The Mechanism of Membrane Targeting of Human Sphingosine Kinase 1*

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Sphingosine 1-phosphate (S1P)1 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 Ca2+ 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 vivo, S1P is excreted into serum 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-α (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-α induced the phosphorylation of Ser225 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-glycero-3-phosphatic acid (POPA), 1-palmitoyl-2-oleoyl-sn-glycero-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-SP (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, tag and purified from the cell extracts using a Ni2+–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 $k_d$ 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 × 1 cm deep) was measured using a Wilhelmy plate attached to a computer-controlled Cahn electrobalance as described previously (23). Five to ten µl 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, 5 mM MgCl₂, and 0.1 mM CaCl₂) to form a monolayer with a given initial surface pressure ($\pi_i$). Once the surface pressure reading of monolayer had been stabilized (after about 5 min), the protein solution (typically 40 µl) 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] = 1.0 µg/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 $\pi$, 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 Oliveira 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 mM d-erythro-SPH-bovine serum albumin adduct (99:7:0.3 in mole ratio), 1 mM [γ-32P]ATP (Amersham Biosciences; 1 Ci), and 500 µM MgCl₂. 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 (80:20:10:20, v/v/v/v) as eluent. Bands corresponding to SPH 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 mM d-erythro-SPH incorporated into phospholipid vesicles of various compositions (5 µM total concentration), 1 mM [γ-32P]ATP (Amersham Biosciences; 1 µCi), and 500 µM MgCl₂. For cellular SK1 activity assays, transfected HEK293 cells were treated with 2 µM PMA for the indicated times, and 10 min before the end of the time point, cells were pulsed with 300 nM d-erythro-[3H]SPH (American Radiolabeled Co.; 1.0 µCi). 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 edcsy tone receptor (Invitrogen) were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37 °C with 5% CO₂ 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 ×63, 1.2 numerical aperture water immersion objective were used for all experiments. Immediately before imaging, induction media was removed and the cells were washed twice with 1 mM HEPES (pH 7.4), containing 2.5 mM MgCl₂, 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 µM 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^{225} 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 acetoni trole/H₂O (1:1, v/v) for 10 min and dehydrated with acetoni trole. Then, the bands were finally washed with 1:1 (v/v) solution of acetoni trole 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 gradient grade trypsin (500 ng) on ice for 45 min. Then, the gel pieces were immersed in 100 µl of 50 mM amni um bicarbonate at 37 °C for 14–18 h. Resulting peptides were extracted sequentially for 20 min with 45% acetoni trole in 20 mM ammonium bicarbonate, 45% acetoni trole in 0.5% trifluoroacetic acid, and 75% ace toni trole 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 H₂O) and loaded onto fused silica capillary columns containing 8 cm of 5-µm particle size Aqua C₁₈ 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 H₂O). Eluted peptides were directly electrospayed 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 database 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

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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, 30, 31). Fig. 1 shows representative sensograms 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 µM, 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/POPG (7:3)). 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/POPA = 80:20 or 85:15), essentially the same PS selectivity was observed (data not shown). Also, the PS selectivity remained unchanged when pH of the binding solution was varied from 7.4 to 8.1 (i.e. $K_d$ for POPC/POPA (7:3)/$K_d$ for POPC/POPS (7:3) = 4.7 at 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 $k_a$, 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. 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 $\pi_0$, the critical surface pressure ($\pi_0$), 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 dyn/cm (36–38), the $\pi_0$ value for a protein that penetrates cell membranes should be above 30 dyn/cm. As shown in Fig. 2A, hSK1 showed weak penetration into the POPC, POPC/POPG (7:3), POPC/POPG (7:3), or POPC/POPA (7:3) monolayer with $\pi_0$ ≈ 26 dyn/cm. However, hSK1 had stronger penetration activity toward the POPC/POPS (7:3) monolayer with $\pi_0$ ≥ 30 dyn/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 dyn/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 whose lipid head group compositions mimic those of the cytoplasmic plasma membrane, the nuclear membrane, and early endosomes, respectively (22). This approach has been successfully used to determine the origin of specific subcellular localization of various

![Representative sensograms for vesicle binding of hSK1. Varying concentrations (1, 2, 4, 8, 16, and 32 nM) of hSK1 were injected at 30 µl/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.](image)

### TABLE ONE

| Proteins | Lipids        | $k_a$  | $k_d$  | $K_d$  | PS* selectivity |
|----------|---------------|--------|--------|--------|-----------------|
| Wild type| POPC/POPS (7:3)| (2.2 ± 0.3) x 10^5 | (9.3 ± 0.7) x 10^-4 | (4.2 ± 0.7) x 10^-9 | 12              |
| Wild type| POPC/POPG (7:3)| (1.4 ± 0.2) x 10^5 | (7.4 ± 0.6) x 10^-4 | (5.2 ± 0.9) x 10^-8 |                 |
| Wild type| POPC/POPA (7:3)| (1.2 ± 0.3) x 10^5 | (8.1 ± 0.7) x 10^-3 | (6.8 ± 3.0) x 10^-8 |                 |
| Wild type| POPC/POA (7:3)| (2.5 ± 0.4) x 10^5 | (6.9 ± 0.9) x 10^-2 | (2.8 ± 0.6) x 10^-8 |                 |
| T54A     | POPC/POPG (7:3)| (9.6 ± 0.3) x 10^4 | (1.8 ± 0.2) x 10^-2 | (1.9 ± 0.2) x 10^-7 | 0.6             |
| T5A      | POPC/POPA (7:3)| (9.2 ± 0.9) x 10^4 | (9.8 ± 1.0) x 10^-2 | (1.1 ± 0.1) x 10^-7 |                 |
| N89A     | POPC/POA (7:3)| (1.1 ± 0.3) x 10^5 | (1.1 ± 0.2) x 10^-2 | (1.0 ± 0.3) x 10^-7 | 1.0             |
| N89A     | POPC/POG (7:3)| (9.7 ± 0.6) x 10^4 | (8.6 ± 0.7) x 10^-4 | (8.9 ± 0.9) x 10^-8 |                 |
| S168A    | POPC/POPS (7:3)| (2.8 ± 0.5) x 10^5 | (9.5 ± 0.9) x 10^-4 | (3.4 ± 0.6) x 10^-9 | 13              |
| S168A    | POPC/POPA (7:3)| (2.0 ± 0.2) x 10^5 | (8.5 ± 0.7) x 10^-3 | (4.3 ± 0.4) x 10^-8 |                 |

*Ratio of (1/$K_d$) for POPC/POPS (7:3) vesicles to (1/$K_d$) for POPC/POPG (7:3) vesicles.
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Binding of hSK1 phosphorylation mutants to cell membrane mimetics
All values represent mean ± S.D. from triplicate determinations. All measurements were performed in 20 mM HEPES, pH 7.4, containing 0.16 mM KCl.

| Proteins                  | $k_a$  | $k_d$  | $K_d$  | Fold* increase in $K_d$ | PM/NM* |
|--------------------------|--------|--------|--------|------------------------|--------|
| Plasma membrane mimetic: |        |        |        |                        |        |
| Wild type                | 2.9 ± 0.3 | 0.55 ± 0.07 | 1.9 ± 0.3 | 1                      | 373    |
| T54A                     | 1.1 ± 0.2 | 9.8 ± 1.0  | 89 ± 20  | 47                     | 22     |
| N89A                     | 1.3 ± 0.3 | 7.7 ± 0.6  | 59 ± 10  | 31                     | 24     |
| S225A                    | 1.4 ± 0.3 | 3.5 ± 0.4  | 25 ± 6   | 13                     | 88     |
| S225E                    | 2.5 ± 0.5 | 0.59 ± 0.4  | 2.4 ± 0.5 | 1.3                    | 329    |
| Nuclear membrane mimetic:|        |        |        |                        |        |
| Wild type                | 0.91 ± 0.08 | 65 ± 6   | 710 ± 90 | 1                      |        |
| T54A                     | 0.45 ± 0.05 | 92 ± 8   | 2000 ± 300 | 2.8                   |        |
| N89A                     | 0.63 ± 0.04 | 86 ± 7   | 1400 ± 300 | 2.0                   |        |
| S225A                    | 0.44 ± 0.06 | 98 ± 15  | 2200 ± 450 | 3.1                   |        |
| S225E                    | 0.86 ± 0.7  | 68 ± 7   | 790 ± 100  | 1.1                   |        |
| Endosomal membrane mimetic:|        |        |        |                        |        |
| Wild type                | 1.1 ± 0.2 | 18 ± 3  | 16 ± 4  |                        |        |

- Fold increase in $K_d$ relative to the binding of hSK1 to each vesicle.
- Ratio of $(1/K_d)$ for the plasma membrane mimetic to $(1/K_d)$ for the nuclear membrane mimetic.

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. This exceptional selectivity of hSK1 for the plasma membrane is not only rich in PS but also the most anionic membrane in all cell membranes. This 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.

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 PKCa 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, Thr54, Ser79, Asn89, Ser165, Ser114, Ser119, Ser166, and Ser168. 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 Thr54 and/or Asn89 might be specifically involved in PS interaction. Interestingly, Asn89 is located within the putative ATP-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-imbedded substrate, SPL. 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 Thr54 and Asn89 is important not only for membrane binding of hSK1 but also for the enzymatic action of hSK1 on membrane-incorporated SPH.

To see if Thr54 and Asn89 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-3H]SPH and monitoring the release of the radioactive S1P into the media. As shown in Fig. 6, PMA induced a 4-fold increase in S1P release over the control for wild type and S168A, whereas little to no increases in S1P release over the controls were detected for T54A and N89A with and without PMA. Collectively, these results indicate that Thr54 and Asn89, 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 Ser225 Phosphorylation on the Membrane Targeting of hSK1—It has been reported that Ser225 of SK1 is phosphorylated by extracellular signal-regulated kinase 1/2 in response to PMA or tumor necrosis factor-α stimulation in HEK293 cells, which leads to PM targeting and activation of SK1 (18). To investigate how Ser225 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 Ser225. To test this notion, we determined the phosphorylation of Ser225 in insect cell-expressed recombinant hSK1 by mass spectrometry. Tandem mass spectrometry data of tryptic peptide fragments containing Ser225 (Fig. 7) consistently showed that Ser225 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 peaks for various peptide fragments containing Ser225 (data not shown) implies that a large portion of Ser225 is phosphorylated.

Most important, S225E had about 10 times higher affinity (i.e. lower $K_d$) for the plasma membrane mimetic than S225A, establishing that Ser225 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 Ser225 phosphorylation may be somehow linked to the PS-mediated membrane interactions of Asn89 and Thr54. 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 $\eta$ 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 Ser225 phosphorylation may regulate the membrane targeting of hSK1 by modulating the specific interaction of Asn89 and Thr54 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 Ser225 phosphorylation regulates the membrane targeting of hSK1 both in vitro and in the cell, presumably by modulating the interactions of Asn89 and Thr54 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 of SK1 derives from its selectivity for the PS head group and the phosphorylation of Ser225 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 Thr54 and Asn89. These data, in conjunction with the fact that the cellular concentration of PS 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. This notion is supported by exceptional selectivity of hSK1 for the plasma membrane mimic 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 and phospholipase C (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.

In the case of PKCa, 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 PKCa but also activates the protein by opening the active site of the enzyme. It is difficult to directly compare the activation mechanisms of PKCa and SK1 because PKCa activation also involves diacylglycerol. A main similarity is, however, that PS enhances the membrane penetra-
tion of both proteins, thereby increasing their residence time (i.e. smaller $k_j$) at and affinity (i.e. smaller $K_j$) 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 Ser225 activates SK1 presumably by promoting its plasma membrane targeting (18). Our results indicate that phosphorylation of Ser225 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 Ser225 modulates the interaction of the two PS-binding residues, Asn89 and Thr54, with the membrane. This notion is supported by our monolayer penetration measurements showing that Ser225 phosphorylation enhances the penetration of hSK1 into the PS-containing monolayer. The relative location of Ser225 and the PS-binding residues in hSK1 are not known at present. If they are located in proximity, Ser225 phosphorylation would regulate the membrane interaction of these residues through short-range electrostatic interactions. If, on the other hand, Ser225 is remote from Asn89 and Thr54, Ser225 phosphorylation would regulate the membrane binding through a long-range conformational change, as seen with other membrane-binding proteins, such as cytosolic phosphatase A5 (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 Ser225 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 Ser225 allows Thr54 and Asn89 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, find its substrate, SPH, and catalyze the phosphorylation in a processive manner. Thr54 and Asn89 may or may not be located in proximity. Likewise, relative location of Ser225 and the two PS-binding residues are not known.

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