Mouse Sphingosine Kinase Isoforms SPHK1a and SPHK1b Differ in Enzymatic Traits Including Stability, Localization, Modification, and Oligomerization*

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Sphingosine kinases catalyze the production of the bioactive lipid molecule sphingosine 1-phosphate. Mice have two isoforms of sphingosine kinase type 1, SPHK1a and SPHK1b. In addition to the previously reported differences in their enzymatic activities, we have found that these isoforms differ in several enzymatic characteristics. First, SPHK1b is unstable, whereas SPHK1a is highly stable. Degradation of SPHK1b occurs at the membrane and is inhibited by a proteasome inhibitor. Second, only SPHK1b exhibits abnormal mobility on SDS-PAGE, probably due to its SDS-resistant structure. Third, SPHK1a and SPHK1b are predominantly detected in the soluble and membrane fractions, respectively, when their degradation is inhibited. Fourth, only SPHK1b is modified with lipid, on its unique Cys residues (Cys-4 and Cys-5). Site-directed mutagenesis at these Cys residues resulted in increased sphingosine kinase activity, suggesting that the modification is inhibitory to the enzyme. Finally, SPHK1b tends to form homo-oligomers, whereas most SPHK1a is presented as monomers. We have also determined that the lipid modification of SPHK1b is involved in its homo-oligomerization. Thus, although these two proteins differ only in a few N-terminal amino acid residues, their enzymatic traits are extremely different.

The bioactive lipid molecule sphingosine 1-phosphate (S1P) regulates several cellular processes such as cell proliferation, cell migration, and differentiation through binding to its cell surface receptors, which are S1P/Edg family members (1–3). In addition to its extracellular action, S1P is presumed to act intracellularly in Ca2+ mobilization, cell proliferation, and apoptosis inhibition (1, 2). S1P is abundant in blood (4) and is physiologically important, especially in the vascular and immune systems (5). Its importance in the vascular system is evident in Sphek1a/Edg1-null mice, which die in utero with severe hemorrhage resulting from impaired vessel integrity due to a deficiency in smooth muscle cell recruitment (6). Its influence in the immune system has been demonstrated using the synthetic immunosuppressant FTY720, which is phosphorylated in vivo and binds to S1P receptors (7, 8). S1P and the S1P1 receptor also have important functions in the egress of lymphocytes from lymphoid organs, and phosphorylated FTY720 induces the down-regulation of S1P1, on lymphocytes and inhibits their recirculation (9, 10).

Sphingosine kinases catalyze the production of S1P from sphingosine and are also responsible for the phosphorylation of FTY720 (11, 12). Although sphingosine kinases possess no apparent enzymatic motif or transmembrane domain, there are five regions, termed C1 to C5, that are conserved among sphingosine kinases (13). The C1 to C3 regions bind to Mg2+-ATP (14), whereas the C4 is involved in sphingosine binding (15). Two mammalian sphingosine kinases are known, SPHK1, which was identified by purification of the enzyme and subsequent sequence determination (13), and SPHK2, which was cloned based on its homology to SPHK1 (16). Both sphingosine kinases are expressed ubiquitously among tissues, although their tissue-specific patterns differ (16). The enzymes may share redundant functions, since the single knock out of either kinase confers no apparent phenotype (12, 17).

Intracellular S1P levels are regulated by a balance between synthesis by sphingosine kinase and degradation by either phosphohydrolase or lase. Sphingosine kinases are activated by stimuli such as treatment with platelet-derived growth factor (18), tumor necrosis factor α (19), or phorbol ester (20) as well as the cross-linking of FcγR1 (21) or FceR1 (22). Additionally, SPHK1 is known to be regulated by protein-protein interactions (23–27), phosphorylation (28, 29), and translocation to the plasma membrane (28, 29). Although such regulation is involved in the stimuli-dependent activation of SPHK1, the precise molecular mechanisms that link the stimuli and the activation still remain largely unknown in most cases.

Two isoforms for mouse SPHK1 have been reported, SPHK1a and SPHK1b (13). Interestingly, the activity of SPHK1b is significantly lower (30–200-fold) than that of SPHK1a (13), although these proteins differ only in a few amino acid residues in their N termini. In the present study we have demonstrated that SPHK1b does not accumulate within the cell due to its low stability. We also found that SPHK1b differs from SPHK1a in several enzymatic characteristics. These include structural resistance toward SDS, membrane localization, protein stability, post-translational modification, and oligomer formation. Thus, the N terminus plays an important role in the determination of the enzymatic properties of SPHK1.

MATERIALS AND METHODS

Cell Culture and Transfection—Mouse F9 embryonal carcinoma cells and human embryonic kidney (HEK) 293T cells were grown in Dulbeccco’s modified Eagle’s medium (D6429; Sigma) containing 10% fetal calf serum and supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin in 0.1% gelatin- and 0.3% collagen-coated dishes, respectively. Transfections were performed using Lipofectamine™ 2000 reagent (Invitrogen) for F9 cells and Lipofectamine Plus™ reagent (Invitrogen) for HEK 293T cells.

Plasmids—The pCE-puro SPHK1a, pCE-puro SPHK1aβ, and pCE-puro SPHK1b plasmids are derivatives of the pCE-puro vector (30) and...
encode SPHK1a, SPHK1a2, and SPHK1b, respectively. The pCE-puro SPHK1a-3×FLAG, pCE-puro SPHK1b-3×FLAG, pCE-puro SPHK1a-Myc, and pCE-puro SPHK1b-Myc plasmids encode SPHK1 proteins tagged with triple FLAG (3×FLAG) or Myc epitopes at the C termini. The pCE-puro 3×FLAG-SPHK1a-Myc and pCE-puro 3×FLAG-SPHK1b-Myc plasmids encode SPHK1 proteins tagged with both 3×FLAG epitopes at their N termini and Myc epitopes at their C termini. The pCE-puro SPHK1a, pCE-puro SPHK1a2, or pCE-puro SPHK1b plasmid was similarly constructed using cloning a 0.48-kilobase EcoRI-SgrAI fragment of pCE-puro SPHK1b plasmid.

The pCE-puro SPHK1a–3×FLAG plasmid was constructed by cloning the ubiquitin cDNA (BD Biosciences Clontech, Palo Alto, CA) using primers 5′-GCCACCAT-GGAGTTGTGTTTTGTTTGTAGTAG-3′ and 5′-CAACCATGCTTCAGGCGGAGTTGTGTTTTGTTTGTAGTAG-3′. The amplified fragment was then cloned into the EcoRI site of the pCE-puro vector.

The pCE-puro SPHK1a-C1, pCE-puro SPHK1a-C2, and pCE-puro SPHK1b-C1C2 plasmids were constructed by site-directed mutagenesis using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) from the pCE-puro SPHK1b plasmid. The primers used are as follows: for pCE-puro SPHK1b-C1, 5′-GACTACAAACAAAACACTACTCCACCACATGGTGG-3′ and 5′-CTACTACAAACAAAACACTACTCCACCACATGGTGG-3′; for pCE-puro SPHK1b-C2, 5′-GACTACAAACAAAACACTACTCCACCACATGGTGG-3′ and 5′-CTACTACAAACAAAACACTACTCCACCACATGGTGG-3′; and for pCE-puro SPHK1b-C1C2, 5′-GACTACAAACAAAACACTACTCCACCACATGGTGG-3′ and 5′-CTACTACAAACAAAACACTACTCCACCACATGGTGG-3′.

The pCE-puro SPHK1b-C1C2–3×FLAG plasmid was constructed by cloning a 0.48-kilobase EcoRI-SgrAI fragment of pCE-puro SPHK1b plasmid. The primers used are as follows: for pCE-puro SPHK1b-C1, 5′-GACTACAAACAAAACACTACTCCACCACATGGTGG-3′ and 5′-CTACTACAAACAAAACACTACTCCACCACATGGTGG-3′; for pCE-puro SPHK1b-C2, 5′-GACTACAAACAAAACACTACTCCACCACATGGTGG-3′ and 5′-CTACTACAAACAAAACACTACTCCACCACATGGTGG-3′; and for pCE-puro SPHK1b-C1C2, 5′-GACTACAAACAAAACACTACTCCACCACATGGTGG-3′ and 5′-CTACTACAAACAAAACACTACTCCACCACATGGTGG-3′.

The pCE-puro SPHK1b-C1C2–3×FLAG plasmid was constructed by cloning a 0.48-kilobase EcoRI-SgrAI fragment of pCE-puro SPHK1b-C1C2 into the EcoRI-SgrAI site of the pCE-puro SPHK1b-3×FLAG. The pCE-puro SPHK1b-C1C2-Myc plasmid was similarly constructed using cloning a 0.48-kilobase EcoRI-SgrAI fragment of pCE-puro SPHK1b-3×FLAG and 5′-GCCACCAT-GGAAACCAGAATGCGCTT(FAM)-G-3′ for SPHK1a and 5′-CTACTACAAACAAAACACTACTCCACCACATGGTGG-3′ (FLAG) or Myc epitopes at the C termini.

The pCE-puro SPHK1b–C1 or pCE-puro SPHK1b–C2 plasmids were transfected with the pCE-puro SPHK1a, pCE-puro SPHK1b, or pCE-puro SPHK1b-C1C2 plasmid. Eighteen hours after transfection, the cell layer was disrupted by a trypsin/EDTA solution, and the cell suspension was divided into 4 or 5 aliquots. Each aliquot was added to a new 30-mm culture dish and incubated at 37 °C for 24 h. Culture medium was then changed to 1 ml of Dulbecco’s modified Eagle’s medium without Met/Cys (D0422, Sigma) and incubated at 37 °C for 1 h. Cells were pulse-labeled with [35S]Met/Cys (22 Ci/dish EXPRESSTM protein labeling mix; PerkinElmer Life Sciences) for 20 min and chased with unlabeled Met (final concentration 0.5 mg/ml) and Cys (final concentration 0.1 mg/ml) in 1 ml of Dulbecco’s modified Eagle’s medium (D6429) containing 10% fetal calf serum. At predetermined times, cells were washed with phosphate-buffered saline (PBS), treated with 1 ml of radioimmune precipitation assay buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor mixture (Complete™ EDTA free; Roche Diagnostics)), and kept on ice. Cells were disrupted by 5 passages through a 21-gauge needle, and debris was removed by centrifugation at 20,000 × g for 5 min at 4 °C. Cell lysates with equal radioactivity were incubated with affinity-purified anti-SHK1 antibodies and protein A-Sepharose (Amersham Biosciences) at 4 °C for 14 h. After 2 washes with 1 ml of radioimmune precipitation assay buffer and 1 with 1 ml of 10 mM Tris-HCl (pH 8.0), beads were suspended in 1 ml of SDS sample buffer (125 mM Tris-HCl (pH 6.8), 8% SDS, 20% glycerol, and a trace amount of bromphenol blue) containing 10% 2-mercaptoethanol and boiled for 3 min. The precipitates were then separated by SDS-PAGE.

Radioimmunoassay associated with SPHK1 were quantified using a Bio-Imaging Analyzer BAS2500 (Fuji Film, Tokyo, Japan).

For detection of SPHK1-ubiquitin conjugations, HEK 293T cells were transfected with pCE-puro 3×FLAG-Ub and either pCE-puro SPHK1a or pCE-puro SPHK1b, then incubated for 40 h at 37 °C. Detection of cell lysates and immunoprecipitation with anti-SHK1 antibodies were performed as described above. Precipitates were separated by SDS-
Different Traits between Two SPHK1 Isoforms

PAGE and subjected to immunoblotting using an anti-FLAG M2 antibody.

To detect homo-oligomer formation of SPHK1, HEK 293T cells were transfected with two plasmids, one carrying 3×FLAG-tagged SPHK1 genes and the other harboring Myc-tagged SPHK1 genes. Twenty-two hours after transfection, 20 μM MG132 was added to inhibit the proteasome-dependent SPHK1 degradation, and the cells were incubated for 4 h at 37 °C. The cells were washed twice with PBS, suspended in buffer A (PBS, 1 mM dithiothreitol, 1× protease inhibitor mixture, and 1 mM phenylmethylsulfonyl fluoride), and sonicated. After a centrifugation at 300 × g for 3 min at 4 °C, the resulting supernatant and pellet were used as soluble and membrane fractions, respectively.

In Vivo [3H]Palmitic Acid Labeling—HEK 293T cells grown on a 30-mm dish were transfected with plasmids and incubated for 23 h at 37 °C. Culture medium was then changed to 1.5 ml of serum-free Dulbecco’s modified Eagle’s medium. After a 30-min incubation at 37 °C, 3 μg/ml cerulenin, which inhibits fatty acid synthesis, and 20 μM MG132 were added to the medium, and the cells were incubated for 30 min at 37 °C. Cells were labeled with 0.2 mCi of [3H]palmitic acid (60 Ci/mmol; American Radiolabeled Chemical, St. Louis, MO) at 37 °C for 3 h. After washing with PBS, cells were treated with 1 ml radioimmune precipitation assay buffer. SPHK1 was immunoprecipitated using anti-SPHK1 antibodies and protein A-Sepharose as described above. Immunoprecipitates suspended in 2× SDS sample buffer containing 10 mM 2-mercaptoethanol were boiled for 3 min, separated by SDS-PAGE, and subjected to immunoblotting using an anti-FLAG M2 antibody or an anti-Myc PL14 antibody.

Preparation of Soluble and Membrane Fractions—HEK 293T cells transfected with plasmids were washed twice with PBS, suspended in buffer A, and sonicated. After removal of cell debris by centrifugation at 300 × g for 3 min at 4 °C, cell lysates were centrifuged at 100,000 × g for 1 h at 4 °C. The resulting supernatant and pellet were used as soluble and membrane fractions, respectively.

Sphingosine Kinase Assay—Sphingosine kinase assays were performed as described elsewhere (33).

RESULTS

Tissue-specific Expression of SPHK1 Isoforms—There are variations in mouse SPHK1 mRNAs due to the differences in transcriptional initiation sites, and as a result, two different polypeptides, SPHK1a and SPHK1b, are produced (34). At least four types of mRNAs, differing in the first exons, encode the same SPHK1a protein, whereas only one mRNA is known for SPHK1b (34). Translation of the SPHK1a protein is initiated near the end of the common second exon (Fig. 1). By searching EST clone databases, we found that certain SPHK1a-encoding mRNAs contain a 3-bp insertion in the coding region. This newly identified variation, named here SPHK1a2, is produced by an altered junction between the second and third exons. Consequently, the SPHK1a2 protein contains an extra Val residue. In contrast, transcription of SPHK1b mRNA is initiated within the intron region between the second and third exons of SPHK1a/SPHK1a2, so the 3′-half of the first exon of the SPHK1b gene is common to the third exon of SPHK1a/SPHK1a2 (34). Translation of the SPHK1b protein is initiated from 28 bp upstream of the 5′-terminal end of the third exon of SPHK1a2 (Fig. 1). Thus, there are three types of SPHK1 proteins, which differ only in their N termini.

The N-terminal sequences specific to each are: SPHK1a1, Met-Glu-Pro; SPHK1a2, Met-Glu-Pro-Val; SPHK1b, Met-Trp-Trp-Cys-Cys-Val-Leu-Phe-Val-Val. Of the 23 EST clones registered, we found that 8 represented SPHK1a1, 12 represented SPHK1a2, and 3 represented SPHK1b, suggesting that SPHK1a1 may be the most abundant isoform, and SPHK1b may be only a minor isoform.

We examined tissue- and embryonic development-specific expression patterns of SPHK1a1a2, and SPHK1b, although we were unable to prepare a specific primer that would distinguish SPHK1a mRNA from SPHK1a2 mRNA. Real-time quantitative PCR analysis revealed that SPHK1a1a2 mRNA is ubiquitously expressed, although levels vary among tissues and embryonic stages (Table 1). However, very little expression of SPHK1a1a2 mRNA was detectable in skeletal muscle, consistent with the extremely low sphingosine kinase activity in this tissue (31). The expression of SPHK1b mRNA was more restricted than that of SPHK1a (Table 1). Little SPHK1b mRNA expression was detected in heart, brain, liver, and skeletal muscle. The expression levels of SPHK1b mRNA were observed to be lower than those of SPHK1a1a2 mRNA regardless of the tissue, which is consistent with the small number of the EST clones carrying SPHK1b. The amount of SPHK1b mRNA was 10–20% that observed for SPHK1a1a2 mRNA in spleen, lung, kidney, and testis. In embryonic stages, days 11–15, the discrepancy was somewhat less, with SPHK1b mRNA levels at 67 and 37% of SPHK1a1a2 mRNA levels. Thus, it would appear that the expression of SPHK1a1a2 mRNA and that of SPHK1b mRNA are regulated differently among tissues and embryonic stages.

Abnormal Mobility of SPHK1b on SDS-PAGE—SPHK1b reportedly has a much lower activity than SPHK1a (13), which we also observed (data not shown), yet the reason was unclear. To study this phenomenon, we cloned each of the three isoforms and expressed them in mouse F9 cells. In immunoblots using anti-SPHK1 antibodies, SPHK1a and SPHK1a2, were detected at ~43 kDa (Fig. 2A), in accordance with their predicted molecular masses (both 42.4 kDa). We found no differences in enzymatic characteristics (activity, gel mobility, membrane localization, stability, and oligomerization) between SPHK1a and SPHK1a2 (Fig. 2A and data not shown), so we described hereafter only SPHK1a.

In contrast, the mobility of SPHK1b deviated from its predicted molecular mass (43.3 kDa) and was instead detected at 34 kDa (Fig. 2A). This mobility was not cell-specific, since it was also observed for HEK 293T cells (Figs. 3–6). An additional band at 68 kDa, which may represent a SDS-resistant dimer (see Fig. 6), was also detected, although its level varied with the experimental conditions. A similar upper band was often observed in SPHK1a blots (Figs. 3B, 4A, and 6A); however, the

![FIGURE 1. Genomic structures of the known mouse SPHK1 isoforms. The second and third exons of SPHK1a and SPHK1a2, and the first exon of SPHK1b are shown. The N-terminal sequences of the respective polypeptides are also shown. Open and closed boxes indicate the untranslated and coding regions, respectively. Nucleotide sequences in the exon are described in uppercase, whereas those in the intron are shown in lowercase.](image-url)
intensity of the band was always lower than that observed for SPHK1b blots.

To examine the possibility that the fast mobility of SPHK1b was due to the proteolytic removal of either the N or C terminus, we prepared SPHK1a and SPHK1b constructs each tagged with 3×FLAG or anti-Myc antibody, 3×FLAG-SPHK1a-Myc was detected at 50 kDa (49.2 kDa). Again, however, the apparent molecular mass of 3×FLAG-SPHK1b-Myc by immunoblot was about 41 kDa, 9 kDa lower than the predicted mass. Importantly, both antibodies detected the 41-kDa band, indicating that SPHK1b was not degraded at either the N terminus or the C terminus; therefore, the observed band represented the full-length protein. Thus, the cause of the abnormally fast gel mobility may be structural as is often observed for multi-span membrane proteins (32, 35, 36). The possibility of an intracellular disulfide bond, however, can be discounted, since we always performed SDS-PAGE under reducing conditions. Although most polypeptides are denatured by SDS and are

### Table 1

Expression levels of SPHK1a/a2 and SPHK1b in various tissues and during embryonic development

| Tissue or embryonic stage | Relative SPHK1 level |
|---------------------------|----------------------|
|                           | SPHK1a/a2 | SPHK1b |
| Heart                     | 2.9 ± 0.40 | 0.96 ± 0.93 |
| Brain                     | 6.4 ± 1.5  | 1.1 ± 0.38  |
| Spleen                    | 190 ± 8.1  | 21 ± 3.2   |
| Lung                      | 240 ± 82   | 48 ± 4.4   |
| Liver                     | 2.4 ± 0.50 | 0.52 ± 0.45 |
| Skeletal muscle           | 0.48 ± 0.030| 0.062 ± 0.053|
| Kidney                    | 19 ± 3.0   | 4.3 ± 0.56  |
| Testis                    | 51 ± 13    | 6.5 ± 6.5  |
| 7 Day                     | 430 ± 24   | 62 ± 2.4   |
| 11 Day                    | 11 ± 1.9   | 7.4 ± 2.5  |
| 15 Day                    | 15 ± 1.7   | 5.5 ± 2.3  |
| 17 Day                    | 26 ± 1.4   | 3.7 ± 1.0  |

### Figure 2

**A** total cell lysates (6 μg) prepared from F9 cells transfected with an empty vector or with a plasmid encoding SPHK1b, SPHK1a, or SPHK1a2 were separated by SDS-PAGE and subjected to immunoblotting with an anti-SPHK1 antibody. (SPHK1b)2 indicates the putative dimer form of SPHK1b. B, HEK 293T cells were transfected with a plasmid encoding 3×FLAG-SPHK1a-Myc or 3×FLAG-SPHK1b-Myc, and total cell lysates were prepared. Proteins (2 μg) were separated by SDS-PAGE followed by immunoblotting with an anti-FLAG or anti-Myc antibody as indicated.

### Figure 3

**A** Differences in protein stability and membrane localization between SPHK1a and SPHK1b. A, HEK 293T cells transfected with a plasmid encoding SPHK1a or SPHK1b were pulse-labeled with [35S]Met/[35S]Cys for 20 min and incubated with excess cold Met/Cys for 0.5, 1, 3, 5, or 7 h. Total cell lysates were prepared, subjected to immunoprecipitation with anti-SPHK1 antibodies, and separated by SDS-PAGE. Gels were fixed, and radiolabeled proteins were detected by autoradiography. B, F9 cells transfected with an empty vector or a plasmid encoding SPHK1a or SPHK1b were incubated for 12 h in the presence or absence of 20 μM MG132, a proteasome inhibitor, then total cell lysates were prepared. Proteins (20 μg) were separated by SDS-PAGE, and SPHK1 proteins were detected by immunoblotting with anti-SPHK1 antibodies. (SPHK1a)2 and (SPHK1b)2 indicate putative dimer forms of SPHK1a and SPHK1b, respectively. C, F9 cells transfected with a plasmid encoding SPHK1a or SPHK1b were treated with or without 20 μM MG132 for 12 h at 37 °C. Total cell lysates were prepared and separated by a 1-h centrifugation at 100,000 × g into membrane (M) and soluble (S) fractions. Each fraction was separated by SDS-PAGE, SPHK1, calnexin (a membrane protein marker), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (a soluble protein marker) were then detected by immunoblotting with appropriate antibodies as detailed under “Materials and Methods.” D, HEK 293T cells transfected with a plasmid encoding SPHK1a or SPHK1b were incubated for 13 h with 15 μM MG132, then immunostained with anti-SPHK1 antibodies and examined on a fluorescence microscope. Bar, 10 mm.
in their extended form in SDS-containing solutions, some proteins, including SPHK1b, may be resistant to SDS and, therefore, remain compact.

Degradation of Membrane-bound Forms of SPHK1s—The total amount of SPHK1b expressed in the F9 cells was much lower than that of SPHK1a/SPHK1a₂ (Fig. 2A), suggesting that the SPHK1b protein is unstable. Therefore, we performed pulse-chase experiments using [35S]Met/Cys. As shown in Fig. 3A, SPHK1a was highly stable, and only a slight decrease was detected at the 7-h chase point. In contrast, about half of the SPHK1b was already degraded after a 3-h incubation, indicating that SPHK1b is unstable.

We next examined the effect of MG132, a proteasome inhibitor, on the total amount of each SPHK1. The amount of SPHK1a slightly increased after treatment with MG132 (Fig. 3B). MG132 also caused an increase in the SPHK1b levels, and in this case the effect was much more prominent. These results suggest that the proteasome is involved in the degradation of SPHK1s. To further investigate this finding, the kinases were fractionated by centrifugation into soluble or membrane-bound forms. In the absence of MG132, SPHK1a was only recovered in the soluble fraction (Fig. 3C). Treatment with MG132 resulted in the appearance of membrane-bound SPHK1a with almost no change in the amount of soluble SPHK1a. Similar results were obtained for SPHK1b; MG132 caused a large increase only in the membrane fraction (Fig. 3C). These results suggest that only membrane-bound SPHK1s are susceptible to degradation by the proteasome. Furthermore, in the presence of MG132 the greater share of SPHK1a (~80%) was found in the soluble fraction, whereas ~90% of SPHK1b was in the membrane fraction; thus, a greater tendency to associate with the membrane may account for the lower stability of SPHK1b. The membrane-bound forms of both SPHK1a and SPHK1b were found to be localized in the plasma membrane by indirect immunofluorescence microscopy. Both proteins were detected in the cytosol and the plasma membrane (Fig. 3D).

Proteins are generally modified by ubiquitin before degradation by the proteasome. To examine the degree of its ubiquitination, each SPHK1 was expressed in HEK 293T cells together with 3×FLAG-ubiquitin, immunoprecipitated with anti-SHK1 antibodies, and subjected to immunoblotting with an anti-FLAG antibody. Bands were detected on the immunoblots of cells expressing either SPHK1a or SPHK1b, indicating ubiquitination (Fig. 4B). Considering the molecular mass of 3×FLAG-ubiquitin (11.8 kDa), it is conceivable that the broad upper bands represent polyubiquitinated SPHK1s, and the bands at the lower end monoubiquitinated proteins. Although the total amount of SPHK1b was much lower than that of SPHK1a (Fig. 4A), the ubiquitinated bands were more prominent (Fig. 4B), consistent with the high susceptibility of SPHK1b to proteasome-dependent degradation.

Lipid Modification of SPHK1b within Its Unique N-terminal Sequence—We recently demonstrated that the yeast long-chain base kinase Lcb4p is palmitoylated (37). Although there are no exact motifs for palmitoylation, different traits between two SPHK1 isoforms.

**Different Traits between Two SPHK1 Isoforms**

**FIGURE 4. Ubiquitination of SPHK1.** HEK 293T cells were transfected with a plasmid encoding 3×FLAG-ubiquitin together with one encoding no protein, SPHK1a, or SPHK1b. A, total cell lysates were prepared, separated by SDS-PAGE, and subjected to immunoblotting with anti-SHK1 antibodies. B, SPHK1 and any associated proteins were immunoprecipitated with anti-SHK1 antibodies. The precipitates were separated by SDS-PAGE followed by immunoblotting with an anti-FLAG antibody.

**FIGURE 5. Lipid modification of SPHK1b at the N-terminal two Cys residues.** HEK 293T cells were transfected with plasmid encoding SPHK1b, SPHK1b-C1, SPHK1b-C2, or SPHK1b-C1C2. A, total lysates (2 μg) were separated by SDS-PAGE followed by immunoblotting with anti-SHK1 antibodies. WT, wild-type. B, cells were labeled with [35S]Met/Cys for 20 min, then incubated with excess cold Met/Cys for 0.5, 1.5, 3, or 6 h. Total lysates were prepared and subjected to immunoprecipitation with anti-SHK1 antibodies, and the precipitates were separated by SDS-PAGE. Radioactivities associated with SPHK1 were quantified using a Bio-Imaging Analyzer BAS2500 and are expressed as a percentage of those at the 0.5-h chase point.
Different Traits between Two SPHK1 Isoforms

![FIGURE 6. Homo-oligomerization of SPHK1s.](image)

**A**. HEK 293T cells were transfected with a combination of plasmids encoding no protein (vector), SPHK1a-Myc, SPHK1a-3×FLAG, SPHK1b-Myc, or SPHK1b-3×FLAG as indicated and incubated with 20 μM MG132 for 4 h. Total cell lysates were subjected to immunoprecipitation with an anti-FLAG antibody. The precipitates were separated by SDS-PAGE followed by immunoblotting with an anti-FLAG or anti-Myc antibody. (IP, immunoprecipitation; *, nonspecific background; (1a)2, putative SPHK1a dimer; (1b)2, putative SPHK1b dimer; (1b)n, putative multimers of SPHK1b. B. HEK 293T cells were transfected with plasmids encoding SPHK1b-3×FLAG and SPHK1b-Myc or SPHK1b-C1C2-3×FLAG and SPHK1b-C1C2-Myc and incubated with 20 μM MG132 for 4 h. Total cell lysates were immunoprecipitated with an anti-FLAG antibody. The supernatant (S) and precipitate (P) were separated by SDS-PAGE followed by immunoblotting with an anti-FLAG or anti-Myc antibody.

many proteins are palmitoylated at Cys residues (often in clusters) near the N terminus (38, 39), and in fact Lcb4p is palmitoylated at Cys-43 and Cys-46 residues (37). The N-terminal sequence that is unique to SPHK1b (and not found in SPHK1a) contains two Cys residues (Cys-4 and Cys-5); therefore we investigated the possibility that SPHK1b is modified by palmitoylation on these Cys residues. We created Cys-to-Ser mutants for Cys-4 and Cys-5 in the N-terminal region.

SPHK1a and SPHK1b, possess completely different molecular characteristics such as mobility on SDS-PAGE, stability, membrane localization, and oligomerization. Moreover, we found two regulatory mechanisms that are common to both SPHK1s. First, both SPHK1s are degraded after associating with membrane; however, a difference in the abilities of SPHK1a and SPHK1b to interact with the membrane may account for the difference in their stability. Second, both SPHK1s can form oligomers, although SPHK1b exhibits a much higher tendency to oligomerize. This is the first report describing the oligomerization of sphingosine kinases, although homo-oligomerization of a related lipid kinase, diacylglycerol kinase, has been reported (43).

SPHK1b exhibited an aberrant, fast mobility on SDS-PAGE. The principle that proteins of the same size migrate equally on SDS-PAGE relies on the supposition that the native structures of proteins are unfolded by SDS, which binds to the hydrophobic regions of proteins. Because proteins of the same size generally contain similar numbers of such regions, they are thought to bind nearly equal amounts of SDS molecules at levels so great that the overall negative charge of the SDS
Different Traits between Two SPHK1 Isoforms

overwhelms the intrinsic charges of the proteins, making the proteins roughly equal in charge. Considering this premise, three possible reasons are conceivable for the aberrant mobility of SPHK1b; they are an extraordinarily high intrinsic charge that cannot be overwhelmed by SDS, excess binding of SDS, and an SDS-resistant structure. The first possibility can be ruled out since SPHK1b differs from normally migrating SPHK1 in only a few amino acid residues, which carry no charge. The second possibility is also unlikely, since the sequence unique to SPHK1b (a nona-peptide) is too small to be covered by excess SDS. One might imagine that the palmitic acid (or its derivative) that modifies SPHK1b would bind a large amount of SDS and cause faster mobility; however, palmitoylation usually causes slower migration compared with the unmodified protein because it increases the molecular mass (44, 45). Indeed, we found no examples of palmitoylation causing faster mobility. Thus, we conclude that the last possibility, an SDS-resistant dimer of SPHK1b was reduced in the C1C2 mutant, which is not lipid-modified, and the aberrant mobility was also partially restored (Fig. 5A).

We recently reported that the yeast long-chain base kinase Lcb4p is anchored to the membrane through palmitoylation (37). In the present study we demonstrated that two reasons are responsible for the lower activity of SPHK1b. First, SPHK1b cannot accumulate in the cells due to its instability (Fig. 3A). Second, the N-terminal palmitoylation inhibits the enzyme activity (Fig. 5C). Although information regarding the three dimensional structure of sphingosine kinase is not available, it is likely that the N terminus is located adjacent to the catalytic site and regulates the activity. Human SPHK1 also has three isoforms differing in the length of their N termini (11). Therefore, it is possible that their N termini differentially regulate their activities. Future studies using x-ray crystallography or NMR analysis will be required to reveal the structure of SPHK1 and the relationship between structure and regulation.

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