Phosphorylation-dependent translocation of sphingosine kinase to the plasma membrane drives its oncogenic signalling

Stuart M. Pitson,[1] Pu Xia,[1,2] Tamara M. Leclercq,[1] Paul A.B. Moretti,[1] Julia R. Zebol,[1] Helen E. Lynn,[1] Binks W. Wattenberg,[1] and Mathew A. Vadas[1,2]

[1]Hanson Institute and Division of Human Immunology, Institute of Medical and Veterinary Science, Adelaide SA 5000, Australia
[2]Department of Medicine, University of Adelaide, Adelaide SA 5000, Australia

Sphingosine kinase (SK) 1 catalyzes the formation of the bioactive lipid sphingosine 1-phosphate, and has been implicated in several biological processes in mammalian cells, including enhanced proliferation, inhibition of apoptosis, and oncogenesis. Human SK (hSK) 1 possesses high instrinsic catalytic activity which can be further increased by a diverse array of cellular agonists. We have shown previously that this activation occurs as a direct consequence of extracellular signal–regulated kinase 1/2–mediated phosphorylation at Ser225, which not only increases catalytic activity, but is also necessary for agonist-induced translocation of hSK1 to the plasma membrane. In this study, we report that the oncogenic effects of overexpressed hSK1 are blocked by mutation of the phosphorylation site despite the phosphorylation-deficient form of the enzyme retaining full instrinsic catalytic activity. This indicates that oncogenic signaling by hSK1 relies on a phosphorylation-dependent function beyond increasing enzyme activity. We demonstrate, through constitutive localization of the phosphorylation-deficient form of hSK1 to the plasma membrane, that hSK1 translocation is the key effect of phosphorylation in oncogenic signaling by this enzyme. Thus, phosphorylation of hSK1 is essential for oncogenic signaling, and is brought about through phosphorylation-induced translocation of hSK1 to the plasma membrane, rather than from enhanced catalytic activity of this enzyme.

CORRESPONDENCE
Stuart M. Pitson:
stuart.pitson@imvs.sa.gov.au

Sphingosine kinases (SKs) catalyze the formation of sphingosine 1-phosphate (S1P), a bioactive lipid that regulates a diverse range of cellular processes, including cell growth, survival, differentiation, motility, and cytoskeletal organization (1, 2). Some of these cellular processes are mediated by five S1P-specific G protein–coupled receptors, whereas other effects appear controlled by intracellular S1P, through as yet unidentified intracellular targets (2, 3).

Two human SK isoforms exist (SK1 and SK2), which differ in their tissue distribution, developmental expression, catalytic properties, and somewhat in their substrate specificity (4, 5). Although these two enzymes appear to have differing roles (6), several studies have shown the effects of SK1 in enhancing cell proliferation and supressing apoptosis (7–9). Furthermore,

we have shown that overexpression of human SK (hSK) 1 in NIH3T3 fibroblasts results in acquisition of the transformed phenotype and the ability to form tumors in mice, demonstrating the oncogenic potential of this enzyme (8). Recent work has also demonstrated the involvement of hSK1 in estrogen-dependent regulation of breast tumor cell growth and survival (10, 11), whereas other studies have shown elevated hSK1 mRNA in a variety of human solid tumors and inhibition of tumor growth in vivo by SK inhibitors (12). Thus, the involvement of hSK1 in cell growth, survival, and tumorigenesis is well established. Less clear, however, are the mechanisms whereby hSK1 brings about these effects. Recent studies have indicated it is independent of G protein–coupled receptors (13), being mediated solely by intracellular S1P generated by SK activity. Although the direct targets of intracellular S1P are unknown, hSK1 has been

B. Wattenberg's present address is James Graham Brown Cancer Center, Louisville, KY 40202.

implicated in several proproliferative and prosurvival pathways, such as activation of extracellular signal–regulated kinase (ERK) 1/2 (14), phosphatidylinositol-3-kinase (15), NF-κB (16), and inhibition of caspase activation (9).

We have shown recently that activation of hSK1 occurs, at least in response to TNFα and phorbol esters, as a direct consequence of phosphorylation at Ser225 by ERK1/2 (17). The effects of this single phosphorylation are unusual since it not only directly increases the catalytic activity of hSK1 but is also necessary for agonist-induced translocation of this protein from the cytosol to the plasma membrane. Notably, hSK1 is phosphorylated to a moderate extent when overexpressed in cells even in the absence of external stimuli. In the current study, we report that the ability of overexpressed hSK1 to support enhanced proliferation, survival, and transformation is blocked by mutation of the phosphorylation site. This indicates that it is the phosphorylated form of hSK1 that is responsible for these biological effects. Furthermore, we have shown through constitutive localization of hSK1 to the plasma membrane that these biological effects are brought about through the phosphorylation-dependent translocation of hSK1 to the plasma membrane rather than from the observed phosphorylation-induced increases in hSK1 catalytic activity.

RESULTS AND DISCUSSION
Phosphorylation of hSK1 is required for enhanced cell proliferation and survival

Overexpression of wild-type hSK1 is known to significantly enhance cell proliferation and survival (7–11, 13), although the precise molecular mechanisms whereby this occurs are unknown. These effects are dependent on the catalytic activity of hSK1 being blocked by the SK inhibitor, N,N-dimethylsphingosine (7–9), and appear independent of G protein–coupled S1P receptors (7, 13). Thus, the enhanced growth and survival appear due to the action of S1P on as yet unidentified intracellular targets. To date, it has been considered that an increase in total S1P levels in the cell, as a consequence of the high intrinsic catalytic activity of the overexpressed hSK1 (4), was sufficient to induce these biological effects (7–11). However, we have noted that even without external stimulation, overexpressed hSK1 is phosphorylated to a moderate extent (Fig. 1 and reference 17). Therefore, we tested whether this phosphorylation was important for the biological consequences of hSK1 overexpression.

To test whether phosphorylation of hSK1 is important in proliferation and survival, we examined the ability of the nonphosphorylatable hSK1^{S225A} mutant to promote these
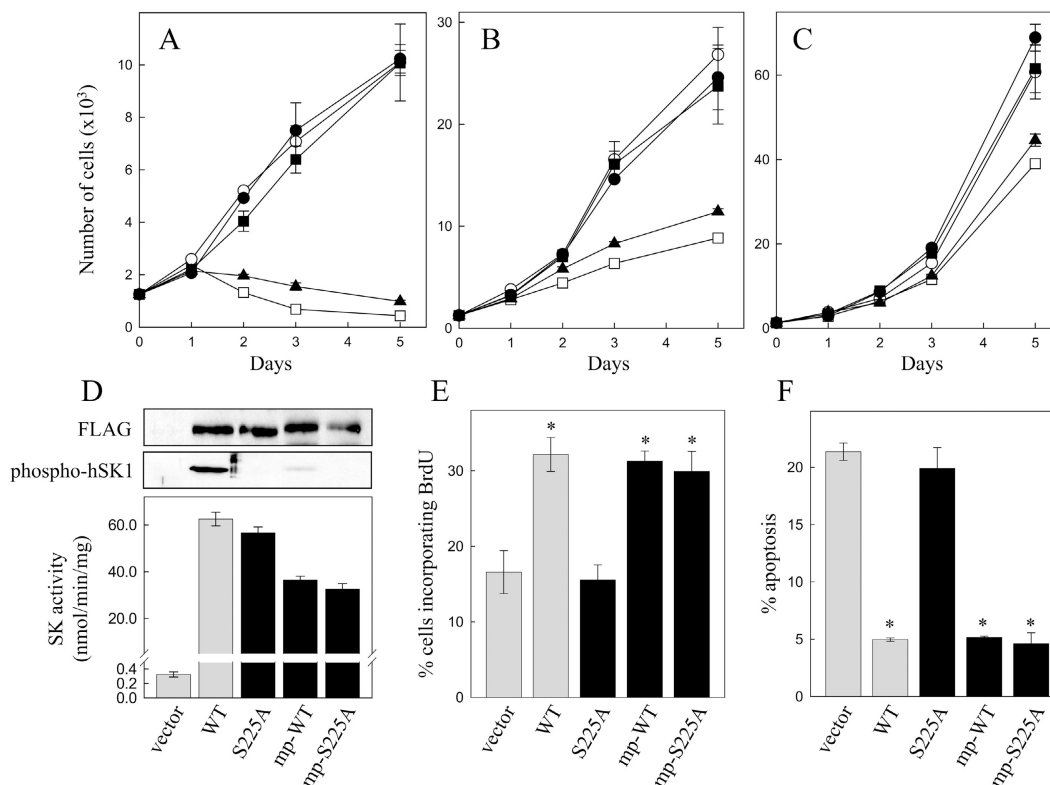

Figure 1.   **Phosphorylation and plasma membrane localization of hSK1 enhances cell proliferation and survival.** Growth of NIH3T3 cells stably transfected with plasmids encoding for wild-type hSK1 (○), hSK1^{S225A} (▲), mp-hSK1 (●), and mp-hSK1^{S225A} (■) or the empty vector (□) over 5 d in (A) serum-free medium (containing 0.1% BSA), (B) medium containing 1% FCS, or (C) 5% FCS. (D) SK activity in these cells after serum deprivation for 16 h. Protein expression and phosphorylation status of the various hSK1 constructs was determined by Western blot via anti-FLAG and anti–phospho-hSK1 antibodies, respectively. (E) Cell proliferation of these cells as measured by BrdU incorporation into nacent DNA. (F) Serum deprivation–induced apoptosis of these cells as measured by nuclear condensation and fragmentation. Data are mean (± SD) from three independent experiments (*P < 0.05 compared with vector control).

processes. Consistent with our previous findings (8), overexpression of wild-type hSK1 not only markedly enhanced the growth of NIH3T3 cells in media containing either 1 or 5% serum but also conferred to these cells the ability to survive and grow in the absence of serum (Fig. 1, A–C). In contrast, however, cells overexpressing hSK1^{S225A} displayed no such enhanced growth or serum independence (Fig. 1, A–C). Notably both wild type and hSK1^{S225A} were expressed at similar level, and extracts of the cells possessed comparable overall SK activities (Fig. 1 D).

Previous studies have shown that the increased growth rates from overexpression of wild-type hSK1 result from a combination of both increased cellular proliferation and reduced apoptosis (7, 9, 13). Consistent with these studies, assays for cellular proliferation by bromodeoxyuridine (BrdU) incorporation into nascent DNA showed overexpression of wild-type hSK1 had a significant effect in increasing cell proliferation (Fig. 1 E). In contrast, however, cells overexpressing hSK1^{S225A} displayed no such enhanced proliferation, showing comparable incorporation of BrdU as control cells (Fig. 1 E). Similarly, overexpression of wild-type hSK1 dramatically reduced serum deprivation–induced apoptosis, as measured by condensation and fragmentation of nuclei (Fig. 1 F). Again, however, overexpression of hSK1^{S225A} showed markedly different results, providing cells with no such protection against apoptosis (Fig. 1 F). Therefore, in stark contrast to wild-type hSK1 overexpression of the nonphosphorylatable hSK1^{S225A} mutant neither increases proliferation, nor protects against apoptosis despite both transfected proteins generating similar cellular SK activities. Thus, phosphorylation of hSK1 imparts a qualitative change in the enzyme that is critical for the signaling processes leading to enhanced proliferation and survival.

It has been well established that hSK1 translocates from the cytosol to the plasma membrane upon exposure of cells to certain agonists (17, 18–21). Although the molecular mechanism whereby translocation of hSK1 occurs is unknown, we have shown recently that it is dependent on phosphorylation of hSK1 at Ser225 (17). Therefore, it was important to test whether translocation was the phosphorylation-dependent process required for hSK1 signaling. We predicted that if this was true, then artificially directing hSK1^{S225A} to the plasma membrane would restore its ability to promote proliferation and transformation. Plasma membrane–localized hSK1 proteins were created through addition of the 10–amino acid Lck tyrosine kinase myristoylation/palmitylation motif to the N terminus of wild-type hSK1 and hSK1^{S225A}, generating mp-hSK1 and mp-hSK1^{S225A}, respectively. Overexpression of these proteins in NIH3T3 cells produced slightly lower cellular SK activities than that observed with overexpression of hSK1 and hSK1^{S225A} (Fig. 1 D). Consistent with previous studies that have shown this motif is sufficient to target proteins to the plasma membrane (22), we observed a substantial localization of mp-hSK1 and mp-hSK1^{S225A} to the membrane fraction (Fig. 2 A). Further immunofluorescence analysis (Fig. 2 B) showed clear localization of mp-hSK1 and mp-
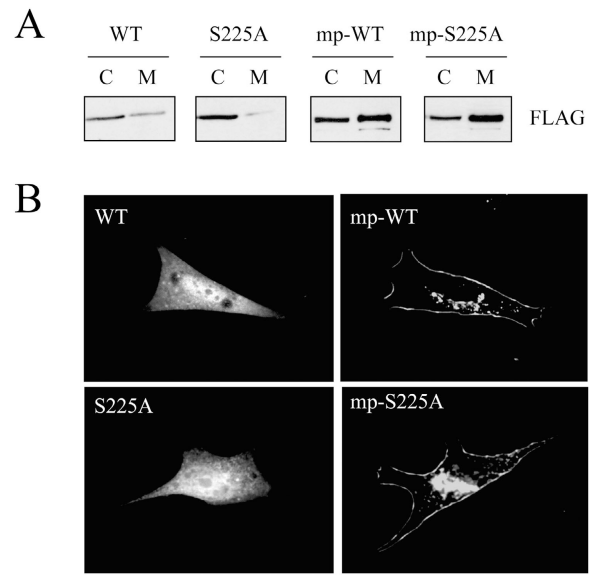
Figure 2. Localization of hSK1 to the plasma membrane by the Lck tyrosine kinase N–terminal myristoylation/palmitylation motif. (A) Lysates from NIH3T3 cells transfected with wild-type hSK1, hSK1^{S225A}, mp-hSK1, and mp-hSK1^{S225A} were fractionated into cytosol (C) and membranes (M) and probed via Western blot with anti-FLAG. Data are representative of three independent experiments. (B) Fluorescence microscopy of the same transfected NIH3T3 cells. Images are representative of >50% of cells observed in three independent experiments.

hSK1^{S225A} to the plasma membrane, whereas hSK1 and hSK1^{S225A} were mainly present in the cytoplasm. Interestingly, localization of wild-type hSK1 to the plasma membrane in this manner substantially reduced its basal phosphorylation (Fig. 1 D). This is presumably due to partial sequestration of hSK1 away from cytosolic ERK1/2, its upstream activating protein kinases (17).

Strikingly, and in stark contrast to hSK1^{S225A}, overexpression of mp-hSK1^{S225A} in NIH3T3 cells conferred an enhancement of growth and survival in serum-deprived conditions (Fig. 1, A–C). As expected, mp-hSK1 also had these effects (Fig. 1, A–C). Further examination of these cells showed that, like wild-type hSK1, both mp-hSK1 and mp-hSK1^{S225A} increased cell growth through enhancing cellular proliferation and reducing serum deprivation–induced apoptosis (Fig. 1, E and F). Similar results were also observed in HEK293 cells (not depicted). Therefore, localization of hSK1 to the plasma membrane is sufficient to enhance cellular proliferation and protect against apoptosis irrespective of the phosphorylation status of the enzyme. This strongly suggests that phosphorylation of hSK1 mediates these observed biological effects through inducing translocation of hSK1 to the plasma membrane rather than as a result of the associated increase in catalytic activity.

Phosphorylation–induced plasma membrane localization of hSK1 mediates oncogenic signaling

Since we had established that Ser225 phosphorylation of hSK1 was essential for its effects in enhancing cell prolifera-

tion and survival, we further investigated its effects on cell transformation. As described previously (8), wild-type hSK1 exhibited considerable transforming activity when overexpressed in NIH3T3 cells, as assayed by colony formation in soft agar (Fig. 3). In contrast, however, overexpression of similar levels and catalytic activity of hSK1[S225A] resulted in remarkably less transformation of these cells (Fig. 3). Notably, these cells expressing hSK1[S225A] had considerably higher SK activity than what we have previously shown necessary for transformation of NIH3T3 cells by wild-type hSK1 (8). Therefore, like the situation for enhanced proliferation and survival, these experiments demonstrate that it is not simply elevated levels of SK activity that are responsible for cell transformation but instead indicate that another aspect of the phosphorylated, activated state of the protein is responsible for these effects. We have demonstrated previously that transformation of NIH3T3 cells by oncogenic H-Ras (V12-Ras) is partially blocked by a catalytically inactive, dominant-negative form of hSK1, indicating that hSK1 is critically involved in Ras-induced cell transformation (8). Strikingly, here we find that the nonphosphorylatable hSK1[S225A] also reduced Ras-induced cell transformation, further confirming the requirement of hSK1 activation in this pathway (Fig. 3). Therefore, in this context hSK1[S225A] is apparently acting as a dominant-negative form of the protein, despite possessing full catalytic activity.

Since plasma membrane localisation of hSK1 mediated the enhanced proliferation and survival resulting from hSK1 overexpression, we next examined its effects on cell transfor-

mation. Like wild-type hSK1, the overexpression of both mp-hSK1 and mp-hSK1[S225A] in NIH3T3 cells resulted in the formation of vigorous colonies in soft agar (Fig. 3). Although some background colonies where observed in empty vector control cells, mp-hSK1 and mp-hSK1[S225A]–overexpressing cells produced 20–30-fold greater colonies which were considerably larger in size. Indeed, the colonies generated by mp-hSK1 and mp-hSK1[S225A] overexpression were also larger and more numerous than those observed in cells overexpressing wild-type hSK1 (Fig. 3). Thus, like the situation for enhanced proliferation and survival, localization of hSK1 to the plasma membrane is sufficient to enhance cell transformation irrespective of the phosphorylation status of the enzyme.

To examine the oncogenic effects of hSK1 phosphorylation and translocation in animals, NIH3T3 cells stably expressing the various forms of hSK1 were subcutaneously injected into NOD/SCID mice. Unlike wild-type hSK1-transfected cells, which we have previously reported generate tumors in these mice (8), no tumors formed in six mice injected with cells expressing hSK1[S225A]. In contrast, and consistent with the in vitro experiments, tumors were observed in mice injected with cells expressing mp-hSK1 (five out of six) and mp-hSK1[S225A] (four out of six).

Phosphorylation and plasma membrane localization of hSK1 enhances S1P generation

Sphingosine, the substrate of hSK1, is concentrated in the plasma membrane (23). Therefore, one possible mechanism
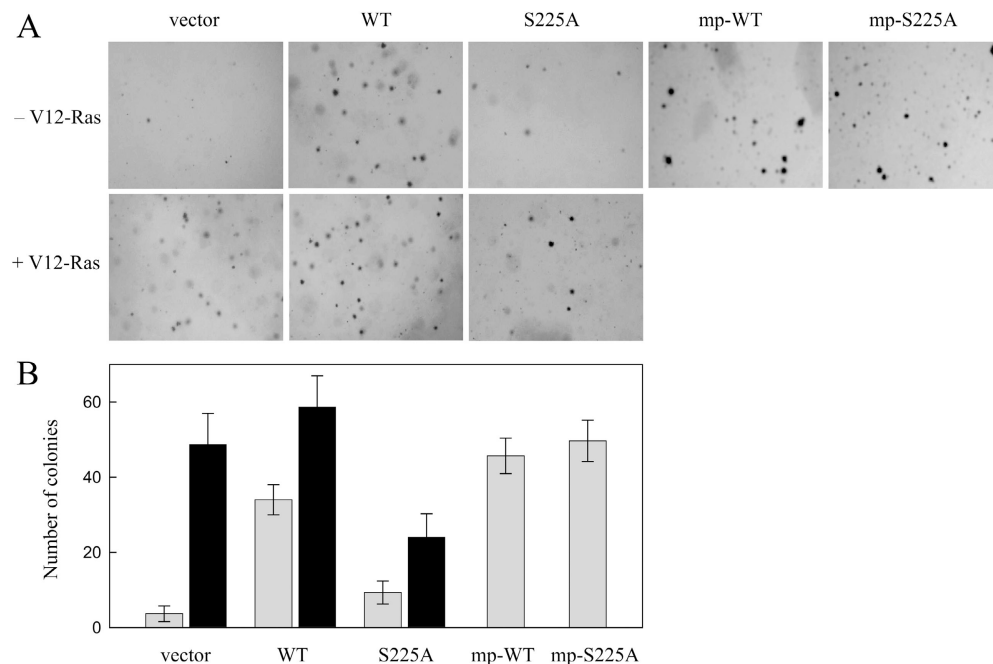

Figure 3. Phosphorylation and plasma membrane localization of hSK1 leads to transformation. (A) NIH3T3 cells stably transfected with empty vector, or plasmids encoding for wild-type hSK1, hSK1[S225A], mp-hSK1, or mp-hSK1[S225A] either alone or cotransfected with plasmid encoding for an activated mutant H-Ras (V12-Ras) were cultured on soft agar. Colonies formed after 3 wk were visualized by MTT staining as described previously (8). (B) Quantitation of colony formation in soft agar for NIH3T3 cells overexpressing hSK1 proteins alone (gray bars) or in combination with V12-Ras (black bars). Data are mean (± SD) from three independent experiments.

for the observed dramatic biological effects of hSK1 localization to the plasma membrane is in enhancing S1P generation by directing hSK1 to its substrate. Therefore, we measured the effect of plasma membrane translocation of hSK1 on levels of its product, S1P. Overexpression of both mp-hSK1 and mp-hSK1[S225A] resulted in similar increases in intracellular S1P and enhanced S1P release into the media. These increases were substantially greater than that observed with either wild-type hSK1 or hSK1[S225A] (Fig. 4). These findings are consistent with our previous reports that Ser225 phosphorylation of hSK1 results in increased cellular S1P and enhanced S1P release from cells (17). This, again, suggests that phosphorylation-induced translocation of hSK1 to the plasma membrane is critical for enhanced S1P generation and the observed subsequent biological effects.

Conclusions and implications of this study

The involvement of hSK1 in tumorigenesis is becoming increasingly evident, with several studies suggesting that elevated hSK1 levels may be important in this process (8–12). In this study, however, we have shown that rather than total hSK1 levels, phosphorylation and subsequent translocation of hSK1 to the plasma membrane are the critical factors involved in the oncogenic effects of this protein. We have proposed previously that the substantial basal activity of hSK1 may reflect an essential housekeeping role that is distinct from its signaling role (4, 14, 17). Agonist-dependent translocation may accomplish the differentiation of these two roles of hSK1. Thus, our studies indicate that disregulation of hSK1 phosphorylation and localization may be key ele-
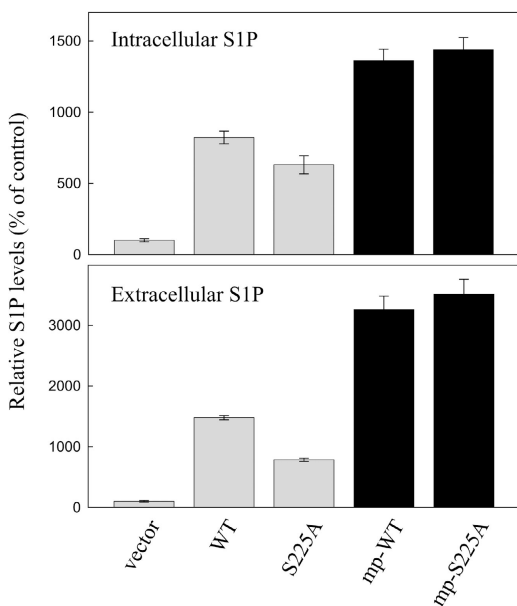
ments in the acquisition of malignant phenotypes through provoking the enhancement of proliferation and, perhaps most importantly, protection from the apoptotic mechanisms that normally target mutated cells for destruction. This suggests that targeting the mechanisms driving hSK1 phosphorylation and/or translocation may provide precise avenues for future anticancer therapies.

MATERIALS AND METHODS

Cell culture, transfection, and cell fractionation. Human embryonic kidney (HEK293T) cells and NIH3T3 fibroblasts were cultured and harvested as described previously (14). Stable and transient transfections were performed using the calcium phosphate precipitation method for HEK293T cells and Lipofectamine 2000 (Invitrogen) for NIH3T3 cells. Stable transfectants were selected for G418 resistance and pooled to avoid the phenotypic artifacts that may arise from the selection and propagation of individual clones from single transfected cells. For subcellular fractionation, postnuclear supernatants of cell lysates were separated into cytosol and membrane fractions as described previously (17).

Antibodies. The M2 anti-FLAG antibody was from Sigma-Aldrich, anti–H-Ras polyclonal antibody was from Santa Cruz Biotechnology, and HRP-conjugated anti–mouse and anti–rabbit IgG were from Pierce Chemical Co. Anti-hSK1 and anti–phospho-hSK1 antibodies have been described previously (17).

SK assays and S1P levels. SK activity was determined as previously described (14). S1P levels were determined following metabolic labeling of cells with [^{32}P]orthophosphate as outlined previously (17).

Generation of plasma membrane–localized hSK1 constructs. A plasma membrane–targeted hSK1 construct (mp-hSK1) containing the myristoylation/palmitylation motif of the Lck tyrosine kinase (MGCGC-SSHPE) (22) was generated by PCR with oligonucleotide primers 5′-TAGAATTCGCCACCATGGGCTGTGGCTGCAGCTCACACCCG-GAAGATCCAGCGGGCGGCC-3′ and SP6 using pcDNA3-hSK1 (4) as template DNA. The resultant product was then cloned into pcDNA3 (Invitrogen) by digestion with EcoRI. The orientation was determined by restriction analysis, and sequencing verified the integrity of the FLAG-tagged mp-hSK1 cDNA sequence. To generate FLAG-tagged mp-hSK1[S225A], the KpnI–PmlI fragment representing the wild-type 5′ end of pcDNA3-hSK1[S225A] (17) was replaced with the 150-bp KpnI–PmlI pcDNA3-mp-hSK1 DNA fragment that contains the Lck myristoylation/palmitylation motif.

Immunofluorescence. Cells were plated onto fibronectin-coated 8-well glass chamber slides at 10^4 cells/well and incubated for 24 h. The cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS, and incubated with M2 anti-FLAG antibody in PBS containing 3% BSA and 0.1% Triton X-100 for 1 h. The immunocomplexes were then detected with FITC-conjugated anti–mouse IgG. Fluorescence microscopy was 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 (Photometrics).

Cell growth, bromodeoxyuridine incorporation, staining of apoptotic nuclei, and tumorigenesis assays. Assays for cell growth were performed by incubating cells in 48-well plates (2,500 cells per well) in medium containing 5 or 1% FCS, or serum free medium (containing 0.1% BSA) as described previously (8). Cell numbers were determined at the indicated times using the MTS assay (Promega). BrdU incorporation into nascent DNA were used as a measure of cell proliferation. Cells were plated onto 8-well glass chamber slides (Nalge Nunc) coated with fibronectin at 10^4 cells/well and grown for 24 h in DMEM with 2% FCS. Cells were then incubated with 10 μM BrdU for 3 h, and then fixed and stained for its incor-

Figure 4. Phosphorylation and plasma membrane localization of hSK1 lead to increased intracellular and extracellular S1P levels. Intracellular and extracellular S1P levels were determined in NIH3T3 cells stably transfected with empty vector, or plasmids encoding for wild-type hSK1, hSK1[S225A], mp-hSK1, and mp-hSK1[S225A]. Data are mean (± SD) from three independent experiments.

poration using an anti–BrdU-FLUOS antibody (Roche) following the manufacturer's protocol. Cells positive for BrdU incorporation were visualized with an Olympus BX-51 fluorescence microscope with at least 300 cells scored per point. Apoptosis was assessed by staining cells with 1 μg/ml DAPI in methanol for 15 min at room temperature. Apoptotic cells were identified by condensation and fragmentation of nuclei using fluorescence microscopy and expressed as a percentage of the total cells counted. A minimum of 300 cells were scored per point. Colony formation in soft agar and tumorigenesis assays in mice were performed as detailed previously (8) with approval from the Institute of Medical and Veterinary Science Animal Ethics Committee.

REFERENCES

1. Pyne, S., and N.J. Pyne. 2000. Sphingosine 1-phosphate signalling in mammalian cells. *Biochem. J.* 349:385–402.
2. Spiegel, S., and S. Milstien. 2002. Sphingosine 1-phosphate, a key cell signaling molecule. *J. Biol. Chem.* 277:25851–25854.
3. Watterson, K., H. Sankala, S. Milstein, and S. Spiegel. 2003. Pleiotropic actions of sphingosine-1-phosphate. *Prog. Lipid Res.* 42:344–357.
4. Pitson, S.M., R.J. D'Andrea, L. Vandeleur, P.A.B. Moretti, P. Xia, J.R. Gamble, M.A. Vadas, and B.W. Wattenberg. 2000. Human sphingosine kinase: purification, molecular cloning and characterisation of the native and recombinant enzymes. *Biochem. J.* 350:429–441.
5. Liu, H., M. Sugiura, V.E. Nava, L.C. Edsall, K. Kono, S. Poulton, S. Milstien, T. Kohama, and S. Spiegel. 2000. Molecular cloning and functional characterization of a novel mammalian sphingosine kinase type 2 isoform. *J. Biol. Chem.* 275:19513–19520.
6. Liu, H., R.E. Toman, S.K. Goparaju, M. Maceyka, V.E. Nava, H. Sankala, S.G. Payne, M. Bektas, I. Ishii, J. Chun, et al. 2003. Sphingosine kinase type 2 is a putative BH3-only protein that induces apoptosis. *J. Biol. Chem.* 278:40330–40336.
7. Olivera, A., T. Kohama, L. Edsall, V. Nava, O. Cuvillier, S. Poulton, and S. Spiegel. 1999. Sphingosine kinase expression increases intracellular sphingosine-1–phosphate and promotes cell growth and survival. *J. Cell Biol.* 147:545–558.
8. Xia, P., J.R. Gamble, L. Wang, S.M. Pitson, P.A.B. Moretti, R.J. D'Andrea, B.W. Wattenberg, and M.A. Vadas. 2000. An oncogenic role of sphingosine kinase. *Curr. Biol.* 10:1527–1530.
9. Edsall, L.C., O. Cuvillier, S. Twitty, S. Spiegel, and S. Milstein. 2001. Sphingosine kinase expression regulates apoptosis and caspase activation in PC12 cells. *J. Neurochem.* 76:1573–1584.
10. Nava, V.E., J.P. Hobson, S. Murthy, S. Milstien, and S. Spiegel. 2002. Sphingosine kinase 1 promotes estrogen-dependent tumorigenesis of breast cancer MCF-7 cells. *Exp. Cell Res.* 281:115–127.
11. Sukocheva, O.A., L. Wang, N. Albanese, S.M. Pitson, M.A. Vadas, and P. Xia. 2003. Sphingosine kinase transmits estrogen signaling in human breast cancer cells. *Mol. Endocrinol.* 17:2002–2012.
12. French, K.J., R.S. Schrecengost, B.D. Lee, Y. Zhuang, S.N. Smith, J.L. Eberly, J.K. Yun, and C.D. Smith. 2003. Discovery and evaluation of inhibitors of human sphingosine kinase. *Cancer Res.* 63:5962–5969.
13. Olivera, A., H.M. Rosenfeldt, M. Bektas, F. Wang, I. Ishii, J. Chun, S. Milstien, and S. Spiegel. 2003. Sphingosine kinase type 1 induces $G_{12/13}$-mediated stress fiber formation, yet promotes growth and survival independent of G protein-coupled receptors. *J. Biol. Chem.* 278:46452–46460.
14. Pitson, S.M., P.A.B. Moretti, J.R. Zebol, P. Xia, J.R. Gamble, M.A. Vadas, R.J. D'Andrea, and B.W. Wattenberg. 2000. Expression of a catalytically inactive sphingosine kinase mutant blocks agonist-induced sphingosine kinase activation: a dominant-negative sphingosine kinase. *J. Biol. Chem.* 275:33945–33950.
15. Osawa, Y., Y. Banno, M. Nagaki, D.A. Brenner, T. Naiki, Y. Nozawa, S. Nakashima, and H. Moriwaki. 2001. TNF-α–induced sphingosine 1-phosphate inhibits apoptosis through a phosphatidylinositol 3-kinase/Akt pathway in human hepatocytes. *J. Immunol.* 167:173–180.
16. Xia, P., L. Wang, P.A.B. Moretti, N. Albanese, F. Chai, S.M. Pitson, R.J. D'Andrea, J.R. Gamble, and M.A. Vadas. 2002. Sphingosine kinase interacts with TRAF2 and dissects TNF signalling. *J. Biol. Chem.* 277:7996–8003.
17. Pitson, S.M., P.A.B. Moretti, J.R. Zebol, H.E. Lynn, P. Xia, M.A. Vadas, and B.W. Wattenberg. 2003. Activation of sphingosine kinase 1 by ERK1/2–mediated phosphorylation. *EMBO J.* 22:5491–5500.
18. Rosenfeldt, H.M., J.P. Hobson, M. Maceyka, A. Olivera, V.E. Nava, S. Milstien, and S. Spiegel. 2001. EDG-1 links the PDGF receptor to Src and focal adhesion kinase activation leading to lamellipodia formation and cell migration. *FASEB J.* 15:2649–2659.
19. Johnson, K.R., K.P. Becker, M.M. Facchinetti, Y.A. Hannun, and L.M. Obeid. 2002. PKC-dependent activation of sphingosine kinase 1 and translocation to the plasma membrane. Extracellular release of sphingosine-1-phosphate induced by phorbol 12-myristate 13-acetate (PMA). *J. Biol. Chem.* 277:35257–35262.
20. Melendez, A.J., and A.K. Khaw. 2002. Dichotomy of Ca^{2+} signals triggered by different phospholipid pathways in antigen stimulation of human mast cells. *J. Biol. Chem.* 277:17255–17262.
21. Young, K.W., J.M. Willets, M.J. Parkinson, P. Bartlett, S. Spiegel, S.R. Nahorski, and R.A.J. Challiss. 2003. Ca^{2+}/calmodulin-dependent translocation of sphingosine kinase: role in plasma membrane translocation but not activation. *Cell Calcium*. 33:119–128.
22. Zlatkine, P., B. Mehul, and A.I. Magee. 1997. Retargeting of cytosolic proteins to the plasma membrane by the Lck protein tyrosine kinase dual acylation motif. *J. Cell Sci.* 110:673–679.
23. Slife, C.W., E. Wang, R. Hunter, S. Wang, C. Burgess, D.C. Liotta, and A.H. Merrill. 1989. Free sphingosine formation from endogenous substrates by a liver plasma membrane system with a divalent cation dependence and a neutral pH optimum. *J. Biol. Chem.* 264:10371–10377.