

# The Mechanism of Membrane Targeting of Human Sphingosine Kinase 1\*

Received for publication, July 13, 2005, and in revised form, October 11, 2005 Published, JBC Papers in Press, October, 2005, DOI 10.1074/jbc.M507574200

Robert V. Stahelin <sup>‡1</sup>, Jeong H. Hwang <sup>‡1</sup>, Jin- Hahn Kim <sup>‡</sup>, Zee- Young Park <sup>§</sup>, Korey R. Johnson <sup>¶</sup>, Lina M. Obein <sup>¶</sup>, and Wonhwa Cho <sup>‡2</sup>

From the Departments of <sup>‡</sup> Chemistry, University of Illinois, Chicago, Illinois 60607, the <sup>¶</sup> Department of Medicine and Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425, and the <sup>§</sup> Department of Life Science, Kwangju Institute of Science and Technology, Gwang- Ju, 500- 712 Korea

**Sphingosine 1- phosphate is a bioactive sphingolipid that regulates cell growth and suppresses programmed cell death. The bio- synthesis of sphingosine 1- phosphate is catalyzed by sphingosine kinase (SK) but the mechanism by which the subcellular localization and activity of SK is regulated in response to various stimuli is not fully understood. To elucidate the origin and structural determinant of the specific subcellular localization of SK, we performed biophysical and cell studies of human SK1 (hSK1) and selected mutants.**

palmitoyl- 2- oleoyl- *sn*- glycerol- 3- phosphoglycerol; POPI, 1- palmitoyl- 2- oleoyl- *sn*- glycerol- 3- phosphoinositol; POPS, 1- palmitoyl- 2- oleoyl- *sn*- glycerol- 3- phosphoserine; PKC, protein kinase C; PS, phosphatidylserine; SK, sphingosine kinase; SPH, sphingosine; SPR, surface plasmon resonance; HEK, human embryonic kidney; POPA, 1- Palmitoyl- 2- oleoyl- *sn*- glycerol- 3- phosphatidic acid.

*In vitro* measurements showed that hSK1 selectively bound phosphatidylserine over other anionic phospholipids and strongly preferred the plasma membrane- mimicking membrane to other cellular membrane mimetics. Mutational analysis indicates that conserved Thr<sup>54</sup> and Asn<sup>89</sup> in the putative membrane- binding surface are essential for lipid selectivity and membrane targeting both *in vitro* and in the cell. Also, phosphorylation of Ser<sup>225</sup> enhances the membrane affinity and plasma membrane selectivity of hSK1, presumably by modulating the interaction of Thr<sup>54</sup> and Asn<sup>89</sup> with the membrane. Collectively, these studies suggest that the specific plasma membrane localization and the activation of SK1 is mediated largely by specific lipid- protein interactions.

\*This work was supported by National Institutes of Health Grants GM52598, GM53987, GM68849 (to W. C.), and GM062887 (to L. M. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. Section 1734 solely to indicate this fact.

<sup>1</sup>Both authors contributed equally to this work.

<sup>2</sup>To whom correspondence should be addressed: M/C 111, 845 West Taylor St., Chicago, IL 60607-7061. Tel.: 312-996-4883; Fax: 312-996-2183; E-mail: [wcho@uic.edu](mailto:wcho@uic.edu).

<sup>3</sup>The abbreviations used are S1P, sphingosine 1- phosphate; EGFP, enhanced green fluorescent protein; hSK1; human sphingosine kinase 1; PA, phosphatidic acid; PMA, phorbol 12- myristate 13- acetate; POPC, 1- palmitoyl- 2- oleoyl- *sn*- glycerol- 3- phosphocholine; POPE, 1- palmitoyl- 2- oleoyl- *sn*- glycerol- 3- phosphoethanolamine; POPG, 1-

Sphingosine 1-phosphate (S1P)<sup>3</sup> is a recently identified bioactive lipid that can act both extracellularly and intracellularly as a first and a second messenger, respectively (1). It has been shown that S1P is excreted into serum from platelets and binds members of the endothelial differentiation gene receptor family (S1P1-5) to activate cellular processes such as differentiation, migration, and mitogenesis (2). Intracellularly, S1P has been implicated in signaling cascades that lead to cytoskeletal changes, motility, release of intracellular Ca<sup>2+</sup> stores, and protection from apoptosis (3-6). S1P is formed from sphingosine (SPH) by sphingosine kinase (SK) and is degraded by S1P lyase and S1P phosphatases (1). In the resting state of cells, the balance between S1P formation and degradation maintains the low basal levels of S1P. However, cellular S1P levels have been shown to increase rapidly and transiently by agonists that activate SK, such as tumor necrosis factor- $\alpha$  (7, 8), platelet-derived growth factor (9), nerve growth factor (10, 11), muscarinic acetylcholine agonists (12), or phorbol esters (13, 14).

Two types of mammalian SKs (SK1 and SK2) have been characterized so far (15, 16), both of which are primarily cytosolic proteins. A recent study suggested that a phorbol ester, phorbol 12-myristate 13-acetate (PMA), induces the protein kinase C (PKC)-mediated phosphorylation and the localization of SK1 to the plasma membrane in human embryonic kidney (HEK) 293 cells (17), which leads to enhanced release of S1P to the media. Subsequently, it was reported that PMA and tumor necrosis factor- $\alpha$  induced the phosphorylation of

Ser<sup>225</sup> of SK1 through the activation of mitogen-activated protein kinase, extracellular signal-regulated kinase 1/2, which resulted in plasma membrane localization and activation of SK1 (18). It has been also reported that the subcellular localization of SK1 is mediated by interactions with putative adapter proteins (19, 20). However, it is still unknown how SK is specifically targeted to the plasma membrane and how phosphorylation triggers the subcellular localization of SK. To elucidate the origin and structural determinants of its specific subcellular localization of SK, we performed biophysical and cell studies of human SK1 (hSK1) and selected mutants. The results from this study provide new insight into how the membrane recruitment of this important protein is regulated in the cell.

## EXPERIMENTAL PROCEDURES

*Materials*- 1-Palmitoyl- 2-oleoyl-*sn*-glycerol- 3-phosphatidic acid (POPA), 1-palmitoyl- 2-oleoyl-*sn*-glycerol- 3-phosphocholine (POPC), 1-palmitoyl- 2-oleoyl-*sn*-glycero- 3-phosphoethanolamine (POPE), 1-palmitoyl- 2-oleoyl-*sn*-glycero- 3-phosphoglycerol (POPG), 1-palmitoyl- 2-oleoyl-*sn*-glycero- 3-phosphoinositol (POPI), 1-palmitoyl- 2-oleoyl-*sn*-glycero- 3-phosphoserine (POPS), and D-*erythro*-SPH (C20) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. PMA was from Sigma. Silica thin layer chromatography G-60 plate was from Merck.

*Protein Expression and Purification*- All SK1 proteins were expressed in

baculovirus- infected Tn5 cells as soluble proteins with a C- terminal His<sub>6</sub> tag and purified from the cell extracts using a Ni<sup>2+</sup>- nitrilotriacetic acid- agarose column (Qiagen) according to the manufacturer's protocol. All proteins were >90% pure electrophoretically. Protein concentration was determined by bicinchoninic acid method (Pierce).

#### *Surface Plasmon Resonance*

*Analysis-* The kinetic SPR measurements were performed at 23 °C in 20 mM HEPES, pH 7.4, containing 0.16 M KCl using a lipid- coated L1 chip in the BIACORE X system as described previously (21). Cell membrane- mimicking vesicles were prepared as described (22). The control sensor surface was coated with 100% POPC vesicles for which all hSK1 proteins have extremely low affinity, and the active sensor surface was coated with vesicles indicated in TABLES ONE and TWO. All data were analyzed using BIAevaluation 3.0 software (Biacore) to determine  $k_a$  and  $k_d$ , and equilibrium dissociation constant ( $K_d$ ) was then calculated using an equation,  $K_d = k_d/k_a$  assuming 1:1 binding: *i.e.* protein + (protein binding site on vesicle) (complex) (21). ↔

*Monolayer Measurements-* Surface pressure ( $\pi$ ) of solution in a circular Teflon trough (4 cm diameter x 1 cm deep) was measured using a Wilhelmy plate attached to a computer- controlled Cahn electrobalance as described previously (23). Five to ten  $\mu$ l of phospholipid solution in ethanol/ hexane (1:9 (v/v)) was spread onto 10 ml of subphase (20 mM HEPES, pH 7.4, containing 0.16 M KCL, and either 0.1 mM EGTA or 0.1 mM CaCl<sub>2</sub>) to form a

monolayer with a given initial surface pressure ( $\pi_0$ ). Once the surface pressure reading of monolayer had been stabilized (after about 5 min), the protein solution (typically 40  $\mu$ l) was injected into the subphase through a small hole drilled at an angle through the wall of the trough and the change in surface pressure ( $\Delta\pi$ ) was measured as a function of time. Typically, the  $\Delta\pi$  value reached a maximum after 20 min. The maximal  $\Delta\pi$  value at a given  $\pi$  depended on the protein concentration and reached a saturation value (*i.e.* [hSK1]  $\geq$  1.0  $\mu$ g/ ml). Protein concentrations in the subphase were therefore maintained above such values to ensure that the observed  $\Delta\pi$  represented a maximal value. The critical surface pressure ( $\pi_c$ ) was determined by extrapolating the  $\Delta\pi$  versus  $\pi_0$  plot to the x-axis (24).

*SK1 Activity Assay- In vitro* hSK1 activity was measured in the absence or presence of lipid vesicles according to the procedure by Olivera and Spiegel (25). For the assays without lipid vesicles, the recombinant hSK1 was added to 20 mM Tris buffer (pH 7.4) containing 0.16 M KCl, 5  $\mu$ M D- erythro- SPH- bovine serum albumin adduct (99.7:0.3 in mole ratio), 1 mM [ $\gamma$ -<sup>32</sup>P]ATP (Amersham Biosciences; 1  $\mu$ Ci), and 500  $\mu$ M MgCl<sub>2</sub>. After the reaction mixture was incubated at 23 °C for 30 min, lipids were extracted with 2.5 volumes of chloroform/ butanol/ HCl (50:50:1, v/v/v), and the organic layer was dried under a gentle stream of nitrogen. SPH and S1P were separated by thin layer chromatography using butanol/ methanol/ acetic acid/ water (80:20:12:20, v/v/v/v) as eluent. Bands corresponding to S1P were

scraped and the radioactivity measured using a scintillation counter. Activity assays in the presence of lipid vesicles were performed in 20 mM Tris buffer (pH 7.4) containing 0.16 M KCl, 5  $\mu$ M D- *erythro*-SPH incorporated into phospholipid vesicles of various compositions (5  $\mu$ M total concentration), 1 mM [ $\gamma$ -<sup>32</sup>P]ATP (Amersham Biosciences; 1  $\mu$ Ci), and 500  $\mu$ M MgCl<sub>2</sub>. For cellular SK1 activity assays, transfected HEK293 cells were treated with 2  $\mu$ M PMA for the indicated times, and 10 min before the end of the time point, cells were pulsed with 300 nM D- [*erythro*-<sup>3</sup>H]SPH (American Radiolabeled Co.; 1.0  $\mu$ Ci). The secreted S1P was extracted and counted as described above.

*Cell Culture, Transfection and Confocal Microscopy*- Wild type hSK1 and its mutants were subcloned in-frame with a C-terminal enhanced green fluorescent protein (EGFP) into the pIND vector. Stable HEK293 cells expressing ecdysone receptor (Invitrogen) were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37 °C with 5% CO<sub>2</sub> and 98% humidity. The cell transfection and the protein expression were performed as described elsewhere (22). Confocal imaging was performed using a four channel Zeiss LSM 510 laser scanning microscope. EGFP was excited using the 488-nm line of an argon/krypton laser. A 505-nm line pass filter and a x 63, 1.2 numerical aperture water immersion objective were used for all experiments. Immediately before imaging, induction media was removed as the cells were washed twice with 1 mM HEPES (pH 7.4), containing 2.5 mM MgCl<sub>2</sub>, 140 mM NaCl, 5

mM KCl, and 6 mM sucrose. The cells were then overlaid with the same buffer and imaged. Translocation experiments were monitored by scanning every 30 s following treatment of the cells with 2  $\mu$ M PMA. The time lapse changes in EGFP intensity ratio at the plasma membrane (= plasma membrane / [plasma membrane + cytoplasm]) were determined as described previously (22).

*Determination of Ser<sup>225</sup> Phosphorylation by Mass Spectrometry*- The hSK1 bands were excised from the Coomassie Brilliant Blue-stained sodium dodecyl sulfate-polyacrylamide gel. The excised bands were washed three times with acetonitrile/H<sub>2</sub>O (1:1, v/v) for 10 min and dehydrated with acetonitrile. Then, the bands were finally washed with 1:1 (v/v) solution of acetonitrile and 100 mM ammonium bicarbonate and dried under vacuum. Proteins contained in gel pieces were reduced by using 10 mM Tris (2-carboxyethyl) phosphine hydrochloride in 0.1 M ammonium bicarbonate at 56 °C for 45 min and then alkylated with 55 mM iodoacetamide in 0.1 M ammonium bicarbonate at room temperature for 30 min. The alkylated sample was washed as described above, dried, and soaked in sequencing-grade trypsin (500 ng) on ice for 45 min. Then, the gel pieces were immersed in 100  $\mu$ l of 500 mM ammonium bicarbonate at 37 °C for 14-18 h. Resulting peptides were extracted sequentially for 20 min with 45% acetonitrile in 20 mM ammonium bicarbonate, 45% acetonitrile in 0.5% trifluoroacetic acid, and 75% acetonitrile in 0.25% trifluoroacetic acid

with agitation. Pooled peptide extracts were evaporated under vacuum. Digested peptides were dissolved in Solvent A (5% acetonitrile and 0.1% formic acid in H<sub>2</sub>O) and loaded onto fused silica capillary columns containing 8 cm of 5-  $\mu$ m particle size Aqua C<sub>18</sub> reverse- phase column material. The column was placed in- line with an Agilent HP 1100 quaternary LC pump and a splitter system was used to achieve a flow rate of 250 nl/ min, with a 90- min gradient of Solvent A and Solvent B (80% acetonitrile and 0.1% formic acid in H<sub>2</sub>O). Eluted peptides were directly electrosprayed into an LCQ Deca XP Plus mass spectrometer (ThermoFinnigan, Palo Alto, CA) by applying 2.3 kV of DC voltage. A data-dependent scan consisting of one full mass scan (400- 1400  $m/z$ ) and three data-dependent tandem mass scans were used to generate tandem mass spectra of eluted peptides. Normalized collision energy of 35% was used throughout the data acquisition. Tandem mass spectra were searched against an in- house protein data base using TurboSequest and phosphorylation modification (+ 80 on Ser, Thr, and Tyr) was considered in the differential modification search. Bioworks version 3.1 was used to filter the search results and the following Xcorr values were applied to different charge states of peptides: 1.8 for singly charged peptides, 2.2 for doubly charged peptides, and 3.2 for triply charged peptides. Tandem mass spectra for phosphorylated peptides were generated using Xcalibur version 3.1 and manual assignment of fragment ions was performed to confirm the search results.

## RESULTS

### *Lipid Selectivity of hSK1-*

Conflicting results have been reported on the lipid selectivity of SK. An earlier report showed that phosphatidylserine (PS) is the most effective activator of SK (26), whereas a recent report suggested that SK1 has the high affinity for phosphatidic acid (PA) (27). To resolve this controversy and determine the exact role of these lipids in membrane targeting of SK1, we first measured the *in vitro* membrane binding parameters of recombinant hSK1 using vesicles with various compositions by means of the SPR analysis. The SPR analysis of membrane- protein interactions offers an advantage over other methods in that membrane association ( $k_a$ ) and dissociation ( $k_d$ ) rate constants can be directly determined (21, 28- 30). We have recently shown that nonspecific electrostatic interactions driven by ionic residues mainly effect  $k_a$ , whereas short- range specific interactions and hydrophobic interactions, which result from the membrane penetration of hydrophobic residues, largely influence  $k_d$  (21, 20, 31). Fig. 1 shows representative sensorgrams for hSK1- vesicle binding from which  $k_a$ ,  $k_d$ , and  $K_d$  were determined.

hSK1 did not bind to Zwitterionic POPC or POPE vesicles with protein concentrations up to 5 $\mu$ M, but showed higher affinity for vesicles containing anionic phospholipids. Because the inner plasma membrane of mammalian cells contains about 30 mol % of total anionic lipids, we first determined  $K_d$  values for mixed vesicles containing 30 mol % of various anionic lipids (*i.e.* POPC/POPX (70:30)). The comparison of  $K_d$  values (TABLE ONE)

shows that hSK1 has definite selectivity for PS over other anionic phospholipids. Although hSK1 bound to PA slightly better than phosphatidylglycerol and phosphatidylinositol, it still showed 5- fold higher affinity for PS than for PA. When lower concentrations of anionic lipids were employed (*e.g.* POPC/POPX = 80:20 or 85:15), essentially the same PS selectivity was observed (data not shown).

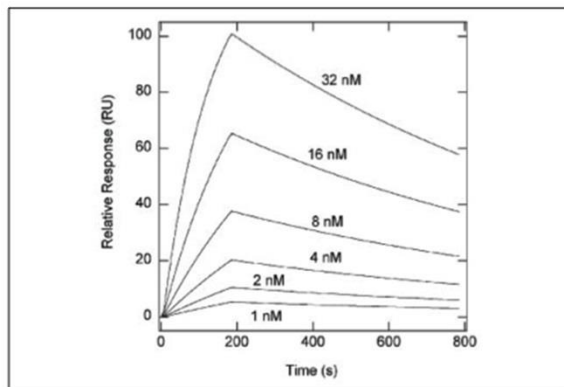


FIGURE 1. Representative sensorgrams for vesicle binding of hSK1. Varying concentrations (1, 2, 4, 8, 16, and 32 nM) of hSK1 were injected at 30  $\mu\text{l}/\text{min}$  to the L1 chip coated with POPC/POPS (7:3) vesicles and the subsequent association and dissociation were monitored. 10 mM HEPES buffer (pH 7.4) containing 0.16 M KCl was used for all measurements.

pH 8.1), precluding the possibility that selectivity of hSK1 for PS over PA affinity reflects incomplete ionization of PA under our experimental conditions. Higher pH could not be used because of a loss of hSK1 activity at pH > 8.3. Furthermore, the addition (up to 3 mol %) of SPH, PMA, diacylglycerol, or any phosphoinositides to POPC/POPS (70:30) vesicles had little effect on the affinity of hSK1 (data not shown). Higher affinity (*i.e.*  $K_d$ ) of hSK1 for PS derives mainly from smaller  $k_d$ , which suggests that the PS head group specifically interacts with hSK1 and may also induce membrane penetration of hSK1 to slow the membrane dissociation.

It has been shown for many membrane- targeting domains and their host proteins that binding of their cognate lipid ligand leads to membrane penetration of the protein (31- 34). To see if PS specifically induces the membrane penetration of hSK1, we measured the penetration of hSK1 into various lipid monolayers at the air- water interface.

TABLE ONE					
PS selectivity of hSK1 and mutants determined by SPR analysis					
All values represent mean $\pm$ S.D. from triplicate determinations. All measurements were performed in 20 mM HEPES, pH 7.4, containing 0.16 M KCl.					
Proteins	Lipids	$k_a$	$k_d$	$K_d$	PS <sup>a</sup> selectivity
		$M^{-1} s^{-1}$	$s^{-1}$	$M$	
Wild type	POPC/POPS (7:3)	$(2.2 \pm 0.3) \times 10^5$	$(9.3 \pm 0.7) \times 10^{-4}$	$(4.2 \pm 0.7) \times 10^{-9}$	12
Wild type	POPC/POPG (7:3)	$(1.4 \pm 0.2) \times 10^5$	$(7.4 \pm 0.6) \times 10^{-3}$	$(5.2 \pm 0.9) \times 10^{-8}$	
Wild type	POPC/POPI (7:3)	$(1.2 \pm 0.3) \times 10^5$	$(8.1 \pm 0.7) \times 10^{-3}$	$(6.8 \pm 3.0) \times 10^{-8}$	
Wild type	POPC/POPA (7:3)	$(2.5 \pm 0.4) \times 10^5$	$(6.9 \pm 0.9) \times 10^{-3}$	$(2.8 \pm 0.6) \times 10^{-8}$	
T54A	POPC/POPS (7:3)	$(9.6 \pm 0.3) \times 10^4$	$(1.8 \pm 0.2) \times 10^{-2}$	$(1.9 \pm 0.2) \times 10^{-7}$	0.6
T54A	POPC/POPG (7:3)	$(9.2 \pm 0.9) \times 10^4$	$(9.8 \pm 1.0) \times 10^{-3}$	$(1.1 \pm 0.1) \times 10^{-7}$	
N89A	POPC/POPS (7:3)	$(1.1 \pm 0.3) \times 10^5$	$(1.1 \pm 0.2) \times 10^{-2}$	$(1.0 \pm 0.3) \times 10^{-7}$	1
N89A	POPC/POPG (7:3)	$(9.7 \pm 0.6) \times 10^4$	$(8.6 \pm 0.7) \times 10^{-3}$	$(8.9 \pm 0.9) \times 10^{-8}$	
S168A	POPC/POPS (7:3)	$(2.8 \pm 0.5) \times 10^5$	$(9.5 \pm 0.9) \times 10^{-4}$	$(3.4 \pm 0.6) \times 10^{-9}$	13
S168A	POPC/POPG (7:3)	$(2.0 \pm 0.2) \times 10^5$	$(8.5 \pm 0.7) \times 10^{-3}$	$(4.3 \pm 0.4) \times 10^{-8}$	

<sup>a</sup> Ratio of  $(1/K_d)$  for POPC/POPS (7:3) vesicles to  $(1/K_d)$  for POPC/POPG (7:3) vesicles.

Also, the PS selectivity remained unchanged when pH of the binding solution was varied from 7.4 to 8.1 (*i.e.*  $[K_d \text{ for POPC/POPA (7:3)}] / [K_d \text{ for POPC/POPS (7:3)}] = 4.7$  at

The phospholipid monolayer was spread at a constant area and the change in surface pressure ( $\Delta\pi$ ) was monitored after injection of protein into the subphase. In general,  $\Delta\pi$  is inversely proportional to  $\pi_0$  of the lipid monolayer and an extrapolation of  $\Delta\pi$

versus  $\pi_0$  plot yields the critical surface pressure ( $\pi_c$ ), which specifies the upper limit of  $\pi_0$  of a monolayer that a protein can penetrate into (24, 35). Because the surface pressure of cell membranes has been estimated to be in the range of 30- 35 dyne/cm (36- 38), the  $\pi_c$  value for a protein that penetrates cell membranes should be above 30 dyne/cm. As shown in Fig. 2A, hSK1 showed weak penetration into the POPC, POPC/ POPG (7:3), POPC/ POPI (7:3), POPC/ POPA (7:3) monolayer with  $\pi_c < 26$  dyne/cm. However, hSK1 had stronger penetration activity toward the POPC/ POPS (7:3) monolayer with  $\pi_c \geq 30$  dyne/cm. This suggests that PS specifically induces the membrane penetration of hSK1 and thus allows its favorable hydrophobic interaction with cell membranes. It should be noted that large unilamellar vesicles used in our SPR

measurements are known to have the surface pressure above 30 dyne/cm (36- 38). This explains why hSK1 shows no affinity for zwitterionic POPC or POPE vesicles and weaker affinities for non- PS- containing anionic vesicles through nonspecific electrostatic interactions in the SPR measurements.

Our recent studies have shown that peripheral proteins with PS selectivity have a high tendency to be localized at the cytoplasmic face of the plasma membrane (22, 39, 40), either in response to specific signal or constitutively, because of high PS content of this membrane. Thus, the reported PMA- induced translocation of hSK1 to the plasma membrane (17) might derive, at least in part, from its preference for PS in the plasma membrane. To test this notion, we measured the binding of hSK1 to vesicles

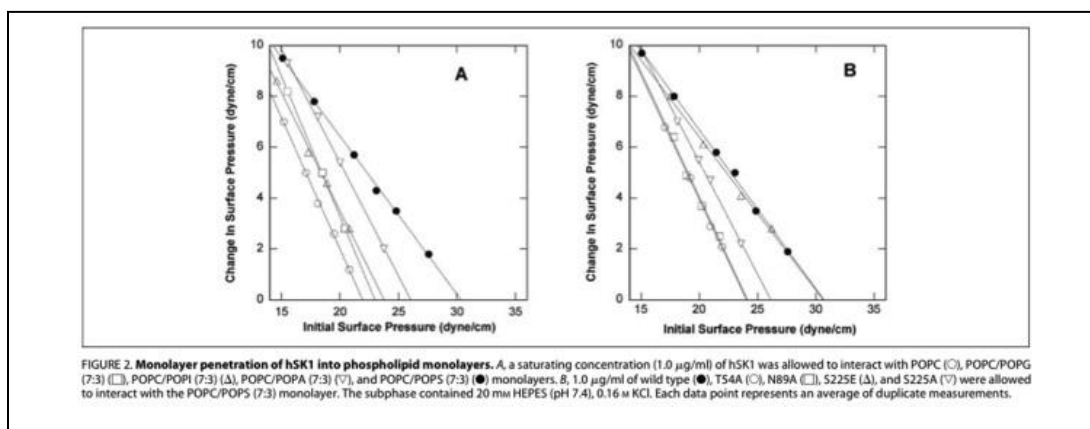


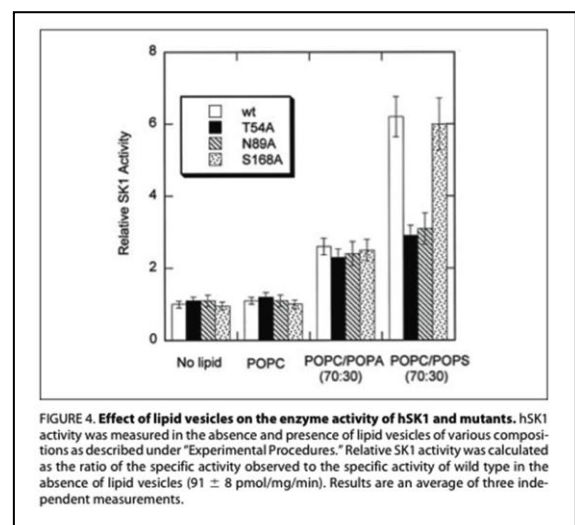
TABLE TWO					
Binding of hSK1 phosphorylation mutants to cell membrane mimetics					
All values represent mean $\pm$ S.D. from triplicate determinations. All measurements were performed in 20 mM HEPES, pH 7.4, containing 0.16 M KCl.					
Proteins	$k_{on}$	$k_{off}$	$K_d$	Fold <sup>a</sup> increase in $K_d$	PM/NM <sup>b</sup>
	$10^5 \text{ M}^{-1} \text{ s}^{-1}$	$10^{-3} \text{ s}^{-1}$	$\text{RM}$		
Plasma membrane mimetic: POPC/POPE/POPS/POPI/cholesterol (12:35:22:9:22)					
Wild type	2.9 $\pm$ 0.3	0.55 $\pm$ 0.07	1.9 $\pm$ 0.3	1	373
T54A	1.1 $\pm$ 0.2	9.8 $\pm$ 1	89 $\pm$ 20	47	22
N89A	1.3 $\pm$ 0.3	7.7 $\pm$ 0.6	59 $\pm$ 10	31	24
S225A	1.4 $\pm$ 0.3	3.5 $\pm$ 0.4	25 $\pm$ 6	13	88
S225E	2.5 $\pm$ 0.5	0.59 $\pm$ 0.4	2.4 $\pm$ 0.5	1.3	329
Nuclear membrane mimetic: POPC/POPE/POPS/POPI/cholesterol (61:21:4:7:7)					
Wild type	0.91 $\pm$ 0.08	65 $\pm$ 6	710 $\pm$ 90	1	
T54A	0.45 $\pm$ 0.05	92 $\pm$ 8	2000 $\pm$ 300	2.8	
N89A	0.63 $\pm$ 0.04	86 $\pm$ 7	1400 $\pm$ 300	2.0	
S225A	0.44 $\pm$ 0.06	98 $\pm$ 15	2200 $\pm$ 450	3.1	
S225E	0.86 $\pm$ 0.7	68 $\pm$ 7	790 $\pm$ 100	1.1	

hSK1	1	-----MDPAGGPRGVLPRPCRVLVLLNPRGGKGAQLQFRSHVQPLLAEEAISFTLMLTERRNHARE-LVRSEELG	70
mSK1a	1	-----MEPECPRGLLP RPCRVLVLLNPPGGKGAQLQFQSRVQPLEEAEITFKLLITERKNHARE-LVCAEELG	69
rSK1	1	-----MQPADCPRGLLP RPCRVLVLLNPRGGKGAQLQFQSRVPLEEAEVSKLMLTERQNHARE-LVCAEELG	70
rSK1a	1	-----MQPADCPRGLLP RPCRVLVLLNPRGGKGAQLQFQSRVPLEEAEVSKLMLTERQNHARE-LVCAEELG	70
mmSK1	12	-----CPRGLLP RPCRVLVLLNPPGGKGAQLQFQSRVQPLEEAEITFKLLITERKNHARE-LVCAEELG	74
LCB5	252	DL---VEEILKRSYKNTNRNKSIFVLIINPFGGKGAKKLFMTKAKPLLLASRCSIEVVYTKYPGHAIE-IAEMDID	321
S.p.	90	----FCEYLLDVAYKGIKRSRRFVFINPFGGKGAKHWESEAEVVFSSAHSICEVVLTRRKHAKS-IAKNLDVG	159
C.e.	63	ENEQLTSVILSRKPPPQEQCRGNLLVFINPNSGTGKSLFTANTVGPDKSLIRYEVVVTTGNHARNVLMTRKADLG	132
* * * * *			
hSK1	71	RWDALVVMGGDGLMHEVVNGLMERPDWETAI-QKPLCSLPGGSGNALAASVNHYAGYEQVTNEDLLINCTL	140
mSK1a	70	HWDALAVMGGDGLMHEVVNGLMERPDWETAI-QKPLCSLPGGSGNALAASVNHYAGYEQVTNEDLLINCTL	139
rSK1	71	HWDALAVMGGDGLMHEVVNGLMERPDWETAI-QKPLCSLPGGSGNALAASLNYYAGYEQVTNEDLLINCTL	140
rSK1a	71	HWDALAVMGGDGLMHEVVNGLMERPDWETAI-QKPLCSLPGGSGNALAASLNYYAGYEQVTNEDLLINCTL	140
mmSK1	75	HWDALAVMGGDGLMHEVVNGLMERPDWETAI-QKPLCSLPGGSGNALAASVNHYAGYEQVTNEDLLINCTL	144
LCB5	322	KYDTIACASGGDGIPEVINGLYQRPDHVKAFNNIAITEIPCSSGNAMSVS-----CHWTNNPSYSTL	391
S.p.	160	SYDGIILSVGGDGLFHEVINGLGERDDYLEAF-KLPVCMIPGGSGNAFSGYN-----ATGQKLPALTAL	229
C.e.	133	KFNGVLILSGDGLVFEALNGILCREDAFRIFPTLPIGIVPSGSGNGLCSV--LSKYGTMNEKSVMERAL	202
* * * * *			
hSK1	141	LLCRR---LLSPMNLSSLHTASGLRLFSVLSLAWGFIADVLESEKYRR-LGEMRFTLGTFLR	199
mSK1a	140	LLCRR---RLSPMNLSSLHTASGLRLYSVLSLSWGFVADVLESEKYRR-LGEIRFTVGTFFR	198
rSK1	141	LLCCR---QLSPMNLSSLHTASGRQLYSVLSLSWGFVADVLESEKYRS-LGEIRFTVGTFFR	199
rSK1a	141	LLCCR---QLSPMNLSSLHTASGRQLYSVLSLSWGFVADVLESEKYRS-LGEIRFTVGTFFR	199
mmSK1	145	LLCRR---RLSPMNLSSLHTASGLRLYSVLSLSWGFVADVLESEKYRR-LGEIRFTVGTFFR	205
LCB5	392	CLIKSIETRIDLCCSQPSYAREHPKLSFLSQTYGLIAETDINTE-FIRWMPARFELGVAFN	448
S.p.	230	EILKGRPTSFDMTFEQ----KGGKAYSFLTANYGIADCDIGTE-NWRPFGENRAYLGFFLR	281
C.e.	203	EIATSPTAKAESVALYSVKTDNQ-SYASFLSIGWGLMADIDIDSEKWRKSLGHHRFTVMGFI	271

FIGURE 3. **Amino acid sequence alignment of hSK1 with SKs from different species.** Comparison of the deduced hSK1 sequence with the sequences of mouse SK1 (*mSK1a*), rat SK1 and SK1a (*rSK1* and *rSK1a*), house mouse SK1 (*mmSK1*), *Saccharomyces cerevisiae* SK (LCB5), and putative SKs from *Schizosaccharomyces pombe* (S.p.) and *Caenorhabditis elegans* (C.e.). Only the first 200 residues are shown here. Conserved residues are shown in **bold** and mutated residues are indicated by dots. The conserved ATP-binding region is underlined. Multiple sequence alignment was performed with CLUSTALW.

has been successfully used to determine the origin of specific subcellular localization of various cellular proteins (31). As shown in TABLE TWO, hSK1 had striking selectivity for the plasma membrane mimetic over other cell membrane mimetics. In particular, it showed almost 400- fold higher affinity for the plasma membrane mimetic than for the nuclear membrane mimetic. It should be noted that this plasma membrane selectivity is much more dramatic than the PS selectivity determined above because the plasma membrane is not only rich in PS but also the most anionic membrane in all cell membranes. This exceptional selectivity of hSK1 for the plasma membrane mimetic strongly suggests the lipid head group selectivity be a main force governing its specific subcellular localization. This also implies that the agonist- induced plasma membrane translocation of this protein be regulated by modulating the lipid- protein interactions, whose lipid head group compositions mimic those of the cytoplasmic plasma membrane,

*Structural Determinants of PS Selectivity and Cellular Membrane Targeting and Activation-* The structural information on the stereospecific recognition of the PS head group by its effector protein is limited. IN the case of the C2 domain of PKC $\alpha$  that was crystallized with a PS molecule, the side chain of an Asn residue





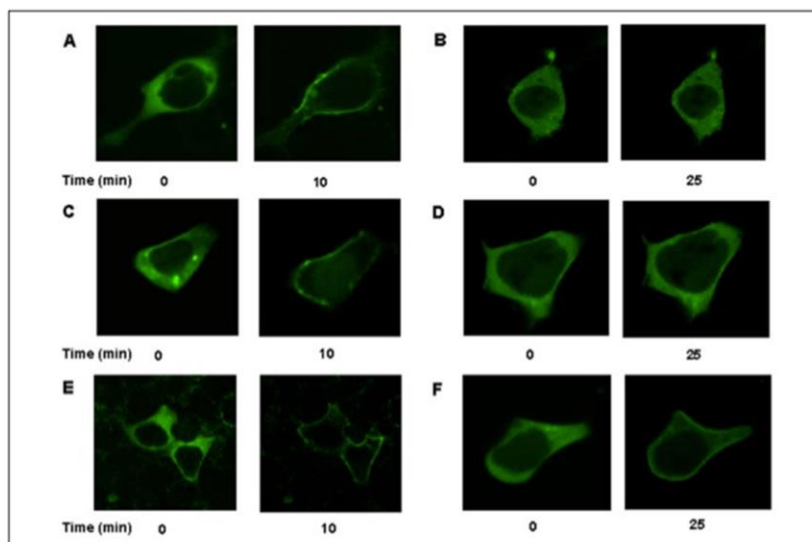
directly interacts with the serine head group of PS, whereas the side chain of a Thr residue interacts with other polar moieties of PS (41). To identify potential PS binding residues in hSK1, we searched polar residues capable of interacting with the PS head group, *i.e.* Asn, Ser, or Thr, which are conserved among SKs (Fig. 3). The search identified at least eight highly conserved residues, including, Thr<sup>54</sup>, Ser<sup>79</sup>, Asn<sup>89</sup>, Ser<sup>112</sup>, Asn<sup>114</sup>, Ser<sup>119</sup>, Ser<sup>165</sup>, and Ser<sup>168</sup>. To see if any of these residues are involved in PS head group recognition, we mutated them to Ala individually and measured the effects on PS selectivity. For the sake of simplicity, we determined the PS selectivity of mutants by comparing their affinity for POPC/POPS (7:3) and POPC/POPG (7:3) vesicles. Interestingly, T54A and N89A showed dramatically reduced PS selectivity (TABLE ONE), whereas S168A (TABLE ONE) and other mutants (data not shown) either behaved like wild type or were not functionally expressed, implying that Thr<sup>54</sup> and/or Asn<sup>89</sup> might be specifically involved in PS interaction. Interestingly, Asn<sup>89</sup> is located within the putative ATO-binding region (between residue 82 and residue 103) (Fig. 3) (42), which should be located near the membrane-binding surface for hSK1 to be able to phosphorylate the membrane-embedded substrate, SPH. Reduced PS selectivity of T54A and N89A was not because of deleterious structural changes caused by mutations because these mutants showed only slightly reduced affinity for POPC/POPG (7:3) vesicles (TABLE ONE). Consistent with their reduced PS selectivity, T54A and N89A also had much lower (*i.e.* 22- to 24- fold) selectivity for the plasma membrane mimetic over the nuclear membrane mimetic than the wild type (373-fold; TABLE TWO). It should be noted that despite their lack of PS selectivity T54A and N89A still prefer the plasma membrane mimetic to other cell membrane mimetics

because the highly anionic nature of the plasma membrane mimetic allows favorable nonspecific electrostatic interactions with these proteins.

To determine whether these PS-binding residues are also important for the enzymatic activity of hSK1, we performed *in vitro* activity assays for wild type, T54A, N89A, and S168A in the absence and presence of lipid vesicles of various compositions. When the activity was measured in the absence of lipid vesicles, wild type and all mutants had comparable specific activities (Fig. 4), indicating the mutations did not disrupt the structural integrity of the active site of the enzyme. When the activity was measured using SPH incorporated into zwitterionic PC vesicles, no increase in activity was observed for any protein (Fig. 4). When anionic POPC/POPA (7:3) vesicles were used in the activity assay, wild type and all mutants uniformly showed about a 2.5- fold enhanced activity (Fig. 4). In the presence of POPC/POPS (7:3) vesicles, however, wild type and S168A showed significantly higher activity than T54A and N89A (Fig. 4). Interestingly, T54A and N89A showed similar activities in the presence of POPC/POPA (7:3) and POPC/POPS (7:3) vesicles, indicating that the PA activation is largely because of nonspecific electrostatic binding to anionic membranes, whereas the PS activation is a specific process.

These data thus indicate that specific PS binding of hSK1 mediated by Thr<sup>54</sup> and Asn<sup>89</sup> is important not only for membrane binding of hSK1 but also for the enzymatic action of hSK1 on membrane-incorporated SPH.

**FIGURE 5. Subcellular localization of hSK1 and its mutants in HEK293 cells.** hSK1 and its mutants tagged with EGFP at their C termini were transiently transfected into HEK293 cells and their subcellular localization was monitored by confocal microscopy. Cell images before and after 2  $\mu$ M PMA stimulation are shown for hSK1 wild type (A), T54A (B), S168A (C), N89A (D), S225E (E), and S225A (F).

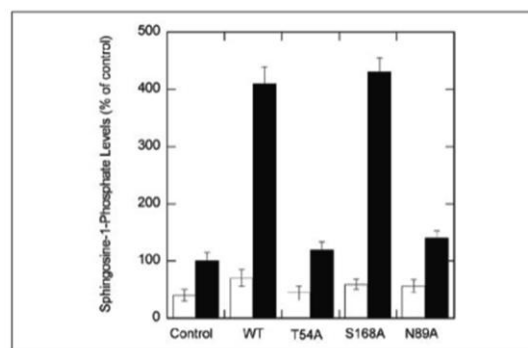


To see if Thr<sup>54</sup> and Asn<sup>89</sup> also play a key role in the subcellular localization of hSK1, we measured the cellular membrane translocation of hSK1 and mutants in HEK293 cells. The Western blotting analysis using a hSK1- specific antibody (17) indicated that all proteins were expressed in comparable levels (data not shown). The C- terminal EGFP- tagged hSK1 transiently transfected into HEK293 cells was recruited to the plasma membrane within 10 min in response to PMA stimulation (Fig. 5A). In stark contrast, EGFP- T54A (Fig. 5B) and EGFP- N89A (Fig. 5D) showed no detectable translocation under the same conditions. Unlike these mutants, EGFP- S168A with wild type- like membrane binding properties readily translocated to the plasma membrane upon PMA treatment (Fig. 5C).

We then measured the cellular activity of these proteins by labeling the aforementioned transfected HEK293 cells with D- [erythro- <sup>3</sup>H]SPH and monitoring the release of the radioactive SIP into the media. As shown in Fig. 6, PMA induced a 4- fold increase in SIP release over the control for wild type and S168A, whereas little to no increases in SIP release over the controls were detected for T54A and N89A with and without PMA. Collectively, these

results indicate that Thr<sup>54</sup> and Asn<sup>89</sup>, located in the putative membrane- binding surface of hSK1, play an essential role in its PS head group recognition, subcellular localization, and cellular activity. This also supports the notion that the subcellular localization of hSK1 is governed in large part by the lipid-protein interactions.

*Effect of Ser<sup>225</sup> Phosphorylation on the Membrane Targeting of hSK1-* It has been reported that Ser<sup>225</sup> of SK1 is phosphorylated by extracellular signal-regulated kinase 1/2 in response to PMA or tumor necrosis factor-  $\alpha$  stimulation in HEK293 cells, which leads to PM targeting and activation of SK1 (18).



**FIGURE 6. Cellular activities of hSK1 and mutants in HEK293 cells.** HEK293 cells were treated in the absence (white bars) or presence (black bars) of 2  $\mu$ M PMA for 1 h and pulsed the last 10 min with 300 nM D- [erythro- <sup>3</sup>H]SPH (4 mCi/mmol). Secreted [<sup>3</sup>H]SIP levels were measured using a scintillation counter as described under "Experimental Procedures." Controls were HEK293 cells transfected with the vehicle. Data are reported as a percentage of PMA-treated control samples. The data are representative of mean  $\pm$  S.D. of four independent experiments. WT, wild type.

To investigate how Ser<sup>225</sup> phosphorylation activates hSK1, we prepared phosphorylation and dephosphorylation- mimicking mutants, S225E and S225A, and measured their binding to cell membrane mimetics. As summarized in TABLE TWO, the membrane affinity of S225E was comparable with that of wild type, implying that a large population of hSK1 molecules expressed in insect cells are phosphorylated at Ser<sup>225</sup>. To test this notion, we determined the phosphorylation of Ser<sup>225</sup> in insect cell-expressed recombinant hSK1 by mass analysis. Tandem mass spectrometry data of tryptic peptide fragments containing Ser<sup>225</sup> (Fig. 7) consistently showed that Ser<sup>225</sup> was phosphorylated in hSK1 expressed in insect cells.

Although quantification is impractical with the current mass spectrometry analysis, the relative height of phosphorylated and non-phosphorylated.

Most important, S225E had about 10 times higher affinity (i.e. lower  $K_d$ ) for the plasma membrane mimetic than S225A, establishing that Ser<sup>225</sup> phosphorylation greatly enhances the affinity of hSK1 for the plasma membrane. Interestingly, the S225A mutation lowered the membrane affinity primarily by increasing  $k_d$ , which is reminiscent of the effect of the T54A and N89A mutations. This implies that Ser<sup>225</sup> phosphorylation may be somehow linked to the PS-mediated membrane interactions of Asn<sup>89</sup> and Thr<sup>54</sup>.

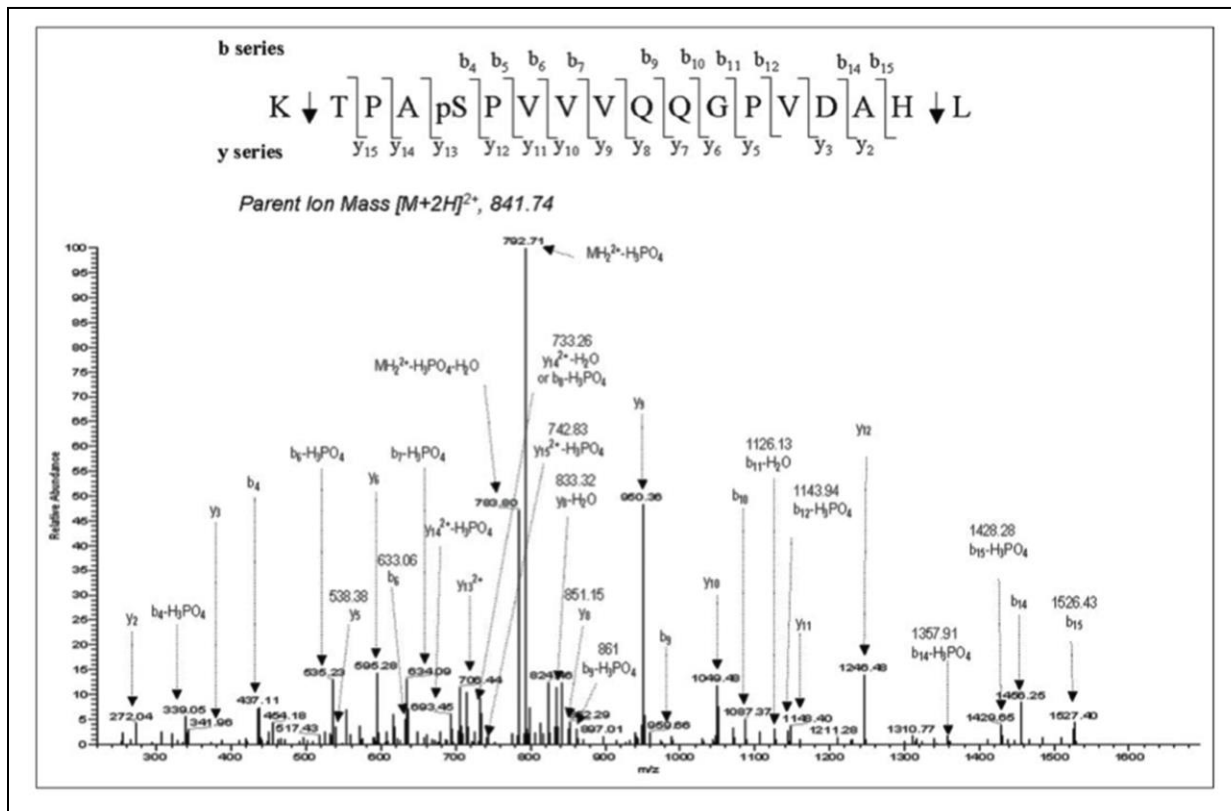


FIGURE 7. Ion trap tandem mass spectra of a hSK1 peptide containing phosphorylated Ser<sup>225</sup>. The fragmentation pattern was matched by the data base searching program, TurboSequest, to the sequence shown. Also shown are the predicted b- and y-type fragment ions (singly charged) and acquired tandem mass spectra of a Ser<sup>225</sup>-phosphorylated peptide. Arrows in the sequence indicate the enzymatic cleavage sites. Notice that the peptide sequence shown here is not a full-length tryptic peptide containing Ser<sup>225</sup>. It has been reported that a non-tryptic (often chymotrypsin-like) cleavage can occur to produce "half-tryptic" peptides when an excess amount of trypsin is used to digest proteins, which is usually the case for the in-gel digestion (46, 47). This peptide fragment was predominantly identified in our analysis, because the full-length tryptic peptide was too large (i.e. 70-amino acid long) to be readily detected by the tandem mass spectrometry analysis. A mass value is assigned to a fragment ion when the mass peak does not have a software-assigned mass value or when the theoretical value does not fully match the experimental value.

This notion is supported by the finding that S225A, like T54A and N89A, had significantly reduced selectivity for the plasma membrane mimetic over the nuclear membrane mimetic than did S225E and wild type (TABLE TWO).

Furthermore, in a monolayer penetration assay using the POPC/POPS (7:3) monolayer, S225A, T54A, and N89A all had significantly lower  $\pi_c$  values than S225E and wild type (Fig. 2B). In particular, the penetration of S225A into the POPC/POPS (7:3) monolayer was comparable with the penetration of wild type hSK1 into non-PS containing monolayers (Fig. 2A). These results suggest that Ser<sup>225</sup> phosphorylation may regulate the membrane targeting of hSK1 by modulating the specific interaction of Asn<sup>89</sup> and Thr<sup>54</sup> with the PS-containing membrane.

We also measured the cellular membrane targeting of C-terminal EGFP-tagged hSK1, S225A, and S225E transiently transfected into HEK293 cells. When transfected cells were stimulated with PMA, S225E translocated to the plasma membrane as well as wild type (Fig. 5E), whereas S225A translocated significantly more slowly than wild type (Fig. 5F), in accordance with its lower *in vitro* membrane affinity. Collectively, these results indicate that Ser<sup>225</sup> phosphorylation regulates the membrane targeting of hSK1 both *in vitro* and in the cell, presumably by modulating the interactions of Asn<sup>89</sup> and Thr<sup>54</sup> with the membrane.

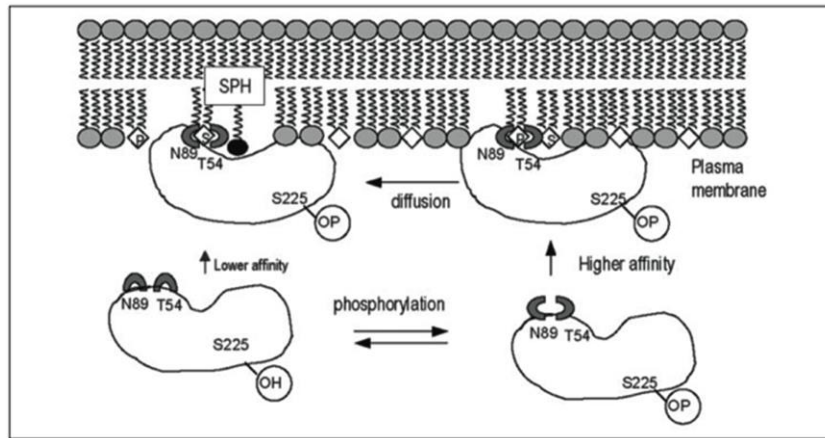
## DISCUSSION

This study systematically investigates the mechanism by which subcellular localization and activation of SK1 is regulated. Because the main physiologic substrate of SK1 is the membrane lipid SPH and SK1 is normally a cytosolic protein, the activation of SK1

should involve its membrane recruitment either through protein-lipid and/or protein-protein interactions. Although some proteins have been reported to interact with SK1 (19, 20), the physiological relevance of protein-mediated subcellular localization of SK1 has not been established. The present study provides new evidence supporting the notion that the lipid-protein interactions play a major role in regulation of membrane translocation and activation of SK1: *i.e.* specific plasma membrane targeting SK1 derives from its selectivity for the PS head group and the phosphorylation of Ser<sup>225</sup> activates SK1 by enhancing its plasma membrane affinity.

Although direct structural information is not available for any SK at present, the sequence comparison of SKs from different species allowed us to identify the residues essential for the PS specificity of hSK1. Our *in vitro* vesicle binding measurements of wild type and mutants indicate that hSK1 specifically interacts with PS via the side chains of Thr<sup>54</sup> and Asn<sup>89</sup>. These data, in conjunction with the fact that the cellular concentration of OS is much higher than PA, strongly dispute the notion that SK1 might act as a specific PA effector in the cell (27). The PS specificity of hSK1 would allow the protein to translocate selectively to the cytoplasmic leaflet of the plasma membrane that is rich in PS. The notion is supported by exceptional selectivity of hSK1 for the plasma membrane mimetic over other cell membrane mimetics, which is dramatically reduced by the T54A or N89A mutations. Thus, along with the C2 domains of protein kinase C $\alpha$  and phospholipase C $\delta$ 1 (22, 39), hSK1 belongs to an expanding group of cellular signaling proteins that are targeted to the plasma membrane from the cytosol because of their selectivity for the PS head group.

**FIGURE 8. A proposed model of SK1 membrane targeting and activation.** Phosphorylation of Ser<sup>225</sup> may induce a conformational change or electrostatic switch of SK1 that allows Thr<sup>54</sup> and Asn<sup>89</sup> to specifically interact with PS (shown with a diamond head group) in the plasma membrane. This induces partial membrane penetration of SK1 and greatly enhances the membrane affinity of SK1. Slower membrane dissociation and elongated membrane residence of SK1 allows SK1 to diffuse in the plane of membrane to find its substrate, SPH, and catalyze the phosphorylation in a processive manner. Thr<sup>54</sup> and Asn<sup>89</sup> may or may not be located in proximity. Likewise, relative location of Ser<sup>225</sup> and the two PS-binding residues are not known.



In the case of PKC $\alpha$ , PS has been reported to specifically induce the conformational change and membrane penetration of the protein (43, 44). This PS- mediated process not only enhances the membrane affinity of PKC $\alpha$  but also activates the protein by opening the active site of the enzyme. It is difficult to directly compare the activation mechanisms of PKC $\alpha$  and SK1 because PKC $\alpha$  activation also involves diacylglycerol. A main similarity is, however, that PS enhances the membrane penetration of both proteins, thereby increasing their residence time (*i.e.* smaller  $k_d$ ) and affinity (*i.e.* smaller  $K_d$ ) for densely packed lipid bilayers including cell membranes. Because little is known about the enzyme activation mechanism of SK1, it is unclear whether the PS- induced membrane penetration of SK1 would simply enhance the membrane affinity or also cause the enzyme activation at the membrane surface. More studies are needed to address this important issue.

The mechanism by which phosphorylation regulates the membrane targeting and activation of SK has not been elucidated. It has been reported that phosphorylation of Ser<sup>225</sup> activates SK1 presumably by promoting its plasma membrane targeting (18). Our results

indicate that phosphorylation of Ser<sup>225</sup> not only enhances the overall membrane binding affinity of hSK1 but also increases its selectivity for the plasma membrane. This raises an interesting possibility that phosphorylation of Ser<sup>225</sup> modulates the interaction of the two PS-binding residues, Asn<sup>89</sup> and Thr<sup>54</sup>, with the membrane. This notion is supported by our monolayer penetration measurements showing that Ser<sup>225</sup> phosphorylation enhances the penetration of hSK1 into the PS- containing monolayer. The relative location of Ser<sup>225</sup> and the PS- binding residues in hSK1 are not known at present. If they are located in proximity, Ser<sup>225</sup> phosphorylation would regulate the membrane interaction of these residues through short- range electrostatic interactions. If, on the other hand, Ser<sup>225</sup> is remote from Asn<sup>89</sup> and Thr<sup>54</sup>, Ser<sup>225</sup> phosphorylation would regulate the membrane binding through a long- range conformational change, as seen with other membrane- binding proteins, such as cytosolic phospholipase A<sub>2</sub> $\alpha$  (45).

On the basis of these results and other available data, we propose a mechanism by which the membrane targeting and activation of SK1 is regulated in the cell (Fig. 8). When Ser<sup>225</sup> is not phosphorylated, SK1 cannot fully exert its

PS- specific binding and therefore only weakly interacts with the anionic plasma membrane through nonspecific electrostatic interactions. Phosphorylation of Ser<sup>225</sup> allows Thr<sup>54</sup> and Asn<sup>89</sup> to specifically interact with PS in the plasma membrane, which induces partial membrane penetration of SK1 and greatly enhances the membrane affinity of SK1. Slower membrane dissociation and elongated membrane residence of SK1 caused by specific PS binding will allow SK1 to laterally diffuse in the plane of membrane, finding its substrate SPH, to catalyze the phosphorylation in a processive manner. Our hypothetical model is based on the premise that the activation of SK1 is regulated mainly at the stage of its membrane binding: *i.e.* cell stimuli activate SK1 mainly by enhancing its membrane affinity. This notion is consistent with the good quantitative correlation between the *in vitro* affinities of hSK1 and mutants for the plasma membrane mimetic and their cellular properties. Such a correlation would not be expected if, for instance, Ser<sup>225</sup> phosphorylation mediated protein- protein interactions. It should be noted that this model is still largely hypothetical and further structural and functional studies are necessary to test the model. For instance, there are no possibilities that some cellular agonists of SK1 and other types of SK1 phosphorylation may mediate protein- protein interactions. Nonetheless, this study provides new insights into the mechanism by which the membrane recruitment of this important protein is regulated and also lays the groundwork for more systematic mechanistic and functional studies.

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