8000 dilution of horseradish peroxidase-conjugated anti-mouse or rabbit IgG F(ab′)$_2$ fragment (Amersham Pharmacia Biotech.). The blot was washed again with TBS-T and detection was performed with an Enhanced Chemiluminescence Detection Kit (ECL, Amersham Pharmacia Biotech).

*Platelet preparation.* Platelets were isolated from the blood of C57BL mice as previously described [17]. To obtain platelet rich plasma (PRP), the blood was collected in 0.2 vol of the anti-coagulant ACD (0.8% citric acid, 2.2% sodium citrate, and 2.45% glucose) and centrifuged without breaking at 112*g* for 15 min at room temperature. Then, 1.0 vol of Buffer A (138 mM NaCl, 3.3 mM $NaH_2PO_4$, 2.9 mM KCl, 1 mM $MgCl_2$, 20 mM Hepes–NaOH (pH 7.4), and 1 mg/mL glucose) and 0.2 vol of ACD were added to the PRP fraction, followed by centrifugation at 1107*g* for 10 min at room temperature. Then, the platelets were washed with buffer A containing 1% fatty acid-free bovine serum albumin (BSA, Sigma). The platelets were resuspended in buffer A and sonicated, and aliquots were stored at −80 °C for further assays.

*Subcellular fractionation.* Hundred microgram protein from each tissue homogenate was diluted to an equal volume with lysis buffer, followed by centrifugation at 100,000*g* for 60 min at 4 °C to separate soluble and membrane fractions. The membrane fractions (pellets) were resuspended in lysis buffer by sonication. A final concentration of 1% Triton X-100 was added to the membrane fractions and the samples were solubilized by rotating for 60 min at 4 °C. The membrane fractions were centrifuged at 100,000*g* for 30 min at 4 °C to remove the insoluble proteins. The soluble and the membrane fractions were subjected to a sphingosine kinase assay. Where indicated in the text, immunoprecipitation was performed using the soluble and membrane fractions.

## Results

### *SPHK1 is the major sphingosine kinase in most mouse tissues*

The distribution of sphingosine kinase activity in mouse tissues was determined by an in vitro sphingosine kinase assay. Sphingosine kinase activity was detected in all tissues (Table 1). The levels of kinase activity varied greatly among the tissues. Sphingosine kinase activity was highest in lung, followed by thymus, then small intestine, spleen, and kidney. Brain, heart, colon, liver, pancreas, stomach, and testis all displayed moderate levels of kinase activity, whereas skeletal muscle exhibited very low activity.

To determine the contribution of SPHK1 to the total sphingosine kinase activity in the mouse tissues, SPHK1

Table 1
Tissue distribution of sphingosine kinase activity

| Sphingosine kinase activity in mouse tissues (pmol/min/mg protein) | |
|---|---|
| Brain | 58 |
| Heart | 54 |
| Colon | 48 |
| Thymus | 161 |
| Spleen | 88 |
| Kidney | 52 |
| Liver | 32 |
| Small intestine | 85 |
| Lung | 235 |
| Pancreas | 46 |
| Stomach | 39 |
| Testis | 65 |
| Skeletal muscle | 2 |
| Platelet | 62 |

One hundred microgram of protein of each tissue homogenate was incubated with sphingosine and [$\gamma$-$^{32}$P]ATP for 30 min at 37 °C. Lipids were then extracted and separated by TLC. Radiolabeled bands were visualized and quantified by a Bio-Imaging Analyzer. Data are representatives of two independent experiments.

was immunoprecipitated from each tissue and its activity was determined. For this purpose, we generated an anti-mSPHK1 antibody against full-length mouse SPHK1a fused to MBP. Immunoblotting analysis demonstrated that the anti-SPHK1 antibody specifically detected the FLAG-SPHK1a protein in whole cell extracts prepared from CHO cells stably expressing FLAG-SPHK1a (Fig. 1A). This antibody was able to detect about 1 ng of recombinant SPHK1 (data not shown), so it retains a high titer of protein. Next, the capacity of the antibody to immunoprecipitate SPHK1 was determined using the CHO cells stably expressing FLAG-SPHK1a (Fig. 1B). This antibody proved useful for immunoprecipitation experiments, since the FLAG-SPHK1a protein, recognizable by an anti-FLAG antibody, was effectively recovered in the immunoprecipitated fraction (lane 4).

Using the anti-SPHK1 antibody, SPHK1 was immunoprecipitated from tissue lysates and then sphingosine kinase activity was determined for the immunoprecipitates. Activity was detected in immunoprecipitates from all tissues (Fig. 2A). Again, lung and thymus exhibited the highest activity, whereas skeletal muscle displayed the lowest activity. This distribution pattern of SPHK1 activity correlates well with the Northern blot analysis reported previously [10], indicating that the activity reflected the mRNA expression level of this protein. Next, we measured sphingosine kinase activity in tissue lysates immunodepleted for SPHK1. Although little sphingosine kinase activity was detected in the SPHK1-depleted lysates of brain, heart, colon, or skeletal muscle, other tissues, especially lung, small intestine, spleen, and platelets did exhibit sphingosine kinase activity not attributable to SPHK1 (Fig. 2B). This activity was also not

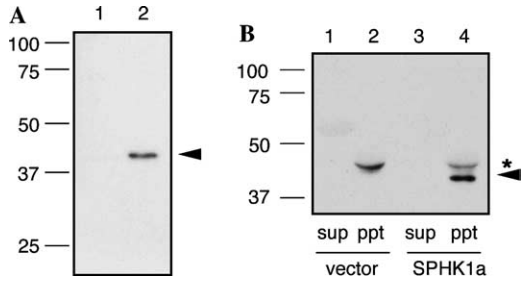


Fig. 1. Anti-mSPHK1 antibody is useful for both immunoblotting and immunoprecipitation assays. (A) Whole cell lysates from CHO cells stably expressing vector (lane 1) or FLAG-SPHK1a (lane 2) were analyzed by immunoblotting using an anti-mSPHK1 antibody. (B) Using an anti-mSPHK1 antibody, SPHK1a was immunoprecipitated from the lysates of CHO cells stably expressing vector or FLAG-SPHK1a. The immunoprecipitate (ppt) and its supernatant (sup) were separated by SDS–PAGE and then analyzed by immunoblotting using an anti-FLAG antibody.

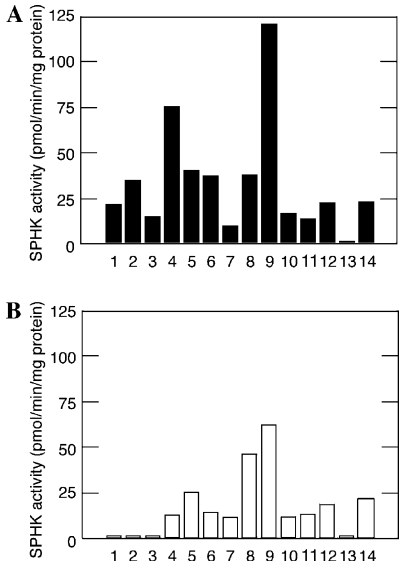


Fig. 2. Distribution pattern of sphingosine kinase activities in mouse tissue and the contribution of SPHK1. Tissue homogenates were solubilized with 1% Triton X-100 and SPHK1 was immunoprecipitated with an anti-mSPHK1 antibody. (A) Immunoprecipitates or (B) their SPHK1-immunodepleted fractions (supernatants) were incubated with sphingosine and [$\gamma$-$^{32}$P]ATP for 30 min at 37 °C. Lipids were then extracted and separated by TLC. Radiolabeled bands were visualized and quantified using a Bio-Imaging Analyzer. Lane 1, brain; lane 2, heart; lane 3, colon; lane 4, thymus; lane 5, spleen; lane 6, kidney; lane 7, liver; lane 8, small intestine; lane 9, lung; lane 10, pancreas; lane 11, stomach; lane 12, testis; lane 13, skeletal muscle; and lane 14, platelets. Data are representatives of two independent experiments.

attributable to SPHK2, since it was not depleted by an anti-SPHK2 antibody (data not shown). Moreover, the immunoprecipitates from that experiment, which contained the SPHK2, displayed only low sphingosine kinase activity (>100-fold lower activity than SPHK1 in most tissues). These results imply the existence of other unidentified sphingosine kinases.

*Kinase activities in SPHK1-immunodepleted fractions exhibit a different substrate specificity from that of SPHK1*

To characterize the sphingosine kinase activities in the lysates immunodepleted for SPHK1, in vitro kinase assays using PHS as a substrate were performed. A previous study reported that SPHK1 cannot phosphorylate PHS [10]. Indeed, we also confirmed this using CHO cells stably expressing FLAG-SPHK1a. The FLAG-SPHK1a immunoprecipitated by the anti-mSPHK1 antibody displayed very low activity against PHS, less than 1% compared to that using Sph (data not shown). We examined the kinase activity toward PHS in tissue lysates immunodepleted for SPHK1 (Fig. 3). Relatively strong activities for PHS, compared to those for Sph, were found in small intestine (14%), in lung (14%), and, especially, in platelets (28%). The stronger kinase activity toward PHS found in platelets correlate well with a previous report, which used human and pig platelets [18]. These results confirmed that the sphingosine kinase activities in SPHK1-immunodepleted lysates are not derived from SPHK1. Moreover, these results suggest the presence of other sphingosine kinases in those tissues which exhibited higher activity toward PHS than that of SPHK1.

*Subcellular distribution of sphingosine kinases*

We also characterized this undefined sphingosine kinase activity in the SPHK1-depleted lysate using a subcellular fractionation experiment. It has been reported that SPHK1 is a soluble protein and approximately 70–80% of the activity is collected in the soluble fraction [10]. In our assay conditions, more than 70% of FLAG-SPHK1a was recovered in the soluble fraction (Fig. 4A). Additionally, a previous study reported the existence of one membrane-bound and two cytosolic sphingosine kinases in human platelets, although which of them corresponded to SPHK1 was unclear [13].

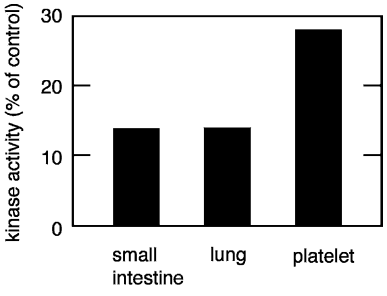


Fig. 3. Kinase activity toward PHS in SPHK1-immunodepleted fractions. SPHK1 was immunoprecipitated from lung, small intestine or platelets, and the SPHK1-immunodepleted fractions were assayed for kinase activity toward PHS. Data are expressed as a percent of the control activity obtained in kinase assays using Sph as substrate. Two independent experiments gave similar results and a representative result is shown.

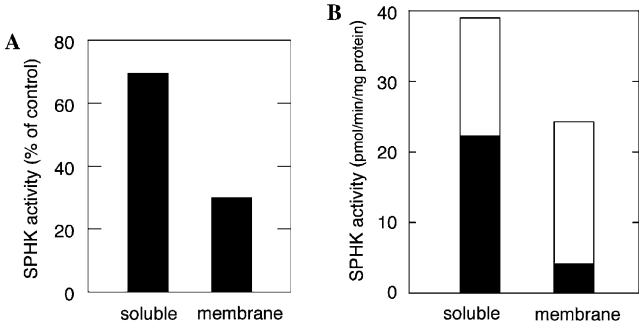


Fig. 4. Characterization of sphingosine kinases in platelets. (A) Cell lysates from CHO cells stably expressing FLAG-SPHK1a were centrifuged for 60 min at 100,000*g*, then the soluble and membrane-associated fractions were collected. After solubilization with 1% Triton X-100, the samples were subjected to in vitro sphingosine kinase assays. (B) Platelets were sonicated and centrifuged for 60 min at 100,000*g*. Then, the soluble and membrane-associated fractions were solubilized with 1% Triton X-100 and SPHK1 was immunoprecipitated with an anti-mSPHK1 antibody. Sphingosine kinase activities in both the total and immunodepleted fractions were determined by in vitro kinase assays. The unfilled portions of the bars indicate sphingosine kinase activities in SPHK1-immunodepleted fractions and the filled portions of the bars indicate SPHK1 activities.

Thus, we performed immunoprecipitation experiments using both the soluble and membrane fractions (Fig. 4B). In platelets, the soluble fraction had a higher kinase activity (62%) than the membrane-associated fraction (38%). About 60% of the sphingosine kinase activity in the soluble fraction was derived from SPHK1, whereas most of the sphingosine kinase activity associated with membrane was not immunodepleted with the SPHK1 antibody. These results suggest that an unidentified, membrane-bound sphingosine kinase(s) exists in platelets.

Since SPHK1 contributed a relatively low proportion of the total sphingosine kinase activity in small intestine, we examined the subcellular distribution pattern of sphingosine kinase in this tissue as well. In contrast to CHO cells stably expressing FLAG-SPHK1a, the membrane-associated activity was dominant in small intestine (63%, data not shown). Thus, small intestine may also contain unidentified sphingosine kinase(s) with different subcellular localization from SPHK1.

## Discussion

In this report, we determined the contribution of SPHK1 to the total sphingosine kinase activity in several mouse tissues. We found SPHK1 to be a major sphingosine kinase in all tissues examined. However, its contribution varied among tissues. SPHK1 contributed approximately 40–70% to the total kinase activity in small intestine, platelets or spleen, whereas it was a dominant contributor in brain, heart, and colon. Our results suggest that unidentified sphingosine kinase(s),

which exhibit a different substrate specificity and subcellular localization, exist in some tissues.

SPL exhibits a strong activity in small intestine [19]. The strong activities of both SPHK and SPL, the enzymes that regulate the steps in the synthesis and degradation of S1P, suggest active sphingolipid turnover in small intestine.

Phyto-type sphingolipids are reportedly found in some tissues, including lung, skin, small intestine, and kidney [20], and are slowly being recognized as molecules which, in mammals, may be involved in signal transduction pathways analogous to those found in yeast. A previous study demonstrated that Edg-6/$S1P_4$ receptors, which are distributed throughout the lymphoid tissues, bind to PHS 1-phosphate (PHS1P) with greater affinity than to S1P [21]. PHS1P may become another important lipid mediator with diverse biological implications, in addition to the now established S1P. Importantly, though, SPHK1 cannot phosphorylate PHS efficiently. Therefore, unidentified sphingosine kinase(s) that can phosphorylate PHS, as suggested by our data, may be responsible for the formation of PHS1P.

Consistent with a previous report that suggested the existence of sphingosine kinases different from SPHK1 [13], we found that 40% of sphingosine kinase activity in platelets is not attributable to SPHK1. Since activated platelets are known to be a principal source for S1P in the blood [9], it will be important to identify the protein that accounts for the remaining activity. Recently, the phosphorylated form of an immunosuppressive drug FTY720 was shown to bind to S1P/Edg receptors to elicit its action [22,23]. Although FTY720 and Sph are similar in structure, SPHK1 phosphorylates FTY720 at a much lower rate compared to Sph (<1% when compared to Sph, data not shown). Therefore, it is possible that other kinase(s) are responsible for generating the phosphorylated FTY720. In some tissues, SPHK1-immunodepleted fractions displayed higher kinase activities toward FTY720 than SPHK1-containing fractions (data not shown). These data indicate that unidentified sphingosine kinase(s) may play important roles in the FTY720 phosphorylation. Identification of other sphingosine kinase(s) and their physiological roles should be determined in future studies. Higher activity towards PHS may be a useful marker in distinguishing the activity of a novel sphingosine kinase from that of SPHK1, when purifying the protein. Understanding the activities of sphingosine kinases in tissues may ultimately provide new insight into the physiological importance of the product S1P in particular tissues.

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