

# Extracellular export of sphingosine kinase-1a contributes to the vascular S1P gradient

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Sphingosine 1-phosphate (S1P), produced by Sphks (sphingosine kinases), is a multifunctional lipid mediator that regulates immune cell trafficking and vascular development. Mammals maintain a large concentration gradient of S1P between vascular and extravascular compartments. Mechanisms by which S1P is released from cells and concentrated in the plasma are poorly understood. We recently demonstrated [Ancellin, Colmont, Su, Li, Mittereder, Chae, Stefansson, Liao and Hla (2002) *J. Biol. Chem.* **277**, 6667–6675] that Sphk1 activity is constitutively secreted by vascular endothelial cells. In the present study, we show that among the five Sphk isoforms expressed in endothelial cells, the Sphk-1a isoform is selectively secreted in HEK-293 cells (human embryonic kidney cells) and human umbilical-vein endothelial cells. In sharp contrast, Sphk2 is not secreted.

The exported Sphk-1a isoform is enzymatically active and produced sufficient S1P to induce S1P receptor internalization. Wild-type mouse plasma contains significant Sphk activity ( $179 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ ). In contrast, *Sphk1*<sup>-/-</sup> mouse plasma has undetectable Sphk activity and approx. 65% reduction in S1P levels. Moreover, human plasma contains enzymatically active Sphk1 ( $46 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ ). These results suggest that export of Sphk-1a occurs under physiological conditions and may contribute to the establishment of the vascular S1P gradient.

**Key words:** extracellular export, platelet-poor plasma, platelet-rich plasma, sphingosine kinase, sphingosine 1-phosphate, sphingosine 1-phosphate (S1P) gradient.

## INTRODUCTION

Sphingosine 1-phosphate (S1P), a product of sphingomyelin metabolism, is an important mediator of cell growth, proliferation and migration *in vitro*. In addition, it regulates angiogenesis, cardiogenesis and trafficking of immune cells *in vivo* [1]. S1P is generated by the phosphorylation of sphingosine by Sphks (sphingosine kinases) 1 and 2, which share a conserved catalytic domain [2]. In mammals the concentration of S1P in plasma varies from 0.1 to 0.6  $\mu\text{M}$ , while in serum it is 0.4–1.1  $\mu\text{M}$  [3–5]. In contrast, tissue S1P levels are generally low (0.5–75 pmol/mg) [6–9]. Therefore a large concentration gradient of S1P exists between blood (plasma) and interstitial fluids in mammals. This may have an important functional role in the vascular and immune systems, which are constantly bathed in plasma.

The source of plasma S1P is widely assumed to be the cells of the haemopoietic system. For example, platelets, neutrophils, mast cells and mononuclear cells are capable of secreting S1P [10]. However, S1P is synthesized by most mammalian cells; many non-haemopoietic cells are also capable of S1P secretion [11]. In addition, cells of the vasculature, such as HUVEC (human umbilical-vein endothelial cells), also secrete S1P [12]. A recent paper examined the mechanisms involved in the secretion of S1P from platelets [13]. The authors proposed a mechanism by which ABC transporters (ATP-binding-cassette transporters) are involved in the intramembrane flipping of S1P from the cytosolic surface to the extracellular surface.

We previously proposed an alternative mechanism for the generation of S1P, in which enzymatically active Sphk1 is constitutively secreted from vascular endothelial cells [12]. This phenomenon of Sphk1 secretion was also observed in airway smooth-muscle cells [14]. Tani et al. [15] also observed secreted Sphk activity in Chinese-hamster ovary cells. This extracellular export of Sphk1 requires the actin cytoskeleton and does not involve the classical ER (endoplasmic reticulum)–Golgi vesicular secretory pathway [12]. The physiological relevance of secreted Sphk is not clear, since the magnitude of secreted Sphk is small and not all cells are capable of secreting active Sphk.

Chromatographically distinct Sphk isoforms are present in human platelets [16] and rat tissues [17]. Such isoforms are differentially responsive to salt, heat treatment, detergent and the inhibitor DMS (*N,N'*-dimethylsphingosine). Heterogeneous biochemical properties could be accounted for by subtle genetic differences in Sphk1 and Sphk2 isoenzymes, post-translational modifications or both. Indeed, biochemical differences in the two isoforms have been described; Sphk2 is able to phosphorylate a wider range of sphingoid base substrates and shows differential sensitivity to salt and detergents [18]. This is also evident from the studies on phosphorylation of the immunomodulator drug FTY720 by Sphk2 [19–21]. As for genetic differences, database searches identified various isoforms of Sphk isoenzymes in mammals. For example, rat Sphk1 is predicted to have at least six isoforms with heterogeneity at the N-terminus [22]. Two isoforms of murine Sphk1 have been cloned which differ in the N-terminus

Abbreviations used: Acc. No., accession number; CM, conditioned medium; DMEM, Dulbecco's modified Eagle's medium; DMS, *N,N'*-dimethylsphingosine; ER, endoplasmic reticulum; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GST, glutathione S-transferase; HEK-293 cell, human embryonic kidney cell; HUVEC, human umbilical-vein endothelial cells; NRS, non-immune rabbit serum; PPP, platelet-poor plasma; PRP, platelet-rich plasma; RT, reverse transcriptase; Q-RT-PCR, quantitative RT-PCR; siRNA, small interfering RNA; S1P, sphingosine 1-phosphate; S1PR, S1P receptor; Sphk, sphingosine kinase; hSphk, human Sphk.

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[23]. hSphk1 (human Sphk1) and hSphk2 also have N-terminally distinct isoforms. However, the physiological functions of Sphk1 and Sphk2 isoforms are not understood.

In the present study, we investigated the Sphk1 and Sphk2 isoforms for their ability to act as an ectokinase. We show that the Sphk-1a isoform is preferentially secreted into the CM (conditioned medium). In addition, Sphk-1a is active in the medium and produces sufficient S1P to activate and internalize the S1PR1 (S1P receptor 1). Finally, we demonstrate for the first time that mouse plasma exhibits DMS-sensitive Sphk activity, which is absent in Sphk1 null mice, suggesting that secretory Sphk1 activity contributes to the high levels of S1P in the plasma.

## MATERIALS AND METHODS

### Reagents

D-Erythro C<sub>18</sub> sphingosine was either from Biomol Research Laboratory (Plymouth Meeting, PA, U.S.A.) or from Avanti Polar Lipids (Alabaster, AL, U.S.A.). D-Erythro S1P was from Matreya Lipid Biochemicals (Pleasant Gap, PA, U.S.A.); D-erythro C<sub>17</sub> sphingosine and C<sub>17</sub>-S1P were from Avanti Polar lipids. DMS and prostaglandin E<sub>1</sub> were from Cayman Chemicals (Ann Arbor, MI, U.S.A.). Redivue [ $\gamma$ -<sup>32</sup>P]ATP (6000 Ci/mmol), Redivue [<sup>35</sup>S]methionine and cysteine, cell labelling grade (1000 Ci/mmol), Redivue [<sup>35</sup>S]methionine and Protein A–Sepharose were purchased from Amersham Biosciences (Piscataway, NJ, U.S.A.). TLC plates were from Whatman (Brentford, U.K.); Lipofectamine<sup>TM</sup> and Lipofectamine<sup>TM</sup> 2000 were from Invitrogen (Carlsbad, CA, U.S.A.). Heparin and protease inhibitor cocktail were from Calbiochem (La Jolla, CA, U.S.A.). PMSF was from Sigma (St. Louis, MO, U.S.A.). Monoclonal GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody was from Abcam (Cambridge, MA, U.S.A.).

### Cell culture

HUVEC were purchased from Clonetics (San Diego, CA, U.S.A.) and cultured up to passage 8 in M199 medium (Gibco-BRL; supplemented with 10% fetal bovine serum, 1× antibiotics and antimycotics + 150 µg/ml of endothelial cell growth factor from bovine brain extract + 5 units/ml of heparin) on human fibronectin-coated plates [25]. HEK-293 cells were obtained from A.T.C.C. (Manassas, VA, U.S.A.; CRL1573) and cultured in DMEM (Dulbecco's modified Eagle's medium) having 10% (v/v) FBS (Invitrogen).

### Mice

C57Bl/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, U.S.A.). *Sphk1* null mice [7] were backcrossed five generations into the C57Bl/6 background.

### Plasmids and cDNA cloning of Sphk 1 and 2 isoforms

Full-length hSphk1 and hSphk2 cDNA clones were purchased from either A.T.C.C. or from Proteintech Group (Chicago, IL, U.S.A.). The GenBank<sup>®</sup> nucleotide accession numbers (Acc.No.) for Sphk1 cDNA clones were: IMAGE ID: 3831657, Acc. No. (accession number) BC008040 for hSphk-1a, 384-amino-acid isoform; IMAGE ID: 3506924, Acc. No. BC009419 for hSphk-1b, 398-amino-acid isoform; and IMAGE ID: 5213270, Acc. No. BC030553 for hSphk-1c, 470-amino-acid isoform. The GenBank<sup>®</sup> nucleotide accession numbers for hSphk2 cDNA clones were: IMAGE ID: 3870258, Acc. No. BC010671 for Sphk-2a, 618-amino-acid isoform; and IMAGE: 3677636, Acc. No.

BC006161 for Sphk-2b, 654-amino-acid isoform. The Sphk-1c (470-amino-acid isoform) and Sphk-2a (618-amino-acid isoform) cDNA clones were in pCMV-SPORT6 vector, while all the other cDNA inserts were released upon EcoRI and XhoI restriction digestion followed by ligation of the released insert to pcDNA 3.0 mammalian expression vector at the same restriction sites.

### Hairpin siRNA (small interfering RNA) construct for Sphk1

Endogenous Sphk1 expression was down-regulated with sequence-specific pSilencer-siSphK1 851 (Clone1) and 1118 (Clone2). Complementary oligonucleotides (sense 851: GATCC-AAATCTCCTTCACGCTGATTTCAAGAGAATCAGCGTGA-AGGAGATTTTTTTTTGGAAA; antisense 851: AGCTTTT-CCAAAAAATAATCTCCTTCACGCTGATTCTCTTGAAAT-CAGCGTGAAGGAGATTTG; sense 1118: GATCCGTGAC-CAACTGCACGCTATTCTCAAGAGAAATAGCGTGCAGTT-GGTCATTTTTTTGGAAA; antisense: AGCTTTTCCAAAAA-ATGACCAACTGCACGCTATTCTCTTGAGAATAGCGTG-CAGTTGGTCAAG). They were annealed and ligated into the pSilencer vector according to the manufacturer's instructions (Ambion). pSilencer vector was used as a control.

### Transfections

Transfections were carried out using either the calcium phosphate method [24] or by using Lipofectamine<sup>TM</sup> or Lipofectamine<sup>TM</sup> 2000 according to the manufacturer's instructions.

### Q-RT-PCR [quantitative RT (reverse transcriptase)-PCR] analysis

HUVEC were grown to confluency in human fibronectin-coated dishes as described in [25]. Total RNA was isolated from HUVEC using RNA STAT 60 as per the manufacturer's instructions. Total RNA (1 µg) was subjected to first strand cDNA synthesis using random primers with MMLV (Moloney-murine-leukaemia virus) RT. Subsequently, first strand cDNA was subjected to quantitative PCR using SYBR Green I DNA-binding dye technology (Foster City, CA, U.S.A.), using sequence-specific primers for various isoforms of hSphk1 and hSphk2. hSphk-1a isoform was amplified using 5'-GTCGAGGTTATGGATCCAGCG and 5'-TTCCGCCGCTCAGTGAGCAT; Sphk-1b isoform was amplified using 5'-ATGGATCCAGTGGTTCGGTTGCG and 5'-TTC-CGCCGCTCAGTGAGCAT; and Sphk-1c isoform was amplified by 5'-ATGTCCGCTCAAGTTCTGGGAT and 5'-TCCTGCCTT-CAGCTCCTTAT respectively. hSphk-2a isoform was amplified using AGATGAATGGACACCTTGAAGC and CTGGGTAG-GAGCCAAACTCG and Sphk-2b isoform was amplified with GCCACCATGGCCCCGCCCCACC GCCACT and CAGGGT-GCAGCAGCCTGAGAC respectively. Duplicate SYBR Green PCR reactions for each sample were done using an ABI 7900HT instrument. Results were expressed relative to the internal control gene GAPDH.

### Subcellular fractionation of HUVEC

Confluent HUVEC were washed three times with 5 ml of ice-cold PBS. All the subsequent operations were carried out at 4°C. Cells were scraped in 20 mM HEPES/KOH (pH 7.4), 10 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM EGTA with 1 mM PMSF and 1× protease inhibitor cocktail (Calbiochem). Cells were disrupted with Dounce's homogenizer 15–20 times. The homogenates were first centrifuged at 300 g for 5 min and the supernatant was subjected to 1000 g for 5 min to obtain the nuclear pellet and post-nuclear supernatant. The supernatant fraction was subsequently subjected to ultracentrifugation at 60000 rev./min for 60 min at

4°C using Beckman TLA 100.2 rotor in Beckman™ ultima TL ultracentrifuge to obtain the cytosol.

### Sphk assay

Sphk assay for homogenates and CM was performed as reported earlier [12]. In a typical assay, 10 µg of total homogenate and 0.25 ml of CM were incubated with 20 µM sphingosine in a total volume of 0.4 ml. The phosphorylation of sphingosine was initiated by addition of 10 µCi [ $\gamma$ -<sup>32</sup>P]ATP diluted in 500 µM ATP for 30 min at 37°C. In some experiments, CM obtained from Sphk-1a-transfected cells were subjected to a low-speed centrifugation (1000 g for 5 min) followed by an ultracentrifugation at 60000 rev./min for 60 min at 4°C using a Beckman TLA 100.2 rotor in a Beckman™ ultima TL ultracentrifuge. Pellets were washed twice with ice-cold PBS and the pellets were resuspended in 0.1 ml of homogenization buffer (20 mM Hepes/KOH, pH 7.4, 10 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 1 mM PMSF and 1× Calbiochem protease inhibitor cocktail) by repeated pipetting. All the fractions were assayed for Sphk activity as mentioned above. Extraction of [ $\gamma$ -<sup>32</sup>P]S1P was followed as described in [11], with slight modification. The organic phase containing [ $\gamma$ -<sup>32</sup>P]S1P was washed twice with 10 mM HCl and re-extracted in chloroform and finally dried under a stream of N<sub>2</sub>. Dried lipids were loaded on to TLC plates and developed using 3:1:1 (by vol.) 1-butanol/acetic acid/water solvent system. Images were developed in a Molecular Dynamics PhosphorImager and radioactive bands corresponding to [ $\gamma$ -<sup>32</sup>P]S1P were scraped and quantified in a Wallac liquid-scintillation counter.

### Isolation of blood and plasma

Whole blood was collected in acid-citrate dextrose (20 mM citric acid, 110 mM sodium citrate and 5 mM dextrose) and heparin. Murine blood was collected via cardiac puncture following Avertin anaesthesia. Human whole blood was obtained from healthy volunteers. Prostaglandin E<sub>1</sub> (1 µM; final concentration) was added to prevent the activation of platelets. The unclotted blood was centrifuged at 310 g for 15 min to obtain PRP (platelet-rich plasma). Subsequently, PPP (platelet-poor plasma) was obtained by a series of centrifugation steps as described by Castellot et al. [26] with modifications. First, PRP was centrifuged once at 1000 g for 15 min, 5000 g for 30 min and finally 20000 g for 30 min to obtain PPP. Freshly obtained 25 µl of blood, PRP and PPP were subjected to Sphk assay as described above. For inhibition of Sphk activity, 50 µM DMS was used. Heat inactivation of plasma was carried out at 95°C for 15 min. In addition, ultracentrifugation of PPP was also carried out at 60000 rev./min for 60 min at 4°C using Beckman TLA 100.2 rotor in Beckman™ ultima TL ultracentrifuge and the Sphk activity was determined for supernatant obtained from ultracentrifuged PPP fractions as described above.

### Sphk activity for human PPP

Ultracentrifuged plasma (0.5 ml) was first precleared with Protein A beads for 1 h. Plasma was collected into new tubes after a brief spin and 2.5 µg of Sphk1 antibody was added, and immune complex was allowed to form at 4°C for 2 h. Next, immune complex was pulled down with Protein A beads for 3 h. Subsequently, beads were washed with ice-cold PBS five times and the beads were directly used for the determination of Sphk activity as mentioned above. NRS (non-immune rabbit serum) was used as a control.

### Antibody production against Sphk1 and Sphk2

Rabbit polyclonal antisera for GST (glutathione S-transferase)–Sphk1 and GST–Sphk2 fusions were developed by Proteintech Group, and the IgG fraction was purified as described below. First, serum was precleared with GST beads coated with GST protein. Next, the precleared serum was affinity purified on a Protein A–Sepharose column. Purified IgG aliquots were stored at –20°C until further usage.

### Pulse–chase analysis of Sphk1 isoforms in transiently transfected HEK-293 cells

HEK-293 cells were cultured to approx. 80% confluency on human fibronectin-coated 60 mM tissue culture dishes. Then, 5 µg of various isoforms of hSphk1 plasmids was transfected by Lipofectamine™-mediated transfection. After 24 h of transfection, cells were washed twice with plain DMEM (devoid of methionine and cysteine) medium and incubated with the same medium for 20 min at 37°C to deplete the intracellular pools of methionine and cysteine. Next, cells were pulsed with a medium containing 0.1 mCi/2 ml [<sup>35</sup>S]methionine/cysteine for 1 h. Subsequently, pulse medium was removed and the cells were chased for 1 h in 2 ml of plain DMEM. After 1 h chasing, the chase medium was collected, while the cells were washed twice with ice-cold PBS and scraped in 0.5 ml of lysis buffer (20 mM Tris/HCl, pH 7.4, with 150 mM NaCl, 1% Nonidet P40, 1 mM PMSF and 1× Calbiochem protease inhibitor cocktail). Cellular proteins were extracted for 30 min. Insoluble proteins were pelleted at 20000 g for 15 min. The CM was subjected to centrifugation at 1000 g for 5 min to discard the dead cells. Immunoprecipitation was carried out for total cellular extracts and CM with 5 µg of anti-Sphk1 antibody. Immune complexes were collected into 25 µl of Protein A–Sepharose beads, washed three times with the lysis buffer and the proteins were separated by gel electrophoresis. The SDS gel was dried and autoradiography was performed for 2 days.

For analysis of down-regulation of endogenous Sphk1 in HEK-293 cells, 3 µg of Sphk1 pSilencer plasmids was transfected in HEK-293 cells. After 2 days of transfection, HEK-293 cells were pulse-labelled with [<sup>35</sup>S]methionine/cysteine and immune complex analysis of lysate was carried out with anti-Sphk1 antibody as mentioned above.

### Pulse–chase analysis of endogenous Sphk1 in HUVEC

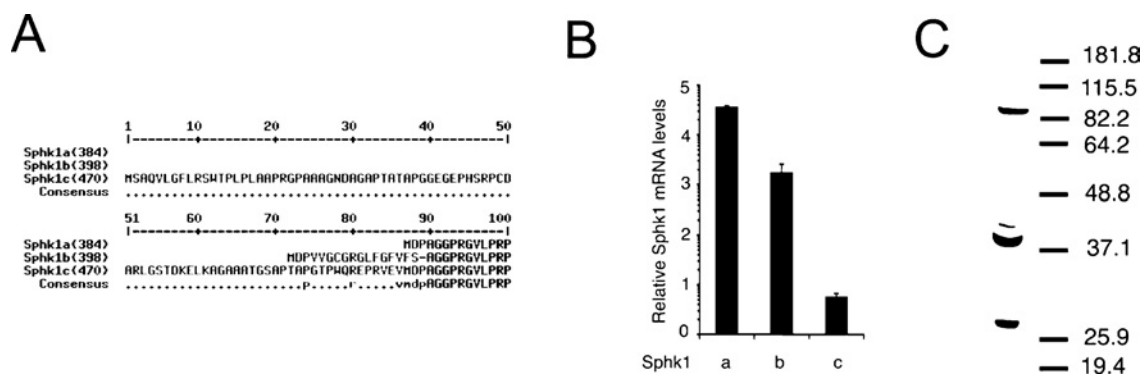
HUVEC were cultured to confluency in 10 cm dishes, and intracellular pools of methionine and cysteine were depleted and labelled with 0.5 mCi of [<sup>35</sup>S]methionine/cysteine per dish for 2 h. Chase was carried out for different length of times with 4 ml of chase medium that had 2% low IgG serum in opTIMEM supplemented with growth factors. This is necessary to keep the HUVEC viable during the chase period. Immunoprecipitation on both total homogenates and CM was done as described above.

### Immunoblotting

Proteins were separated by PAGE under denaturing conditions and transferred on to a nitrocellulose membrane for 1.5 h at 100 V. Subsequently, the membranes were probed with Sphk1 or Sphk2 antibody at 1:2000 and 1:1000 dilutions respectively and detected by horseradish peroxidase-conjugated sheep anti-rabbit secondary antibody. Blots were developed with the Western blot development kit from Amersham.

### Immunostaining and confocal microscopy

HEK-293 cells were plated on human fibronectin-coated glass bottom dishes and transfected with 200 ng of various isoforms of



**Figure 1** Isoforms of hSphk1

(A) Amino acid sequence alignment of hSphk1 isoforms. Sphk isoform 1a has 384 amino acids, isoform 1b has 398 amino acids and isoform 1c has 470 acids. A short stretch of the N-terminus is shown. Identical amino acid residues are indicated in upper-case, while similar amino acid residues are shown in lower-case. (B) RT-PCR analysis of Sphk1 transcripts in HUVEC. Bars a, b and c represent the expression of 384-, 398- and 470-amino-acid isoforms. Results are normalized to GAPDH mRNA levels. Transcripts were not detected in the absence of reverse transcription (results not shown). (C) Immunoblot analysis of the expression of endogenous Sphk1 polypeptides in HUVEC cytosolic fraction is shown. Cytosolic fraction (50  $\mu$ g) was electrophoresed and probed with rabbit polyclonal anti-Sphk1 antibody.

hSphk1 by the Lipofectamine<sup>TM</sup> method. After 24 h of transfections, cells were fixed in 4% (w/v) paraformaldehyde for 15 min and permeabilized with 0.2% Triton for 5 min. Localization was performed with Sphk1 antibody and FITC-conjugated goat anti-rabbit secondary antibody in a Zeiss LSM510 confocal microscope. Immunolocalization of endogenous Sphk1 and Sphk2 in HUVEC was also carried out using respective antibodies as described above.

#### Internalization of GFP (green fluorescent protein)-S1PR1

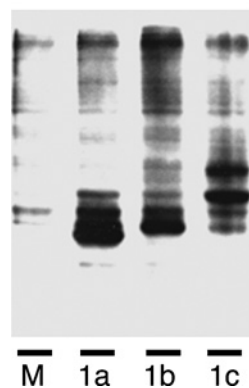
HEK-293 cells stably expressing S1PR1 tagged with GFP [27] were cultured in DMEM with 2% charcoal-stripped serum for 2 days. For internalization of GFP-S1PR1 experiments, cells were washed in plain DMEM and subsequently incubated for 1 h at 37°C with CM obtained from transiently transfected HEK-293 cells that were pre-incubated in the presence and absence of 1  $\mu$ M sphingosine and 0.5 mM ATP for 1 h. Subsequently, cells were fixed in 4% paraformaldehyde and internalization of GFP-S1PR1 was evaluated by confocal microscopy using a Zeiss LSM510 confocal microscope. Quantification of GFP fluorescence on membrane was carried out on seven to ten cells using Metamorph software and the results were presented as intensity of GFP-S1PR1 on membrane. Formation of S1P in the CM used for internalization of GFP-S1PR1 was determined by HPLC analysis as described in [8]. C17-S1P was used as an internal standard.

## RESULTS

### Expression of Sphk1 isoforms in HUVEC

HUVEC express Sphk1 abundantly and secrete enzymatically active Sphk1 into the CM [12]. RT-PCR analysis of HUVEC showed that mRNA for all three Sphk1 isoforms was expressed and that the Sphk-1a isoform was most abundant (Figure 1B). Immunoblot analysis of HUVEC cytosol detected approx. 27, 38, 39 and 85 kDa immunoreactive bands (Figure 1C). The major immunoreactive bands (~38–39 kDa) and a larger species that corresponds to the dimeric form (~85 kDa) were competed by the Sphk1 antigen. The Sphk1 bands were not detected in Sphk1 siRNA-transfected cells (see Supplementary Figure 1 at <http://www.BiochemJ.org/bj/397/bj3970461add.htm>).

### A. Intracellular



### B. Extracellular



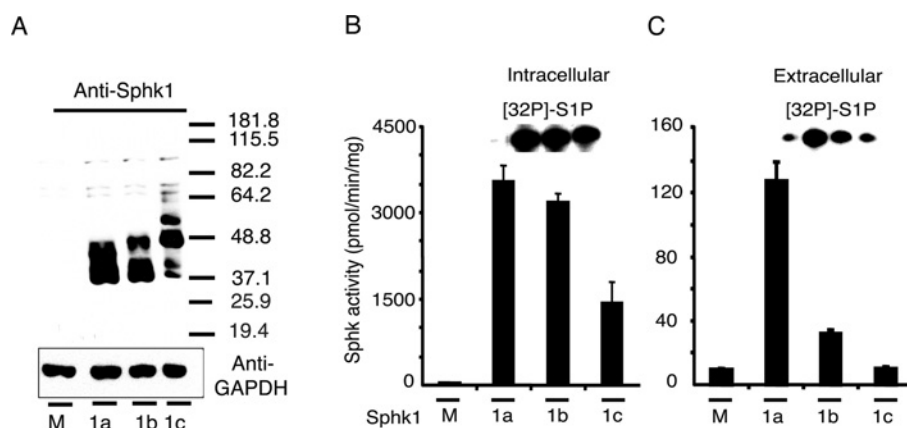
**Figure 2** Pulse-chase analysis of Sphk1 isoforms in HEK-293 cells

HEK-293 cells transiently transfected with pcDNA 3.0 (M), Sphk-1a (1a), Sphk-1b (1b) and Sphk-1c (1c) plasmids were pulsed with [<sup>35</sup>S]methionine/cysteine. (A) Total cellular extracts (intracellular) and (B) CM (extracellular) were subjected to immunoprecipitation with anti-Sphk1 antibody.

The approx. 27 kDa band is likely to represent a non-specific immunoreactive band. These results suggest that HUVEC express multiple Sphk1 isoforms endogenously.

### Extracellular export of hSphk-1a isoform

We next expressed the hSphk1 isoforms separately in HEK-293 cells. Transient transfection of Sphk-1a, Sphk-1b and Sphk-1c plasmids, followed by metabolic labelling with [<sup>35</sup>S]methionine/cysteine and immunoprecipitation analysis showed that approx. 38, 39 and 47 kDa polypeptide species were detected in the cytosol, which corresponded to the calculated molecular masses based on primary sequence (Figures 2A and 3A). In addition, higher molecular mass immunoreactive polypeptides were also detected in cells overexpressing each of the Sphk1 isoforms, which could result from oligomerization and/or post-translational modification. A minor approx. 37 kDa species in the Sphk-1c isoform could result from lipid-modification and/or proteolysis. Analysis of the CM showed that Sphk-1a was the predominantly secreted isoform (Figure 2B), although the Sphk-1b isoform was



**Figure 3** Expression and enzymatic activity of transiently transfected Sphk1 isoforms in HEK-293 cells

(A) Immunoblot analysis of Sphk1 isoforms in HEK-293 cells. HEK-293 cells were transfected with 3  $\mu$ g of pcDNA 3.0 (M), hSphk-1a (1a), hSphk-1b (1b) and hSphk-1c (1c) plasmids and 10  $\mu$ g of the total homogenate was subjected to immunoblot analysis with rabbit polyclonal Sphk1 antibody. (B, C) Phosphorylation of sphingosine by Sphk1 isoforms. Total cellular extract (10  $\mu$ g) (B) and 0.25 ml of CM (C) from corresponding isoforms were directly assayed for Sphk activity *in vitro* by addition of sphingosine and [ $^{32}$ P]ATP, and the formation of [ $^{32}$ P]S1P was analysed by TLC. TLC bands corresponding to [ $^{32}$ P]S1P are shown in (B, C). Results shown are the means  $\pm$  S.E.M. ( $n = 3$ ).

**Table 1** Sphk activity in intracellular and extracellular fractions of HEK-293 cells transfected with Sphk-1a

Homogenates, CM centrifuged at low speed, ultracentrifuged CM and the ultracentrifuged pellet were assayed for Sphk activity in the presence and absence of 20  $\mu$ M sphingosine and 10  $\mu$ Ci of [ $^{32}$ P]ATP in 500  $\mu$ M ATP as described in the Materials and methods section. Formation of [ $^{32}$ P]S1P was evaluated by TLC analysis. Formation of [ $^{32}$ P]S1P was not detected in the absence of sphingosine. Results are means  $\pm$  S.E.M for duplicate assays carried out on two independent transfections ( $n = 4$ ).

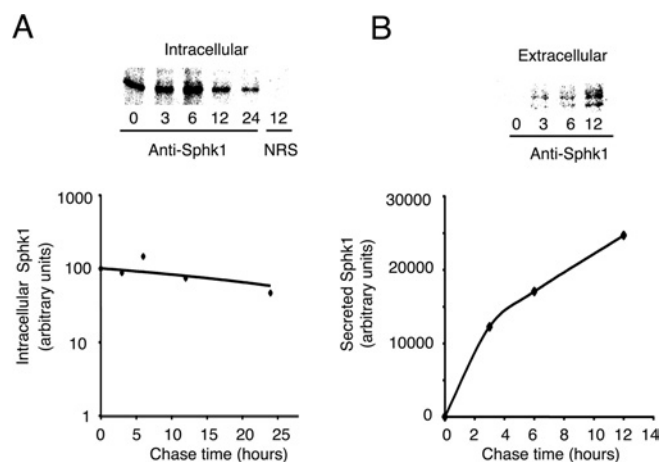
	Sphk activity (pmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$ )			
	Homogenate	Low speed (CM)	High speed (CM)	High speed (pellet)
Mock	55 $\pm$ 4.8	4.7 $\pm$ 0.6	6.3 $\pm$ 0.6	0.3 $\pm$ 0.0
Sphk-1a	1923.6 $\pm$ 145.0	60.4 $\pm$ 5.6	49.4 $\pm$ 5.4	1.3 $\pm$ 0.2

present to a smaller extent (12 % for Sphk-1a and 2.8 % for Sphk-1b of secreted/cytosol). However, the Sphk-1c isoform was not detected in the CM.

A large increase in Sphk activity was detected in HEK-293 cells transfected with each of the isoforms ( $\sim$ 70-fold increase for Sphk-1a and Sphk-1b isoforms and  $\sim$ 30-fold increase for Sphk-1c) when compared with mock-transfected cells (Figure 3B). Analysis of the CM for Sphk activity showed that the Sphk-1a isoform exhibited the highest activity (Figure 3C). Approx. 3.5 % of total Sphk-1a activity was secreted into the media in 2 h. In contrast, less than 1 % of Sphk-1b and Sphk-1c activity was secreted. The secreted Sphk1 activity was not pelleted by ultracentrifugation at 60 000 rev./min for 60 min, suggesting that it is not associated with membranous vesicular structures (Table 1). These results suggest that the Sphk-1a isoform is exported preferentially into the extracellular milieu in transfected cells.

### Secretion of Sphk1 in HUVEC

We next analysed the secretion of endogenous Sphk1 polypeptide species in HUVEC using pulse-chase analysis. Immunoprecipitation of [ $^{35}$ S]methionine/cysteine-labelled HUVEC extracts using anti-Sphk1 antibody detected the 38 kDa species, corresponding to the Sphk-1a isoform (Figure 4A). The 38 kDa isoform was also found in the CM within 3 h and accumulated thereafter



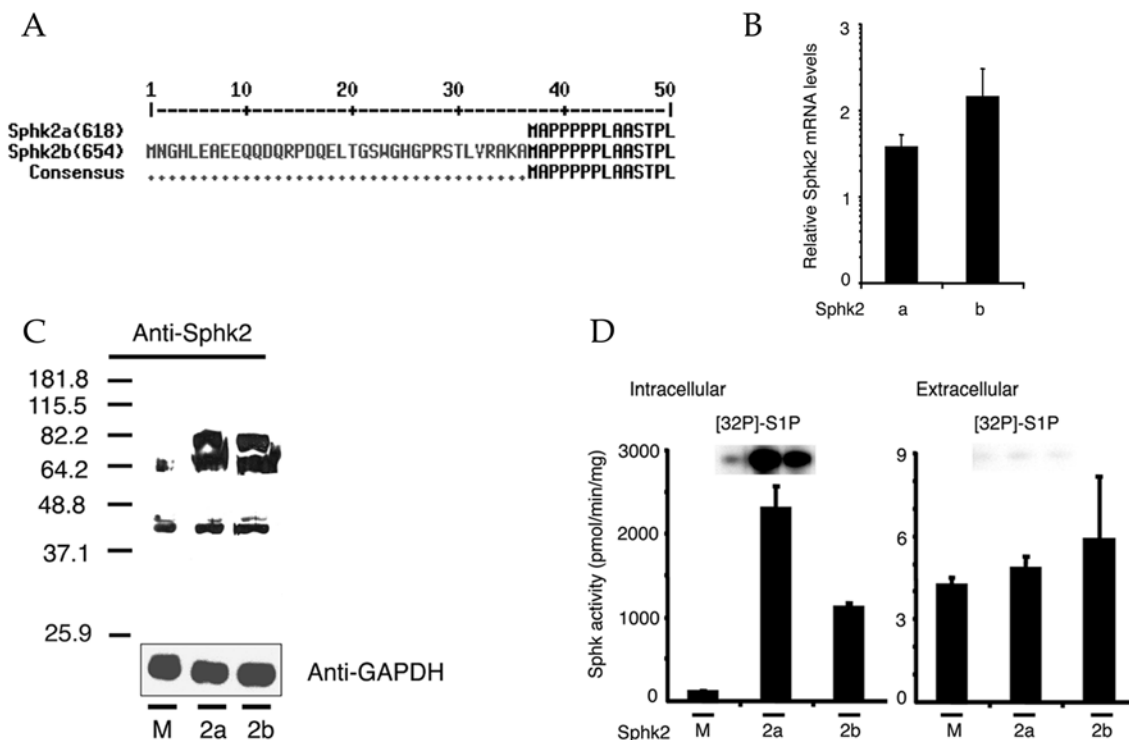
**Figure 4** Pulse-chase analysis of endogenous Sphk1 polypeptides in HUVEC

(A) [ $^{35}$ S]methionine/cysteine-labelled HUVEC extracts (intracellular) and (B) CM (extracellular) were immunoprecipitated with anti-Sphk1 antibody or NRS for the indicated time and [ $^{35}$ S]-labelled Sphk1 was quantified by a phosphorimager. Decay of intracellular Sphk-1a is plotted on a semi-logarithmic scale. Results shown are from a representative experiment that was repeated three times.

(Figures 4A and 4B). The slow rate of decay in the cytosol suggests a long half-life of the Sphk1 polypeptide (Figure 4A).

### Lack of secretion of Sphk2 isoforms

Two N-terminally divergent Sphk2 isoforms exist in genome databases (Figure 5A) [20]. HUVEC also express Sphk2, as determined by Q-RT-PCR (Figure 5B). In order to determine whether Sphk2 isoforms could also be secreted into the CM, we transiently transfected each isoform of Sphk2 in HEK-293 cells. We detected approx. 64 and 80 kDa species in cells expressing Sphk-2a and Sphk-2b isoforms (Figure 5C). A slow moving immunoreactive band might represent a post-translationally modified isoform. Overexpression of Sphk2 isoforms in HEK-293 cells resulted in a large increase in cell-associated enzymatic activity (Figure 5D). Surprisingly, the Sphk activity detected in the CM was less than 0.5 % when compared with cell-associated



**Figure 5** Isoforms of hSphk2

(A) Amino acid sequence alignment of hSphk isoforms. Sphk isoform 2a has 618 amino acids and isoform 2b has 654 amino acids. Conserved residues are shown in upper-case. (B) RT-PCR analysis of Sphk2 transcripts in HUVEC. Bars a and b represent the expression of 618- and 654-amino-acid isoforms. Results are normalized to GAPDH mRNA levels. (C) Immunoblot analysis of overexpressed Sphk2 isoforms in HEK-293 cells. HEK-293 cells were transfected with 3  $\mu$ g of pcDNA 3.0 (M), hSphk-2a (2a) and hSphk-2b (2b) cDNA encoding plasmids. Total homogenate (10  $\mu$ g) was subjected to immunoblot analysis with rabbit polyclonal Sphk2 antibody. (D) Phosphorylation of sphingosine by Sphk2 isoforms was evaluated with 10  $\mu$ g of total cellular extract (intracellular) and 0.25 ml of CM (extracellular) for corresponding isoforms in an *in vitro* kinase assay by addition of sphingosine and [<sup>32</sup>P]ATP, and the formation of [<sup>32</sup>P]S1P was analysed by TLC. TLC bands corresponding to [<sup>32</sup>P]S1P are shown. Results shown are the means  $\pm$  S.E.M. ( $n = 4$ ).

activity, and was not significantly higher than baseline activity from mock-transfected cells, suggesting that Sphk2 isoforms are not secreted (Figure 5D).

#### Subcellular localization of Sphk1 and Sphk2

Next we examined the subcellular localization of Sphk isoforms in transiently transfected HEK-293 cells using confocal immunofluorescence microscopy. All of the Sphk1 and Sphk2 isoforms were localized primarily in the cytosol (Figure 6). In addition, intracellular membrane, vesicular and perinuclear structures were also positive, consistent with the possibility that Sphk isoforms are made in the ER. However, the Sphk-1b and Sphk-1c isoforms show enhanced plasma membrane localization even at the basal state, suggesting that the N-terminal sequences may determine subcellular localization (Figure 6). In addition, Sphk-1c isoform had a granular cytoplasmic appearance, which may be related to the localization in specific cytoplasmic structures such as vesicles or membranes. Sphk-2a isoform is associated with the nucleus in addition to being present in the cytoplasm. This is in contrast with the Sphk-2b isoform, which showed minimal nuclear localization. Plasma membrane localization of Sphk2 isoforms was not observed. These results suggest that while all the Sphk isoforms are cytosolic proteins, subtle changes in the N-termini determine targeting to distinct subcellular compartments.

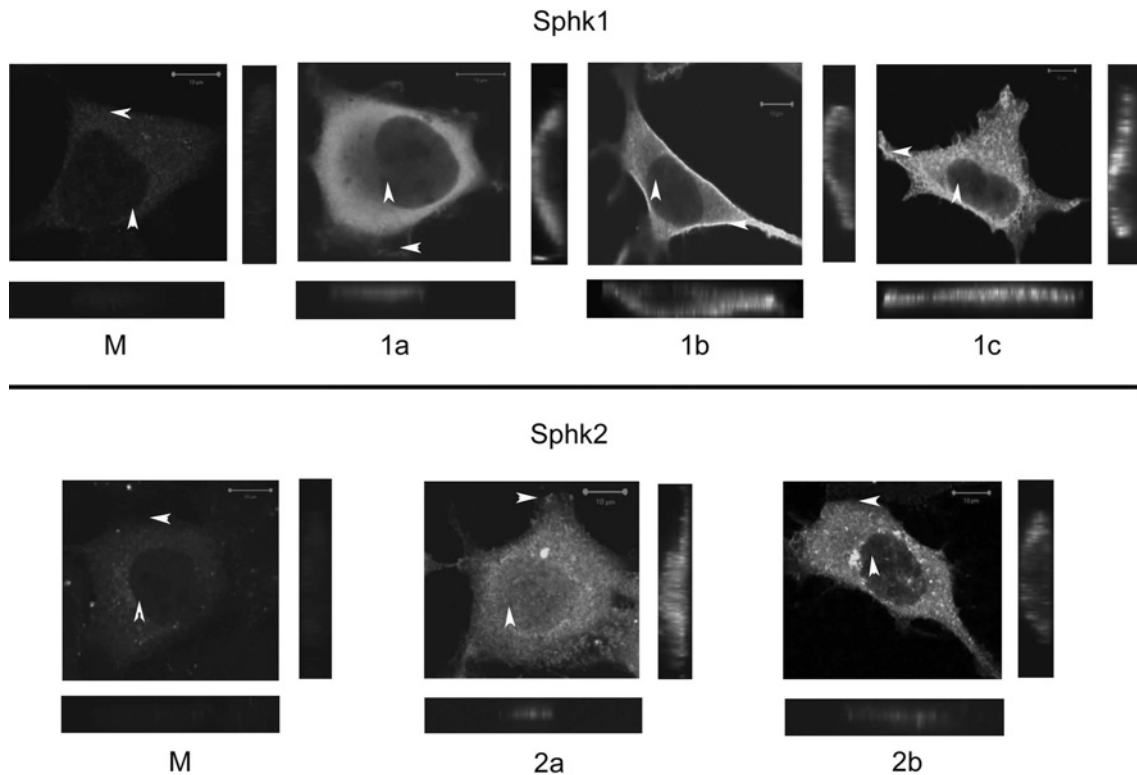
#### Localization of endogenous Sphk1 and Sphk2 in HUVEC

Immunofluorescence microscopy of endogenous Sphk1 and Sphk2 in HUVEC is shown in Figure 7. Endogenous Sphk1 was

present in the cytosol, intracellular membranous compartments and vesicles (Figure 7). In contrast, the subcellular localization of Sphk2 showed that it is present in the cytosol, perinuclear structures and in the nucleus (Figure 7). These results suggest that both Sphk1 and Sphk2 isoforms are localized to distinct compartments in HUVEC and could mediate different cellular functions in various subcellular sites.

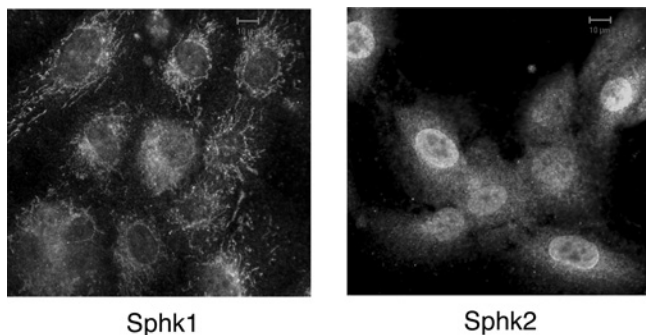
#### Extracellular action of Sphk-1a activates S1P receptors

Internalization of GFP-S1PR1 was carried out in a humidified CO<sub>2</sub> incubator at 37°C. Next we tested if secreted Sphk is enzymatically active. For this, we utilized the internalization of S1PR1 fused to GFP as an indicator of receptor activation [27]. As reported earlier [27], we observed rapid internalization of the S1PR1 when cells were incubated with 100 nM S1P for 30 min (results not shown). CM from HEK-293 cells expressing Sphk1 or Sphk2 isoforms was incubated with 1  $\mu$ M sphingosine and 500  $\mu$ M ATP and exposed to cells expressing S1PR1-GFP for 60 min. As shown in Figure 8, CM from Sphk-1a-expressing cells strongly induced receptor internalization (65% internalization). The other two Sphk1 isoforms only induced minimal receptor internalization (~30%), whereas Sphk2 isoforms were unable to induce this effect. In addition, internalization of S1PR1 was not observed when CM lacked sphingosine or ATP or both (results not shown). These results strongly suggest that the secreted Sphk-1a isoform is functional in the CM. The amount of S1P present in this assay was quantified (Figure 8C). In CM from Sphk-1a-transfected cells, approx. 200 nM total S1P was detected, which



**Figure 6** Subcellular localization of Sphk1 and Sphk2 isoenzymes in HEK-293 cells

Representative confocal images of HEK-293 cells transiently transfected with Mock (pcDNA 3.0), Sphk-1a, Sphk-1b, Sphk-1c, Sphk-2a and Sphk-2b plasmids. Confocal images were collected from fixed cells that were immunostained with polyclonal Sphk1 or Sphk2 antibody. For each construct, the  $x$ - $y$  image (centre),  $x$ - $z$  (box below, section indicated with horizontal arrowhead) and  $y$ - $z$  (right box, section indicated with vertical horizontal arrowhead) images are shown. Note that among the Sphk1 constructs, the b and the c isoforms are more associated with the membrane and the c isoform exhibits a granular localization in the cytoplasm. Sphk-2a is present in the nucleus, whereas the Sphk-2b is not.



**Figure 7** Immunolocalization of endogenously expressed Sphk1 and Sphk2 isoenzymes in HUVEC

HUVEC were immunostained with polyclonal Sphk1 and Sphk2 antibodies and visualized in a confocal immunofluorescence microscope as described. Sphk1 isoforms are localized to cytosol, membranous compartments and vesicle-like structures. In contrast, Sphk2 is localized to nuclear and perinuclear region, in addition to cytosol.

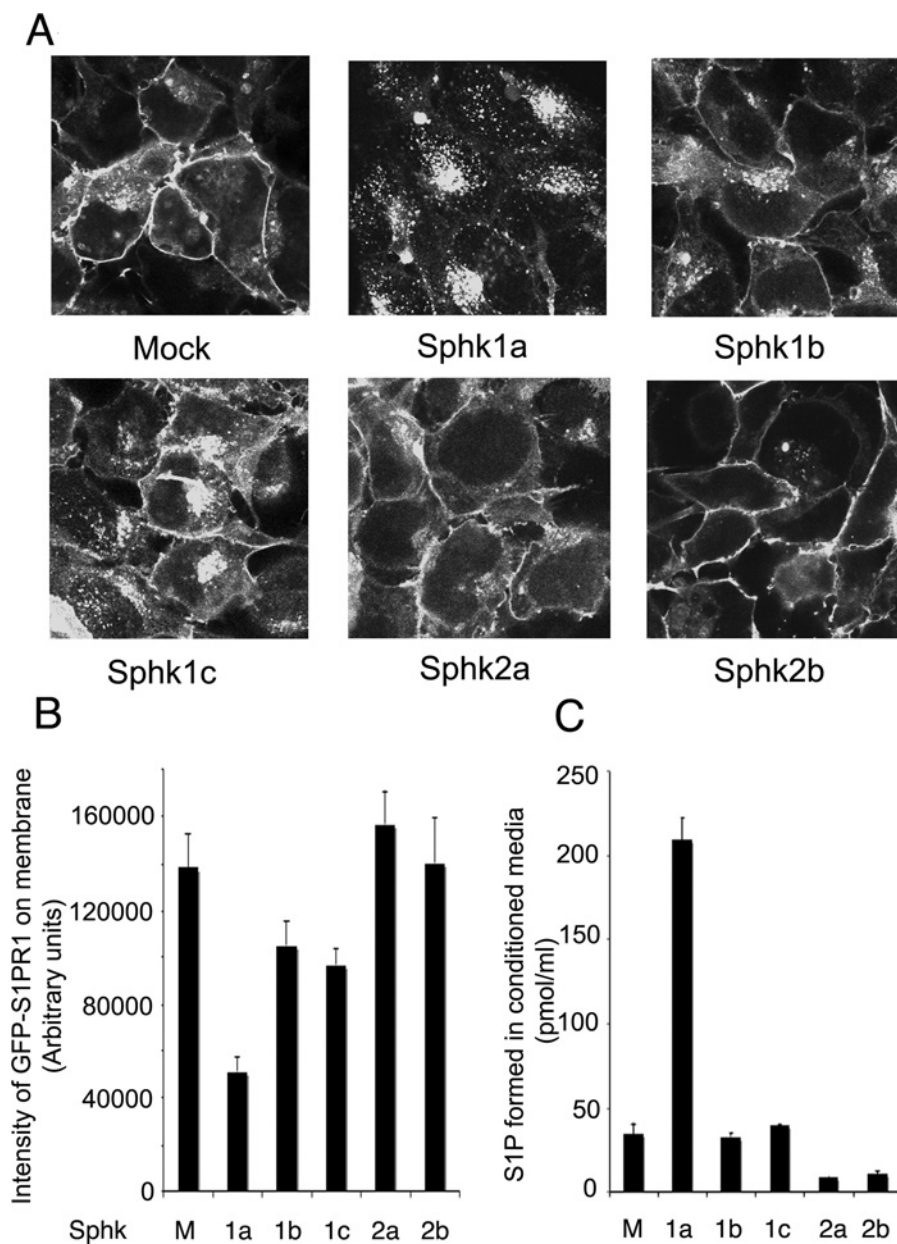
is significantly higher than the amount found in CM from mock-, Sphk-1b-, Sphk-1c- and Sphk2-transfected cells. The reason for the minor internalization of S1PR1-GFP upon incubation with CM from Sphk-1b and Sphk-1c is not clear, but may be related to the presence of low levels of cryptic enzyme in the CM.

### Secreted Sphk1 in plasma

The physiological relevance of secreted Sphk was addressed by assaying for its activity in plasma. We reasoned that the secreted Sphk1 could contribute to plasma S1P levels as we consistently detected secretion of endogenous Sphk-1a from HUVEC and transiently transfected HEK-293 cells [12]. Therefore we tested Sphk activity in mouse whole blood, PRP and PPP.

Freshly collected mouse blood rapidly phosphorylated sphingosine with a specific activity of  $27.6 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$  (Table 2). PRP had significantly less Sphk activity ( $279 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ ), suggesting that haemopoietic cells in the blood express high Sphk activity. Surprisingly, PPP alone had significant Sphk activity ( $179 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ ), suggesting that soluble Sphk activity in plasma is similar in magnitude to that found in platelets. Similar to CM from HEK-293 cells, the plasma-derived Sphk activity is soluble and cannot be pelleted by ultracentrifugation at  $60000 \text{ rev./min}$  for 60 min. However, it was inactivated by heating or DMS treatment (Figure 9 and Table 2).

Analysis of plasma from *Sphk1* null mice indicated that S1P levels were reduced approx. 65% compared with the wild-type counterparts ( $465 \pm 88 \text{ nM}$  versus  $160 \pm 16 \text{ nM}$ ;  $n = 6-16$ ). However, Sphk activity in whole blood was reduced >100-fold, suggesting that Sphk1 is the predominant isoform expressed in the haemopoietic cells. In sharp contrast with the wild-type mice, *Sphk1* null mice did not exhibit significant kinase activity in the plasma (either PRP or PPP).



**Figure 8** Production of S1P in the CM from Sphk-1a-expressing cells

(A) HEK-293 cells were transfected with various isoforms of hSphk1 and hSphk2 plasmids, CM (at 2 h) was collected, and incubated with  $1 \mu\text{M}$  sphingosine and  $500 \mu\text{M}$  ATP to allow the Sphk enzymatic activity to occur. Subsequently, CM was added to HEK-293 cells stably expressing GFP-S1PR1. Internalization of GFP-tagged S1PR1 was monitored after 1 h on fixed cells by confocal microscopy. (B) Intensity of GFP-S1PR1 on the plasma membrane is quantified. Results represent means  $\pm$  S.E.M.;  $n = 7-10$  cells from a representative experiment that was repeated at least twice. (C) Formation of S1P was determined in the respective CM as described in the Materials and methods section. Results represent the means  $\pm$  S.E.M. ( $n = 3$ ).

Although human plasma contains low Sphk activity ( $\sim 9.2 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ ), this activity is Sphk1-immunoreactive (Table 3). The Sphk activity in human plasma was precipitated by Sphk1-antibody-containing Protein A beads but not by beads containing an irrelevant antibody. Together, these results conclusively show that a soluble Sphk1 enzyme is present in human and mouse plasma.

## DISCUSSION

In the present paper, we address the expression and function of Sphk isoforms in vascular endothelial cells. We show that

HUVEC express several isoforms of Sphk1 and Sphk2. Distinct localization of Sphk isoforms in different subcellular compartments implies that cells are endowed with the ability to generate S1P at specific compartments and thereby mediate specific biological outcomes. Indeed, it is known that Sphk1 promotes growth and cellular proliferation, while Sphk2 inhibits growth [28–31]. Moreover, Sphk1 and Sphk2 null mice develop and reproduce normally, which suggests that Sphk1 and Sphk2 compensate for the lack of each other [7,32,33]. However, double deletion of Sphk1 and Sphk2 is lethal to the embryo [32]. These studies indicate that Sphk isoforms could have distinct as well as redundant functions.



**Table 2** Sphk activity in whole blood, platelet-rich and platelet-poor fractions in *Sphk1*<sup>+/+</sup> and *Sphk1*<sup>-/-</sup> mice

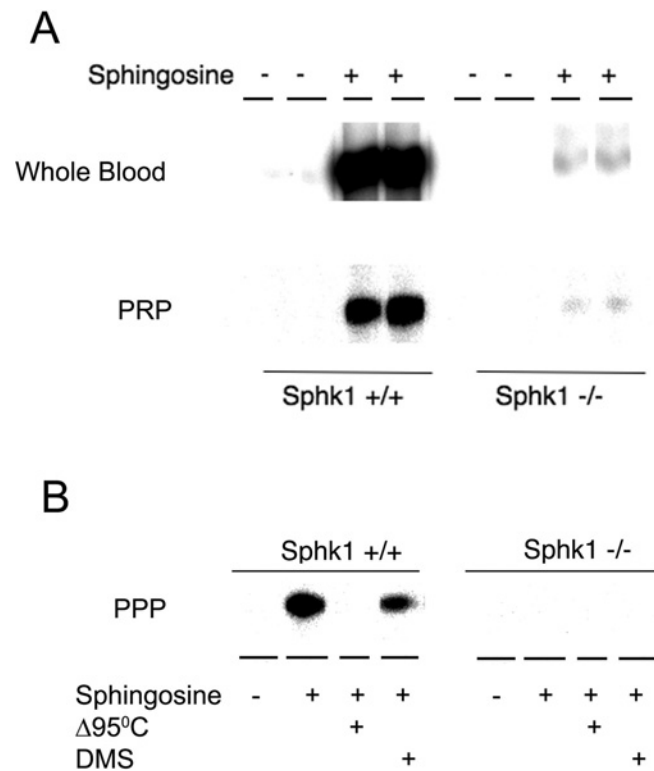
A volume of 0.025 ml of whole blood, PRP and PPP fractions was incubated in the presence and absence of 20  $\mu$ M sphingosine and 10  $\mu$ Ci of [<sup>32</sup>P]ATP in 500  $\mu$ M ATP as described in the Materials and methods section. Formation of [<sup>32</sup>P]S1P was evaluated by TLC analysis. In addition, the formation of [<sup>32</sup>P]S1P was also evaluated with boiled plasma and in the presence of Sphk inhibitor DMS (50  $\mu$ M). Results are means  $\pm$  S.E.M. for two independent assays on three to four animals ( $n=6-8$ ). ND, not detected.

	Specific activity of Sphk (pmol $\cdot$ min <sup>-1</sup> $\cdot$ g <sup>-1</sup> )	
	<i>Sphk1</i> <sup>+/+</sup>	<i>Sphk1</i> <sup>-/-</sup>
Blood - sphingosine	84.9 $\pm$ 14.8	56.4 $\pm$ 7.1
Blood + sphingosine	27581.7 $\pm$ 2823.1	248.4 $\pm$ 20.6
PRP - sphingosine	19.8 $\pm$ 3.2	19.3 $\pm$ 3.9
PRP + sphingosine	279.7 $\pm$ 16.1	24.9 $\pm$ 2.7
PPP - sphingosine	ND	ND
PPP + sphingosine	179.2 $\pm$ 23.9	ND
Boiled PPP + sphingosine	ND	ND
PPP + sphingosine + DMS	64.9 $\pm$ 6.6	ND

**Table 3** Sphk activity in whole blood and PPP and in PPP-Sphk1 antibody immune complex

Human blood, PPP and PPP immunocomplexed with Sphk1 antibody to Protein A beads were assayed in the presence and absence of 20  $\mu$ M sphingosine and 10  $\mu$ Ci of [<sup>32</sup>P]ATP in 500  $\mu$ M ATP as described in the Materials and methods section. Formation of [<sup>32</sup>P]S1P was evaluated by TLC analysis. PPP immunocomplexed with NRS was used as a control. Formation of [<sup>32</sup>P]S1P was not detected in the absence of sphingosine. Results are means  $\pm$  S.E.M. ( $n=3$ ).

	Sphk activity (pmol $\cdot$ min <sup>-1</sup> $\cdot$ g <sup>-1</sup> )	Sphk activity in whole blood (%)
Blood	1629.5 $\pm$ 101.6	100
PPP	9.2 $\pm$ 2.3	0.56
Sphk1-antibody IP beads	46.8 $\pm$ 5.9	2.87
NRS IP beads	4.0 $\pm$ 1.8	0.24

**Figure 9** TLC analysis of Sphk activity in whole blood, PRP and PPP fractions from *Sphk1*<sup>+/+</sup> and *Sphk1*<sup>-/-</sup> mice

(A) A volume of 25  $\mu$ l of whole blood and PRP and (B) PPP was incubated in the presence or absence of 20  $\mu$ M sphingosine with 10  $\mu$ Ci of [<sup>32</sup>P]ATP in the final concentration 500  $\mu$ M ATP. Formation of [<sup>32</sup>P]S1P was determined by TLC analysis.

Recently, our laboratory proposed an alternate mechanism by which S1P is generated outside the cell by the action of secreted Sphk [12]. Other work has shown that secretory sphingomyelinases [34], acid sphingomyelinase, acid ceramidase [35] and neutral ceramidase [36] are known to be present in the extracellular compartment. Sequential action of sphingomyelinase and ceramidase produces sphingosine, a substrate of Sphk. In the

present study, we characterized the ability of endothelial cell Sphk isoforms to act as extracellular kinases.

The major finding from the present study is that the Sphk-1a isoform is preferentially secreted, as demonstrated by activity measurements, detection of secreted protein and the kinetics of secretion. The structural basis for the preferential secretion of Sphk-1a may be related to the lack of retention domains in the N-terminus. All the isoforms of Sphk share a common diacylglycerol kinase catalytic motif. Sphk2 isoforms possess a proline-rich domain and N-terminal extension, which might be involved in retention in the perinuclear structures and/or blockage of secretion. In addition, all isoforms lack classical signal peptide motifs at the N-terminus. The short N-terminal extensions of Sphk-1b and Sphk-1c contain additional cysteine residues, which may be involved in post-translational acylation and membrane retention. Indeed, recently it was shown that the murine Sphk-1b isoform, which contains a double cysteine residue, is palmitoylated, but the Sphk-1a isoform is not [37]. Our immunofluorescence microscopy indicates that the Sphk-1c isoform exhibits preferential membrane localization. In addition, our previous work showed that Sphk1 export shares some similarity to other non-ER/Golgi secreted factors such as fibroblast growth factor, interleukin-1 and annexin-II [12]. Further studies are needed to determine the detailed steps by which Sphk-1a is secreted.

The secretion of Sphk-1a isoform is specific, as Sphk2 isoforms are not detected in the CM, even in overexpressed HEK-293 cells. In addition, the secreted Sphk-1a is functional, and can produce sufficient S1P in the extracellular compartment to activate the S1PRs, as indicated by the receptor internalization assay. Since sphingomyelinases and ceramidases are found in the extracellular compartments, and significant sphingomyelin, ceramide, sphingosine and ATP are present in plasma, an extracellular S1P-generating system may exist in the vascular compartment.

Mammals are endowed with a large vascular S1P gradient. Previous estimates of S1P in serum and serum and plasma are in the ranges 0.4–1.1 and 0.1–0.6  $\mu$ M respectively. However, the tissue levels of S1P are lower by several orders of magnitude (0.5–75 pmol/mg) [4–8]. It is not clear how this gradient is maintained between vascular and extravascular compartments. It is possible that cells in the vascular compartment are endowed with high Sphk activity and efficient secretion of S1P when compared with the extravascular tissues. Alternatively, S1P degradation may be enhanced in the extravascular tissues. Of course, these two possibilities are not mutually exclusive. We invoke a third possibility whereby the secreted Sphk activity in the vascular compartment contributes to the high vascular S1P levels.

We detected significant Sphk activity in human plasma upon immune complex formation with Sphk1 antibody ( $46 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ ). In addition, mouse plasma also exhibited Sphk activity ( $179 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ ). The mouse genome contains two N-terminally divergent Sphk1 isoforms, which are orthologous to the hSphk1 isoforms. The sources of Sphk1 in plasma could be endothelial cells although other blood-borne or somatic cells could also secrete active forms of Sphk1. The presence of significant Sphk activity found in the human and mouse plasma suggests that the secreted Sphk1 could contribute to high plasma S1P levels. In addition, we also found that the Sphk activity found in PPP fraction and PRP fractions were comparable. This is in contrast with a recent study by Aoki et al. [38], who showed that PRP but not PPP could rapidly phosphorylate [ $^3\text{H}$ ]sphingosine. The reason for this apparent difference is not clear although it should be noted that their activity assays were carried out in the absence of ATP. In vascular endothelial cells, the intracellular Sphk-1a isoform is highly stable, as indicated by pulse-chase experiments, which is supported by the studies of Kihara et al. [37] who recently showed that murine Sphk-1a isoform is highly stable in transiently transfected HEK-293T cells [HEK-293 cells expressing the large T-antigen of SV40 (simian virus 40)]. In addition, the Sphk-1a isoform is found to accumulate in the CM. Thus secreted Sphk-1a may contribute significant amounts of S1P in the plasma. Moreover, plasma from Sphk1 null mice exhibited no Sphk activity compared with wild-type plasma. In Sphk1 null mice, whole blood and PRP contain Sphk activity, albeit at significantly lower levels than the wild-type counterparts. S1P levels are reduced approximately 40% in Sphk1 null serum, as reported by Allende et al. [7] but not in Sphk2 null mice [32]. These results strongly suggest that secreted Sphk-1a contributes to the high plasma S1P levels and thus is a factor in the establishment of the vascular S1P gradient.

In conclusion, we show that vascular endothelial cells express several functional isoforms of Sphk1 and Sphk2 isoenzymes. Such isoforms are localized at different subcellular sites, suggesting coupling with different biological outputs. Interestingly, the Sphk-1a isoform is secreted into the extracellular compartment and produces sufficient S1P to activate the receptors. Furthermore, plasma contains significant secreted Sphk1 activity, suggesting that endothelial cell secretion of Sphk-1a contributes to the high plasma S1P levels and thus maintains the vascular S1P gradient.

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