Human sphingosine kinase: purification, molecular cloning and characterization of the native and recombinant enzymes

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Sphingosine 1-phosphate (S1P) is a novel lipid messenger that has important roles in a wide variety of mammalian cellular processes including growth, differentiation and death. Basal levels of S1P in mammalian cells are generally low, but can increase rapidly and transiently when cells are exposed to mitogenic agents and other stimuli. This increase is largely due to increased activity of sphingosine kinase (SK), the enzyme that catalyses its formation. In the current study we have purified, cloned and characterized the first human SK to obtain a better understanding of its biochemical activity and possible activation mechanisms. The enzyme was purified to homogeneity from human placenta using ammonium sulphate precipitation, anion-exchange chromatography, calmodulin-affinity chromatography and gel-filtration chromatography. This resulted in a purification of over 10⁶-fold from the original placenta extract. The enzyme was cloned and expressed in active form in both HEK-293T cells and *Escherichia coli*, and the recombinant *E. coli*-derived SK

purified to homogeneity. To establish whether post-translational modifications lead to activation of human SK activity we characterized both the purified placental enzyme and the purified recombinant SK produced in *E. coli*, where such modifications would not occur. The premise for this study was that post-translational modifications are likely to cause conformational changes in the structure of SK, which may result in detectable changes in the physico-chemical or catalytic properties of the enzyme. Thus the enzymes were characterized with respect to substrate specificity and kinetics, inhibition kinetics and various other physico-chemical properties. In all cases, both the native and recombinant SKs displayed remarkably similar properties, indicating that post-translational modifications are not required for basal activity of human SK.

Key words: enzyme activation, lipid kinase, second messenger, sphingosine 1-phosphate.

INTRODUCTION

Sphingosine kinase (SK) catalyses the formation of sphingosine 1-phosphate (S1P), a novel lipid messenger that plays important roles in a wide variety of mammalian cellular processes [1–3]. S1P is mitogenic in various cell types and appears to trigger a diverse range of important regulatory pathways including prevention of ceramide-induced apoptosis [4,5], mobilization of intracellular calcium by an inositol trisphosphate-independent pathway [6], stimulation of DNA-binding activity of the transcription factor activator protein-1 [7], activation of mitogen-activated protein kinase pathways [8] and activation of phospholipase D [9]. Recent studies in our laboratory have also shown that S1P is an obligatory signalling intermediate in the inflammatory response of vascular endothelial cells to tumour necrosis factor α (TNF α) [10].

Two areas of S1P research have emerged in recent times and are focused on identification of the direct downstream targets of S1P in the cell, and elucidation of the molecular mechanisms that regulate cellular S1P levels. Some downstream targets have been recognized, with the recent identification of S1P-specific G-protein-coupled cell-surface receptors, with three isoforms now known [11]. These studies have helped to elucidate the mechanism of action and role of extracellular S1P, but it is clear that S1P

also acts as an intracellular messenger [1,2,12], with the direct downstream intracellular target or receptor yet to be identified. Little is also understood of the molecular mechanisms that regulate cellular S1P levels. It is known that S1P levels in the cell are largely mediated by its formation from sphingosine by the activity of SK, and to a lesser extent by its degradation by endoplasmic reticulum-associated S1P lyase [13,14] and S1P phosphatase [2]. Basal levels of S1P in the cell are generally low [2,15], but can increase rapidly and transiently when cells are exposed to various agonists, including TNF α [4,10], platelet-derived growth factor [15], nerve growth factor [16] and phorbol esters [17,18]. This response appears to be invariably correlated with an increase in SK activity in the cytosol and can be prevented by addition of SK inhibitors. This places SK in a central and obligatory role in mediating the observed effects attributed to S1P in the cell. However, despite this enzyme's obvious importance, little is known regarding the catalytic properties and regulation of activity of SK. Recent purification of a rat SK [19] and cloning of the enzymes from mouse [20] and yeast [21] has provided some insight into the properties of this class of enzyme, although the molecular mechanisms of activation and regulation of this enzyme remain to be elucidated. Previous studies have suggested a possible role for protein kinase C (PKC) in SK activation [18], although this does not appear to be a direct

Abbreviations used: SK, sphingosine kinase; hSK, human SK; S1P, sphingosine 1-phosphate; TNF α , tumour necrosis factor α ; HUVEC, human umbilical vein endothelial cell; IPTG, isopropyl β -D-thiogalactoside; GST, glutathione S-transferase; PKC, protein kinase C; DGK, diacylglycerol kinase; EST, expressed sequence tag.

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role since PKC does not stimulate SK activity *in vitro*. Although cell agonists stimulate SK activity, there is considerable basal activity found in unstimulated cells. This may represent activity required for a 'house-keeping' function as part of the sphingomyelin degradative pathway [3,22]. What is unclear is whether this basal SK activity is due to low-level stimulation of the pathway that activates the enzyme during agonist stimulation, or alternatively, reflects an intrinsic basal activity of the enzyme.

In this study we report the purification and cloning of the first human SK (hSK). We also describe the first expression and isolation of an active recombinant *Escherichia coli*-derived SK. Characterization of both the native and recombinant SKs is described and was undertaken in an attempt to identify any differences in catalytic or physico-chemical properties in these enzymes that may be attributable to eukaryotic post-translational modifications. Our data show that both enzymes have remarkably similar properties, indicating that SK is a constitutively active enzyme.

EXPERIMENTAL

Materials

D-erythro-Sphingosine, D-erythro-dihydrosphingosine, D,L-threo-dihydrosphingosine, N,N-dimethylsphingosine, N-acetyl-sphingosine (C₂-ceramide), S1P and Fumonisin B1 were purchased from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). Phytosphingosine, L- α -phosphatidic acid, L- α -phosphatidylinositol, L- α -phosphatidylserine, L- α -phosphatidylcholine, L- α -phosphatidylethanolamine, 1,2-dioctanoyl-sn-glycerol, 1,2-dioleoyl-sn-glycerol, ATP, calmodulin, GSH and BSA were from Sigma. N,N,N-Trimethylsphingosine and ADP were purchased from Calbiochem (Bad Soden, Germany), [γ -³²P]ATP from Geneworks (Adelaide, SA, Australia), TNF α from R&D Systems (Minneapolis, MN, U.S.A.) and isopropyl β -D-thiogalactoside (IPTG) from Promega (Madison, WI, U.S.A.). Prepacked Mono-Q, Superose 75 and HiTrap-Q columns, Q-Sepharose fast flow, calmodulin-Sepharose 4B, GSH-Sepharose 4B, thrombin and gel-filtration molecular-mass protein standards were from Amersham Pharmacia Biotech. SDS/PAGE molecular-mass protein standards, Silver Stain Plus kit, Coomassie Brilliant Blue R250 and Coomassie protein reagent were from Bio-Rad. Centricon concentrators were purchased from Amicon (Beverly, MA, U.S.A.) and bichinchonic acid (BCA) protein reagent was from Pierce (Rockford, IL, U.S.A.).

SK enzyme assay

SK activity was determined routinely using D-erythro-sphingosine and [γ -³²P]ATP as substrates, essentially as described previously [19], except using 1-butanol/ethanol/acetic acid/water (8:2:1:2, by vol.) as the mobile phase for isolation of S1P by TLC. A unit of activity is defined as the amount of enzyme required to produce 1 pmol of S1P/min.

Purification of SK from human placenta

SK was purified from 1240 g of human placenta (four placentas), with all steps performed at 4 °C. The placentas were diced, washed in buffer A (25 mM Tris/HCl buffer, pH 7.4, containing 10% (v/v) glycerol, 0.05% Triton X-100 and 1 mM dithiothreitol), transferred to 1.5 litres of fresh buffer A containing a protease-inhibitor cocktail (Complete[®], Boehringer Mannheim; buffer B) and minced in a Waring blender. The resultant homogenate was stored on ice for 30 min to enhance enzyme extraction, and the soluble fraction of the homogenate then

isolated by centrifugation at 17000 g for 60 min. This preparation was then fractionated by (NH₄)₂SO₄ precipitation by the addition of solid (NH₄)₂SO₄ at pH 7.4 and collection of the precipitated proteins by centrifugation (17000 g, 30 min). The 25–35%-saturated (NH₄)₂SO₄ fraction was then redissolved in buffer B, desalted by extensive dialysis against this same buffer, and centrifuged (17000 g, 30 min) to remove insoluble material. All subsequent chromatographic steps were performed using a FPLC system (Amersham Pharmacia Biotech) at 4 °C.

The dialysed (NH₄)₂SO₄ fraction was applied to a Q-Sepharose fast-flow column (50 mm diameter, 250 ml bed volume) pre-equilibrated with buffer A at a flow rate of 7 ml/min. SK activity was eluted with a NaCl gradient of 0–1 M in buffer A and collected in fractions of 10 ml. Fractions containing SK (SK1) activity were then combined, and CaCl₂ and NaCl added to give final concentrations of 4 and 250 mM, respectively. This pooled extract was then applied to a calmodulin-Sepharose 4B column (16 mm diameter, 10 ml bed volume), pre-equilibrated with buffer A containing 2 mM CaCl₂, at a flow rate of 1 ml/min. The column was then washed with several column volumes of equilibration buffer, followed by buffer A containing 4 mM EGTA, and then SK eluted with buffer A containing 4 mM EGTA and 1 M NaCl. The fractions containing highest SK activity were pooled, desalted on a Sephadex G25 column, and applied at a flow rate of 1 ml/min to a Mono-Q column (5 mm diameter, 1 ml bed volume) pre-equilibrated with buffer A. SK activity was eluted with a NaCl gradient of 0–1 M in buffer A. NaCl (to 500 mM) was added immediately to the 1-ml fractions collected to stabilize enzyme activity.

The Mono-Q fractions containing the highest SK activity were combined and desalted on a Sephadex G25 column. ATP and MgCl₂ were then added to the pooled fractions to final concentrations of 1 and 5 mM, respectively, before re-application at a flow rate of 1 ml/min to the Mono-Q column pre-equilibrated with buffer A containing 1 mM ATP and 5 mM MgCl₂. SK activity was eluted with a NaCl gradient of 0–1 M in the equilibration buffer. Again, NaCl (to 500 mM) was immediately added to the fractions (1 ml) collected to stabilize enzyme activity.

The ATP-Mono-Q fractions containing highest SK activity were pooled and concentrated 10-fold to a final volume of 200 μ l in a Centricon-10 concentrator and applied at a flow rate of 0.4 ml/min to a Superdex 75 column (10 mm diameter, 20 ml bed volume) pre-equilibrated with buffer A containing 500 mM NaCl. SK activity was eluted with the same buffer and 0.4-ml fractions collected. The molecular mass of the enzyme was estimated from this column by comparison with the elution volumes of ribonuclease A, chymotrypsinogen A, ovalbumin and BSA.

Cloning of hSK

The hSK cDNA was amplified from a human umbilical vein endothelial cell (HUVEC) λ Zap cDNA library using PCR primers derived from human expressed-sequence-tag (EST) sequences (GenBank[®] accession numbers D31133, W63556, AA026479, AA232791, AA081152, A1769914 and A1769914) aligned to the murine SKs [20]. Two primers, spanning a central *Sac*II site (P1, 5'-CGGAATTCCTCCAGTCGGCCGCGGTA-3', and P2, 5'-TAGAATTCTACCGCGCCGACTGGCT-3'), were used in combination with T3 and T7 primers to generate two overlapping PCR products of 669 and 550 bp that represented the 5' and 3' ends of hSK, respectively. These two PCR products were then cloned separately into pGEM4Z. A 584-bp *Sac*II fragment from the 5' hSK PCR clone was then subcloned in the correct orientation into the *Sac*II site of the 3' hSK PCR clone, to generate a 1130-bp partial hSK cDNA clone. A full-

length clone encoding hSK was then generated by subcloning a 120-bp *EcoRI*/*StuI* fragment from the 669-bp 5' hSK clone into this pGEM4Z 1130-bp clone digested with *EcoRI*/*StuI*. Sequencing the cDNA clone in both directions verified the integrity of the hSK cDNA sequence.

For bacterial expression, the hSK cDNA was subcloned into pGEX4T2. The pGEM4Z-hSK clone was digested with *Bam*HI and blunted with 3 units of *Pfu* polymerase in $1 \times$ *Pfu* buffer/50 μ M dNTPs at 72 °C for 30 min. The 1163-bp hSK cDNA was gel purified following digestion with *Sal*I and the blunt/*Sal*I fragment was then ligated to pGEX4T2 *Sma*I/*Xho*I. For mammalian cell expression the hSK cDNA was FLAG-epitope (DYKDDDDK) tagged at the 3' end by *Pfu* polymerase PCR with oligonucleotide primers T7 and 5'-TAGAATTCACTGTCATCGTCGTCCTTGTAAGGGCTCTTCTGGC-GGT-3'. This FLAG-tagged hSK cDNA was then cloned into pcDNA3 by digestion with *Eco*RI. The orientation was determined by restriction analysis and sequencing verified the integrity of the hSK-FLAG cDNA sequence.

SK amino acid-sequence analysis

The hSK amino acid sequence was searched against non-redundant amino acid- and nucleotide-sequence databases at the Australian National Genome Information Service using the blastp and tblastn algorithms [23].

Cell culture

HUVECs were isolated as described previously [24] and cultured on gelatin-coated culture flasks in medium M199 with Earle's salts supplemented with 20 % fetal calf serum, 25 μ g/ml endothelial growth supplement (Collaborative Research) and 25 μ g/ml heparin. The cells were passaged three times and grown to 80 % confluency before treatment and harvesting. Human embryonic kidney cells (HEK-293T, A. T. C. C. CRL-1573) were cultured in Dulbecco's modified Eagle's medium containing 10 % fetal calf serum, 2 mM glutamine, 0.2 % (w/v) sodium bicarbonate, 1.2 mg/ml penicillin and 1.6 mg/ml gentamycin and transiently transfected using the calcium phosphate precipitation method [25]. Treatment of HUVECs and HEK-293T cells with TNF α (1 ng/ml) was performed as described previously [10].

Expression and isolation of recombinant hSK from *E. coli*

The pGEX4T2-hSK vector was transformed into *E. coli* BL21 by electroporation. Overnight cultures (100 ml) of transformed isolates were grown with shaking (200 revs/min) at 30 °C in Superbroth medium (20 g/l glucose, 35 g/l tryptone, 20 g/l yeast extract and 5 g/l NaCl, pH 7.5) containing ampicillin (100 mg/l). The cultures were diluted 1:20 into fresh medium and grown at 30 °C with shaking to a D_{600} of 0.7–0.9. Expression of the glutathione S-transferase (GST)-coupled SK was then induced by addition of 0.1 mM IPTG and further incubation of the cultures at 30 °C for 3 h. After this time the bacterial cells were then harvested by centrifugation at 6000 *g* for 20 min at 4 °C and resuspended in 20 ml of buffer B containing 250 mM NaCl. The cells were then lysed with lysozyme at a final concentration of 0.3 mg/ml for 15 min at 25 °C followed by sonication, consisting of three cycles of 20-s ultrasonic pulses followed by 1 min of cooling. The lysate was then clarified by centrifugation at 50000 *g* for 45 min at 4 °C, followed by filtration through 0.22- μ m filters. The filtered supernatant was then incubated with 0.2 vols of 50 % (w/v) GSH-Sepharose 4B, which was washed and pre-equi-

librated with buffer B, for 60 min at 4 °C with constant mixing. After this time the mixture was poured into a glass chromatography column (10 mm diameter) and the beads (with bound GST-SK) washed with 10 column vols of buffer B at 4 °C. The GST-SK was then eluted from the column in 10 ml of buffer B containing 10 mM GSH. Cleavage of the GST away from SK was then performed by incubation with 20 μ g of thrombin for 3 h at 25 °C. The released SK was then purified by application of the cleavage mix to a calmodulin-Sepharose 4B column and then a Mono-Q anion-exchange column as described for the purification of the SK from human placenta.

Characterization of SKs

The effect of pH on the activity of the isolated SKs was determined over the pH range 4.0–11.0 in buffers at 50 mM (sodium acetate, pH 4.0–5.0; Mes, pH 6.0–7.0; Hepes, pH 7.0–8.2; Tris/HCl, pH 8.2–10.0; Caps, pH 10.0–11.0) at 37 °C. pH stability was determined by assaying the residual activity after pre-incubation of the enzymes in the same buffers for 5 h at 4 °C. Similarly, thermal stabilities were determined by assaying the residual activity after pre-incubation of the enzymes at various temperatures (4–80 °C) for 30 min at pH 7.4 (50 mM Tris/HCl containing 10 % glycerol, 0.5 M NaCl and 0.05 % Triton X-100). Substrate kinetics were analysed using Michaelis–Menten kinetics with a weighted non-linear regression program [26]. Since sphingosine and its analogues were added to the enzyme assays in mixed micelles with Triton X-100, where they exhibit surface-dilution kinetics [27], all K_m and K_i values obtained for these molecules were expressed as mol % of Triton X-100, rather than as bulk solution concentrations. For assays to determine the effect of calcium/calmodulin on SK activity, calcium and calmodulin were added to the standard assay mixtures containing 20 nM of isolated SK at final concentrations of 4 mM and 0.6 μ M, respectively.

Other analytical methods

Protein was determined using either the Coomassie Brilliant Blue R250 or BCA reagents using BSA as standard. In some cases protein estimations were performed after concentration and removal of detergent by precipitation [28] to increase the sensitivity and accuracy of the determinations. SDS/PAGE was performed according to the method of Laemmli [29] using 12 % acrylamide gels. Protein bands on gels were visualized with either Coomassie Brilliant Blue R250 or silver staining. Molecular mass was estimated by comparison with the electrophoretic mobility of myosin, β -galactosidase, BSA, ovalbumin, carbonic anhydrase, soya bean trypsin inhibitor, lysozyme and aprotinin.

RESULTS AND DISCUSSION

Purification of hSK

Human placenta was selected as a source of hSK due to the availability of large quantities of fresh tissue, and because the specific enzyme activity in human placenta was comparable with that of other animal tissues previously used for attempts at SK purification (results not shown). Approximately half of the total SK activity in human placenta was present in the cytosol after tissue homogenization. This is a similar distribution to many other animal cells and tissues, including rat kidney, the source used for the only other successful purification of a SK [19].

The purification of SK from human placenta is summarized in Table 1. The soluble fraction from the human placenta homogenate was initially subjected to ammonium sulphate precipi-

Table 1 Purification of SK from human placenta

SK was purified from 1240 g of fresh human placenta (four placentas); 1 unit of SK activity is defined as that needed for 1 pmol of S1P to be formed from sphingosine and ATP/min.

Step	Activity (units $\times 10^3$)	Protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Soluble fraction of homogenate	7943	123 600	58	100	—
Ammonium sulphate (25–35%)	7723	3527	1966	97	33
Q-Sepharose anion exchange	4048	1098	4597	63	79
Calmodulin-Sepharose	3197	18.23	1.75×10^5	40	3.0×10^3
Mono-Q anion exchange	1706	2.921	5.84×10^5	21.5	1.0×10^4
ATP–Mono-Q anion exchange	1133	0.419	2.70×10^6	14.3	4.6×10^4
Superdex 75 gel filtration	549	0.008	6.64×10^7	6.9	1.1×10^6

Figure 1 Anion-exchange chromatography of hSK

Anion-exchange chromatography with Q-Sepharose fast flow of the $(\text{NH}_4)_2\text{SO}_4$ -precipitated fraction of the human placenta extract showing two peaks of SK activity. Extracts were applied in buffer A and SK activity (●) was eluted with a NaCl gradient of 0–1 M (---). Protein eluted was followed by absorbance at 280 nm (—).

tation which resulted in a remarkably good purification of SK (33-fold). It should be noted that, unlike the situation in rat kidney [19], it was not necessary to ultracentrifuge the human placenta extracts prior to ammonium sulphate precipitation since the precipitate resuspended easily with almost no loss of SK activity. For anion-exchange chromatography the ammonium sulphate fraction was rapidly desalted with the use of a Sephadex G25 column and the desalted fraction loaded immediately on to a Q-Sepharose fast-flow column. The speed of this desalting step appeared critical since the SK activity appeared very unstable at NaCl concentrations below about 0.2 M, meaning slower desalting steps, such as dialysis, resulted in substantial losses of enzyme activity. Q-Sepharose fast-flow chromatography resulted in two peaks of SK activity, eluting at approx. 0.15 and 0.6 M NaCl, and were designated SK1 and SK2, respectively (Figure 1). Similarly, two SK activities have previously been observed after anion-exchange chromatography of human platelet [30] and bovine brain extracts [31], although the significance of these two activities has yet to be elucidated. For this study SK1 was selected for further purification due to its greater abundance and stability; SK2 activity appeared very unstable and, unlike SK1, could not be stabilized by the addition of 0.5 M NaCl, 10% glycerol and 0.05% Triton X-100. SK1 was also chosen since it

appeared to be the isoform present in HUVECs, as discussed later.

Fractions from the Q-Sepharose fast-flow column containing SK1 were then affinity purified by application to a calmodulin-Sepharose 4B column in the presence of CaCl_2 , and elution of SK1 performed with EGTA and NaCl, resulting in further substantial purification (38-fold) and high enzyme yields. Like the rat kidney enzyme [19], the SK1 could not be eluted from the calmodulin-Sepharose 4B column with EGTA alone, indicating an unusual association of the enzyme with this affinity matrix. The active fractions that eluted from the calmodulin-Sepharose 4B column were then desalted and applied to two subsequent steps of analytical anion-exchange chromatography on a Mono-Q column, with the second step performed in the presence of ATP and MgCl_2 (Figure 2). This type of purification, utilizing ATP, has been used previously for some other ATP-dependent enzymes, particularly PKC [32]. It aids purification since the elution of the SK1 and the SK1–ATP complex differ, with the active peak of SK1 eluting at approx. 150 and 75 mM NaCl in the absence and presence of ATP, respectively. Like PKC [32], this difference in elution is probably due to a substrate (ATP)-induced conformational change in SK1, and not an increase in negative charge caused simply by the presence of bound ATP,

Figure 2 Purification of human placenta SK1 by anion-exchange chromatography on a Mono-Q column is enhanced by reapplication and elution from the same column in the presence of ATP

(A) The fractions from the calmodulin-Sepharose 4B column containing SK activity were pooled, desalted and applied to the Mono-Q column in buffer A. (B) Active fractions from the Mono-Q column were desalted and reappplied to the Mono-Q column in buffer A with 1 mM ATP and 5 mM MgCl_2 . In both cases SK activity (●) was eluted with a NaCl gradient of 0–1 M (---), with protein elution followed by absorbance at 280 nm (—).

since SK1 is actually bound less strongly to the anion-exchange matrix, eluting at a lower NaCl concentration (Figure 2). The active fractions resulting from these anion-exchange steps were then applied to gel-filtration chromatography with a Superdex 75 column as a final purification step. SK1 eluted from this column as a single peak with a molecular mass corresponding to 44 kDa. Analysis of the active fraction from this final column by SDS/PAGE with silver staining (Figure 3) revealed a single band of molecular mass 45 kDa, indicating a homogeneous protein that had been purified over 10^6 -fold from the original placenta extract with a remarkably good yield of 7% of the original SK activity (Table 1).

SK isoforms in HUVECs

Since two SK activities were identified in human placenta (Figure 1), other human tissue [31] and cultured human cells [30], we examined the multiplicity of this enzyme activity in HUVECs by the use of preparative anion-exchange columns. In contrast to other human tissues and cells, application of HUVEC extracts to these columns resulted in the appearance of only a single SK peak that eluted at the same point as the human placenta SK1

(Figure 4). Similarly, only a single SK peak eluted after application of HUVEC extracts in which SK activity had been stimulated by a 10-min treatment of the cells with $\text{TNF}\alpha$ [4,10]. These results would indicate that SK1, the human placenta SK isolated in this study, is probably the main isoform found in HUVECs, and that $\text{TNF}\alpha$ treatment results in an increase in the activity of this enzyme, rather than the activation of another, otherwise latent, isoform.

Cloning of hSK and transient expression in HEK-293T cells

A hSK cDNA was generated from a HUVEC λ Zap cDNA library using primers designed from human ESTs aligned with the published murine SK sequence [20]. The cDNA generated had an open reading frame coding for 384 amino acids. It should be noted that the sequence lacked a recognizable Kozak consensus motif, raising the possibility that the actual initiation sequence may not be included in this cDNA. The SK cDNA encodes for a protein (hSK) with a predicted isoelectric point of 6.64 and a molecular mass of 42 550 kDa, consistent with the molecular mass determined for the purified human placenta SK

Figure 3 SDS/PAGE of purified human placenta SK

The fraction from the Superdex 75 column containing the highest SK activity was applied to SDS/PAGE with silver staining, yielding a single band of 45 kDa.

(SK1). Subcloning into pcDNA3 and transient expression of hSK in HEK-293T cells resulted in a 3200-fold increase in SK activity in these cells (Figure 5), compared with untransfected HEK-293T cells or HEK-293T cells transfected with empty vector, indicating that the generated hSK cDNA encodes a genuine SK. Interestingly, although hSK-transfected HEK-293T cells had 3200-fold higher levels of SK activity, treatment of these cells with TNF α resulted in a rapid (10 min) increase in SK activity by a similar proportion (approx. 2-fold) to that seen in untransfected HEK-293T cells (Figure 5) [10]. Similar results were also recently reported with platelet-derived growth factor stimulation of NIH 3T3 fibroblasts expressing a murine SK [33]. This indicates that the high levels of over-expressed SK are not saturating the as yet unknown TNF α -mediated activation mechanism in these cells. This, combined with the extremely rapid activation (10 min), suggests that the activating molecule is in high abundance in the cell compared with the endogenous levels of SK. This may point away from an activation mechanism that requires direct interaction with a specific activating protein, such as a 'SK kinase', since this type of molecule may be expected to be in relatively low abundance in the cell. Instead, the SK-activating mechanism is more likely to involve a molecule that has more diverse functions in the cell, and which therefore is expressed at higher levels in the cell. The identity of the directly activating molecule and activation mechanism of SK in response to TNF α and other cell agonists remains to be elucidated.

hSK sequence analysis

Sequence comparison of hSK with the two reported murine SK sequences, mSK1a and mSK1b [20], revealed very high similarity, with 81 and 80% amino acid-sequence identity, respectively. A search of the database showed that hSK also shares amino acid-sequence similarity (28–36% identity) with two recently identified *Saccharomyces cerevisiae* SKs [21] and several other ESTs encoding putative SK proteins from *Schizosaccharomyces pombe*,

Figure 4 Only a single chromatographically identified SK isoform is present in HUVECs

Preparative anion-exchange chromatography with HiTrap-Q columns of (A) human placenta, (B) HUVECs and (C) TNF α -treated HUVEC extracts. Cells were harvested, lysed and the soluble extracts applied to the HiTrap-Q column in buffer A. Total SK activities in human placenta, HUVECs and TNF α -treated HUVEC extracts were 51, 78 and 136 units/mg of protein, respectively. SK activity (●) was eluted with a NaCl gradient of 0–1 M (---).

Caenorhabditis elegans and *Arabidopsis thaliana*. Multiple sequence alignment of hSK with these homologues (Figure 6) revealed several regions of highly conserved amino acids throughout the protein, but particularly towards the N-terminus.

A search of the domain structures of hSK sequence revealed three calcium/calmodulin-binding motifs [34], one of the 1-8-14 type A [(F/I/L/V/W)XXX(F/A/I/L/V/W)XX(F/A/I/L/V/W)XXXXX(F/I/L/V/W)] with a net charge of +3 to +6 spanning residues 290–303, and two of the 1-8-14 type B [(F/I/L/V/W)XXXXX(F/A/I/L/V/W)XXXXX(F/I/L/V/W)] with a net charge of +2 to +4 that overlap between residues 134 and 153. These regions align with the calcium/calmodulin-binding motifs found in mSK1a and are consistent with the observation that both hSK and mSK1a [19] bind strongly to calmodulin–Sepharose in the presence of calcium. Further analy-

Figure 5 Expression and TNF α stimulation of hSK activity in HEK-293T cells

HEK-293T cells transiently transfected with either (A) empty pcDNA3 expression vector alone or (B) pcDNA3 containing hSK cDNA. Transfected cells were either untreated or treated with TNF α for 10 min, harvested and SK activity in cell lysates determined. Data are means $\pm$ S.D. of duplicates and are representative of three independent experiments.

hSK	1	MDPAGGPRGVLP	PCRVLLNPRGGKGKALQL	FRSHVQPLLAEEISFTLML	TERRNHARE	LVRSEELGRWDALVVM	SGDGLMHEVVNGLMERPD	WETAI	.QKPLCSLPAG						
mSK1a	1	MEPECPRGLLP	PCRVLLNPNQGGKGKALQL	FSRVQPFLEEEAETFKLIL	TERKNHARE	LVC AEELGHWDALAVM	SGDGLMHEVVNGLMERPD	WETAI	.QKPLCSLPAG						
mSK1b	1	MWWCCVL	FVVECPRGLLP	PCRVLLNPNQGGKGKALQL	FSRVQPFLEEEAETFKLIL	TERKNHARE	LVC AEELGHWDALAVM	SGDGLMHEVVNGLMERPD	WETAI	.QKPLCSLPAG					
Yeast LCB4	206	NISSGTVEEILEKSYENSKRNRSIL	VIINPHGGKGIAKNLFLTKAR	PILVESGCKIEIAYTKYARH	AID	IAKDLDISKYDTIACASGDG	IPYEVIINGLYRRDRVD	AFNKLAVTQL	PCG						
Yeast LCB5	252	DL....VEEILKRSYKNTRRNKSIF	VIINPHGGKGKAKKLFMTKAKP	LLASRCSIEVVYTKYPGHAIE	IAREMDIDKYDTIACASGDG	IPYEVIINGLYRRDRVD	AFNKLAVTQL	PCG							
S. pombe	90	FCEYLLDVAYKGIKRSRRFI	VFIPNHGGKGKAKHIWESEAE	PVFSSAHSICEVVLTRRKDHAKS	IAKNLDVGSYDGLSVG	GDGLFHEVIINGLYRRDRVD	AFNKLAVTQL	PCG							
C. elegans	63	ENEQLTSVILSRKPPPQEQCRGNLL	VFIPNPSGCKSLETTANTVGP	PKLDKSLIRYEVVVTGPNHARN	VLMTKADLGKFNGLVIL	SGDGLVFEALNGLICRED	AFRIFPTLP	IGIVPSG							
Consensus		*.....R.....*V.*NP.GGKGKA..*F.*...P.L.*.....**T....HA.*.*.....**D.*.....SGDG*..EV*NGL..R.D....A.....**.*P.G													
hSK	112	SGNALAAS	LNHYAGYEQVTNEDLL	TNCTLLCRR..	LLSPMNL	LSLHTASGLRL	FSVLSLAWCFIADVD	LESEKYRR	.LGEMRFTLCTFLRLAALRTYRGRL						
mSK1a	111	SGNALAAS	VNHYAGYEQVTNEDLL	INCTLLCRR..	RLSPMNL	LSLHTASGLRL	YSVLSLWCFVADVD	LESEKYRR	.LGEIRFTVCTFFRLASLRIYQGQL						
mSK1b	118	SGNALAAS	VNHYAGYEQVTNEDLL	INCTLLCRR..	RLSPMNL	LSLHTASGLRL	YSVLSLWCFVADVD	LESEKYRR	.LGEIRFTVCTFFRLASLRIYQGQL						
Yeast LCB4	325	SGNAMSIS	CHWTNNPSYAAL	CLVKSIETRIDLMCCSQPSY	MNEWPRLSFLS	QTYGVIAESDINTE	FIRWMGPVRFNGLGVAFNII	QKKYPC	EVFVKYAAKSKKELKVHFL						
Yeast LCB5	368	SGNAMSIS	CHWTNNPSYSTL	CLIKSIETRIDLMCCSQPSY	AREHPKLSFLS	QTYGLIAETDINTE	FIRWMGPVRFELGVAFNII	QKKYPC	EIVVKYAAKSKKELKNHYL						
S. pombe	203	SGNAFSYN	ATGQLKPALTA	EILKGRPTSF	DLMTFEQ...KGKKAYS	FLTANYGIIADCDIGTE	NWRFMGENRAYL	CFFLRLFQKPD	WKCSIEMDVVSSDRTEIKHMY						
C. elegans	184	SGNGLLS	GV..LSKYGTFKMNEKSV	MRALAIATSP	TAKAESVALYSVK	TDNQ..SYASFLSIGW	CLMADIDIDSEKWRK	SLGHHFTVMG	FIRSCNLSYKGR						
Consensus		SGNA*..S.....*L.*.....*M.....*S.LS.**G.*A.*D**E.*R.*G..RF.*G.....*Y.....*P.....*													
hSK	211	AYLP	VGRVGS	KTPASPVV	VQCPVDAHL	VPLEEPVPSHW	TVVPD	EDFVL	VLVLLHSHL	GSEMFAAPMGRCA					
mSK1a	210	AYLP	VGTVASKR	PAST.LVQK	GPVDTHL	VPLEEPVPSHW	TVVPE	QDFVL	VLVLLHSHL	SSELFAAPMGRCE					
mSK1b	217	AYLP	VGTVASKR	PAST.LVQK	GPVDTHL	VPLEEPVPSHW	TVVPE	QDFVL	VLVLLHSHL	SSELFAAPMGRCE					
Yeast LCB4	435	ENKDKNKGCLTFEP	..NPSPNSSPD	LLSKNNINN	STKDE.....LSPN	FLNEDNFKL	KYPMTEPVPRD	WEKMDSEL	TDNLTIFYTG	KMPYIAKDTKFFPAALPA					
Yeast LCB5	478	EH..KNKGSLE	FQHITMKN	DNEDCDN	YNYENYE	TENEDEDEDAD	ADDEDSHLIS	RDLADSSADQ	KEEDFKI	KYPLDEGIPSDWERLDPNISNNLGIFYTGKMPYV	AADTKFFPAALPS				
S. pombe	308	EKS	KNLAPMS	ESSDS	SDK.....TV	STSPESH	LLTFEI..NDLS	IFCAGLL	PYIAPDA	KMFPAASND					
C. elegans	284	TYR	PKPGFHP	SSNVF	SVYEKT	TQQRID	DSKVKTNG	SVSDSEET	METKFN	WTLPDSDET	LAGSSDLEETVIE.....DNFVN	NIYAVT	LSHIAADG	FFAPSAKLE	
Consensus		*.....													

Figure 6 Sequence comparison of hSK with other known and putative SKs

Comparison of the deduced hSK amino acid sequence with the amino acid sequences of the murine (mSK1a and mSK1b) [20] and *S. cerevisiae* (LCB4 and LCB5) [21] SKs, and EST sequences of putative SKs from *S. pombe* and *C. elegans* (GenBank® accession numbers Z98762 and Z66494, respectively). Although the amino acid sequence similarity to hSK was high (36% identity), the *A. thaliana* putative SK sequence (accession number AL022603) gave relatively poor alignment and, for clarity, is not shown. The consensus sequence represents amino acids that are conserved in at least six of the seven aligned sequences, while conservation of structurally similar amino acids are denoted with an asterisk. Multiple sequence alignment was performed with CLUSTALW.

Figure 7 Expression of recombinant hSK in *E. coli* BL21

E. coli BL21 was transformed with the pGEX4T2-hSK expression construct and expression of the GST-SK fusion protein analysed after induction with 100 μ M IPTG. **(A)** SK activity in uninduced and IPTG-induced *E. coli* cell extracts. Means $\pm$ S.D. are shown. **(B)** Coomassie Brilliant Blue-stained SDS/PAGE gel showing GST-SK fusion protein expression in *E. coli* cell lysates. **(C)** Purity of the isolated recombinant hSK. The fraction from the Mono-Q column containing SK activity was applied to SDS/PAGE with silver staining and yielded a single band of 45 kDa.

sis of the hSK sequence revealed a possible N-myristoylation site close to the N-terminus (at Gly⁵) that may be applicable if the protein is subject to proteolytic cleavage. Also identified were one putative casein kinase II phosphorylation site (at Thr¹³⁰) and four putative PKC phosphorylation sites (at Thr⁵⁴, Ser¹⁸¹, Thr²⁰⁵ and Ser³⁷¹). Again, these putative phosphorylation sites are also found in both murine SK isoforms, although the mouse enzymes also display six more possible phosphorylation sites, four for PKC, and one each for casein kinase II and protein kinase A, that do not occur in hSK.

A search of signalling-domain sequences using the SMART search tool [35] revealed similarity in residues 16–153 of hSK with the putative diacylglycerol kinase (DGK) catalytic domain. hSK showed an overall 36% identity to the consensus sequence of the DGK catalytic domain family, and possessed 17 of the 24 very highly conserved amino acids of this domain. hSK, however, showed only low similarity to the proposed ATP-binding motif of this domain (GXGXXGX_nK), although it should be noted that the applicability of this protein kinase ATP-binding-site motif to DGKs remains contentious [36,37]. Further sequence

analysis of the human and related SKs also failed to find regions showing any marked similarity to the proposed nucleotide-binding motifs found in other protein families [38,39]. Apart from the similarity with the DGK catalytic domain, hSK shows no similarity with other lipid-binding enzymes, and does not appear to have any recognizable lipid-binding domains, like PKC C2 or pleckstrin homology domains. There are also no other obvious regulatory domains, with the possible exception of a proline-rich region at the C-terminus which shares some similarity with SH3-binding domains [40,41].

Expression in *E. coli* and isolation of recombinant SK

The hSK cDNA was subcloned into the pGEX4T2 plasmid and hSK expressed as a GST fusion protein in *E. coli* by IPTG induction (Figure 7). After the GST-SK fusion protein was partially purified using GSH-Sepharose 4B, and the GST removed by thrombin cleavage, the hSK was further purified by subsequent elutions from calmodulin-Sepharose and Mono-Q anion-exchange columns. This resulted in high recovery of SK activity (greater than 70% of the originally induced activity), and an electrophoretically pure SK (Figure 7). However, only low protein yields of the recombinant enzyme could be obtained since a large proportion of the IPTG-induced, thrombin-cleaved hSK protein did not bind to the calmodulin-Sepharose column (Figure 8). This non-binding form of hSK also had no demonstrable catalytic activity, suggesting that it was incorrectly folded.

Characterization of native and *E. coli*-derived recombinant SKs

To determine if eukaryotic post-translational modifications are required for, or modulate, SK activity, we compared the physico-chemical and catalytic properties of the purified native human placenta SK with those of the recombinant SK expressed in *E. coli*, where such modifications would not occur. Maximum activity of the native and recombinant SKs was observed at pH 7.4, with both enzymes showing greater than 60% of maximum activity in the pH range 6.8–7.4 (Figure 9). Both SKs retained more than 90% of their original activity after 5 h of incubation at 4 °C in the pH range 6–7.8 (Figure 9). At pH 7.4 in the presence of 10% glycerol, 0.5 M NaCl and 0.05% Triton X-100, both enzymes were stable for 30 min at temperatures up to 37 °C (Figure 9). The enzymes were much less stable in buffers lacking glycerol, NaCl and Triton X-100 (results not shown), consistent with previous observations of the bovine brain and rat kidney SKs [19,31]. Both hSKs showed a requirement for divalent metal ions since the presence of EDTA in assays eliminated activity (Figure 9). Like other SKs examined [19,21,22,27,31], both human enzymes showed highest activity with Mg²⁺, somewhat lower activity with Mn²⁺, and only very low activity with Ca²⁺. Other divalent metal ions examined, including Zn²⁺, Cu²⁺ and Fe²⁺, did not support SK activity (Figure 9).

The native and recombinant SKs have very similar, and narrow, substrate specificities (Figure 10), with both showing greatest activity with the naturally occurring mammalian substrate *D-erythro*-sphingosine as well as *D-erythro*-dihydrosphingosine. Low activity was also detected for both enzymes against phytosphingosine, while a range of other sphingosine derivatives and related molecules were not phosphorylated. These included *D,L-threo*-dihydrosphingosine, *N,N*-dimethylsphingosine, *N,N,N*-trimethylsphingosine, *N*-acetylsphingosine (*C*₂-ceramide), diacylglycerol (1,2-dioctanoyl-*sn*-glycerol and 1,2-dioleoyl-*sn*-

Figure 8 Purification of recombinant hSK with calmodulin-Sepharose 4B allows the separation of active and inactive enzyme

The GST-SK fusion protein was partially purified using GSH-Sepharose 4B, cleaved by thrombin and applied to the calmodulin-Sepharose 4B column in buffer B containing 4 mM CaCl_2 . Elution ($\downarrow$) of the active SK bound to the column was performed with buffer A containing 2 mM EGTA and 1 M NaCl. (A) SDS/PAGE analysis of fractions eluted from the calmodulin-Sepharose 4B column. (B) SK activity in column fractions showing most of the recombinant hSK protein did not bind to the column and displayed no catalytic activity.

glycerol) and phosphatidylinositol. Similar substrate specificities have been observed with the partially purified rat brain SK [22,27] and the recombinant mouse SKs [20], although the latter do not phosphorylate phytosphingosine. *D-erythro*-Sphingosine is also the best substrate for the *S. cerevisiae* SKs, but these enzymes appear to phosphorylate phytosphingosine much more efficiently than their mammalian counterparts [21,42], an observation that is perhaps not so surprising considering that this lipid is much more abundant than sphingosine in yeast [43].

Further analysis of the hSKs with *D-erythro*-sphingosine and *D-erythro*-dihydrosphingosine revealed Michaelis-Menten kinetics over the concentration range used (Figure 10), with both isolated native and recombinant SKs showing very similar kinetic properties and slightly higher affinity for *D-erythro*-sphingosine over *D-erythro*-dihydrosphingosine (Table 2). Both enzymes also displayed similar kinetics when sphingosine was supplied as a sphingosine-BSA complex, although presentation of the substrate in this manner resulted in a lower k_{cat} value for both enzymes (28 and 39 s^{-1} for the native and recombinant SKs, respectively) compared with its presentation in Triton X-100 mixed micelles, as used for all the other assays performed in this study. Sphingosine supplied as a BSA complex gave K_m values of 16 ± 4 and $17 \pm 2 \text{ mM}$ for the native and recombinant SKs, respectively. Both the native and recombinant SKs had the same affinity for ATP (K_m of approx. $80 \mu\text{M}$; Table 2), and were very similar to K_m values found for the rat [19] and recombinant mouse [20] SKs. Other studies have reported SK K_m values for ATP ranging between 25 and $310 \mu\text{M}$ [21,31,44], but since these studies were performed with either crude or only partially purified

enzymes, their relevance to the true K_m values for these enzymes is not known.

Both the native and recombinant SK were inhibited by *D,L-threo*-dihydrosphingosine, *N,N*-dimethylsphingosine and *N,N,N*-trimethylsphingosine (Figure 10), with all three of these molecules displaying competitive inhibition with respect to sphingosine. Although the inhibition constants for these molecules were quite similar, *N,N,N*-trimethylsphingosine gave slightly more efficient inhibition than *D,L-threo*-dihydrosphingosine, which was a marginally more efficient inhibitor than *N,N*-dimethylsphingosine (Table 2). Similar results for *N,N*-dimethylsphingosine and *D,L-threo*-dihydrosphingosine have been reported for the rat [19] and recombinant mouse [20] SKs, whereas, surprisingly, studies with crude platelet extracts appear to show *N,N*-dimethylsphingosine as a considerably more effective inhibitor of SK than either *D,L-threo*-dihydrosphingosine or *N,N,N*-trimethylsphingosine [30,45]. Interestingly, studies *in vivo* with platelets [45] also showed *N,N*-dimethylsphingosine to be a much more effective inhibitor of SK activity than *D,L-threo*-dihydrosphingosine, while very little inhibition is seen with *N,N,N*-trimethylsphingosine. Presumably, these results reflect the differential uptake of these molecules into the cell, although application of *N,N,N*-trimethylsphingosine to HUVECs inhibits nuclear factor κB activation and consequent E-selectin expression [46], presumably through inhibition of SK activity which is known to be involved in regulation of this pathway [10].

ADP also showed weak competitive inhibition, with respect to ATP (Table 2). In all cases remarkably similar inhibition constants were observed for the native and recombinant SKs

Figure 9 Physico-chemical properties of the native and recombinant SKs

(A) pH optima. The effect of pH on SK activity was determined by assaying the activity over the pH range of 4–11 in 50 mM buffers (for details see the Experimental section). (B) pH stability. Shown is the SK activity remaining after preincubation of the enzymes at various pH at 4 °C for 5 h. (C) Temperature stability. Shown is the SK activity remaining after preincubation of the enzymes at various temperatures (4–80 °C) for 30 min at pH 7.4 (50 mM Tris/HCl containing 10% glycerol, 0.5 M NaCl and 0.05% Triton X-100). (D) Metal-ion requirement. The various metal ions or EDTA were supplied in the assay mixture at a final concentration of 10 mM. In all cases the maximum activities of the native (● and filled bars) and recombinant (○ and open bars) SKs were arbitrarily set at 100% and correspond to 2.65 and 7.43 k-units, respectively. Data are means $\pm$ S.D.

Figure 10 Substrate specificity and kinetics of the native and recombinant SKs

(A) Substrate specificity of the native (filled bars) and recombinant (open bars) SKs with sphingosine analogues and other lipids supplied at 100 μ M in 0.25% Triton X-100. The rates of phosphorylation of sphingosine by the native and recombinant SKs were arbitrarily set at 100% and correspond to 2.65 and 7.43 k-units, respectively. Activity against other potential substrates was expressed relative to the activity against sphingosine. No phosphorylation was observed with *D,L*-threo-dihydrosphingosine, *N,N*-dimethylsphingosine, *N,N,N*-trimethylsphingosine, *N*-acetylsphingosine (*C*₂-ceramide), diacylglycerol (1,2-dioctanoyl-*sn*-glycerol and 1,2-dioleoyl-*sn*-glycerol) and phosphatidylinositol. (B) Substrate kinetics of the recombinant hSK with sphingosine (●) and *D*-erythro-dihydrosphingosine (○) as substrates. (C) Kinetics of inhibition of the recombinant hSK with *N,N,N*-trimethylsphingosine at 5 (○) and 25 μ M (▼), and in its absence (●). Inset: Lineweaver-Burk plot. Data are means $\pm$ S.D.

Table 2 Substrate and inhibition kinetics of the native and recombinant SKs

Substrate kinetics were determined by supplying substrates over the concentration range 0.5–200 μM (0.0125–5 mol%) for sphingosine analogues, and 5–1000 μM for ATP. Inhibition kinetics were determined by the use of inhibitors over the concentration range of 2–50 μM (0.05–1.25 mol%) for sphingosine analogues, and 0.1–5 mM for ADP. For comparison with previous studies, all K_m and K_i values for sphingosine and its derivatives are expressed as both bulk solution concentrations and mol% of Triton X-100, where Triton X-100 was present in all assays at a final concentration of 0.25% (w/v). All kinetic values and S.E.M. values were obtained from non-linear regression analysis [26].

Substrate kinetics	Native SK	Recombinant SK
Sphingosine		
K_m (mol%)	0.35 ± 0.05	0.30 ± 0.07
K_m (μM)	14 ± 2	12 ± 3
k_{cat} (s^{-1})	50	85
k_{cat}/K_m ($10^{-5} \text{ M}^{-1} \cdot \text{s}^{-1}$)	36	71
Dihydrosphingosine		
K_m (mol%)	0.50 ± 0.05	0.48 ± 0.05
K_m (μM)	20 ± 2	19 ± 2
k_{cat} (s^{-1})	35	76
k_{cat}/K_m ($10^{-5} \text{ M}^{-1} \cdot \text{s}^{-1}$)	18	39
ATP		
K_m (μM)	77 ± 11	86 ± 12
Inhibitor kinetics	Native SK	Recombinant SK
<i>N,N</i> -Dimethylsphingosine		
K_i (mol%)	0.20 ± 0.03	0.19 ± 0.02
K_i (μM)	7.8 ± 1	7.5 ± 1
<i>D,L</i> -threo-Dihydrosphingosine		
K_i (mol%)	0.15 ± 0.02	0.14 ± 0.03
K_i (μM)	5.9 ± 1	5.7 ± 1
<i>N,N,N</i> -Trimethylsphingosine		
K_i (mol%)	0.12 ± 0.03	0.10 ± 0.02
K_i (μM)	4.6 ± 1	3.8 ± 1
ADP		
K_i (mM)	1.1 ± 0.3	0.9 ± 0.2

(Table 2). No product feedback inhibition of the SKs was observed by S1P at concentrations up to 10 μM , nor was any inhibition seen with 1 mM *N*-acetylsphingosine or 1 mM Fumonisin B1, a ceramide synthase inhibitor (results not shown).

The findings that hSK binds to a calmodulin-affinity column and has putative calcium/calmodulin-binding motifs in its amino acid sequence raised the intriguing prospect that calmodulin may have some functional role with these enzymes. Since calcium/calmodulin is a well-known modulator of the activity of some protein kinases [47], we examined its effect on SK activity. However, under the assay conditions used, calcium/calmodulin had no effect on the activity of either the native or recombinant hSKs (results not shown). While this result indicates a lack of SK activity regulation by calmodulin, the possibility remains that calmodulin may be involved in some other function with SK, such as regulating its subcellular localization.

Some previous studies [18,48] have indicated that acidic phospholipids can stimulate SK activity in crude enzyme preparations, while other studies have not [27,30]. Since the interpretation of these observations is difficult due to the wide range of other proteins and lipids in these crude, or partially purified, enzyme preparations, this phenomenon was examined further with the purified hSKs. Whereas the addition of the neutral phospholipids phosphatidylcholine and phosphatidylethanolamine to the enzyme assay mixture did not result in any detectable differences in hSK activity, marked increases in activity

Figure 11 Acidic phospholipids stimulate the activity of the native and recombinant SKs

The effects of various phospholipids on the activity of the native and recombinant SKs were determined by assaying the activity under standard conditions in the presence of these phospholipids at 10 mol% of Triton X-100. The activities of the native (filled bars) and recombinant (open bars) SKs in the absence of phospholipids were arbitrarily set at 100% and correspond to 2.65 and 7.43 k-units, respectively. PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid. Data are means $\pm$ S.D.

(1.6–2-fold) were observed with the acidic phospholipids phosphatidylserine, phosphatidylinositol and phosphatidic acid (Figure 11). Both the native and recombinant SKs were activated in a similar manner by these acidic phospholipids, with kinetic analyses revealing that all three phospholipids caused an increase in the enzymes' k_{cat} values, while the K_m values remained unchanged. The basis for this acidic-phospholipid-mediated stimulation of SK activity is not yet known. Acidic phospholipids are known to form electrostatic interactions with sphingosine which is largely protonated at physiological pH [49]. It is possible, therefore, that negatively charged phospholipids may stimulate SK activity through either neutralization of the sphingosine positive charge or by altering the conformation of sphingosine [48,49]. However, this interaction may be expected to affect the K_m value for sphingosine, and not just result in the observed increase in k_{cat} . Alternatively, acidic phospholipids may interact directly with SK to stimulate activity, as is the case in several other cell-signalling enzymes, including PKC [50] and some DGKs [51], although it should be noted that there are no obvious phospholipid-binding domains within the SK amino acid sequence. Further elucidation of these findings awaits more detailed analysis of possible enzyme–acidic-phospholipid binding and the solving of the SK crystal structure.

Also of interest, in light of our results, are the observations of Desai et al. [9] showing that S1P increases cellular levels of phosphatidic acid via activation of phospholipase D. Increased levels of phosphatidic acid would appear to provide a positive-feedback loop to SK, and an obvious mechanism for amplification of SK-mediated cell signalling. Equally, the observed *in vitro* stimulation of SK activity by phosphatidic acid and other acidic phospholipids makes it tempting to speculate that they may be largely responsible for SK activation in response to TNF α and other cell agonists. This is particularly relevant when, as discussed earlier, our data suggest that the SK-activating molecule(s) are at relatively high levels in the cell, and that

modification and mobilization of the highly abundant cellular phospholipids in response to various cell agonists is well known [52–54]. It should also be noted that the extent of stimulation of SK activity seen in this study with acidic phospholipids is similar to the increase in activity that occurs with various cell agonists.

Conclusions

Characterization of both the purified recombinant and native SKs revealed that these enzymes have remarkably similar properties and therefore establish that eukaryotic post-translational modifications are not required for activity of the hSK. This finding indicates that the basal SK activity, seemingly always present in the cell, is due to the presence of a constitutively active SK and not a result of low-level activation of this enzyme. This, combined with the observations that basal SIP levels in the cell are very low [2,15], probably due to the presence of SIP lyase [13], and that SK activity and SIP levels are rapidly increased by many cell agonists [2], suggests that SK has dual roles in the cell. The basal activity is likely to be involved in sphingomyelin turnover, assisting in clearing the cell of sphingosine and ceramide, two molecules with apparent pro-apoptotic functions [3,55]. In contrast, it would appear reasonable that the higher levels of SK activity seen upon cell stimulation with various cell agonists would confer the more recently described cell-signalling role for this enzyme through the production of SIP [1,2].

The apparent dual role of SK raises the intriguing question of how these very different functions are controlled in the cell. An obvious mechanism is subcellular compartmentalization of the enzyme where the different roles are accomplished in different locations in the cell. The common observations that SK activity partitions between the cytosolic and insoluble fractions of the cell [30,33,56] may support this hypothesis further.

In conclusion, we report here the first successful purification, molecular cloning and characterization of a hSK. We also report here the first isolation and characterization of an *E. coli*-derived recombinant hSK which has remarkably similar properties to the native human enzyme. This study has also given further insight into the regulation and roles of hSK, and has provided the tools required for further elucidation of these questions.

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