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Cloning and characterization of rat sphingosine kinase 1 with an N-terminal extension

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Abstract

Sphingosine kinase (SK) is an enzyme that converts sphingosine to sphingosine 1-phosphate, a lysophospholipid with various cellular functions. Here, we report cloning and characterization of a novel isoform of rat SK1 (rSK1) with an N-terminal extension (long-rSK1). Long-rSK1 is 458 amino acid-long and has a calculated molecular weight of 49.8 kDa. Deduced amino acid sequences of rat and human long-SK1 have high homology (71.3% identity and 78.9% similarity). Long-rSK1 mRNA is widely expressed throughout the tissues including brain, heart, lung, liver, kidney and spleen, whereas mRNA encoding rSK1 has been shown not to be expressed in heart or liver. When the long-rSK1 was transfected in COS-7 cells, SK activity was 53-fold increased. Substrate specificity and dependency on divalent cations of long-rSK1 were similar to those of rSK1. Taken together, the fact that long-rSK1 with similar enzymatic characteristics to rSK1 displays differential tissue distribution may suggest an additional role of long-rSK1.

Keywords

Rat long-sphingosine kinase 1; Sphingosine 1-phosphate; Cloning; Tissue distribution

Introduction

Sphingosine 1-phosphate (S1P), a ligand for a family of five specific G protein-coupled receptors (S1P₁₋₅) and a ligand for an intracellular target to be identified, is implicated in various biological processes including calcium homeostasis, growth, survival, differentiation, cytoskeleton rearrangements, motility, angiogenesis, and immunity (reviewed in ref. [1,2]). The levels of S1P are tightly regulated by the balance between synthesis and cleavage of S1P. Sphingosine kinase (SK) catalyzes the formation of S1P from sphingosine and S1P lyase [3] and S1P phosphatase [4–6] are responsible for degradation and dephosphorylation of S1P, respectively [7]. SKs are highly conserved throughout various organisms from plants to mammals [8]. Two isoforms, SK1 and SK2, have been cloned and characterized so far in

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mammals [9-14]. Although these two enzymes have a high degree of sequence similarity, they are different in kinetic properties and tissue distribution and originate from different genes [2,15]. In addition, they differ in their physiological functions [16]. While SK1 stimulates growth and survival [17,18], SK2, which contains putative BH3 domain, inhibits cell growth and enhances apoptosis [19].

In addition to the two isoforms, many variants of SK1 and SK2 have been identified [15]. SK1 was initially isolated from rat kidney as a 49 kDa protein [13]. Subsequently, two SK1s, SPHK1a and SPHK1b, were cloned from mice [9]. Although these two isoforms are different only in a few N-terminal amino acid residues, they show different characteristics including stability, intracellular distribution, and tissue distribution [20]. With respect to human SK1 [11,12,14], two additional variants were reported and enzymatic activity of these variants has been shown to be similar to that of SK1 [15]. While sequencing rat genomic DNA and cDNA, Imamura et al. identified 8 alternatively spliced mRNAs with alternative first exons within a 3.7-kb CpG island [21,22]. Interestingly, all these spliced isoforms encode the same protein homologous to mouse SPHK1a. The transcript homologous to mouse *sphk1b* is missing in rats [21]. It appears that the presence of the alternatively spliced first exons and a CpG island at the 5' end is common with both human and mouse SK1 [22].

Here we show cloning of an rSK1 isoform with an additional 75 amino acids at the N-terminal end (long-rSK1). We compared the enzymatic characteristics of long-rSK1 with rSK1 and tissue distribution of mRNA expression of long-rSK1 was determined.

Materials and methods

Materials

D-erythro-sphingosine, D-erythro-dihydrosphingosine (DHS), DL-threo-dihydrosphingosine (threoDHS), and N,N-dimethylsphingosine (DMS) were from Biomol (Plymouth Meeting, PA); N-acetyl-D-sphingosine from Sigma (St. Louis, MO); [γ - 32 P]ATP from PerkinElmer (Boston, MA); Silica gel TLC plates from Bodman (Aston, PA). Materials for cell culture were obtained from Invitrogen (Gaithersburg, MD).

Cloning of long-rSK1

In order to obtain predicted mRNA sequences of spliced variants of rSK1, we performed a search by scanning genomic sequences of rat chromosome 10 (accession no. NW_047343), using Genescan 1.0 program (<http://gmen.mit.edu/cgi-bin/genscaw.cgi>). The search gave a candidate mRNA, which would encode rSK1 with an N-terminal extension. To confirm the expression of such mRNA, total RNA was isolated from rat kidney and rat basophilic leukemia (RBL)-2H3, a rat mast cell line, using TRIzol[®] reagent (Invitrogen). The cDNAs were produced using a gene-specific primer (5'-GGGCAGCATCGGTGGCTCCT-3') and N-terminal portion of long-rSK1 was amplified using two primers (sense (P1): 5'-GGTTCTTCGTCCAGAGTA-3', antisense (P2): 5'-AGAGCGCCC-TCGTCATTCCC-3') (Product #1, Fig. 1C). The rest of rSK1 was cloned using two primers (sense (P3): 5'-ATCTGGCCGCCTCCGGAAT-3' antisense (P4): 5'-GGCAGATCCTCATATTGGTTC-3') and as a template, rSK1f cDNA (accession No., BC081738), purchased from Invitrogen (Product #2, Fig. 1C). The full-length long-rSK1 (Product #3, Fig. 1C) was cloned by PCR using two primers (sense (P5): 5'-TCTGGGAAGCTTATGCAACTG-3' antisense: P4) and products #1 and #2 as templates. The coding region of rSK1 was also cloned by PCR using two primers (sense (P6): 5'-CGGGACAAGCTTATGCAACCA-3' antisense: P4) and rSK1f as a template (Product #4, Fig. 1C). PCR products were subcloned into the pCR[®]2.1-TOPO[®] TA cloning vector. Correct sequences of the PCR products were confirmed by sequencing analysis (Johns Hopkins CORE

DNA Analysis Facility). Finally, cDNAs of long-rSK1 and rSK1 were subcloned into the pFlag vector using the HindIII/EcoRI restriction enzymes.

RT-PCR

Wister rat tissues were washed twice with ice-cold phosphate buffered saline and quickly frozen in liquid N₂. Frozen tissues were ground while being kept frozen by continuously adding liquid N₂. Total RNA was isolated from these tissues using TRIzol[®] reagent (Invitrogen). The cDNAs were produced using a random primer. The N-terminal portion of long-rSK1 (Product #1, Fig. 1C) was amplified using two primers, P1 and P2 (Fig. 1C). β -actin was amplified using another pair of primers (sense: 5'-GTGGGCCGGTCTAGGCACCAA-3', antisense: 5'-CTCTTTG-ATGTGACGCACGA-TTTC-3').

Cell culture and transfection

COS-7 cells were maintained as monolayer cultures in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μ g/ml). COS-7 cells were transfected with pFlag-long-rSK1 or pFlag-rSK1, using Lipofectamine[®] according to the manufacturer's instruction. Expressing cells were selected with 2 mg/ml of G418 for 12 days before using for experiments. RBL-2H3 cells were maintained as monolayer cultures in Earle's modified Eagle's medium supplemented with 15% fetal bovine serum, 2 mM glutamine, penicillin (1000 units/ml) and streptomycin (100 μ g/ml).

Western blot analysis

COS-7 cells transfected with pFlag-long-rSK1 or pFlag-rSK1 were lysed by addition of ice-cold lysis buffer, containing 0.5% Triton X-100, 200 mM boric acid (pH 8.0), 150 mM NaCl, 5 mM EDTA, 5 mM NaF, 1 mM Na₃VO₄, 10 μ g/ml aprotinin and pepstatin A and 5 μ g/ml leupeptin. Lysates were left on ice for 30 minutes and centrifuged for 30 minutes at 12,000 $\times$ g at 4°C. The supernatant was used for Western blot analysis using anti-human SK1 antibody raised against C-terminal 20 amino acids of human SK1.

In vitro sphingosine kinase assay

Cytosolic SK activity was measured by incubating various concentration of D-erythro-sphingosine in the reaction mixture containing 0.125% Triton X-100, 10 mM MgCl₂, and [γ -³²P]ATP (5 μ Ci, 1 mM) at 30 °C for 20 minutes as previously described [9].

Results and discussion

Cloning of long-rSK1

As previously mentioned, there has been 8 alternatively spliced mRNAs of rSK1 identified so far with all but b form encoding the same protein [21,22]. We were interested in finding out whether there might be an alternatively spliced mRNA that encodes long form of rSK1 as in the case of human SK1 [15]. We performed a search for spliced variants by scanning genomic sequences of rat chromosome 10, using Genescan 1.0 program. The analysis identified a novel alternatively spliced mRNA, the first two exons of which were derived from the first exon of isoform f (Fig. 1A). To confirm the expression of such mRNA, two PCR primers (a sense primer from exon 1 and an antisense primer from exon 2 shown by two arrowheads in Fig. 1A) were designed and RT-PCR was performed using total RNAs obtained from rat kidney or rat mast cell line RBL-2H3. As shown in Fig. 1B, products corresponding to mRNA f and the long-form were detected. Interestingly, one additional band was produced, which might suggest an additional spliced variant. The sequences of the long-rSK1 from rat kidney and RBL-2H3 cells were confirmed to be identical and the same as the predicted one. To clone the rest of cDNA of the novel isoform, we performed PCR using primers P3 and P4 and rSK1f

(product #2, Fig. 1C). The full-length long-rSK1 (Product #3) and rSK1 (Product #4) were cloned as described in Materials and Methods.

Deduced amino acid sequences of long-rSK1 and its tissue distribution

Deduced amino acid sequences of the long-rSK1 (accession no. DQ486894) were aligned with those of human long-SK1 (Fig. 2). The two sequences have high homology (71.3% identity and 78.9% similarity). The N-terminal region (75 amino acids before initiating methionine of rSK1) of long-rSK1 was also highly homologous to that of human long-SK1 (43.3% identity and 48.9% similarity).

The tissue distribution of the expression of long-rSK1 mRNA in adult rat tissues was analyzed by RT-PCR (Fig. 2B). Long-rSK1 mRNA was expressed in all the tissues tested including brain, heart, lung, liver, kidney, spleen and RBL-2H3 cells. Ratio of long-rSK1 over β -actin indicates highest expression of long-rSK1 in spleen followed by Kidney. The tissue distribution pattern was different from that of *sphk1* mRNAs previously reported by Imamura et al [21], in which *sphk1* mRNAs were expressed in rat adult brain, kidney, spleen and lung but not in heart or liver.

Kinetics of long-rSK1

To ensure that the cloned long-rSK1 was indeed a functional SK1, we transfected COS-7 cells with pFlag-long-rSK1 or pFlag-rSK1. Expression of these proteins was confirmed by Western blot analysis (Fig. 3A). The relative expression levels of long-rSK1 and rSK1 were similar as measured by Western blot analysis and the ratio of rSK1 expression over that of long-rSK1 was 0.93 ± 0.08 (mean $\pm$ SD). SK activity in lysates of the transfected cells was measured after adjusting the amounts of the lysates to keep the same amounts of overexpressed enzymes. SK activity in long-rSK1- or rSK1-transfected cells was 53- and 20-fold increased, respectively. Kinetics of long-rSK1 and rSK1 were determined using *D-erythro*-sphingosine as a substrate over the concentration range of 5-100 μ M. The enzyme reaction showed a typical Michaelis-Menten kinetics reaching maximal velocity with increasing concentrations of *D-erythro*-sphingosine (Fig. 3B). The corresponding double reciprocal plots of long-rSK1 and rSK1 indicate that both enzymes have similar K_m values of 6.1 μ M and 4.4 μ M, respectively and V_{max} for long-rSK1 is about 2.5 folds higher than that for rSK1 (Fig. 3C).

Substrate specificity and requirements for divalent cations

The substrate specificity of long-rSK1 and rSK1 was very similar to those of previously purified rat [13] and cloned mouse and human SK1s [12,14]. The best substrate was *D-erythro*-sphingosine, followed by DHS (Fig. 3D). Long-rSK1 appeared not to discriminate DHS from *D-erythro*-sphingosine so much compared to rSK1 and showed only 20% less activity towards DHS while rSK1 showed about 40% less activity. No phosphorylation was observed with threoDHS and DMS, which are known to be competitive inhibitors of SK1. *N*-acetyl-*D*-sphingosine, also known as C_2 -ceramide, was not a substrate. As in the case with *D-erythro*-sphingosine (Fig. 3B, C), the activity of long-rSK1 towards DHS was about 3.6 fold higher than that of rSK1 (Fig. 3D). Both isoforms required divalent cations for their activity and the presence of EDTA in the reaction mixture eliminated activity. Consistent with previous report with human SK1 [14], both enzymes showed highest activity with Mg^{2+} , followed by Mn^{2+} and Ca^{2+} (Fig. 3E). In our experimental conditions, Mn^{2+} was almost equally effective as Mg^{2+} in supporting SK activity of both enzymes.

Competitive inhibition of long-rSK1

SK activity of long-rSK1 and rSK1 was determined with varying concentrations (2.5-50 μ M) of *D-erythro*-sphingosine in the absence or presence of 20 μ M or 40 μ M DMS. As expected,

the activity of both enzymes was inhibited by DMS (Fig. 4A, B). Double reciprocal plots suggested that DMS displayed competitive inhibition with increased K_m values for long-rSK1 to 39.7 μM and 52.0 μM with 20 μM and 40 μM of DMS, respectively and those for rSK1 to 16.7 μM and 43.8 μM (Fig. 4C, D). K_i values for long-rSK1 and rSK1 were 5.8 μM and 0.33 μM of DMS, respectively.

To summarize, long-rSK1 mRNA is a novel isoform of rat *sphk1* that uniquely encodes an additional 75 amino acids at the N-terminal end of rSK1, whereas the 7 other isoforms encodes rSK1. It is plausible to think that the N-terminal region of long-rSK1 may serve as a regulatory domain, which receives signals from upstream or play a role in localization of the enzyme in different subcellular organelles. In fact, our study with human long-SK1 fused with green fluorescent protein indicated that long-SK1 is partially associated with the endoplasmic reticulum (ER) and further translocated to the ER upon receptor stimulation (unpublished observation, Jung, ID and Choi, OH). This might suggest that long-rSK1 is involved in cell signaling.

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References

1. Spiegel S, Milstien S. Functions of the Multifaceted Family of Sphingosine Kinases and Some Close Relatives. *J Biol Chem* 2007;282:2125–2129. [PubMed: 17135245]
2. Spiegel S, Milstien S. Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol* 2003;4:397–407. [PubMed: 12728273]
3. Saba JD, Nara F, Bielawska A, Garrett S, Hannun YA. The BST1 gene of *Saccharomyces cerevisiae* is the sphingosine-1-phosphate lyase. *J Biol Chem* 1997;272:26087–26090. [PubMed: 9334171]
4. Mandala SM, Thornton R, Galve-Roperh I, et al. Molecular cloning and characterization of a lipid phosphohydrolase that degrades sphingosine-1-phosphate and induces cell death. *Proc Natl Acad Sci U S A* 2000;97:7859–7864. [PubMed: 10859351]
5. Ogawa C, Kihara A, Gokoh M, Igarashi Y. Identification and Characterization of a Novel Human Sphingosine-1-phosphate Phosphohydrolase, hSPP2. *J Biol Chem* 2003;278:1268–1272. [PubMed: 12411432]
6. Le Stunff H, Peterson C, Liu H, Milstien S, Spiegel S. Sphingosine-1-phosphate and lipid phosphohydrolases. *Biochim Biophys Acta* 2002;1582:8–17. [PubMed: 12069805]
7. Saba JD, Hla T. Point-counterpoint of sphingosine 1-phosphate metabolism. *Circulation Research* 2004;94:724–734. [PubMed: 15059942]
8. Leclercq TM, Pitson SM. Cellular signalling by sphingosine kinase and sphingosine 1-phosphate. *IUBMB Life* 2006;58:467–472. [PubMed: 16916784]
9. Kohama T, Olivera A, Edsall L, Nagiec MM, Dickson R, Spiegel S. Molecular cloning and functional characterization of murine sphingosine kinase. *J Biol Chem* 1998;273:23722–23728. [PubMed: 9726979]
10. Liu H, Sugiura M, Nava VE, et al. Molecular cloning and functional characterization of a novel mammalian sphingosine kinase type 2 isoform. *J Biol Chem* 2000;275:19513–19520. [PubMed: 10751414]
11. Melendez AJ, Carlos-Dias E, Gosink M, Allen JM, Takacs L. Human sphingosine kinase: molecular cloning, functional characterization and tissue distribution. *Gene* 2000;251:19–26. [PubMed: 10863092]
12. Nava VE, Lacana E, POULTON S. Functional characterization of human sphingosine kinase-1. *FEBS Lett* 2000;473:81–84. [PubMed: 10802064]
13. Olivera A, Kohama T, Tu Z, Milstien S, Spiegel S. Purification and characterization of rat kidney sphingosine kinase. *J Biol Chem* 1998;273:12576–12583. [PubMed: 9575218]

14. Pitson SM, D'andrea RJ, Vandeleur L, et al. Human sphingosine kinase: purification, molecular cloning and characterization of the native and recombinant enzymes. *Biochem J* 2000;350:429–441. [PubMed: 10947957]
15. Billich A, Bornancin F, Devay P, Mechtcheriakova D, Urtz N, Baumruker T. Phosphorylation of the Immunomodulatory Drug FTY720 by Sphingosine Kinases. *J Biol Chem* 2003;278:47408–47415. [PubMed: 13129923]
16. Maceyka M, Sankala H, Hait NC, et al. SphK1 and SphK2, Sphingosine Kinase Isoenzymes with Opposing Functions in Sphingolipid Metabolism. *J Biol Chem* 2005;280:37118–37129. [PubMed: 16118219]
17. Olivera A, Kohama T, Edsall L, et al. Sphingosine kinase expression increases intracellular sphingosine-1-phosphate and promotes cell growth and survival. *J Cell Biol* 1999;147:545–558. [PubMed: 10545499]
18. Olivera A, Rosenfeldt HM, Bektas M, et al. Sphingosine Kinase Type 1 Induces G12/13-mediated Stress Fiber Formation, yet Promotes Growth and Survival Independent of G Protein-coupled Receptors. *J Biol Chem* 2003;278:46452–46460. [PubMed: 12963721]
19. Liu H, Toman RE, Goparaju SK, et al. Sphingosine Kinase Type 2 Is a Putative BH3-only Protein That Induces Apoptosis. *J Biol Chem* 2003;278:40330–40336. [PubMed: 12835323]
20. Kihara A, Anada Y, Igarashi Y. Mouse Sphingosine Kinase Isoforms SPHK1a and SPHK1b Differ in Enzymatic Traits Including Stability, Localization, Modification, and Oligomerization. *J Biol Chem* 2006;281:4532–4539. [PubMed: 16368679]
21. Imamura T, Ohgane J, Ito S, et al. CpG Island of Rat Sphingosine Kinase-1 Gene: Tissue-Dependent DNA Methylation Status and Multiple Alternative First Exons. *Genomics* 2001;76:117–125. [PubMed: 11560121]
22. Imamura T, Miyauchi-Senda N, Tanaka S, Shiota K. Identification of genetic and epigenetic similarities of SPHK1/Sphk1 in mammals. *J Vet Med Sci* 2004;66:1387–1393. [PubMed: 15585953]

Fig. 1.

Cloning of long-rSK1. (A) Comparison of mRNA of long-rSK1 with those of 7 other known SK1 subtypes. Lines and boxes indicate introns and exons, respectively. Numbers in the boxes indicate exon numbers and first exons and part of first introns are truncated for SK1a, c, d, e, g and h. The arrows shown above SK1a or below the long-form indicate the translation initiation site for all 7 subtypes or long-form, respectively. The arrow heads indicate the location of PCR primers. (B) PCR products, produced using primers indicated with two arrow heads in panel A, were separated on 1.2% agarose gel (lane 1, 100 bp DNA ladder; 2, rat kidney; 3, RBL-2H3 cells). (C) Schematic description of cloning of full-length long-rSK1 and rSK1. P1, P2, P3, P4, P5 and P6 indicate sites for primers and Products #1, #2, #3 and #4 indicate PCR products made using indicated pairs of primers.

A

		1		60
Rat	(1)	-----MQLDSPPTSKDLAASGNDEGALTAQG-GGEGEPHGQSP	TRLGNTDKQL	
Human	(1)	MSAQVLGFLRSWTPLPLAAPRGPAAGNDAGAPTATAPGGEGEPHSR	PCDARLGSTDKEL	
		61	*	120
Rat	(49)	HTGAAVTSSSAGAAASVAPCOREPRDLAMQPADCP	RGILLPRPCRVLLNPRGGKGLKAL	
Human	(61)	KAGAAATGS-APTAPGTPWQREPRVEVMDPAGG	PRGVLPRPCRVLLNPRGGKGLKAL	
		121		180
Rat	(109)	FQSRVRPLLEAEVSFKLMLTERONHARELVCAEELGHWDALA	VMSGDGLMHEVVNGLME	
Human	(120)	FRSHVQPLLAEEISFTLMLTERNHARELVRSEELGRWDALV	VMSGDGLMHEVVNGLME	
		181		240
Rat	(169)	RPDWESAIQKPLCSLPGSGNALAASLNYYAGHEQVTNEDLL	INCTLLLCRQLSPMNLL	
Human	(180)	RPDWETAIQKPLCSLPAGSGNALAASLNHYAGYEQVTNEDLL	TNCTLLLCRRLSPMNLL	
		241		300
Rat	(229)	SLHTASGRQLYSVLSLSWGFVADVLESEKYRS	LGELIRFTVGTFFRLASLRIYQQLAYL	
Human	(240)	SLHTASGLRLFSVLSLAWGFIADVLESEKYRRLGEMRFTL	GTFLRLAALRTYRRLAYL	
		301		360
Rat	(289)	PVGKAASKIPASSLA-QKGPANTYLVPLEEPVPPHWT	VVPEQDFVLVLVLLHHLSTEMF	
Human	(300)	PVGRVGSKTASPVVVQQGFVDAHLVPLEEPVPSHWT	VVPEDEDFVLVLALLHSHLGSEMF	
		361		420
Rat	(348)	AAPMGRCEAGVMHLFYIRAGVSRAMLVRLFLAMQ	KGKHMDDLCPYLHVHPVVAFRLEPRN	
Human	(360)	AAPMGRCAAGVMHLFYVRAGVSRAMLLRLFLAME	KGRHMEYECPYLVYVPVVAFRLEPKD	
		421		471
Rat	(408)	QRGVFSVDGELMVCEAVQGQVHPNYLWMVSGSSDS	PSGRDSQRRPPPEEPI	
Human	(420)	GKGVFAVDGELMVSEAVQGQVHPNYFWMVSGC	VEPEPPSWKPPQMPPEEPL	

B **Fig. 2.**

Alignment of deduced amino acid sequences of rat and human long-SK1 and the tissue distribution of the expression of long-rSK1 mRNAs. (A) Sequence alignment of deduced amino acid sequences of rat and human long-SK1 was performed by ClustalW. Identical sequences were shaded in dark grey and similar sequences in grey background. The initiation methionines of rat and human SK1s are indicated with *. (B) Tissue distribution of expression of long-rSK1 mRNAs. Tissue-specific expression of long-rSK1 mRNAs in adult rat tissues was analyzed by RT-PCR. cDNAs corresponding to 0.3 µg of total RNA of brain, heart, lung, liver, kidney, spleen and RBL-2H3 cells were produced using a random primer. Using gene-specific primers described in the Materials and methods, long-rSK1 (L-rSK1) and β-actin were

amplified. Numbers indicate the ratio of the intensities of long-rSK1 over those of β -actin. The depicted data are representative of four experiments with similar results.

Fig. 3. Kinetics, substrate specificity, and divalent cation requirements of long-rSK1 and rSK1. (A) Western blot analysis of long-rSK1 and rSK1. Total cell lysates of COS-7 cells (equivalent to 8,000 cells/lane) transfected with pFlag-long-rSK1 (lane 1) or pFlag-rSK1 (lane 2) were separated on a 10% acrylamide gel. (B) Kinetics of long-rSK1 (■) and rSK1 (▲) with D-erythro-sphingosine. [³²P]S1P spots on autoradiogram were quantified by densitometry. The strongest spot which obtained either with 50 μM or 100 μM D-erythro-sphingosine and long-rSK1 was set to be 100%. Values are mean ± SD (n=3). (C) The corresponding double reciprocal plots. (D) Substrate specificity of the long-rSK1 (open bars) and rSK1 (filled bars) with sphingosine analogues at 50 μM. The rates of phosphorylation of D-erythro-sphingosine (Sph) by the long-rSK1 were arbitrarily set at 100%. Activity against other potential substrates was expressed relative to the activity against Sph. acetylSph, N-acetyl-D-sphingosine. Values are means ± SD (n=3). (E) Requirements of divalent cations. The various divalent cations or EDTA were included in the assay mixture at a final concentration of 10 μM. The amounts of [³²P]S1P produced by long-rSK1 in the presence of MgCl₂ was set at 100%. Values are means ± SD (n=3).

Fig. 4. Inhibition Kinetics of long-rSK1 and rSK1. (A, B) Saturation curves with long-rSK1 (A) and rSK1 (B) in the presence of DMS at 0 μM (■), 20 μM (▲) and 40 μM (▼). The strongest [³²P]S1P spot obtained either with 25 μM or 50 μM *D-erythro*-sphingosine and long-rSK1 was considered to be 100%. Values are mean ± SD (n=3). (C, D) The corresponding double reciprocal plots.