Sphingosine kinases catalyze the formation of sphingosine 1-phosphate, a bioactive lipid involved in many aspects of cellular regulation, including the fundamental biological processes of cell growth and survival. A diverse range of cell agonists induce activation of human sphingosine kinase 1 (hSK1) and, commonly, its translocation to the plasma membrane. Although the activation of hSK1 in response to at least some agonists occurs directly via its phosphorylation at Ser225 by ERK1/2, many aspects governing the regulation of this phosphorylation and subsequent translocation remain unknown. Here, in an attempt to understand some of these processes, we have examined the known interaction of hSK1 with calmodulin (CaM). By using a combination of limited proteolysis, peptide interaction analysis, and site-directed mutagenesis, we have identified that the CaM-binding site of hSK1 resides in the region spanned by residues 191–206. Specifically, Phe197 and Leu198 are critically involved in the interaction because a version of hSK1 incorporating mutations of both Phe197 → Ala and Leu198 → Gln failed to bind CaM. We have also shown for the first time that human sphingosine kinase 2 (hSK2) binds CaM, and does so via a CaM binding region that is conserved with hSK1 because comparable mutations in hSK2 also ablate CaM binding to this protein. By using the CaM-binding-deficient version of hSK1, we have begun to elucidate the role of CaM in hSK1 regulation by demonstrating that disruption of the CaM-binding site ablates agonist-induced translocation of hSK1 from the cytoplasm to the plasma membrane, while having no effect on hSK1 phosphorylation and catalytic activation.

Sphingosine kinases are important signaling enzymes because of their role in the synthesis of the bioactive lipid sphingosine 1-phosphate (S1P).3 Many studies have shown that S1P can affect a diverse array of biological processes, including calcium mobilization, mitogenesis, apoptosis, atherosclerosis, inflammatory responses, cell motility, and angiogenesis (1–4). Although some of these varied effects of S1P result from its action as a ligand for S1P-specific cell-surface G-protein-coupled receptors (5), significant evidence now exists that indicates S1P can also function intracellularly as a second messenger, particularly in the regulation of cell proliferation and apoptosis (6).

Two sphingosine kinases exist in humans (hSK1 and hSK2), with most studies to date focusing on hSK1. Although these two enzymes originate from different genes and differ in size, tissue distribution, developmental expression, substrate specificity, specific activity, and possibly in their cellular roles (7–10), their polypeptide sequences possess a high degree of similarity. In fact, almost all of the hSK1 sequence aligns with regions of the larger hSK2 sequence with 80% overall similarity (45% identity) (7). However, hSK2 also possesses two additional polypeptide regions at its N terminus and within the central region of its sequence that are quite distinct from hSK1.

Although hSK1 has considerable intrinsic catalytic activity (11), its activity can be enhanced by exposure of cells to various growth factors and other agonists (6). This agonist-induced activation of hSK1 results in an increase in cellular levels of S1P, which appears critical in mediating the cellular effects attributed to S1P (12). We have recently identified that activation of hSK1 occurs directly via its phosphorylation at Ser225 by ERK1/2 (13). We have also observed that this phosphorylation and, in particular, the subsequent phosphorylation-dependent translocation of hSK1 to the plasma membrane are critical steps for the pro-proliferative, anti-apoptotic effects of this enzyme in the cell (14). Thus, considerable insight into the regulation of hSK1 has been recently gained. However, much is still not known regarding the exact molecular mechanisms controlling the activation and cellular localization of this enzyme.

Numerous studies have shown that both human and mouse SK1 associate with calmodulin (CaM) in a Ca2+-dependent manner (11, 15–18). Because CaM is a common regulatory protein controlling the activity and function of many signaling enzymes (19), its potential role in the regulation of hSK1 has long been speculated (11, 20, 21). Although we have shown previously that neither Ca2+-bound nor Ca2+-free CaM (Ca2+/CaM and apoCaM, respectively) had an effect on hSK1 activity in vitro (11), other studies have suggested it may play a role in Ca2+-dependent translocation of hSK1 to the plasma membrane because this process is inhibited by the Ca2+/CaM antagonist W-7 (22). Further elucidation of the direct role of CaM in the regulation of hSK1 has, however, been limited by the lack of molecular tools to specifically ablate CaM binding to hSK1. In this study we have identified the CaM-binding sites of hSK1 and hSK2. This has enabled the generation of CaM-binding-deficient versions of the proteins and allowed further
molecular examination of the role of CaM in sphingosine kinase regulation.

**EXPERIMENTAL PROCEDURES**

Cell Culture and Transfection—Human embryonic kidney cells (HEK293T) were cultured in Dulbecco’s modified Eagle’s medium (JR Biosciences, Lenexa, KS) containing 10% bovine calf serum (JR Biosciences), 2 mM glutamine, 0.2% (w/v) sodium bicarbonate, penicillin (1.2 mg/ml), and streptomycin (1.6 mg/ml). Cells were transiently transfected using the calcium phosphate precipitation method, harvested, and lysed by sonication as described previously [12]. Protein concentrations in cell homogenates were determined with Coomassie Brilliant Blue reagent (Sigma) using bovine serum albumin as standard. Assays for colony formation in soft agar were performed as detailed previously [23].

Construction of Sphingosine Kinase Mutants—hSK1 cDNA (GenBank accession number AF200328) was FLAG epitope tagged at the N terminus with enhanced green fluorescent protein (EGFP) by using methods described previously [24]. hSK2 was also tagged at the N terminus with EGFP. Briefly, EGFP was PCR-amplified from pEGFP-1 (Clontech) with the primers 5′-ATGAGAAGCAATTGCACAACCT-3′ and 5′-GCGGCTGCTGACCCCTG-3′. The cDNA was subsequently subcloned into pcDNA3 (Invitrogen) for transient transfection into HEK293T cells.

The hSK1F197A/L198Q was tagged at the N terminus with enhanced green fluorescent protein (EGFP) by using methods described previously for wild type hSK1 (13). hSK2 was PCR-amplified from the hSK1 cDNA (GenBank accession number AF200328) with the primers 5′-GCGGCTGCTGACCCCTG-3′ and 5′-GCGGCTGCTGACCCCTG-3′. The cDNA was subsequently subcloned into pcDNA3 (Invitrogen) for transient transfection into HEK293T cells.

For studies to elucidate the role of hSK1 phosphorylation on its association with CaM, purified recombinant hSK1 generated in E. coli was phosphorylated in vitro at Ser225 by ERK2 as described previously [13].

Generation of GST Peptides and Pull-down Analyses—The sequences encoding peptides containing the putative CaM binding (PBox) regions of hSK1 were PCR-amplified from the hSK1 cDNA with the following primers: PBox1/2, 5′-TAGATGATGCCCGCCGCTACCGACGGCCATTACGCG-3′ and 5′-TAGATGATGCCCGCCGCTACCGACGGCCATTACGCG-3′; PBox3, 5′-TAGATGATGCCCGCCGCTACCGACGGCCATTACGCG-3′ and 5′-TAGATGATGCCCGCCGCTACCGACGGCCATTACGCG-3′; and PBox4, 5′-TAGATGATGCCCGCCGCTACCGACGGCCATTACGCG-3′ and 5′-TAGATGATGCCCGCCGCTACCGACGGCCATTACGCG-3′. The products were subsequently digested with EcoRI and cloned into pGEX2T. Enzymes of Escherichia coli JM109 transformed with these pGEX2T-PBox plasmids were grown overnight in Luria broth containing 100 mg/liter ampicillin at 37 °C with shaking. The culture was then diluted 1 in 10 into the same medium and grown at 37 °C for 1 h with shaking until reaching an A<sub>600</sub> of ∼0.6. Expression of the GST-PBox peptides was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM, and the culture was then harvested for analysis.

The H11032 peptide was expressed in E. coli and purified as described previously [25]. The sequence of the peptide is 5′-AGGTGCGCCATCGTCTGGTG-3′. The H11032 peptide was used as substrate for the assay.

Measurement of S1P—Measurement of cellular S1P levels were performed using the enzyme immunoassay described by Edsall et al. [24].

Calmodulin Binding Assays—Assays to assess CaM binding of sphingosine kinase were performed as detailed previously [17]. Brieﬂy, HEK293T cells overexpressing wild type or mutant hSK1 or hSK2 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 CaM-Sepharose 4B (Amersham Biosciences) pre-equilibrated with binding buffer composed of 50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 10% (w/v) glycerol, 0.05% (w/v) Triton X-100, 1 mM dithiothreitol, 1 mM MgCl₂, and 0.1% NP-40, and protease inhibitors (Complete<sup>™</sup>, Roche Applied Science) and incubated with either 5 mM CaCl₂ or 5 mM EGTA for 30 min at 4 °C with continuous mixing. The CaM-Sepharose 4B beads were then pelleted by centrifugation (5000 × g, 5 min at 4 °C) and washed twice with binding buffer. Bound hSK1 or hSK2 was then resolved by SDS-PAGE and visualized by Western blotting via the FLAG epitope. Sepharose CL-4B (Amersham Biosciences) was used as a control for nonspecific binding to CaM-Sepharose 4B.
glutathione-Sepharose (Amersham Biosciences) was added, and the mixture was incubated at 4 °C for 1 h with constant agitation. After this time the glutathione-Sepharose was washed three times with cold phosphate-buffered saline, and the GST peptides were eluted with 20 mM glutathione in 50 mM Tris/HCl, pH 8.5, for 10 min at 4 °C. Pull-down analyses with CaM-Sepharose and the GST peptide fusion proteins was performed as described above using ~1 μg of each purified GST peptide or GST alone. Peptide binding to CaM-Sepharose was detected using an anti-GST antibody.

**Limited Proteolysis and N-terminal Sequencing**—Recombinant hSK1 was generated and purified from Sf9 cells as described previously (17). Limited proteolysis of this hSK1 (1.5 μg in 15 μl) was performed in the presence or absence of a 3-fold molar excess of purified bovine CaM (Sigma) by the addition of 2 or 5 ng of trypsin (Roche Applied Science) in 100 mM Tris/HCl, pH 8.5. The mixture was then incubated at 37 °C for 60 min, stopped by the addition of 1.5 μl of 100 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (Roche Applied Science), and incubated for an additional 5 min at 37 °C. Tryptic cleavage products were then resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane. Following Coomassie staining of the membrane, bands that were protected in the presence of CaM were excised, and their N-terminal sequences were determined by 6 cycles of automated Edman degradation using an Applied Biosystems 494 Procise Protein Sequencing System at the Australian Proteome Analysis Facility.

**Western Blotting**—SDS-PAGE was performed on cell lysates using 12% acrylamide gels. Proteins were transferred to nitrocellulose, and the membranes were blocked overnight at 4 °C in phosphate-buffered saline containing 5% skim milk powder and 0.1% (v/v) Triton X-100. hSK1 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 horseradish peroxidase anti-mouse (Pierce) IgG using an enhanced chemiluminescence kit (ECL, Amersham Biosciences).

**Fluorescence Microscopy and Subcellular Fractionation**—HEK293T cells expressing EGFP fusion proteins were plated onto poly-L-lysine-coated (Sigma) 8-well glass chamber slides and incubated for 24 h. Following incubation for 30 min with or without PMA, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 min. Epifluorescence microscopy was then performed on an Olympus BX-51 microscope equipped with a fluorescein excitation filter (494 nm), acquired to a Cool Snap FX charge-coupled device camera (Photomet- rics). COS-7 cells expressing EGFP fusion proteins were analyzed in a similar manner, except instead of PMA treatment, the effect of cell density on hSK2 cellular localization was determined by plating the cells at a wide range of cell densities. Cell lysates were fractionated into cytosol and membrane fractions as described previously (13).

**RESULTS**

**Mutagenesis of Predicted CaM binding sites in hSK1**—hSK1 and its murine orthologue have been shown to bind CaM in a Ca2+-dependent manner (11, 15–18). Previous analysis of the hSK1 polypeptide sequence indicated the presence of three potential Ca2+/CaM binding regions (11) as follows: two that overlap between residues 134 and 153 (termed here PCB1 and PCB2), and one spanning residues 290–303 (PCB4) (Fig. 1). All three of these putative Ca2+/CaM-binding sites are conserved in the mouse SK1 sequence (27). Further analysis of the hSK1 polypeptide sequence using the CaM target data base (26) also revealed another putative Ca2+/CaM-binding site spanning residues 191–206 (PCB3) (Fig. 1). Again, this putative Ca2+/CaM-binding region is also conserved in murine SK1.

To examine the potential role of the putative Ca2+/CaM-binding sites in the interaction of hSK1 with CaM, we performed site-directed mutagenesis of hSK1 in these regions. In particular, this mutagenesis concentrated on the conserved hydrophobic residues within these motifs that are generally critical in Ca2+/CaM binding (28–30), changing them to structurally conservative hydrophilic residues. PCB1 and PCB2 were targeted by mutations of Leu147 → Gln (hSK1L147Q) and Leu153 → Gln (hSK1L153Q), respectively. PCB3 was targeted by mutations of Leu193 → Gln (hSK1L193Q) and Leu200 → Gln (hSK1L200Q), and PCB4 was targeted by a Phe203 → His mutation (hSK1L200Q). These versions of hSK1 were then expressed in HEK293T cells and analyzed for both their ability to bind CaM-Sepharose and their catalytic activity, as a measure of retained gross protein folding. Although these hSK1 mutants all retained at least some catalytic activity (Fig. 2B), somewhat surprisingly, all five bound Ca2+/CaM with similar efficiency to wild type hSK1 (Fig. 2A). This suggested that these predicted Ca2+/CaM binding regions of hSK1 may not be involved in CaM binding. Unfortunately, further mutagenesis of other conserved hydrophobic residues within these putative Ca2+/CaM binding regions (i.e. Leu134 → Gln and Val290 → Asn) yielded catalytically inactive hSK1 proteins (Fig. 2B) that were therefore not further analyzed because of the likelihood that the mutations caused disruption to the gross folding of these proteins.

**Direct Identification of the CaM-binding Site in hSK1**—Because our mutagenesis experiments suggested that the Ca2+/CaM-binding sites of hSK1 predicted from sequence analysis were not responsible for CaM binding, we performed further experiments to directly identify the CaM binding region in hSK1. This was initially performed using limited proteolysis of purified recombinant hSK1 and identifying cleavage sites in hSK1 that were protected by the presence of Ca2+/CaM. Such an approach has been used previously to identify the CaM-binding sites of other proteins (31, 32). Limited proteolysis of purified recombinant hSK1 with trypsin generated several detectable cleavage products rang-
Calmodulin-binding Site of Sphingosine Kinase

FIGURE 2. Site-directed mutagenesis of predicted CaM binding regions of hSK1. A, selective binding of the hSK1 mutants to CaM-Sepharose (CaM) was examined using extracts from HEK293T cells expressing the various hSK1 mutants (Load). Bound hSK1 proteins were visualized by Western blotting via their FLAG epitope. Binding to Sepharose CL-4B (CL4B) was used as a control to account for any nonspecific binding to the CaM-Sepharose beads. B, relative catalytic activity of the hSK1 mutants. Data are mean (±S.D.) from three independent experiments. WT, wild type.

in size from ~9 to 32 kDa (Fig. 3A). Inclusion of Ca\(^{2+}\)/CaM during this limited proteolysis, however, resulted in the loss of a number of hSK1-derived products in the 17–22-kDa range and the accumulation of larger hSK1-derived polypeptides (Fig. 3A). The two most notable polypeptides not generated during limited proteolysis in the presence of Ca\(^{2+}\)/CaM had approximate molecular masses of 21 and 22 kDa (Fig. 3A). These polypeptides represented C-terminal fragments of hSK1 because they retained the His tag that resides at this end of the intact recombinant hSK1 (Fig. 3B), and they were presumably not generated because of the presence of bound Ca\(^{2+}\)/CaM in close proximity to at least two tryptic cleavage sites within the central region of hSK1. Thus, to identify these cleavage sites protected by Ca\(^{2+}\)/CaM, we performed N-terminal sequencing of the two peptides. The sequences obtained (FTLTGTF- and LAALRRTYR-) indicated that the two protected tryptic cleavage sites reside at Arg\(^{191}\)/Phe\(^{192}\) and Arg\(^{199}\)/Leu\(^{200}\). Interestingly, these two tryptic cleavage sites reside within the PCB3 region predicted to be a potential Ca\(^{2+}\)/CaM-binding site by the CaM target data base. Despite the results obtained with our mutagenesis experiments, this suggested that this region may indeed constitute the CaM binding region.

To further elucidate the potential CaM-binding sites in hSK1, we examined the Ca\(^{2+}\)/CaM interaction with 30 amino acid peptides, based on the PCB1/2, PCB3, and PCB4 regions of hSK1, that were fused to GST. Because Ca\(^{2+}\)/CaM generally interacts with distinct amphipathic \(\alpha\)-helices of ~20 residues (26, 29, 33), such an approach has been used previously to successfully identify CaM-binding sites in a number of proteins (34–36). The three peptides were prepared as fusion proteins with GST and were then assessed for their ability to bind CaM-Sepharose. Consistent with the results obtained above using limited proteolysis, only the fusion protein containing the PCB3 peptide displayed an association with Ca\(^{2+}\)/CaM (Fig. 4). Combined, these data strongly indicate that PCB3 is the CaM recognition region of hSK1.

**Generation of an hSK1 Mutant Deficient in CaM Binding**—Although mutations of either Leu\(^{197}\) or Leu\(^{200}\) within PCB3 did not alter the binding of hSK1 with Ca\(^{2+}\)/CaM (Fig. 2), the results obtained from limited proteolysis and peptide binding studies provided the impetus to further examine other residues in this region that may be critical for the association. Thus, we generated versions of hSK1 containing mutations in several individual hydrophobic residues within PCB3, including Leu\(^{194}\) → Gln (hSK1\(^{194}\)Q), Phe\(^{197}\) → Ala (hSK1\(^{197}\)A), and Leu\(^{200}\) → Gln (hSK1\(^{200}\)Q). Again, these versions of hSK1 were then expressed in HEK293T cells and analyzed for their ability to bind Ca\(^{2+}\)/CaM.

Although all three appeared to associate with CaM-Sepharose, the results demonstrate that they did so with somewhat reduced efficiency compared with wild type hSK1 (Fig. 5). This is despite all of three variant proteins retaining at least some catalytic activity, suggesting the mutations were not affecting gross protein folding. In light of these findings we generated a version of hSK1 containing both the Phe\(^{197}\) → Ala and Leu\(^{198}\) → Gln mutations (hSK1\(^{197A/198Q}\)), and we examined its ability to associate with Ca\(^{2+}\)/CaM. These two mutations completely ablated the binding of hSK1 to Ca\(^{2+}\)/CaM (Fig. 5). Furthermore, this double mutant of hSK1 retained considerable catalytic activity indicating that this defect in its ability to associate with Ca\(^{2+}\)/CaM was not a result of a disruption ingross folding of the protein. Thus, these results firmly establish that the CaM binding region of hSK1 resides within the PCB3...
region of hSK1 (residues 191–206) and that Phe197 and Leu198 are critically involved in the interaction of hSK1 with Ca\(^{2+}\)/CaM.

To further characterize the CaM-binding site of hSK1, we also targeted a basic region toward the N-terminal end of PCB3 because, in addition to the critical involvement of hydrophobic residues in Ca\(^{2+}\)/CaM binding, such clusters of basic residues are commonly involved in the electrostatic stabilization of the interaction of Ca\(^{2+}\)/CaM with its targets (29). We generated a version of hSK1 containing Ala at both Arg185 and Arg186 (hSK1 R185A/R186A), and we examined its ability to associate with CaM-Sepharose. Somewhat surprising, based on our previous analyses and comparison with other CaM-binding sites (29), hSK1 R185A/R186A retained the capacity to bind Ca\(^{2+}\)/CaM (Fig. 5), indicating that these basic residues are not required for this interaction.

**Role of CaM Binding in Sphingosine Kinase Regulation**—We have recently established that activation of hSK1 through phosphorylation at Ser225 results in the subsequent translocation of hSK1 to the plasma membrane (13). Studies by Young et al. (22) suggested a potential role for CaM in the Ca\(^{2+}\)-dependent translocation of hSK1 to the plasma membrane because treatment of cells with the CaM antagonist W-7 inhibited this process. However, it was not clear from these studies whether interaction between CaM and hSK1 was required for the translocation or whether CaM was indirectly involved in the process. Also unclear was the potential involvement of CaM in the translocation of hSK1 to the plasma membrane observed to be induced by various other cellular stimuli, including platelet-derived growth factor (37), anaphylatoxin C5a (38), phorbol esters (13, 39), and cross-linking of the Fc\(\varepsilon\)R1 immunoglobulin receptor (40). The identification of the CaM-binding site and generation of a CaM-binding deficient version of hSK1 in the current study enabled us to examine further the direct role of CaM in the cellular localization of hSK1. Thus, we examined the involvement of CaM in the well established phorbol ester-induced translocation of hSK1 to the plasma membrane. Wild type hSK1 and hSK1 F197A/L198Q were expressed in HEK293T cells as fusion proteins with EGFP, and their localization was examined following cell exposure to PMA. As reported previously (13, 39), a shift in the localization of wild type hSK1 from the cytosol to the plasma membrane was observed following this treatment (Fig. 6A). In stark contrast, no redistribution of hSK1 F197A/L198Q was observed in response to PMA (Fig. 6A). Comparable results were also observed by immunofluorescence (data not shown) and subcellular fractionation (Fig. 6B) using versions of hSK1 lacking the EGFP fusion partner. Together, the data strongly indicate an important role for the CaM-binding site in translocation of hSK1.

We have previously observed the critical requirement of phosphorylation of hSK1 at Ser225 in PMA-induced translocation of this enzyme (13). Therefore, we examined the phosphorylation and activation of hSK1 F197A/L198Q in response to cell exposure to PMA and TNF\(\alpha\) to ensure that the mutations to the CaM-binding site were not inhibiting translocation via an indirect effect on this process. Indeed, we found that this was not the case because treatment of cells with either PMA or TNF\(\alpha\) resulted in enhanced phosphorylation and catalytic activity of hSK1 F197A/L198Q in a comparable manner to that observed with wild type hSK1 (Fig. 6C). This demonstrates that CaM binding is not involved in the phosphorylation and catalytic activation of hSK1, and suggests that disruption of hSK1 translocation by mutations of the CaM-binding site occurs by directly altering protein/protein interactions at this site.

We also examined the effect of hSK1 phosphorylation on its association with CaM. The results show that *in vitro* phosphorylation of recombinant hSK1 at Ser225 with ERK2 yielded a phosphoprotein that had with similar binding efficiency to CaM-Sepharose as nonphosphorylated recombinant hSK1 (Fig. 6D).

Notably, the PMA-induced increase in cellular S1P levels observed with cells overexpressing wild type hSK1 was strongly attenuated in cells expressing hSK1 F197A/L198Q (Fig. 7A), further supporting the role of hSK1 translocation to the plasma membrane in its signaling functions. Interestingly, however, ablation of CaM binding appeared to have little effect on the ability of hSK1 to induce neoplastic transformation of NIH3T3 cells, as measured by the formation of colonies in soft agar (Fig. 7B).

**CaM Binding Is Conserved between hSK1 and hSK2**—Although no previous studies had examined the ability of hSK2 to bind CaM, sequence analysis demonstrated that the identified CaM-binding site of hSK1 is conserved in hSK2 and resides within a region predicted to form an \(\alpha\)-helix in both proteins (Fig. 8A). Furthermore, analysis of the hSK2 polypeptide sequence using the CaM target data base (26) also identified this same region as a putative Ca\(^{2+}\)/CaM-binding site. Thus, we examined whether CaM could also bind hSK2. Indeed, as with hSK1, we found that hSK2 associated with CaM (Fig. 8B). Although this interaction between hSK2 and CaM was enhanced by the presence of Ca\(^{2+}\),
unlike the situation with hSK1, considerable binding of (Ca\(^{2+}\)/H\(^{11001}\)-free) apoCaM to hSK2 was observed in the absence of Ca\(^{2+}\)/H\(^{11001}\) (Fig. 8B).

Although hSK2 interacted with CaM, addition of Ca\(^{2+}\)/CaM or apoCaM to enzyme assays of recombinant hSK2 indicated that, like the situation for hSK1 (11), Ca\(^{2+}\)/CaM does not alter catalytic activity of hSK2 in vitro (data not shown). Thus, the physiological role of this interaction of CaM with hSK2 remains to be determined.

To generate a version of hSK2 deficient in CaM binding, we performed mutagenesis on the residues in this protein that were conserved with those critical for CaM binding of hSK1. This version of hSK2 (hSK2\(^{F197A/L198Q}\)) was then expressed in HEK293T cells and analyzed for both its ability to bind CaM-Sepharose and catalytic activity, as a measure of retained gross protein folding. Consistent with our findings with hSK1, this hSK2 mutant retained high catalytic activity (Fig. 8D) but did not interact with either Ca\(^{2+}\)/CaM (Fig. 8C) or apoCaM (data not shown). Thus, these studies firmly establish that hSK1 and hSK2 not only associate with CaM but that both enzymes do so via a conserved binding site.

We next examined the molecular basis of the differential requirement of Ca\(^{2+}\) for interaction of CaM with the conserved regions of hSK1 and hSK2. Nitric-oxide synthase-I and -II also display differential binding to Ca\(^{2+}\)/CaM and apoCaM, respectively (42). Because this differential binding of nitric-oxide synthase-I and -II to CaM appears mediated by just one charged amino acid within the conserved CaM-binding site of these proteins (42), we examined whether a similar situation may occur with the sphingosine kinases. Thus, we generated versions of hSK1 and hSK2 where the CaM-binding sites were swapped (incorporating the seven variations outlined in Fig. 8A to generate hSK1hSK2-CBS and hSK2hSK1-CBS). These versions of hSK1 and hSK2 were then examined for their ability to associate with CaM in the presence and absence of Ca\(^{2+}\). Somewhat surprisingly, however, in light of the situation with the nitric-oxide synthase isoforms, swapping the CaM-binding sites between hSK1 and hSK2 did not alter the effect of Ca\(^{2+}\) on their respective interactions with CaM (Fig. 8E). Clearly, other determinants within these proteins, outside of the immediate CaM-binding sites, must play a role in regulating the Ca\(^{2+}\) dependence of their association with CaM.

Because the CaM-binding site of hSK1 appears to be involved in the agonist-dependent translocation of hSK1 to the plasma membrane, we examined whether the CaM-binding site of hSK2 also plays a role in the cellular distribution of this enzyme. Wild type hSK2 and hSK2\(^{F197A/L198Q}\) with either FLAG epitope tags or as fusion proteins with EGFP were expressed in HEK293T cells, and their localization was examined following cell exposure to PMA. Consistent with previous studies (8, 9, 43), wild type hSK2 was present largely in the cytoplasm of...
the untreated cells, with hSK2V327A/L328Q also showing a similar localization (data not shown). Unlike hSK1, exposure of the cells to PMA did not result in detectable redistribution of hSK2. Similar results were also recently reported for hSK2 following epidermal growth factor treatment of cells, which was shown to activate hSK2 (43), suggesting that, unlike hSK1, hSK2 may not change cellular localization in an agonist-dependent manner. Previous studies have suggested that hSK2 resides within various cellular compartments, including the cytosol, nucleus, plasma membrane, and other membrane fractions, with some apparent differences between cell types (8, 9, 43). Interestingly, Igarashi et al. (9) have suggested that in COS-7 cells, hSK2 redistributes from the cytoplasm to the nucleus when cells are grown at high density. Therefore, we attempted to establish if the CaM-binding site of hSK2 is involved in this process. Unlike the previous study (9), however, and despite repeated attempts, we were unable to observed a correlation between cell density and nuclear localization of hSK2 in COS-7 cells (data not shown). The reasons for the discrepancy between our work and the previous study (9) are not currently clear.

**DISCUSSION**

In this study we have elucidated the CaM-binding site in hSK1 using a combination of approaches, including protection from limited proteolysis, peptide binding analysis, and site-directed mutagenesis. The CaM-binding site of hSK1, which is also conserved in hSK2, occurs in a region of the proteins predicted to form an α-helix. This is consistent with many CaM target domains that generally form basic, amphiphilic α-helices (29).

Sequence analysis would suggest that the CaM-binding site of hSK1 and hSK2 belongs to the general “1-8-14” class of Ca²⁺/CaM-binding motifs (26), designated by the conserved positions of large hydrophobic residues in the primary sequence that reside on one face of a presumed α-helix (i.e. Leu¹⁸⁷, Leu¹⁹⁴, and Leu²⁰⁶ of hSK1). Furthermore, this type of analysis would suggest that the CaM binding domain of hSK1 belongs to a further subset of this class, the “basic 1-8-14” Ca²⁺/CaM-binding motifs (26), because the hydrophobic residues are immediately preceded in the sequence by basic residues (i.e. Arg¹⁸⁵ and Arg¹⁸⁶). However, the observed lack of substantial effects on CaM binding by either mutation of the defining hydrophobic residues to hydrophilic residues or the removal of the positive charged residues (Figs. 2 and 5) may cast some doubt over classification of these CaM binding domains in this manner. Indeed, it should be noted that considerable diversity exists in the structures of the CaM target domains and the mechanisms of CaM
binding to its targets (29), making predictions of likely CaM targets by such sequence analysis difficult.

This study has also established that the CaM-binding site of hSK1 is critical in the agonist-induced translocation of this protein from the cytosol to the plasma membrane. This is in keeping with previous findings that pharmacological antagonists of CaM blocked Ca\(^{2+}\)-dependent translocation of murine SK1 in cells (22). Together, these data suggest a direct role for Ca\(^{2+}\)/CaM, or possibly a closely related protein, in regulating the cellular localization of SK1. Notably, we have shown previously that translocation of hSK1 to the plasma membrane is also dependent on its activation by phosphorylation at Ser\(^{225}\) (13). Because numerous studies have demonstrated that S1P can mediate increases in intracellular Ca\(^{2+}\) (reviewed in Ref. 44), it is possible that the enhanced intracellular Ca\(^{2+}\) that results from activation of hSK1, via elevated S1P, leads to Ca\(^{2+}\)/CaM binding to hSK1 and subsequent translocation of the complex to the plasma membrane (Fig. 9). Ca\(^{2+}\)-dependent translocation of cytosolic CaM in cells has been observed previously (19), and although the nucleus appears the main destination for this redistributed CaM, some studies have observed distinct localization of CaM near the plasma membrane during cell division (45). The mechanism whereby CaM translocation occurs is not currently understood, however. Thus, although the current findings firmly implicate the involvement of the CaM-binding site of hSK1, and most likely CaM, in agonist-induced translocation of hSK1 to the plasma membrane, further studies are required to establish the molecular mechanisms whereby this process is accomplished.

Previous studies have suggested that PA may also play a role in membrane localization of SK1 through the direct binding of SK1 with membrane-associated PA formed following phospholipase D activation by protein kinase C (46). More recent studies, however, have suggested that hSK1 associates more strongly in vitro with PS than PA, and therefore, this phospholipid may be more a likely candidate to mediate the membrane association of hSK1 (47). Notably, the association of hSK1 with PS within artificial membranes was substantially enhanced by phosphorylation of hSK1 at Ser\(^{225}\) (25). Although these previous findings provide an important explanation for prolonged association of hSK1 with the plasma membrane, they do not present a mechanism for the rapid translocation of hSK1 to this site following cell exposure to agonists. This study suggests a mechanism whereby agonist-induced translocation of hSK1 (probably both phosphorylated and nonphosphorylated) occurs via Ca\(^{2+}\)/CaM, although the presence of PS, or possibly PA, in the plasma membrane may then mediate preferential retention of phosphorylated hSK1 at this site (Fig. 9).

Interestingly, although we have shown previously that the ability of hSK1 to induce neoplastic cell transformation is dependent on its phosphorylation and subsequent localization to the plasma membrane (14), these data (Fig. 7B) indicate that this process is not dependent on its association with CaM. This may initially appear a paradox, because of the proposed role of CaM in agonist-induced translocation of hSK1 to the plasma membrane. It should be noted, however, that the observed cellular transformation occurs over an extended time period where accumulation and retention of phosphorylated hSK1 at the plasma membrane may be possible independent of active translocation machinery. In contrast, the CaM-dependent translocation of hSK1 observed in this study occurs rapidly following agonist activation of cells and is more likely to be involved in mediating rapid and transient cellular signaling from these agonists.

The hSK1 CaM-binding site is also present in hSK2, initially suggesting a possible functional conservation in the regulation of both sphingosine kinase isoforms by CaM. Unlike hSK1, however, hSK2 does not appear to alter in its subcellular distribution in response to cell agonists. Thus, despite the conservation in CaM-binding sites, association with CaM appears to have different functional consequences for these two proteins. The reasons for this are not currently clear. Recent studies suggest that, like hSK1, activation of hSK2 occurs in cells following exposure to various cell agonists, including epidermal growth factor (43), interleukin-1β, TNFα (48), or engagement of the high affinity receptor for IgE (49). It is not yet known, however, whether this activation of hSK2 results in intracellular Ca\(^{2+}\) fluxes, as appears the case with activation of hSK1 (38, 50, 51). Such mobilization of intracellular Ca\(^{2+}\) appears necessary for the redistribution of CaM and its target proteins in the cell (19). Another possible reason for the apparent lack of CaM-mediated hSK2 translocation may be associated with the observed substantial binding of apoCaM to hSK2 (Fig. 8A), which could render this protein insensitive to mobilization by Ca\(^{2+}\)/CaM. Alternatively, it has been proposed that distinct pools of CaM exist in the cell that may exert their effects on different CaM targets depending on the location, duration, and magnitude of the intracellular Ca\(^{2+}\) flux (19). Such a proposal may not only provide an explanation for the different effects of CaM on hSK1 and hSK2 but may also help to explain why CaM does not mediate redistribution of hSK1 to the nucleus, rather than the plasma membrane, despite the majority of CaM moving to the nucleus following increases in intracellular Ca\(^{2+}\) (52). Clearly, further studies are required to understand these processes.

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