Functional characterization of human sphingosine kinase-1

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Abstract Sphingosine kinase catalyzes the phosphorylation of sphingosine to form sphingosine 1-phosphate (SPP), a novel lipid mediator with both intra- and extracellular functions. Based on sequence identity to murine sphingosine kinase (mSPHK1a), we cloned and characterized the first human sphingosine kinase (hSPHK1). The open reading frame of hSPHK1 encodes a 384 amino acid protein with 85% identity and 92% similarity to mSPHK1a at the amino acid level. Similar to mSPHK1a, when HEK293 cells were transfected with hSPHK1, there were marked increases in sphingosine kinase activity resulting in elevated SPP levels. hSPHK1 also specifically phosphorylated D-*erythro*-sphingosine and to a lesser extent sphinganine, but not other lipids, such as D,L-*threo*-dihydrosphingosine, *N*,*N*-dimethylsphingosine, diacylglycerol, ceramide, or phosphatidylinositol. Northern analysis revealed that hSPHK1 was widely expressed with highest levels in adult liver, kidney, heart and skeletal muscle. Thus, hSPHK1 belongs to a highly conserved unique lipid kinase family that regulates diverse biological functions.

Key words: Human sphingosine kinase; Sphingosine 1-phosphate

1. Introduction

The metabolic product of sphingosine kinase (SPHK), sphingosine 1-phosphate (SPP), is a lipid signaling molecule that acts both intra- and extracellularly to affect many biological processes. These include mitogenesis [1,2], apoptosis [3], atherosclerosis [4] and inflammatory responses [5,6]. Specific members of the EDG-1 family of G protein-coupled receptors bind SPP (reviewed in [7,8]) and modulate chemotaxis [9,10], angiogenesis [10–12], neurite retraction and cell rounding [13]. Because SPP levels are mainly regulated by the activity of SPHK, cloning and characterization of this enzyme are important for understanding its role in normal and pathological processes. Previously, we purified SPHK to homogeneity from rat kidneys [14] and subsequently identified mouse cDNAs encoding two forms of SPHK, designated mSPHK1a and mSPHK1b, whose predicted proteins differ by only 10 amino acids at their N-terminus [15]. The corresponding mRNAs may arise by alternative splicing. In this study, sequence homologies to the mSPHK1a cDNAs were used to identify and clone the first human homologue, hSPHK1. hSPHK1 is ubiquitously expressed in adult tissues with highest levels in liver, kidney, lung and skeletal muscle. Our results suggest that hSPHK1 belongs to a family of highly conserved enzymes which differ from other known lipid kinases.

2. Materials and methods

2.1. Materials

SPP, sphingosine, and *N*,*N*-dimethylsphingosine (DMS) were from Biomol Research Laboratory Inc. (Plymouth Meeting, PA). All other lipids were purchased from Avanti Polar Lipids (Birmingham, AL). [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Poly-L-lysine was from Boehringer Mannheim (Indianapolis, IN). Alkaline phosphatase from bovine intestinal mucosa, type VII-NT, was from Sigma (St. Louis, MO). Restriction enzymes were from New England Biolabs (Beverly, MA). Lipofectamine Plus was from Life Technologies (Gaithersburg, MD).

2.2. Human sphingosine kinase cDNA cloning

BLAST searches using mSPHK1a sequences identified an EST clone (AA026479) which contained sequences homologous to several conserved domains of mSPHK [15]. To obtain a full-length cDNA, the 5′-end of hSPHK1 was extended by rapid amplification of cDNA ends/polymerase chain reaction (RACE-PCR; Life Technologies). First, cDNA was synthesized from HEK293 poly(A)$^+$ RNA with a gene-specific antisense primer hspk1-GSP1 (5′-ACCATTGTCCAGTGAG). Then two consecutive PCR reactions using LA Taq (TaKaRa) were performed. First PCR: 5′RACE Abridged Anchor Primer and the antisense primer hspk1-GSP2 (5′-TTCCTACAGGGAGGTAGGCC) at 94°C for 2 min followed by 30 cycles of amplification (94°C for 1 min, 55°C for 1 min, 72°C for 2 min) and primer extension at 72°C for 5 min. Second PCR: Abridged Universal Amplification Primer and the antisense primer hspk1-GSP3 (5′-GGCTGCCAGACGCAGGAAGG) using a program similar to the first PCR but with annealing at 65°C. The PCR products were cloned into pCR 2.1 (TA Cloning, Invitrogen) and sequences confirmed by automated sequencing. To make expression constructs, a primer set was designed as follows: sense primer containing a Kozak sequence and ATG start codon, sphk1-GSP4 (5′-GCCACCATGGATCCAGCGGGCGGCCCC); antisense primer, sphk1-GSP5 (5′-TCATAAGGGCTCTTCTGGCGGTGGCATCTG). The PCR reaction was performed using human fetus Marathon-Ready cDNA (Clontech) as template with the above primers, and the amplification product was subcloned into pCR3.1 (Eukaryotic TA Cloning, Invitrogen). In addition, hSPHK1 was tagged at the N-terminus by subcloning into a pcDNA-c-myc vector [2] using high fidelity taq polymerase (Pfu, Stratagene). hSPHK1 accession number is AF238083.

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Abbreviations: BSA, bovine serum albumin; DMS, *N*,*N*-dimethylsphingosine; DHS, D,L-*threo*-dihydrosphingosine; SPHK, sphingosine kinase; SPP, sphingosine 1-phosphate; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends

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2.3. Cell culture and expression of sphingosine kinase

Human embryonic kidney cells (HEK293, ATCC CRL-1573) were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine supplemented with 10% fetal bovine serum [15]. Cells were transfected with either pcDNA3.1 or pCR3.1 containing hSPHK1 using Lipofectamine Plus according to the manufacturer's protocol. Transfection efficiencies were typically about 40%.

2.4. Measurement of sphingosine kinase activity

Cytosolic sphingosine kinase activity was determined with 50 μM sphingosine, dissolved in 5% Triton X-100 (final concentration 0.25%), and [γ-^{32}P]ATP (10 μCi, 1 mM) containing $MgCl_2$ (10 mM) as previously described [15]. In some experiments, sphingosine was added as a complex with bovine serum albumin (BSA) as previously described [15]. Specific activity is expressed as pmol SPP formed per min per mg protein.

2.5. Lipid extraction and measurement of SPP, sphingosine, and ceramide

Cells were washed with phosphate buffered saline and scraped in 1 ml of methanol containing 2.5 μl concentrated HCl. Lipids were extracted by adding 2 ml chloroform/1 M NaCl (1:1, v/v) and 100 μl 3 N NaOH and phases were separated. The basic aqueous phase containing SPP, and devoid of sphingosine, ceramide, and the majority of phospholipids, was transferred to a siliconized glass tube. The organic phases were re-extracted with 1 ml methanol/1 M NaCl (1:1, v/v) plus 50 μl 3 N NaOH, and the aqueous fractions combined. Mass measurements of SPP in the aqueous phase were carried out as previously described [16]. Sphingosine and ceramide in the organic phase were determined by enzymatic methods using sphingosine kinase and diacylglycerol kinase, respectively [17]. Total phospholipids present in lipid extracts were also quantified [17].

2.6. Northern blotting analysis

Poly(A)$^+$ RNA blots containing 2 μg of poly(A)$^+$ RNA per lane from multiple adult human tissues (Clontech) were hybridized with the 0.6 kb *Eco*RV/*Sph*I fragment of pCR3.1-hSPHK1, which was gel-purified and labeled with [^{32}P]dCTP by random priming. Hybridization in ExpressHyb buffer (Clontech) was carried out at 65°C overnight according to the manufacturer's protocol. Blots were reprobed with a human β-actin control probe (Clontech). Bands were quantified using a Molecular Dynamics Phosphoimager.

3. Results and discussion

3.1. Cloning of hSPHK1

BLAST searches of the EST database identified a human homologue of murine SPHK, EST AA026479, with similarity to the 3′ end of mSPHK1a. This sequence was used to design specific primers and 5′ RACE was performed on mRNA extracted from HEK293 cells to obtain the full-length cDNA of hSPHK1. The open reading frame encodes a protein with 384 amino acids, and 85% identity and 92% similarity to mSPHK1a at the amino acid level (Fig. 1). We previously found by sequence alignment that SPHKs from mouse, yeast and *Caenorhabditis elegans* share several conserved blocks of amino acids [15]. Similarly, hSPHK1 contains these conserved regions (C1–C5, Fig. 1), including the invariant positively charged motif, GGKGK, in the C1 domain, which may be part of the ATP binding site of this novel class of lipid kinases.

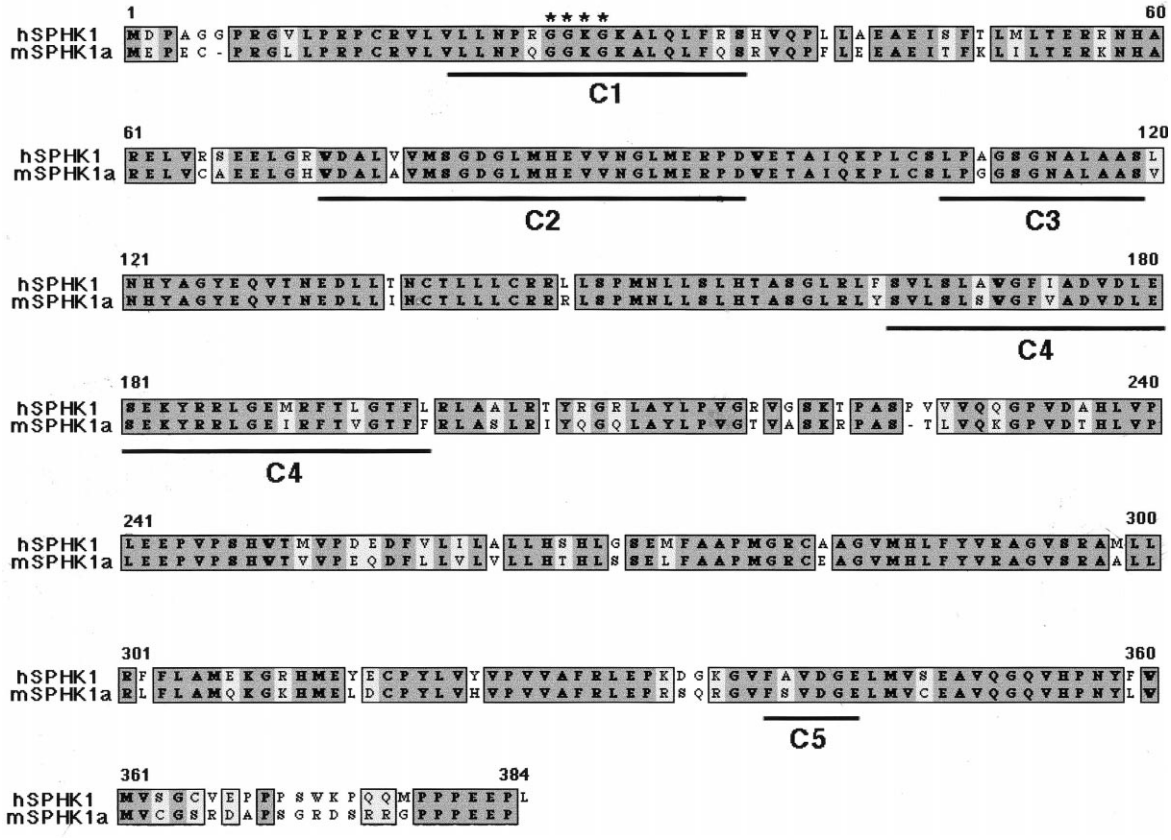

Fig. 1. Predicted amino acid sequence of hSPHK1 and alignment of the conserved domains. ClustalW alignment of SPHKs from mouse and human. Identical and conserved amino acid substitutions are shaded dark and light gray, respectively. The conserved domains (C1–C5) are indicated by lines and the invariant positively charged motif GGKGK by asterisks.

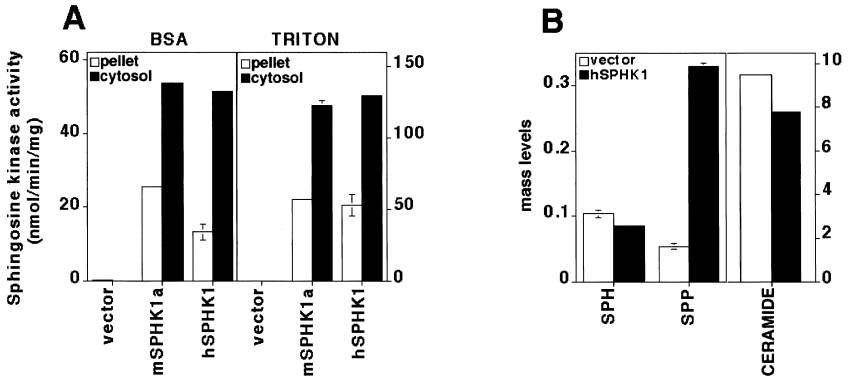

Fig. 2. Activity of hSPHK1 expressed in HEK293 cells. A: HEK293 cells were transiently transfected with empty vector or vector containing either mSPHK1a or hSPHK1. SPHK activity was measured in cytosol (filled bars) and particulate pellet (open bars) 24 h after transfection using sphingosine–BSA complexes or sphingosine–Triton X-100 micelles as substrate as indicated. SPHK activity in vector transfected cells was 84 ± 2 and 134 ± 27 pmol/min/mg using sphingosine–BSA complexes or sphingosine–Triton X-100 micelles as substrate, respectively. Data are means ± S.D. and are representative of two independent experiments performed in triplicate. B: Changes in mass levels of SPP, sphingosine, and ceramide. Mass levels of SPP, sphingosine and ceramide in cells transfected with empty vector (open bars) or vector containing hSPHK1 (filled bars) were measured after 24 h. Data are expressed as pmol/nmol phospholipid and are means ± S.D. of triplicate determinations.

3.2. hSPHK1 encodes a functional sphingosine kinase

HEK293 cells were transfected with expression vectors containing hSPHK1 to determine whether it encodes a bona fide SPHK. Modest levels of endogenous SPHK activity were detected in cells transfected with an empty vector (Fig. 2A). Twenty-four hours after transfection with pcDNA3.1-hSPHK1, the SPHK activity increased approximately 600-fold and remained at this level for at least 2 days. For comparison, a similar increase in activity was observed after transfection with mSPHK1a (Fig. 2A). Similar results were obtained when cells were transfected with hSPHK1 in pCR3.1. In agreement with previous results with mSPHK1a [15], hSPHK1 was stimulated by Triton X-100. Both membrane-associated and cytosolic SPHK activity have been described in mammalian tissues and cell lines [1,18–21]. In cells transfected with hSPHK1, approximately 70% of the SPHK activity was found in the cytosol and only about 30% was membrane-associated (Fig. 2A). Similarly, we previously found that the majority of mSPHK1a activity was also expressed in the cytosol [2,15]. Kyte–Doolittle hydropathy plots did not suggest the presence of any potential hydrophobic membrane spanning domains in the primary structure of hSPHK1.

Transfection of HEK293 cells with hSPHK1 also resulted in changes in levels of sphingolipid metabolites (Fig. 2B). Mass levels of SPP increased 5.7-fold compared to cells transfected with vector alone, with a 18% decrease in levels of both sphingosine and ceramide. However, because intracellular ceramide pools are much larger than sphingosine pools, the absolute decrease of ceramide was greater than the decrease in sphingosine mass. These results suggest that transfected hSPHK1 is active in intact cells, and that kinase overexpression can alter the intracellular balance of sphingolipid metabolites.

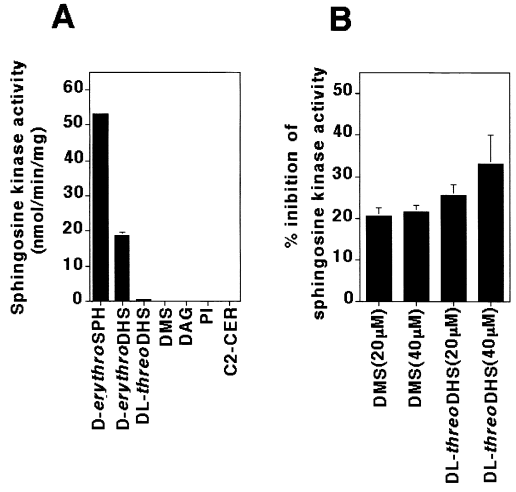

Fig. 3. A: Substrate specificity of hSPHK1. HEK293 cells were transfected with hSPHK1 and SPHK-dependent phosphorylation of various sphingosine analogs or other lipids (50 μM) was measured using cell lysates as enzyme source. DAG, diacylglycerol; PI, phosphatidylinositol; C2-CER, *N*-acetyl-sphingosine. B: DMS and DHS are inhibitors of hSPHK1. SPHK activity in HEK293 cell lysates 24 h after transfection with hSPHK1 was measured with 10 μM SPP in the absence or presence of 20 μM and 40 μM DMS or DHS. Data are means ± S.D. of triplicate determinations and are expressed as percent inhibition.

3.3. Substrate specificity of hSPHK1

The naturally occurring D-(+)-*erythro-trans*-isomer of sphingosine and *erythro*-dihydrosphingosine (sphinganine) were the best substrates for hSPHK1 (Fig. 3A). However, similar to the specificity of mSPHK1a [15], sphingosine was more efficiently phosphorylated than sphinganine. Moreover, other sphingo-

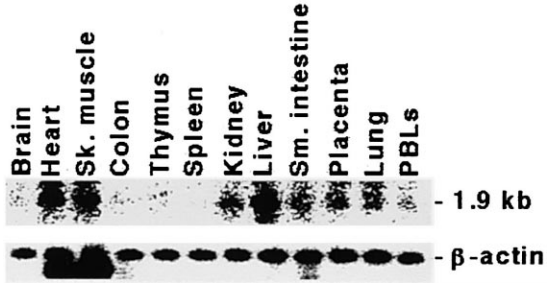

Fig. 4. Tissue-specific expression of hSPHK1 by Northern blot analysis. Top panel: A hSPHK1 probe was hybridized to a poly(A)$^+$ RNA blot with the human tissues indicated at the top of each lane as described in Section 2. Bottom panel: A β-actin probe was used to reprobe the blot.

lipids, including D,L-*threo*-dihydrosphingosine (DHS) and C2-ceramide, as well as diacylglycerol and phosphatidylinositol were not substrates (Fig. 3A). With D-*erythro*-sphingosine as substrate, half-maximal velocity was found at 5 μM, in excellent agreement with K_m values previously determined with rat kidney SPHK [14] and recombinant mSPHK1a [15]. DMS and DHS have previously been used to inhibit SPHK and block increases in SPP induced by various physiological stimuli [1,3,22]. Both of these sphingolipids also inhibited hSPHK1 and similar to their inhibitory effects on mSPHK1a [15], DHS was slightly more potent than DMS (Fig. 3B).

3.4. Tissue distribution of hSPHK1 expression

The tissue distribution of SPHK1 mRNA expression in adult human tissues was analyzed by Northern blotting (Fig. 4). In most tissues, including adult brain, heart, spleen, lung, kidney, and testis, a predominant 1.9 kb mRNA species was detected. Expression was highest in adult liver, heart and skeletal muscle. In comparison, we previously showed that mSPHK1a expression is greatest in mouse spleen, lung, kidney, testis and heart, with much lower expression in skeletal muscle [15].

In summary, hSPHK1 is the human homolog of mSPHK1. Based on EST sequences, hSPHK1 has been localized on chromosome 17q25.2 at the marker stSG28540 (D17S785-D17S836 Reference Interval, UniGene cluster Hs. 68061, URL: http://www.ncbi.nlm.nih.gov/unigene/clust.cgi?org = hs and cid = 68061). hSPHK1 belongs to a conserved family of genes that is distinct from other known lipid kinases. Molecular cloning and characterization of members of the SPHK family should help to clarify their potential roles in various human diseases as their product, SPP, has been implicated as an important regulatory component of biological processes including growth, survival, allergy, chemotaxis, and angiogenesis.

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