A point mutant of human sphingosine kinase 1 with increased catalytic activity

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Abstract Sphingosine kinase (SK) catalyses the formation of sphingosine 1-phosphate, a lipid second messenger that has been implicated in mediating such fundamental biological processes as cell growth and survival. Very little is currently known regarding the structure or mechanisms of catalysis and activation of SK. Here we have tested the functional importance of Gly^{113}, a highly conserved residue of human sphingosine kinase 1 (hSK), by site-directed mutagenesis. Surprisingly, a $Gly^{113} \rightarrow Ala$ substitution generated a mutant that had 1.7-fold greater catalytic activity than wild-type hSK (hSK^{WT}). Our data suggests that the $Gly^{113} \rightarrow Ala$ mutation increases catalytic efficiency of hSK, probably by inducing a conformational change that increases the efficiency of phosphoryl transfer. Interestingly, hSK^{G113A} activity could be stimulated in HEK293T cells by cell agonists to a comparable extent to hSK^{WT}. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

1. Introduction

Sphingosine kinase (SK) catalyses the formation of sphingosine 1-phosphate (S1P), a lipid second messenger that appears to be involved in mediating such diverse and fundamental biological processes as cell growth and survival, as well as calcium mobilisation, and adhesion molecule expression [1–4]. Cultured mammalian cells, in the absence of stimulating factors, have basal SK activity that is a consequence of the intrinsic catalytic activity of human SK1 (hSK) [5]. This SK activity can, however, be rapidly increased by various stimuli, including tumour necrosis factor-α (TNFα) [3,6], phorbol esters [7], platelet-derived growth factor [8], nerve growth factor [9], *N*-formyl-methionyl-leucyl-phenylalanine [10], muscarinic acetylcholine agonists [11], and engagement of the FcϵRI [12] and FcγRI [13] antigen receptors. Stimulation of SK activity

by these agonists results in rapid and transient increases in cellular S1P, the basal levels of which are generally low [14]. This increase in S1P triggers numerous downstream signaling pathways [1,2] that can be blocked by the presence of SK inhibitors [6] or a dominant-negative SK we have recently developed that blocks SK activation [7].

We [5] and others [15,16] have recently cloned hSK. Sequence analysis of hSK [5,7] or other recently cloned SKs [17–20] has provided very little information regarding its structure, location of the substrate-binding sites, or possible activation mechanisms. Residues 16 to 153 of hSK have similarity to the putative diacylglycerol kinase (DGK) catalytic domain [7], possessing 17 of the 24 very highly conserved amino acids of this domain. This includes the residue (Gly^{82} in hSK) that is known to be essential for catalytic activity in DGK [21,22], which we have also recently shown to be essential for the catalytic activity of hSK [7]. However, like SKs, very little else is known of the structure of DGKs. hSK has three putative calcium/calmodulin-binding motifs, consistent with the observation that hSK binds to calmodulin–Sepharose 4B in the presence of calcium [5,23]. However, calcium/calmodulin does not appear to modulate the catalytic activity of hSK in vitro [5]. hSK has no other obvious regulatory domains, with the possible exception of a proline-rich region at the C-terminus which has resemblance to SH3-binding domains. Furthermore, there is no close similarity to any established ATP-binding motifs, or recognisable pockets of hydrophobic residues that may be involved in sphingosine binding. Thus, very little is known of the mechanisms of catalysis or regulation of hSK.

In this study we have described the mutagenesis of Gly^{113} of hSK, a residue highly conserved between the known SKs and many DGKs [7]. We find that introduction of a bulky, negatively charged residue at this site ($Gly^{113} \rightarrow Asp$) substantially decreases the activity of the enzyme but that, remarkably, introduction of a more conservative change ($Gly^{113} \rightarrow Ala$) creates an enzyme that exhibits increased activity relative to the wild-type enzyme.

2. Materials and methods

2.1. Construction and expression of hSK mutants
Wild-type hSK (hSK^{WT}) cDNA [5] (GenBank accession number AF200328) was FLAG epitope-tagged at the 3′-end and subcloned into pALTER (Promega, Madison, WI, USA) site-directed mutagenesis vector, as previously described [7]. Single-stranded DNA was prepared and used as a template for oligonucleotide-directed muta-

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genesis as detailed in the manufacturer's protocol. The mutagenic oligonucleotides 5′-CCCAGCAGGATCCGACAACGCGCT-3′ and 5′-CCCAGCAGGATCCGCCAACGCGCT-3′ were designed to generate the hSK[G113D] (hSK with Gly[113]→Asp mutation) and hSK[G113A] (hSK with Gly[113]→Ala mutation) mutants, respectively. Both mutants were sequenced to verify incorporation of the desired modification. The mutant cDNA were then subcloned into pcDNA3 (Invitrogen, San Diego, CA, USA) for transient transfection into HEK293T cells.

2.2. Cell culture and transfection

Human embryonic kidney cells (HEK293T, ATCC CRL-1573) were cultured on Dulbecco's modified Eagle's medium (CSL Biosciences, Parkville, Australia) containing 10% foetal calf serum, 2 mM glutamine, 0.2% (w/v) sodium bicarbonate, penicillin (1.2 mg/ml), and gentamycin (1.6 mg/ml). Transfections were performed using the calcium phosphate precipitation method [24]. Cells were harvested 24 h after transfection and lysed by sonication (2 W for 30 s at 4°C) in lysis buffer containing 50 mM Tris–HCl (pH 7.4), 10% (w/v) glycerol, 0.05% (w/v) Triton X-100, 150 mM NaCl, 1 mM dithiothreitol, 2 mM Na_3VO_4, 10 mM NaF, 1 mM EDTA and protease inhibitors (Complete™; Boehringer Mannheim). Activation of hSK[G113A] was assessed by treatment of cells with either phorbol 12-myristate 13-acetate (PMA; Sigma) for 30 min, or TNFα (R&D Systems, Minneapolis, MN, USA) for 10 min, as previously described [7]. Protein concentrations in cell homogenates were determined with Coomassie brilliant blue (Sigma) reagent using bovine serum albumin (BSA) as standard.

2.3. SK assays

SK activity was routinely determined using D-erythro-sphingosine (Biomol, Plymouth Meeting, PA, USA) and [γ-^{32}P]ATP (Geneworks, Adelaide, South Australia) as substrates, as described previously [5]. A unit (U) of SK activity is defined as the amount of enzyme required to produce 1 pmol S1P/min. Substrate kinetics were analysed using Michaelis–Menten kinetics with the non-linear regression program, Hyper 1.1 s. For assays examining substrate presentation, sphingosine (2 mM) was solubilised in 2% (w/v) BSA in 50 mM Tris–HCl, pH 7.4 by sonication (4 W for 30 s at 4°C).

2.4. Calmodulin-binding assay for assessing correct folding of SK mutants

To assess the effect of mutations on gross folding of hSK we have developed an assay which exploits the known interaction of hSK with calmodulin, which occurs only with correctly folded hSK protein [5]. HEK293T cells overexpressing hSK[WT] or mutant hSKs were harvested and lysed as described above. The cell lysates were then centrifuged (13 000×g, 15 min at 4°C) to remove cell debris. Aliquots of the supernatants were added to tubes containing calmodulin–Sepharose 4B (Amersham Pharmacia Biotech) pre-equilibrated with binding buffer composed of 50 mM Tris–HCl (pH 7.4), 5 mM $CaCl_2$, 200 mM NaCl, 10% (w/v) glycerol, 0.05% (w/v) Triton X-100, 1 mM dithiothreitol, 2 mM Na_3VO_4, 10 mM NaF and protease inhibitors (Complete™). Further binding buffer was then added and the mixtures incubated for 2 h at 4°C with continuous mixing. The calmodulin–Sepharose 4B beads were then pelleted by centrifugation (3500×g, 5 min at 4°C) and washed twice with binding buffer. hSK bound to the beads were then resolved by sodium dodecyl sulphate–polyacrylamide gel eletrophoresis (SDS–PAGE) and visualised by Western blotting via their FLAG epitope. Binding of the hSK[WT] and mutant hSKs to Sepharose CL-4B (Amersham Pharmacia Biotech) was also assessed as a control for non-specific binding to the Sepharose 4B beads.

2.5. Western blotting

SDS–PAGE was performed on cell lysates according to the method of Laemmli [25] using 12% acrylamide gels. Proteins were blotted to nitrocellulose and the membranes blocked overnight at 4°C in PBS containing 5% skim milk powder and 0.1% (w/v) Triton X-100. hSK expression levels in cell lysates were quantitated over a dilution series of the lysates with the monoclonal M2 anti-FLAG antibody (Sigma), with the immunocomplexes detected with both horseradish peroxidase (HRP) anti-mouse (Pierce) IgG using an enhanced chemiluminescence kit (ECL, Amersham Pharmacia Biotech), and alkaline phosphatase anti-mouse (Selinus/Amrad, Melbourne, Australia) IgG using an ECF kit (Amersham Pharmacia Biotech) and a Molecular Dynamics (Sunnyvale, CA, USA) fluorimager.

3. Results and discussion

3.1. Site-directed mutagenesis of hSK

We have recently cloned and characterised hSK [5]. Our analysis has shown similarity in residues 16 to 153 of the hSK amino acid sequence to the putative catalytic domain of DGKs [7]. Several amino acids are highly conserved in this region between the known SKs and many DGKs [7]. We have recently reported that mutation of one of these conserved residues (Gly[82]→Asp) results in a catalytically inactive hSK [7]. Another highly conserved residue corresponds to Gly[113] of hSK. This residue, like Gly[82] [7], occurs within a pocket of highly conserved residues rich in glycines (Fig. 1), a characteristic often associated with loop regions coordinating nucleotide binding [27,28]. To test the functional importance of this highly conserved residue, we carried out site-directed mutagenesis on Gly[113] in hSK.

Two mutations were performed to study the importance of Gly[113] in hSK. Initially we generated a disruptive Gly[113]→Asp mutation. This mutation increases the size of

Human SK1	106	CSLPAGSGNALAAS	119	
Mouse SK1		CSLPGGSGNALAAS		
Human SK2		GILPCGSGNALAGA		
Mouse SK2		GVLPCGSGNALAGA		
S. cerevisiae LCB4		TQLPCGSGNAMSIS		
S. cerevisiae LCB5		TEIPCGSGNAMSVS		
S. pombe SK		CMIPGGSGNAFSYN		
C. elegans SK		GIVPSGSGNGLLCS		
Arabidosis SK		GMVPAGTGNGMIKS		
Arabidosis SK		GIVPAGSDNSLVWT		
Drosophila SK		GIIPCGSGNGLAKS		
Human DGKα/γ/ε		AVLPLGTGNDLARC		
Human DGKβ/δ		AILPLGTGNDLARC		
Human DGKζ		AILPLGTGNDLART		
Human DGKη		GVLPLGTGNDLARV		
Human DGKθ		AILPLGTGNDLGRV		
Human DGKι		GVLPLGTGNDLART		
Pig DGKα		AVLPLGTGNDLARC		
Hamster DGKδ		GVLPLGTGNDLARV		
Mouse DGKα		AVLPLGTGNDLARC		
Mouse DGKε		AVLPLGTGNDLSNT		
Rat DGKα/γ		AVLPLGTGNDLARC		
Rat DGKβ/ζ		AILPLGTGNDLARC		
Tomato DGK		ATVPLGTGNNLPFA		
Arabidopsis DGK1		AILPAGTGNDLSRV		
Arabidopsis DGK		GVIPLGTGNDLSRS		
Arabidopsis DGK		AVMPLGTGNDLARV		
Synechocystis DGK		GIIPRGTANAFSVA		
M. tuberculosis DGK		AVVPGGSANVLARA		
C. elegans DGK		GIVPLGTGNDLARV		
C. elegans DGK		AILPLGTGNDLARV		
C. elegans DGK		AVLPLGTGNDLARC		
Drosophila DGK1		GVIPLGTGNDLARC		
Drosophila DGK2		GVLPLGTGNDLARA		
Drosophila DGKε		AIMPLGTGNDLSRV		

Fig. 1. Partial sequence alignment of SKs and some DGKs showing high sequence conservation around Gly[113] of hSK. The sequence alignment was from the DGK putative catalytic domain family of the SMART database [26]. Highly conserved residues within this region are highlighted. The marked (●) residue indicates Gly[113] of hSK [5].
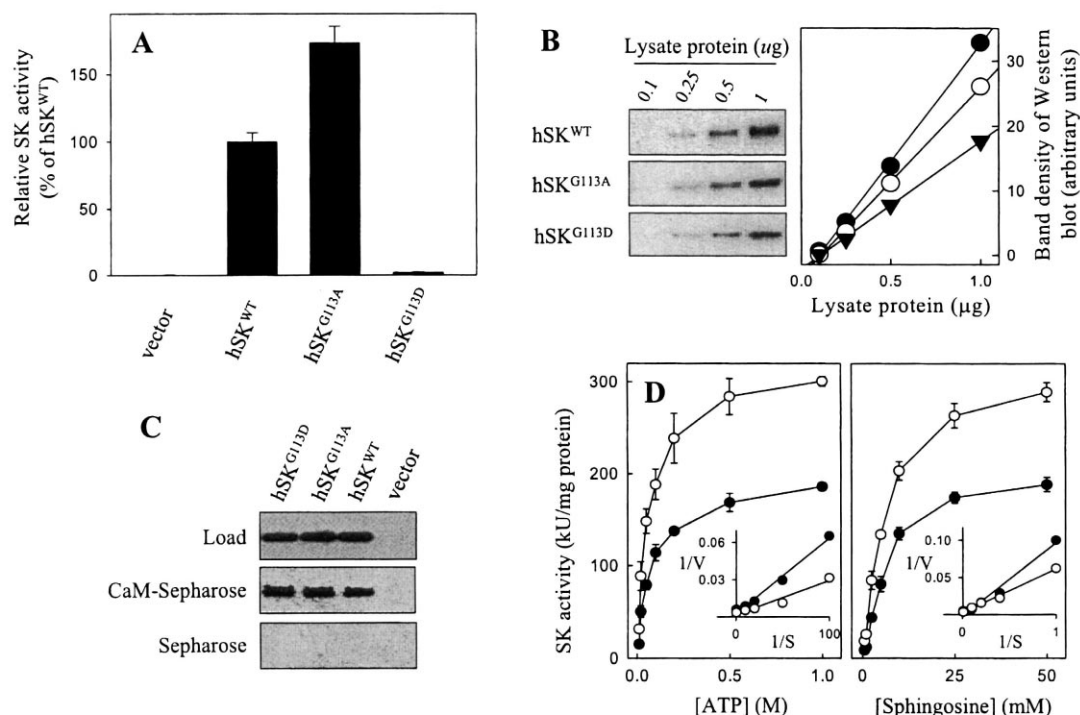
Fig. 2. Site-directed mutagenesis of Gly[113] of hSK. A: HEK293T cells transfected with either pcDNA3-hSK[WT], pcDNA3-hSK[G113A] pcDNA3-hSK[G113D] or empty pcDNA3 vector were harvested and analysed for SK activity. SK activities given are relative to the activity of hSK[WT] and corrected for slight differences in hSK expression levels. B: Expression of the hSKs were quantitated over a dilution range (0.1 to 1 μg protein) by Western blot using the anti-FLAG antibody, anti-mouse HRP and ECL reagent. Band densities for hSK[WT] (●), hSK[G113A] (○) and hSK[G113D] (▼) were quantitated using ImageQant software (Molecular Dynamics), showing linearity in the quantitation over this protein concentration range with correlation coefficients of 0.991, 0.995 and 0.997 for hSK[WT], hSK[G113A] and hSK[G113D], respectively. Quantitation of hSK expression by Western blot using the anti-FLAG antibody, anti-mouse alkaline phosphatase and ECF reagent gave similar results (data not shown). C: Selective binding to calmodulin (CaM)–Sepharose indicated that the mutant hSKs were correctly folded. D: Substrate kinetics with ATP and sphingosine for hSK[WT] (●) and hSK[G113A] (○) indicate that the Gly[113] → Ala mutation increases the catalytic efficiency but does not alter the binding affinity for either substrate. K_m values for ATP of hSK[WT] and hSK[G113A] were 85 ± 12 and 71 ± 10 μM, respectively, while K_m values for sphingosine were 17 ± 3 and 20 ± 4 μM, respectively. V_{max} values for hSK[WT] and hSK[G113A] were 215 ± 24 and 327 ± 31 μU/mg lysate protein, respectively. All data shown are means $\pm$ S.D. of triplicate determinations.

the residue and introduces a negative charge and is expected to be maximally disruptive to short-range interactions of the glycine residue. We then generated a more conservative Gly[113] → Ala mutation to test whether subtle changes in structure at this site would affect catalytic activity. Expression of hSK[G113D], hSK[G113A] and hSK[WT] in HEK293T cells, followed by quantitative Western blotting showed that while all three proteins were present in similar mass levels (Fig. 2B), their SK activities in the cell lysates varied dramatically (Fig. 2A).

As previously reported [5,7], overexpression of hSK[WT] produces high cellular SK activity, with a specific activity of 185 kU/mg protein. This represents an increase in SK activity of approximately 2000-fold over the low endogenous SK activity present in these cells. However, expression of similar mass levels of hSK[G113D] resulted in only very low levels of SK activity (Fig. 2A). In fact, hSK[G113D] showed less than 2% of the activity of hSK[WT], when normalised for protein expression levels. To assess the effect of this mutation on gross folding of hSK we exploited the known interaction of hSK with calmodulin, which occurs only with correctly folded hSK protein [5]. Like hSK[WT], hSK[G113D] bound specifically to calmodulin–Sepharose (Fig. 2C) verifying that the protein was still folded in the correct manner to allow this interaction to exist [5]. Therefore, the Gly[113] → Asp mutation had a pro-

found negative effect on the catalytic activity of hSK, suggesting that Gly[113] has an important role in the catalytic mechanism of hSK. Interestingly, while Gly[113] is highly conserved in SKs and many DGKs, an aspartate occurs at the corresponding position in a SK from *Arabidopsis thaliana* (Fig. 1) [20]. The functional characterisation of this enzyme, however, has not been reported. Therefore, it is not known if this *Arabidopsis* SK has low specific activity, or if a divergence in another region of the protein compensates for the presence of this aspartate.

Surprisingly, expression of hSK[G113A], containing the more conservative Gly[113] → Ala mutation, in HEK293T cells produced considerably higher SK activity than those transfected with hSK[WT] (Fig. 2A). In fact, when normalised for protein expression levels the Gly[113] → Ala mutation increased catalytic activity of hSK[WT] by 1.7-fold under our standard assay conditions. This is a significant increase in catalytic activity given that agonist stimulation of hSK in various cell types results in approximately two-fold increases in SK activity [3,7,12,29]. Substrate kinetic analysis of hSK[G113A] (Fig. 2D) demonstrated that the observed increased activity of this mutant was a consequence of a higher V_{max}, rather than altered substrate affinity, since hSK[G113A] gave comparable K_m values for ATP and sphingosine as hSK[WT].

3.2. hSK^{G113A} has the same stability and substrate accessibility properties as hSK^{WT}

One explanation for increased activity of hSK^{G113A} in cell lysates is that the Gly^{113} → Ala mutation may have conferred increased stability of the protein. Previously, Gly → Ala mutations have been reported to increase the stability of lysozyme and some other proteins by decreasing the entropy of unfolding [30]. To test this we examined the thermostability of both hSK^{WT} and hSK^{G113A}. The results (Fig. 3A) show that both enzymes have almost identical thermostabilities. This indicates that the observed higher activity of hSK^{G113A} is not a consequence of enhanced protein stability over hSK^{WT}.

Next, we considered the possibility that the Gly^{113} → Ala mutation may have enhanced the ability of hSK^{G113A} to access sphingosine in our in vitro enzyme assays. Unlike enzymes that act on soluble substrates, our standard SK assays rely on supplying sphingosine as a mixed micelle with Triton X-100. This means that the SK must interact with the Triton X-100 micelle to gain access to sphingosine, in a comparable manner to how it most likely encounters this substrate in vivo in various cellular membranes [31]. Therefore, a mutation that alters the dynamics of this interaction may alter the observed catalytic efficiency of an enzyme. To test this, we examined the activity of hSK^{WT} and hSK^{G113A} with sphingosine supplied as a BSA-solubilised complex, a widely used alternative substrate-delivery system [32]. The results (Fig. 3B) show that, while the BSA-solubilised sphingosine supported much lower activity than with the mixed micelle assay, hSK^{G113A} still displayed approximately 1.7-fold higher activity than hSK^{WT} under these conditions. This suggests that the increased activity shown by hSK^{G113A} is not a consequence of the substrate presentation conditions, but is truly an increase in catalytic efficiency.

Fig. 4. Activation of hSK^{WT} and hSK^{G113A} by TNFα and PMA. HEK293T cells transfected with either hSK^{WT} or hSK^{G113A} were harvested and assayed for SK activity after treatment with either 1 ng/ml TNFα for 10 min or 100 ng/ml PMA for 30 min. SK activities are expressed relative to protein concentrations of cell lysates and are means ± S.D. of triplicate determinations from three independent experiments.

3.3. The Gly^{113} → Ala mutation does not mimic agonist-dependent hSK activation

The fold increase in SK activity induced by the Gly^{113} → Ala mutation (1.7-fold) is remarkably similar to the increase in SK activity observed upon treatment of cells with agonists like TNFα or PMA (1.5- to 2-fold) [5–7]. Furthermore, like hSK^{G113A} (Fig. 2D), this increase in SK activity observed during agonist-mediated activation arises from an increase in V_{max} of the enzyme [33]. Thus, we considered the possibility that the Gly^{113} → Ala mutation may have induced a conformational change in hSK towards the, as yet unidentified, activated form of this enzyme. To investigate this, we examined the activation of SK activity in hSK^{G113A}- and hSK^{WT}-transfected HEK293T cells in response to TNFα and PMA (Fig. 4). As reported previously [5,7], hSK^{WT} activity was stimulated almost two-fold by these agonists. hSK^{G113A} activity was stimulated by these agonists to the same degree as hSK^{WT} (Fig. 4). If the Gly^{113} → Ala mutation mimicked the agonist-induced activation we would not have expected any stimulatory effect of these agonists on hSK^{G113A} activity. Our observations suggest that the structural change in hSK created by the Gly^{113} → Ala mutation that enhances SK activity is not directly related to conformational changes that increase SK activity during hSK activation.
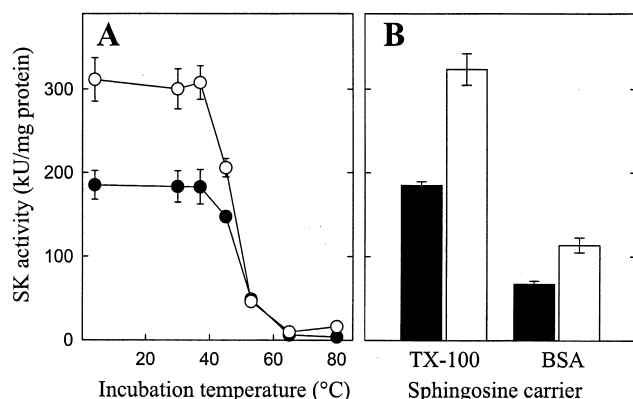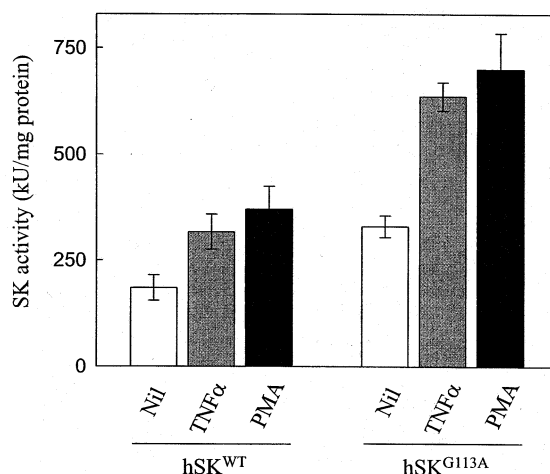
Fig. 3. The increased catalytic activity of hSK^{G113A} is not a result of enhanced stability or substrate accessibility. A: To determine the thermal stability of hSK^{WT} or hSK^{G113A} lysates from HEK293T cells transfected with either hSK^{WT} (●) or hSK^{G113A} (○) were diluted in lysis buffer, incubated at the indicated temperatures for 30 min, and then assayed for residual SK activity. B: To determine the effect of substrate presentation on the activity of hSK^{WT} and hSK^{G113A} lysates from HEK293T cells transfected with either hSK^{WT} (filled bars) or hSK^{G113A} (open bars) were assayed using sphingosine (100 μM) supplied in Triton X-100 (0.25% w/v final Triton X-100 concentration) mixed micelles or as a BSA complex (0.1% w/v final BSA concentration). In all cases SK activities are expressed relative to the protein concentrations of cell lysates and are means ± S.D. of triplicate determinations from three independent experiments.

3.4. Conclusions

Here we have probed the role of Gly^{113}, a residue highly conserved among SKs and DGKs. To accomplish this we have utilised two mutations, one disruptive and one relatively subtle. The disruptive mutation, Gly^{113} → Asp markedly decreased catalytic activity. Surprisingly the more conservative Gly^{113} → Ala mutation resulted in a hSK mutant (hSK^{G113A}) that has 1.7-fold greater catalytic activity than hSK^{WT}. This, together with the very low activity of the hSK^{G113D} mutant indicates Gly^{113} has an important role in the catalytic mechanism of hSK. We have previously reported the generation of a catalytically inactive mutant of hSK through mutagenesis of Gly^{82} → Asp [7]. This Gly^{82} → Asp mutation interferes with

ATP binding (our unpublished observations). This is consistent with the loose similarity between this region and the glycine-rich loop within the ATP-binding site of protein kinases [27]. The current study has shown a further conserved region around Gly[113] is also important in the catalytic activity of hSK. However, unlike Gly[82], it appears that Gly[113] is not directly involved in substrate binding since hSK[G113A] showed similar affinity for ATP and sphingosine as hSK[WT]. Instead, our data suggests that the Gly[113]→Ala mutation increases catalytic efficiency of hSK.

SKs and DGKs represent a novel class of lipid kinases, one for which no structural information has been attained, and where the mechanism of phosphoryl transfer is unknown. The Gly[113]→Ala in hSK increases the efficiency of this transfer without altering the substrate-binding kinetics of this enzyme. This indicates that this mutation may induce a conformational change that increases the efficiency of phosphoryl transfer. Importantly, we have shown that hSK[G113A] can still be activated by cell agonists indicating that the structural change in hSK created by this mutation is not related to any conformational change that may arise during the currently unknown hSK activation mechanism. Ongoing structural and mutagenic studies are aimed at determining the structural basis of catalysis of this novel class of kinases and how Gly[113]→Ala induces a hyper-active conformation.

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