Asp177 in C4 domain of mouse sphingosine kinase 1a is important for the sphingosine recognition

Shinji Yokota, Yuki Taniguchi, Akio Kihara, Susumu Mitsutake, Yasuyuki Igarashi*

Department of Biomembrane and Biofunctional Chemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita 12, Nishi 6, Kita-ku, Sapporo 060-0812, Japan

Received 1 October 2004; revised 20 October 2004; accepted 20 October 2004

Available online 11 November 2004

Edited by Gerrit van Meer

Abstract Sphingosine kinase (SK) is the enzyme that catalyzes the formation of sphingosine 1-phosphate (S1P). Although diverse biological functions have been reported for SK, its recognition site for its substrate sphingosine (Sph) is still unclear. We constructed various mutants of mouse sphingosine kinase 1a (mSK1a), carrying mutations in the C4 domain, which we had expected to encompass the Sph-binding site. We analyzed the influence of these mutations on the SK activity and substrate kinetics. One mutation, $Asp^{177} \rightarrow Asn^{177}$, caused a dramatic decrease in SK activity (to ~6% of wild type) and an increase in the K_m value for Sph (10.1 → 108 μM), with no change in the affinity for ATP. This result suggests that the C4 domain, especially the Asp^{177}, is involved in the specific recognition of Sph. In this report, we are able, for the first time, to provide an account of the Sph-binding site of SK.

Keywords: Sphingosine kinase; Sphingosine; Sphingosine 1-phosphate; Calmodulin

1. Introduction

Sphingosine kinase (SK) catalyzes the formation of sphingosine 1-phosphate (S1P), a lipid second messenger involved in mediating diverse and fundamental biological processes [1]. SK activity can be increased by various stimuli such as tumor necrosis factor-alpha [2,3], phorbol 12-myristate 13-acetate [4], platelet-derived growth factor [5], nerve growth factor [6,7], and muscarinic acetylcholine agonists [8]. Stimulation induced by these agonists results in a rapid and transient increase in cellular S1P, whose basal levels are generally low. Although the intracellular targets of S1P have yet to be determined, extracellular S1P has been implicated as a ligand for the S1P receptors [9], initiating cell proliferation, survival [10], Ca^{2+} mobilization [11,12], and expression of adhesion molecules [12].

SK was originally cloned in budding yeast [13], but members of the SK family have been identified in plant [14], fly [15], mouse [16], rat [17] and human [18]. All known SKs have five highly conserved domains, C1–C5 [16], that are considered to be involved in the maintenance of the protein structure and the recognition of substrates. SK has known substrate specificities phosphorylating D-erythro-sphingosine and to a lesser extent D-erythro-dihydrosphingosine but not phytosphingosine [16,19,20], and the resulting phosphorylation is ATP-dependent [17]. SK activity is also known to be widely distributed in mouse and rat tissues and, at the cellular level, to reside predominantly in the cytosol [16], although some activity has been found in the mitochondrial and microsomal fractions [21].

Recent studies regarding the structural biology of SKs have revealed three putative calmodulin-binding motifs, which appear to be Ca^{2+} and conformation dependent [22]. SKs translocate to the plasma membrane as a result of associating with calmodulin. However, it remains unclear whether calmodulin has any important biological roles in the SK activity, as there was no obvious influence in an in vitro experiment [23]. Additionally, studies have reported the presence of other motifs, such as the nuclear export signal detected in SK1 [24], and the nuclear localization signal [25], proline rich domain [26], and SH3 binding motif detected in the C-terminus region [27] in SK2. To date, though, no studies have been reported regarding the interaction of SKs with sphingosine (Sph). Therefore, we set out to identify the Sph-binding site in mouse sphingosine kinase 1a (mSK1a), using mutagenesis and analysis of the substrate kinetics.

According to a previous report, the C1–C3 domains of SKs exhibit high homology with protein kinases, and the consensus sequence $SGDGx_{17-21}K$ in the C2–C3 domain is an ATP-binding site with similarity to a highly conserved glycine-rich loop [28] found in many such kinases. The C5 domain is highly conserved, not only in the SKs but also in lipid kinases such as the ceramide kinases (CERKs) [29] and the diacylglycerol kinases (DGKs) [30], whereas the C4 domain is highly conserved only in SKs (Fig. 1). Thus, we considered the C4 domain as a specific recognition site for Sph. So we examined whether this domain was involved.

2. Materials and methods

2.1. Construction of mSK1a mutants

Wild-type mSK1a ($mSK1a^{WT}$) (GenBank™ Accession No. AAL07499) was FLAG epitope-tagged at the 5′-end and subcloned into a pcDNA3 site-directed mutagenesis vector (Invitrogen Corp.,

*Corresponding author. Fax: +81 11 706 4986.
E-mail address: yigarash@pharm.hokudai.ac.jp (Y. Igarashi).

Abbreviations: Sph, sphingosine; SK, sphingosine kinase; S1P, sphingosine 1-phosphate; mSK1a, mouse sphingosine kinase 1a; DGK, diacylglycerol kinase; CERK, ceramide kinase; TBS, Tris-buffered saline; CaM-Sepharose, calmodulin/Sepharose 4B

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.10.081

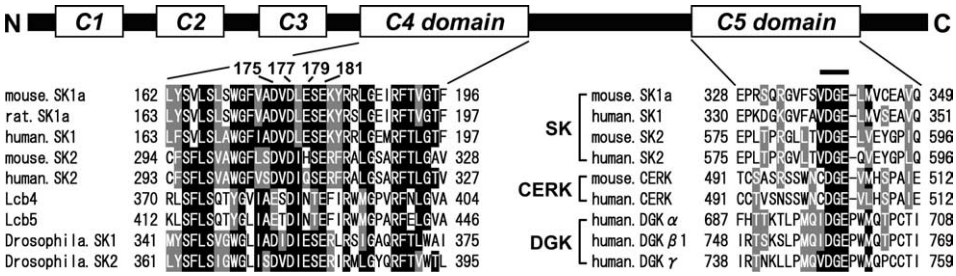

Fig. 1. A partial sequence alignment of SKs. Protein sequences were aligned using the ClustalW method from DNA Data Bank of Japan (DDBJ). Identical amino acids across all aligned proteins are highlighted in black and highly conserved amino acids are highlighted in gray. The left figure shows a sequence alignment of the C4 domain of SKs and the site where we constructed mutants. The right figure shows a sequence alignment between the C5 domains of SKs, CERKs and DGKs.

Carlsbad, CA). Single-stranded DNA was prepared and used as a template for oligonucleotide-directed mutagenesis as detailed in the manufacturer's protocol. The mutagenic oligonucleotides used to generate the point mutant constructs were: for mSK1a^{D175N}, F, 5′-GGCTTTGTTGCTAACGTCGACCTCGAG-3′; R, 5′-CTCGAGGTCGACGTTAGCAACAAAGCC-3′; mSK1a^{D177N}, F, 5′-GTTGCTGACGTCAACCTCGAGAGAGTGAG-3′; R, 5′-CTCACTCTCGAGGTTGACGTCAGCAAC-3′; mSK1a^{E179Q}, F, 5′-GACGTCGACCTCCAGAGTGAGAAGTAC-3′; R, 5′-GTACTTCTCACTCTGGAGGTCGACGTC-3′; mSK1a^{E181Q}, F, 5′-GACCTCGAGAGTCAGAAGTACAGGCGC-3′; R, 5′-GCGCCTGTACTTCTGACTCTCGAGGTC-3′; mSK1a$^{D175N/D177N}$, F, 5′-TTTGTTGCTAACGTCAACCTCGAGAGT-3′; R, 5′-ACTCTCGAGGTTGACGTTAGCAACAAA-3′; mSK1a$^{D177N/E179Q}$, F, 5′-GCTGACGTCAACCTCCAGAGTGAGAAG-3′; R, 5′-CTTCTCACTCTGGAGGTTCACGTCAGC-3′; The mutants were sequenced to verify incorporation of the desired modification.

2.2. Cell culture and transfection

Human embryonic kidney cells (HEK293; ATCC CRL-1573) were cultured on collagen-coated dishes in Dulbecco's modified Eagle's medium D6429 (Sigma–Aldrich Corp., St. Louis, MO) containing 10% (v/v) FBS (Sigma), 100 U/ml penicillin and 100 ng/ml streptomycin (Sigma), at 37 °C in 5% CO_2. Transfections were performed using the LipofectAMINE and LipofectAMINE Plus Reagents (Invitrogen) following the manufacturer's protocol. Cells were harvested and lysed by sonication (2 W for 30 s at 4 °C) in lysis buffer (50 mM Tris–HCl (pH 7.4), 10% glycerol, 0.05% Triton X-100, 150 mM NaCl, 1 mM dithiothreitol, 2 mM Na_3VO_4, 10 mM NaF, 1 mM EDTA and protease inhibitors (Complete™; Roche Diagnostics Corp., Mannheim, Germany)). Protein concentrations of cell lysates were determined with a BCA Protein Assay Reagent Kit (Pierce Biotechnology Inc., Rockford, IL) using bovine serum albumin as a standard.

2.3. Sphingosine kinase assay

Twenty four hours after transfection, cells were harvested as described above. A SK assay was performed as described previously [16] with slight modifications. The reaction was carried out for 30 min at 37 °C in assay buffer (20 mM Tris–HCl (pH 7.4), 25 mM EDTA, 12 mM β-glycerophosphate, 1 mM NaPPi, 5% glycerol, 5 mM Na_3VO_4, 2 mM dithiothreitol, 0.5 mM 4-deoxypyridoxine and protease inhibitors), using the cell lysate as the enzyme and the substrate mixture (50 μM D-erythro-sphingosine (Biomol Research Laboratory Inc., Plymouth Meeting, PA) and 10 μCi of [γ-^{32}P] ATP (10 Ci/mmol; Perkin–Elmer Life Science Inc., Boston, MA) in 1 mM final concentration of ATP in 0.25% Triton X-100). The lipids were separated by thin-layer chromatography on silica gel 60 plates (Merck KGaA, Darmstadt, Germany) with 1-butanol/acetic acid/water (3:1:1, v/v), and the labeled S1P was quantified by a BAS 2500 imaging analyzer (Fuji Photo Film Co., Kanagawa, Japan).

When we performed a SK assay, we used equal amount of protein and the unit of SK activity was defined as the amount of enzyme required to produce 1 pmol of S1P/min. The kinetic data were analyzed using Michaelis–Menten kinetics.

2.4. Calmodulin-binding assay

A calmodulin-binding assay was performed as described previously [22], with slight modifications. HEK293 cells overexpressing mSK1a^{WT} or mSK1a mutants were harvested and lysed as described above. The cell lysates were then centrifuged (100 000 × *g*, 30 min, 4 °C) to remove debris. Aliquots of the supernatants were added to tubes containing calmodulin/Sepharose 4B beads (CaM–Sepharose; Amersham Biosciences Corp., Piscataway, NJ), pre-equilibrated with binding buffer (50 mM Tris–HCl (pH 7.4), 200 mM NaCl, 10% glycerol, 0.2% Triton X-100, 1 mM dithiothreitol, 2 mM Na_3VO_4, 10 mM NaF and protease inhibitors). Additional binding buffer containing 5 mM $CaCl_2$ or 10 mM EGTA was added to each tube, and the mixtures were incubated for 2 h at 4 °C, with continuous mixing. CaM–Sepharose beads were then pelleted by centrifugation (3500 × *g*, 5 min, 4 °C), washed twice with binding buffer containing 5 mM $CaCl_2$ or 10 mM EGTA and, finally, washed in buffer without Triton X-100. Proteins were visualized by Western blotting via their FLAG epitope.

2.5. Western blotting

SDS–PAGE was performed on cell lysates using 12% acrylamide gels according to the method of Laemmli [31]. Proteins were transferred to PVDF membranes (Millipore Corp., Bedford, MA) and the membranes were blocked overnight at 4 °C in Tris-buffered saline with 0.1% Tween 20 (TBS/T), containing 5% skimmed milk powder. This was followed by incubation with a 1:1000 dilution of the monoclonal anti-FLAG antibody M2 (Sigma). The blots were washed with TBS/T, then incubated with a 1:10 000 dilution of horseradish peroxidase-conjugated anti-mouse IgG F(ab′)$_2$ flagment (Amersham). The mSK1a proteins were detected using an enhanced chemiluminescence (ECL) kit (Amersham).

3. Results

3.1. Asp^{177} → Asn^{177} mutation (mSK^{D177N}) decreases the SK activity

We first constructed plasmids of mSK1a mutants to examine the influence of the C4 domain on the catalytic reaction. The constructed plasmids were mutated by substituting Asn or Gln for the negatively charged Asp^{175}, Asp^{177}, Glu^{179} and Glu^{181} residues, which are highly conserved in the C4 domain (Fig. 1). Then, an in vitro kinase assay was performed on each expressed mutant to examine the effects on the enzyme activity. Although all of the mutants tested exhibited decreased activity (mSK1a^{D175N} had only half the activity of the wild type and, mSK1a^{E179Q} and mSK1a^{E181Q} only a quarter), the greatest decrease (to 6% that of wild type) was observed in mSK1a^{D177N} (Fig. 2). Furthermore, we confirmed that the double mutants mSK1a$^{D175N/D177N}$ and mSK1a$^{D177N/E179Q}$, which also lack the Asp^{177} residue, showed an even greater decline in SK activity (Fig. 2). Thus, it appears that the region encompassing Asp^{177} influences the mSK1a activity.

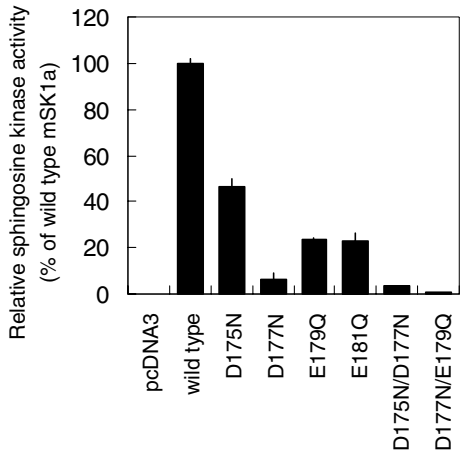

Fig. 2. Relative SK activities of mSK1a mutants. HEK293 cells transfected with empty vector, pcDNA3-mSK1a^{WT}, pcDNA3-mSK1a^{D175N}, pcDNA3-mSK1a^{D177N}, pcDNA3-mSK1a^{E179Q}, pcDNA3-mSK1a^{E181Q}, pcDNA3-mSK1a$^{D175N/D177N}$ or pcDNA3-mSK1a$^{D177N/E179Q}$ were harvested and analyzed for SK activity using D-erythro-sphingosine and ATP as substrates. SK activities given are relative to the activity of mSK1a^{WT}. Data shown are means of three separate experiments with S.D.

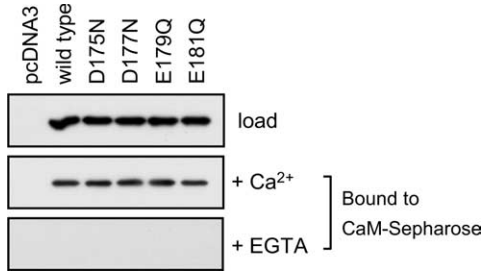

Fig. 3. Selective binding to calmodulin indicates that the mSK1a mutants undergo proper folding. Cell lysates from HEK293 cells expressing each mSK1a mutant (upper frame) were incubated with CaM–Sepharose beads in the presence of 5 mM Ca^{2+} (middle frame) or 10 mM EGTA (bottom frame), which was used as a control to account for any non-specific binding. The beads were then washed and subjected to SDS–PAGE, and each mSK1a protein bound to CaM–Sepharose was visualized by Western blotting via the FLAG epitope.

In a previous report, calmodulin bound only the SK that had undergone proper folding [22]. To examine whether the decline in the SK activity of our mutants occurred as a result of changes in the protein structure, we performed a calmodulin-binding assay in accordance with that report. We determined that calmodulin bound to the SK protein in all the mSK1a mutants. Since this binding was in the presence of Ca^{2+} (Fig. 3), we also executed this assay in the presence of the calcium chelator EGTA as a control. We observed no detectable bands in any of the mutants when Ca^{2+} was absent (Fig. 3), confirming that the bands originally observed were not due to non-specific binding. These results indicated that the mSK1a mutants had undergone proper folding with no collapse in their structure due to the mutagenesis.

3.2. $Asp^{177} \rightarrow Asn^{177}$ mutation ($mSK1a^{D177N}$) diminishes the binding affinity for Sph

In order to examine the affinity of SKs for substrates, SK activities were measured using various concentrations of Sph (final concentrations: 3, 5, 10, 17, 30, 50, 90 and 150 μM). When the results were plotted and K_m values for each enzyme toward Sph were calculated by Lineweaver–Burk plot, we could identify a gentle slope in the plot of mSK1a^{D177N} (Fig. 4B) compared to wild type (Fig. 4A). The K_m values for mSK1a^{WT} and mSK1a^{D177N} toward Sph were 10.1 ± 0.7 and

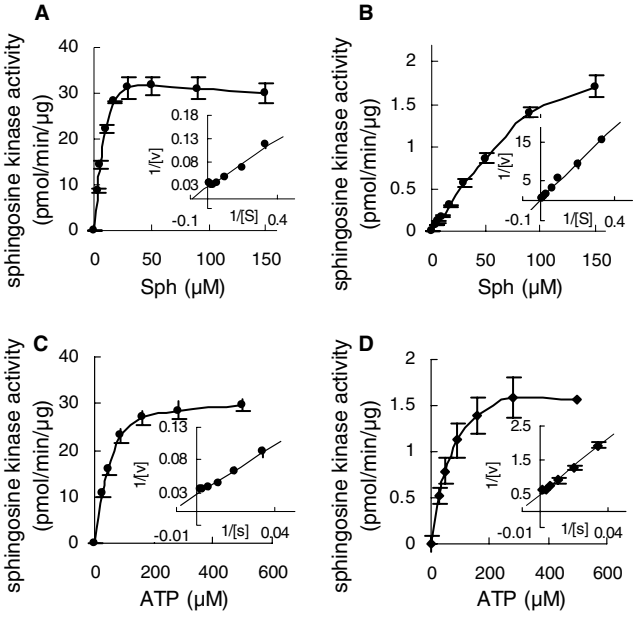

Fig. 4. Michaelis–Menten and Lineweaver–Burk plots for mSK1a proteins. Kinetics of mSK1a^{WT} (A) and mSK1a^{D177N} (B) with Sph as a substrate. SK activities were measured using the various concentrations of Sph and the K_m values toward Sph were calculated by Lineweaver Burk plot (inset plots). Kinetics of mSK1a^{WT} (C) and mSK1a^{D177N} (D) with another substrate, ATP. SK activities at the various concentrations of ATP and the K_m values toward ATP were exhibited. The data shown are means ± S.D. of four independent experiments.

Table 1
Substrate kinetics of mSK1a mutants

mSK1a mutants	V_{max} (pmol/min/μg)	K_m	
		Sph (μM)	ATP (μM)
Wild type	39.1 ± 0.7	10.1 ± 0.7	71.5 ± 10
D175N	18.1 ± 1.3	36.6 ± 8.1	77.4 ± 9.2
D177N	2.4 ± 1.0	108 ± 20	88.4 ± 5.0
E179Q	9.2 ± 0.2	25.7 ± 4.2	62.8 ± 6.5
E181Q	9.0 ± 1.2	10.2 ± 4.1	94.7 ± 13

These data are shown with S.D. by the four independent experiments.

108 ± 20 μM, respectively (Fig. 4A and B), a remarkable rise. We were also able to confirm slight increases in K_m values for mSK1a^{D175N} (36.6 ± 8.1 μM) and mSK1a^{E179Q} (25.7 ± 4.2 μM) (Table 1). This result indicated that a mutation abolishing a negative charge, such as Asp^{177} → Asn^{177}, decreases the recognition ability of the enzyme for Sph, and that the Asp^{177} residue, especially, is involved in the recognition of Sph.

Subsequently, we examined whether Asp^{177} was involved only in the specific recognition of Sph. Using various concentrations of another substrate, ATP (final concentrations: 30, 50, 90, 160, 280 and 500 μM), we again measured the SK activities and calculated the K_m values. No difference was apparent between the K_m values of mSK1a^{WT} and mSK1a^{D177N} for ATP (Fig. 4C and D), indicating that the affinity for ATP remains unchanged after these mutations. We also confirmed that there were no changes in the affinity for ATP in any of the other mutants (mSK1a^{WT}, mSK1a^{D175N}, mSK1a^{E179Q} and mSK1a^{E181Q}, shown in Table 1). Taken together, these results demonstrate that Asp^{177} is involved in the specific recognition of Sph and also support the finding in Fig. 3 that each mutant undergoes proper folding, since all exhibited activity toward ATP.

3.3. Discussion

In this report, we demonstrated that the Asp^{177} residue, which is highly conserved in SKs, is involved in the Sph-recognition. In initial enzyme assays of mSK1a mutants carrying substitutions in the C4 domain, mSK1a^{D177N} carrying the Asp^{177} → Asn^{177} mutation exhibited a great decline in SK activity (Fig. 2). Additionally, the substrate affinity of this mutant towards Sph decreased remarkably, with no change in the affinity towards ATP, indicating that the C4 domain (including the Asp^{177} residue) is involved only in the specific recognition of Sph (Fig. 4 and Table 1). We hypothesize that the negative charge of Asp^{177} may interact with a positive charge of an amino group in Sph, or that Asp^{177} may be involved in a hydrogen bond with a hydroxyl group in Sph. However, the SK activity did not disappear completely. Therefore, it is also probable that in the recognition of Sph not only is the C4 domain involved, but other regions of SK as well. We predicted that the hydrophobic residues in the region adjacent to the C4 domain interact with the hydrophobic part of Sph. In fact, we have confirmed that a mutation in these hydrophobic residues does destroy the SK activity (manuscript in preparation). Although SKs and CERKs are representatives of a novel class of lipid kinases and are not so well understood, we provide for the first time an account of the region involved in the Sph-recognition. However, many things have not yet been explained, such as the mechanism of transferring the phosphate group of ATP to Sph and structural information of the SKs. These remain for future study, perhaps using X-ray crystallographic analysis.

Acknowledgment: This work was supported in part by a Grant-in Aid for Scientific Research on Priority Areas (B) (12140201) from the ministry of Education, Culture, Sports, Science and Technology.

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