The physiologic and pathologic functions of sphingosine kinase (SK) require translocation to specific membrane compartments. We tested the hypothesis that interactions with actin filaments regulate the localization of SK1 to membrane surfaces; including the plasma membrane (PM) and phagosome. Macrophage activation is accompanied by a marked increase in association of SK1 with actin filaments. Catalytically-inactive (CI)- and phosphorylation-defective (PD)-SK1 mutants exhibited reductions in PM translocation, colocalization with cortical actin filaments, membrane ruffling, and lamellipodia formation, compared to wild-type (WT)-SK1. However, translocation of CI- and PD-SK1 to phagosomes were equivalent to WT-SK1. SK1 exhibited constitutive- and stimulus-enhanced association with actin filaments and F-actin-enriched membrane fractions in both intact macrophages and a novel in vitro assay. In contrast, SK1 bound G-actin only understimulated conditions. Actin inhibitors disrupted SK1 localization and modulated its activity. Conversely, reduction of SK1 levels or activity via RNA interference or specific chemical inhibition resulted in dysregulation of actin filaments. Thus, the localization and activity of SK1 are coordinately regulated with actin dynamics during macrophage activation.

INTRODUCTION

Sphingosine kinases (SK), comprise an evolutionarily-conserved enzyme family that regulate essential cellular processes, including proliferation, motility, inflammation, apoptosis, and cancer (1-6). The two mammalian isoforms, SK1 and SK2, exhibit approximately 45% identity and 80% similarity at the amino acid level (7). SK1 (42 kDa) is present in most cells and tissues, especially lung, spleen, heart, liver, intestine, and macrophages (8). The distribution and cell-type specific levels of SK2 (69 kDa) are distinct from SK1, including its absence from macrophages (7;9). Both isoforms are inhibited by dimethylsphingosine (DMS), but only SK1 is inhibited by D, L-threo-dihydrosphingosine (DHS), (3;7;10). SK1 activity can be stimulated by growth factors, cytokines, complement components, immune complexes, phagocytic particles, and phorbol myristate acetate (PMA) (6;11). Although the mechanisms by which cell surface receptors initiate the activation of SK1 are not fully defined, evidence supports the involvement of ERK2, protein kinase C (PKC), the phospholipase D (PLD) product, phosphatidic acid, protein tyrosine kinases, and cytosolic Ca²⁺ (5;6;11-15). Less information is available on agonist-induced stimulation of SK2, though epidermal growth factor (EGF) and IgE receptor ligation have recently been reported to activate this isoform (16;17).

SK catalyzes the phosphorylation of sphingosine to sphingosine-1-phosphate (S1P),
and its important physiologic and pathologic roles reflect this dynamic interconversion of signaling lipids (6;11). Virtually all cell types respond to the extracellular and/or intracellular actions of S1P. The S1P receptor family are the major extracellular receptors that mediate many of the critical effects of this bioactive lipid in tissues and the vasculature (18-23). Although the intracellular receptor(s) for S1P has not been identified, multiple experimental approaches, including microinjection, indicate that intracellular S1P leads to an increase of cytosolic Ca²⁺ via inositol-(1,4,5)-trisphosphate (IP₃)-independent release from the endoplasmic reticulum (24-26).

The involvement of SK1 in a broad range of cellular responses implies a requirement for precise spatiotemporal regulation of enzyme activity. Of note, agonists induce only modest increases in the specific activity of SK1, averaging 1.5 to 3-fold in numerous cell types and experimental conditions (summarized in (11)), further supporting the hypothesis that localization of SK1 to specific subcellular compartments is a critical element of its regulation. The majority of cellular SK activity (>70% in most studies) is localized to the cytosol (3;5;6;10;11;27). In human macrophages, 95% of SK1 activity is cytosolic (28). Therefore, stimulation of its enzymatic activity must be accompanied by targeting of SK1 to specific membranes, the site of its substrate, sphingosine.

Stimulated translocation of SK1 to the plasma membrane has been demonstrated in response to tumor necrosis factor-α (TNF-α), complement fragment C5a, bacterial lipopolysaccharide (LPS), platelet derived growth factor, nerve growth factor, insulin-like growth factor binding protein-3, lysophosphatidic acid, muscarinic agonists, PMA, and Ca²⁺ ionophores (6;11-13;15;23;29-34). We have recently demonstrated a key role for specific localization and activation of SK1 in the human innate immune response, and its evasion by microbial pathogens (28;35). Macrophage phagocytosis triggers the translocation of cytosolic SK1 to the nascent phagosome, which promotes a localized elevation of Ca²⁺ that is required for its maturation to an acidic, microbicidal phagolysosome. The human pathogen, Mycobacterium tuberculosis, inhibits the phagosomal translocation and activation of SK1, resulting in a block in Ca²⁺-dependent phagosome maturation, which is central to the pathogenesis of tuberculosis (28;35-37). These studies underscore the complexity and physiologic/pathologic importance of SK1’s spatiotemporal regulation.

The phagosome represents a temporally- and spatially-regulated platform of signaling components that have specific interactions with other organelles of the endosomal-lysosomal pathway (38-43). Thus, in addition to its direct relevance to immune defense and inflammation, phagocytosis and phagosome maturation serve as a model for characterizing the regulation of vesicular trafficking and its integration with cytoskeletal elements (40;44). In macrophages, SK1 activity and the resultant S1P production have been shown to regulate Ca²⁺-signaling (8;35), vesicle trafficking (9), phagosome maturation (35;37), and cytokine secretion (9;31;45;46). However, the mechanisms that regulate the localization of SK1 to specific membrane compartments, including the phagosome, remain undefined. Utilizing live cell confocal microscopy, we recently described the kinetics of SK1 translocation to nascent phagosomes (28). Interestingly, this transient recruitment of SK1 during phagocytosis is similar to that which has been previously documented for phagosomal actin polymerization and depolymerization (47). Actin polymerizes at the base of the phagocytic cup and within the encircling pseudopods, and actin filaments remain attached to the nascent phagosome for the first few min following ingestion (47). The similarity in the kinetics of SK1’s recruitment and subsequent release from the phagosome (28) suggest that SK1 may interact with actin filaments at early stages of phagocytosis. In support of this hypothesis, several lipid-modifying enzymes, including phosphatidylinositol phospholipase C (PI-PLC) (48-50) and PLD (51-53) bind to actin filaments. Furthermore, actin plays an essential role in several phagocyte functions to which SK1 has been linked, including motility (20;54-56), vesicle trafficking (57-59), phagosome maturation (35;60-62), and secretion (46;57).

The objective of this study was to test the hypotheses: (1) SK1 associates with actin filaments in macrophages; and (2) these
interactions modulate the localization and activity of SK1 and the dynamics of actin polymerization during macrophage activation.

EXPERIMENTAL PROCEDURES

Materials - Unless otherwise stated, materials were from previously published sources (28;35). Polystyrene beads were purchased from Polysciences, Inc. (Eppelheim, Germany), glass chamber slides were from Fisher (Hampton, NH), and FuGENE 6 was from Roche (Indianapolis, IN). The plasmid encoding red fluorescent protein (RFP)-actin was a generous gift of Elaine Fuchs (Rockefeller University, New York).

Antibodies - Polyclonal rabbit anti-SK1 antibodies (Ab) were generated as previously described (13). Murine anti-actin (Ab-1) was purchased from Calbiochem. Murine anti-human LAMP-1 (H4A3) Ab was purchased from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Goat anti-rabbit Texas Red and goat anti-mouse Oregon Green secondary Ab were purchased from Invitrogen.

Phagocytic particles - IgG-coated polystyrene beads and complement-opsonized zymosan (COZ) were used as model phagocytic particles. IgG-coated beads were prepared as previously described (120). Briefly, beads were rotated overnight in 2% bovine serum albumin (BSA; Sigma) at room temperature and opsonized by incubating with 1:200 dilution of rabbit anti-BSA IgG (Molecular Probes) for 2 hr at 37°C. Beads were then washed and resuspended in RAW cell medium. IgG-opsonization was verified by indirect immunofluorescence utilizing Oregon Green-conjugated anti-rabbit IgG (Molecular Probes). COZ was prepared as previously described (35). Briefly, zymosan A was boiled for 15 min at 100°C, washed with PBS, and opsonized in 50% human serum for 30 min at 37°C. COZ was then washed repeatedly with PBS and stored on ice until use.

Macrophage cell lines and transfection - The RAW 264.7 murine macrophage cell line and human THP-1 promonocytic leukemia cells were purchased from American Type Culture Collection and cultured in RPMI containing 10% fetal bovine serum and 1% penicillin/streptomycin (28). Plasmids encoding enhanced green fluorescent protein (EGFP) fusion proteins of wild type, catalytically-inactive (SK1<sup>G82D</sup>) (12;13;32), phosphorylation-defective (SK1<sup>S25A</sup>) (25) and/or RFP-actin (121;122) were transfected into RAW 264.7 or THP-1 cells using FuGENE 6, as per the manufacturer’s instructions. Briefly, cells were passaged onto 25 mm glass coverslips and allowed to adhere. The next day, cells were transfected with 1 µg of plasmid DNA. Confocal microscopy was performed 24 hr after transfection.

RNA interference of RAW macrophages or human THP-1 promonocytes was performed via transfection of short-interfering (si)RNA oligonucleotides to SK1, utilizing a Nucleofector apparatus (Amaxa Inc., Gaithersburg, MD). Macrophages were transfected with one of two distinct siRNA to SK1: SK1-RNAi-1: 5'-GTGGACCTAGAGAGTGAGAAGTATC-3' TGAGAAAGTATC-3' SK1-RNAi-2: 5’-GGGCAAGGCCUUCAGCUCUUGCAGGC-3' (83;87).

A point mutant oligonucleotide that eliminated RNAi-mediated suppression of SK1 levels by SK1-RNAi-2, served as a control: 5’-GGGCAAGGCCUUGCAGGC-3’.

Preparation of primary macrophages - Human MDM were isolated and cultured as previously described (28;35). Briefly, venous blood was drawn from healthy, adult volunteers in accordance with the human subjects guidelines approved by the University of Iowa Institutional Review Board. PBMCs were isolated by density gradient centrifugation on Ficoll-Hypaque and cultured in RPMI, 20% autologous serum in Teflon wells for 5-7 days at 37°C, 5% CO₂. MDM were purified by adherence to tissue culture plates or glass chamber slides for 2 hr at 37°C in RPMI, 20 mM HEPES (pH 7.4), 10% autologous serum. MDM monolayers were washed twice and then incubated in RPMI containing 1% bovine serum albumin, without antibiotics, for use in experiments. Effects of experimental manipulations on MDM viability were assessed by exclusion of trypan blue, and monolayer density was determined by nuclei counting with naphthol blue-black stain (35;36). Purity and viability of MDMs were >95% by
Wright staining and trypan blue exclusion, respectively.

**Determination of sphingosine kinase activity** - RAW 264.7 cells were incubated in jasplakinolide (1 µM, 15 min), latrunculin B (20 µg/mL, 10 min), or buffer control at 37 ºC. PMA (100 nM) was added to select samples. Cells were washed once with ice-cold PBS and scraped into 500 µL of cold Assay Buffer (0.9 mM CaCl₂, 1 mM EGTA, 0.2 M Tris, 0.5 mM deoxypiridoxine, 15 mM sodium fluoride, 0.01% 2-mercaptoethanol, 1 mM sodium orthovanadate, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 10 µg/mL soybean trypsin inhibitor, 40 mM β-glycerolphosphate, 1 mM PMSF, and 10% glycerol) (10). The cells were disrupted by sonication and assessed for efficiency of lysis by microscopy. Aliquots of each whole cell lysate were incubated with sphingosine (1 mM in 5% Triton X-100, final concentrations: 0.25% Triton X-100, 50 µM sphingosine) and [γ-32P]ATP (0.025μCi/sample) for 10 min at 37°C. 32P-labeled sphingosine-1-phosphate was isolated by thin layer chromatography (80 butanol: 20 ethanol: 10 acetic acid: 20 water mobile phase) and quantified by a Typhoon phosphorimager (Amersham Biosciences). SK activity of samples was expressed as the percent of control cells incubated in buffer alone (mean ± range deviation).

**Confocal microscopy** - For fixed cell imaging, MDM or RAW 264.7 macrophages were adhered in 8-chamber glass culture slides at approximately 50% confluence and incubated with appropriate stimuli for the indicated times. Cells were fixed in 3.75% paraformaldehyde for 15 min and permeabilized in ice-cold methanol: acetone (1:1) or acetone alone (for experiments using phalloidin) (36). Samples were sequentially incubated with blocking buffer (PBS, 5% BSA, 10% horse serum), Texas Red-phalloidin (Molecular Probes, 0.17 µM), rabbit polyclonal anti-SK1 Ab (1:100 dilution) and Oregon Green-conjugated goat anti-rabbit IgG 2° Ab, all for 1 hr at 25 ºC. In select experiments, cells were preincubated with 100 nM PMA (in 0.1% DMSO) for 5-30 min. The 0.1% DMSO solvent control exhibited no differences in viability or SK1 localization, compared to cells incubated in buffer alone.

Analysis was performed using a Zeiss 510 laser scanning confocal microscope. Data are representative of 50 cells analyzed/condition for duplicate samples in each experiment.

For live-cell imaging, RAW 264.7 cells were cultured and transfected as described above. On the day of analysis, the coverslips were placed in a heated stage (Warner Instruments, Hamden, CT) at 37 ºC, and analyzed at rest, during phagocytosis of model particles, or after incubation with various pharmacologic agents, using the Zeiss 510 laser scanning microscope and the associated LSM time series imaging software. Z-series confocal images were acquired every 30-45 s for 45 min. Loss of fluorescence at later time points due to photobleaching was compensated by increasing the gain of the photomultiplier tube. Quantitation of differences in fluorescent staining between samples, including the degree of co-localization, were performed using ImageJ software.

**Western blotting** - The association of SK1 with membranes and their Triton X-100-insoluble fraction was analyzed in both intact cells (basal and stimulated) as well as in a cell-free assay. Intact RAW 264.7 cells were incubated with 2 µM PMA or buffer for 30 min at 37 ºC. Cells were disrupted by N₂ cavitation (450 psi, 25 min, 4 ºC), followed by differential centrifugation (900g for 8 min, 150,000g for 60 min) to separate the membrane and cytosol fractions for each sample. The membrane fraction was washed and re-centrifuged at 200,000g for 60 min. Membrane and cytosolic fractions were subjected to 9% SDS-PAGE and transferred to PVDF as described previously (114). The PVDF membrane was blocked with 5% nonfat, dry milk, probed with polyclonal rabbit anti-SK1 Ab (1:100 dilution) followed by immunodetection using horseradish peroxidase-coupled secondary antibody and enhanced chemiluminescence (ECL).

In the cell-free system, membranes and cytosol prepared from resting RAW 264.7 macrophages were incubated with buffer or PMA (2 µM) for 15 min at 37 ºC. Membranes were reisolated by centrifugation (150,000g for 60 min), washed with Lysis Buffer (above) and extracted with 1% Triton X-100 in Lysis Buffer for 1 hr on ice. The detergent-soluble and -
insoluble fractions derived from membranes were separated by centrifugation (150,000g for 60 min) and subjected to SDS-PAGE/western blotting, as noted above (123).

**Immunoprecipitation** - RAW cells were stimulated with 500 nM PMA, COZ (particle: cell ratio of 10:1), or buffer control for 20 min, then washed in RPMI. Cells were scraped, pelleted, and resuspended in 5 mL of Lysis Buffer (25 mM HEPES, 100 mM KCl, 3 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, pH 7.4, 2 mM PMSF, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 1% deoxycholate, 1% Triton X-100, 1% octyl-glucoside, 0.05% SDS), passed through an 18-gauge needle 10 times, and incubated for 1 hr on ice. Lysates were precleared with EZ View Protein G agarose (Sigma) for 2 hr at 4 ºC, then incubated with anti-SK1 or control rabbit IgG preadsorbed to Protein G agarose beads for 3 hrs at 4 ºC. The immunoprecipitates were washed, boiled in sample buffer (0.625 M Tris HCl, 10% glycerol, 2% SDS, 200 mM β-mercaptoethanol, 0.01% bromophenol blue, in water), and loaded onto a 9% SDS-PAGE gel. After transfer to PVDF, blots were blocked in 5% non-fat dry milk, washed and incubated in anti-actin antibody (monoclonal IgM; Calbiochem) overnight at 4 ºC. Detection utilized 2° HRP-conjugated Ab (Calbiochem) and ECL.

**Binding of SK1 to actin.**

**Effect of phalloidin on velocity sedimentation on sucrose density gradients** - Freshly prepared membranes and cytosol from 2 x 10⁸ RAW cells or THP-1 cells were incubated with PMA (100 nM) at 37°C for 30 min. Membranes were re-isolated by centrifugation at 17,500g, for 30 min at 4°C, and washed in H/K buffer (25 mM HEPES, pH 7.4, 100 mM KCl, 3 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, 2 µM leupeptin, 0.5 mM phenylmethylsulfonylfluoride, 1 mM DTT). Phalloidin (10 µM) was added to select samples for the final 15 min of the incubation (126). Membranes were washed and solubilized in 500 µl TEB (1% Triton X-100 in H/K buffer) for 1 h at 4°C and loaded on top of a 4-ml linear sucrose gradient (20-55%) in TEB. A 0.5-ml sucrose cushion (80%) was placed at the bottom of each tube. Samples were centrifuged at 150,000g for 16 h at 8°C, and fractions (400 µl) collected from the top of the gradient, were subjected to SDS-PAGE and probed for SK-1 and actin.

**Co-sedimentation assay** - Cytosol from 10⁶ RAW or THP-1 cells was precleared by ultracentrifugation (150,000g, 30 min, 4°C). Aliquots (100 µg) in H/K buffer were incubated with GTPγS (100 µM) to induce actin polymerization, and jasplakinolide was added at a final concentration of 1 µM to stabilize actin filaments (123). Actin filaments were pelleted by centrifugation at 17,500g, for 30 min, at 4°C), then washed extensively in H/K buffer. Supernatant and pellet fractions were analyzed by 10% SDS-PAGE gels and probed for sphingosine kinase 1 and actin by immunoblotting.

**Co-isolation of sphingosine kinase 1 with G-actin by binding to immobilized DNase I** - Cytosol from 10⁶ RAW or THP-1 cells was dialyzed extensively against actin monomer buffer (AMB, 5mM Tris HCl, pH 8.0, 0.2mM CaCl₂ with 0.2mM ATP, 0.5mM DTT) and then precleared by ultracentrifugation. Aliquots of clarified cytosol (100 µg) were incubated with or without PMA (100 nM) for 30 min at 37°C. Immobilized DNase 1 was added to each fraction and incubated for 4 hr at 4°C (114). The DNase-bound material was pelleted and washed extensively with AMB buffer before subjecting to SDS-PAGE and immunoblotting for SK1 and actin.

**Electron microscopy** - MDM were cultured and purified as described above. Cells were adhered to glass coverslips for 3 hours, after which they were incubated with 25 µM DHS or buffer control for 15 min at 37 °C. Samples were then stimulated with 100 nM PMA for 20 min at 37 °C. Following treatment, the samples were placed in extraction buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 1 µM phallacidin, and 0.75% Triton X-100) for 45 min at room temperature. The samples were then washed and fixed in 1% glutaraldehyde. After fixation, the cells were washed and incubated in 1% osmium tetroxide for 30 min, and progressively dehydrated in ethanol. Images are representative of the results obtained in 3 identical experiments performed in duplicate.
Analysis of data - Data from each experimental group were subjected to an analysis of normality and variance. Differences between experimental groups composed of normally-distributed data were analyzed for statistical significance using Student's t-test. Non-parametric evaluation of other data sets was performed with the Mann-Whitney Rank Sum test (124).

Online supplemental material - Four supplemental movies of live cell confocal microscopy of RAW 264.7 cells accompany the manuscript. Transfected plasmids, stimulation conditions, and related figures are:
- movie1.avi - GFP-WT-SK1, PMA (100 nM) added to cells for 1 min, Fig. 1A-D;
- movie2.avi - GFP-CI-SK1\(^{G82D}\), PMA added to cells for 1 min, Fig. 1E-L;
- movie3.avi - GFP-WT-SK1 and RFP-actin, COZ added 15 min prior to acquiring images, Fig. 4A-D.
- movie4.avi - GFP-WT-SK1 and RFP-actin, IgG-beads for 15 min prior to acquiring images, Fig. 4E-H.

For each video, frames were collected every 45 sec.

RESULTS

The molecular determinants of SK1 localization to the plasma membrane. Activation of SK1 by multiple stimuli, including phorbol esters, calcium ionophores, cytokines, and growth factors, is associated with translocation of this predominantly cytosolic kinase to the plasma membrane (2;12;14;28). To further characterize the molecular mechanisms that regulate agonist-induced localization of SK1 to the plasma membrane, we utilized live cell confocal microscopy of RAW 264.7 murine macrophages expressing GFP-fusion proteins of WT-SK1 and two point mutants. CI-SK1 is a catalytically-inactive mutant with a substitution of the ATP-binding Gly of the active site with Asp (G82D) (12;32). PD-SK1 is a point mutant (S225A) that prevents ERK1/2-mediated phosphorylation and plasma membrane localization following stimulation by PMA or TNF-\(\alpha\) (25).

WT-SK1 exhibited a predominantly cytosolic distribution (Figure 1), which was similar to the distribution of endogenous SK1 protein and the majority of SK activity (~95%) in resting macrophages (28). Treatment with PMA induced the localization of SK1 to the plasma membrane, accompanied by prominent membrane ruffling and extension of lamellipodia (Figure 1A-D, movie1.avi). Endogenous SK1 exhibited similar PMA-induced translocation in both RAW macrophages as well as primary human monocyte-derived macrophages (MDM, not shown). In contrast, the GFP control remained cytosolic following PMA stimulation. These data are concordant with previous reports of PMA-stimulated plasma membrane localization of SK1 in HEK293 and NIH3T3 cells (12;13). Of note, WT-SK1 was particularly enriched in membrane ruffles, sites of enhanced actin polymerization-depolymerization (63).

In contrast to macrophages expressing WT-SK1, cells transfected with CI-SK1 were much less dynamic in response to PMA, exhibited very little ruffling or lamellipodia, and only low levels of CI-SK1 were detected at the plasma membrane (Fig. 1E-H). Quantitation by ImageJ software indicated that the level of PMA-induced plasma membrane localization of CI-SK1 was only 21 +/- 4% that of WT-SK1, after correction for the levels of transgene expression (p<0.001, n=6). The “inert” phenotype and decreased membrane ruffling of CI-SK1-expressing macrophages was most clearly demonstrated by live cell confocal microscopy (movie2.avi).

Cells expressing PD-SK1 exhibited a phenotype that was very similar to that of CI-SK1. Specifically, the levels of PMA-induced, (a) cytosol-to-membrane translocation, (b) membrane ruffling, and (c) lamellipodia formation were markedly reduced in PD-SK1-vs. WT-SK1-expressing macrophages. The level of plasma membrane-localized PD-SK1 was only 30 ± 5% that of WT-SK1, following stimulation with PMA (p<0.003, n=6). The viability of macrophages transfected with WT-SK1, CT-SK1, and PD-SK1 did not differ from control, untransfected cells, or those transfected with a plasmid expressing GFP alone (not shown).

SK1 exhibits basal and stimulus-enhanced colocalization with cortical actin filaments at the plasma membrane. Membrane ruffles are...
The prominent localization of SK1 to plasma membrane ruffles of PMA-stimulated macrophages, led us to hypothesize that activation of SK1 resulted in its association with actin filaments. To test this hypothesis, the localization of SK1 and actin filaments were determined in resting and activated macrophages by confocal microscopy. RAW 264.7 macrophages were adhered to chamber slides and transfected with plasmids expressing GFP-fusion proteins of WT-, CI-, or PD-SK1, as noted above. 24 hr after transfection, cells were stimulated with PMA or buffer for 15 min, fixed, and actin filaments were visualized with Texas Red-conjugated phalloidin. Resting macrophages exhibited a basal level of colocalization of WT-SK1 and F-actin (Figure 2A). Stimulation with PMA induced a 3.6-fold increase in the association of WT-SK1 with actin filaments (range 2.9 - 4.3-fold increase, p<0.01, n=4). (Fig. 2B). The majority of this stimulus-induced colocalization was evident in the cell cortex, with a minor fraction noted in an intracellular, punctuate distribution. GFP alone did not exhibit association with actin filaments in resting or PMA-stimulated cells (not shown). These data support the hypothesis that SK1 exhibits constitutive and stimulus-enhanced association with actin filaments.

In comparison to WT-SK1, CI-SK1 exhibited a lesser degree of colocalization with actin filaments at the plasma membrane of resting macrophages (21% of control, range 18-25%, p<0.01, n=4) (Fig. 2C). In addition, upon stimulation with PMA, CI-SK1 exhibited significantly less colocalization with cortical actin than did WT-SK1 (Fig. 2D). The level of CI-SK1 associated with actin filaments was only 9% (range 5-13%) that of WT-SK1 (p<0.003). Of note, PMA induced no significant change in the level of colocalization of CI-SK1 and actin filaments, compared to resting macrophages (1.13-fold, range 0.87 - 1.25-fold of control). These data suggest that, in stimulated macrophages, catalytic activity is necessary for optimal translocation of SK1 to cortical actin filaments.

Macrophages transfected with PD-SK1 also demonstrated a lower level of basal colocalization of SK1 and actin (Figure 2E) as compared to WT-SK1 (36% of control, range 32-39%, p<0.01, n=4). Upon stimulation with PMA, PD-SK1 exhibited only 24% (range 19-29%) of the WT-SK1’s level of colocalization with actin filaments (p<0.003). Similar to CI-SK1, PMA induced no significant change in the level of colocalization of PD-SK1 and actin filaments, compared to resting macrophages (1.06-fold, range 0.93 - 1.19-fold of control) (Fig. 2F). Interestingly, the majority of colocalization between actin and PD-SK1, in both resting macrophages as well as those stimulated with PMA, occurred at non-cortical sites. These data indicate that PMA induces SK1 translocation from the cytosol to actin-rich areas of the plasma membrane, and support the hypothesis that optimal translocation is dependent on catalytic activity and the phosphorylation of Ser225 (12;64). The levels of PMA-induced colocalization of the SK1 isoforms (WT, CI, and PD) with actin filaments (Fig. 2) was highly correlated with their level of plasma membrane translocation (Fig. 1) (r = 0.947, p < 0.001). This high correlation between membrane localization and co-localization with actin filaments for WT-, CI-, and PD-SK1 support the hypothesis of a causal connection, i.e, that association of SK1 with actin filaments functions in its basal and stimulus-enhanced association with the plasma membrane.

SK1 colocalizes with actin filaments at the phagosome membrane. The molecular determinants that regulate the targeting of SK1 to specific cellular membranes are not fully understood (6). The membrane of phagosomes is a complex and dynamic hybrid derived from several compartments, including the plasma membrane and, progressively, the membranes of early endosomes, late endosomes, and lysosomes (65-71). In specific situations, the endoplasmic reticulum may also contribute, although the data on this point are conflicting (67;71-74). Actin filaments localize strongly to the membrane of the nascent phagosome from its inception at the phagocytic cup, and through the first several min after ingestion (47;75;76).

To determine whether SK1 colocalizes with phagosome-associated actin filaments, RAW 264.7 macrophages were transfected with WT-SK1 and stained with Texas Red-phalloidin for analysis by confocal microscopy. In response
to the model phagocytic particle, complement-opsonized zymosan (COZ), WT-SK1 colocalized with actin filaments at the phagosome membrane (Figure 3A). Similar colocalization of endogenous SK1 and actin filaments occurred in both RAW macrophages and human MDM, whereas GFP alone did not colocalize with phagosomes or their associated actin filaments (28, and not shown). In comparison to WT-SK1, CI-SK1 exhibited a similar degree of colocalization with phagosomal actin filaments (106% of control, range 97-109%, NS, n=5) (Fig. 3B). The similar levels of colocalization of WT-SK1 and CI-SK1 with F-actin at phagosomes contrasted sharply with their distinct levels of F-actin-colocalization at the plasma membrane in PMA-stimulated cells (Fig. 2).

PD-SK1 exhibited levels of colocalization with actin filaments at COZ phagosomes that were not different than WT-SK1 (96% of control, range 92-101%, p<NS, n=5) (Fig. 3C). Taken together, the data in Figs. 2 and 3 indicate that the molecular determinants of SK1 required for its association with actin filaments differ with respect to the plasma membrane versus the membrane of nascent phagosomes. Catalytic activity and phosphorylation at Ser225 are required for association with actin filaments at the plasma membrane, but not at the phagosome membrane. The one aspect of localization of WT and mutant SK1s that is similar at both plasma membrane and phagosomes is the high correlation between membrane translocation of this cytosolic enzyme and its association with filamentous actin in stimulated cells. These data support the hypothesis that the association of SK1 with actin filaments contributes to the regulation of its membrane translocation.

To further characterize the kinetics of phagosomal acquisition of SK1 and actin, we utilized live cell confocal microscopy of macrophages co-transfected with GFP-SK1 and red fluorescent protein (RFP)-actin (77). In response to COZ, actin and SK1 were observed to localize to and dissipate from the phagosome with very similar kinetics (Figure 4A-D, movie3.avi). Specifically, actin and SK1 were sequentially enriched in the phagocytoc cup, pseudopods, and the nascent phagosome for the first 5 min after particle contact with the macrophage surface.

In addition to complement receptors, the other major class of phagocytic receptors are those which bind the Fc portion of IgG, i.e., Fcγ receptors (FcγRs). To determine whether stimulation of FcγRs induce the colocalization of SK1 and actin filaments, RAW 264.7 macrophages expressing GFP-SK1 and RFP-actin were incubated with IgG-coated latex beads and monitored by live cell confocal microscopy. IgG bead phagosomes also exhibited colocalization of actin filaments and SK1, from the formation of the phagocytic cup and for the first 5 min of ingestion (Figure 4E-H, movie4.avi). Specifically, the binding of IgG-beads to the phagosome stimulated an initial burst of RFP-actin followed rapidly by GFP-SK1 (yielding the merged yellow color surrounding nascent phagosomes). The strong temporal correlation between the localization of actin filaments and SK1 to the phagosome during ingestion of both COZ and IgG-coated beads supports the hypothesis of a causal link between these two components. In particular, we hypothesize that actin is recruited first to growing filaments at the base of the phagosomal cup, followed rapidly by co-localization of SK1. Further evaluation of this hypothesis will require improved kinetic resolution of the earliest stages of particle ingestion.

**SK1 exhibits constitutive and stimulus-enhanced association with actin-enriched membrane fractions.** To further evaluate the colocalization of SK1 and actin filaments, we characterized the detergent solubility of SK1 in resting and stimulated macrophages. Detergent-insoluble fractions of membranes are enriched in sphingolipids, cholesterol, actin filaments, and multiple signal transduction components (68;78). Macrophages were incubated with buffer or PMA for 30 min at 37 °C. Cytosol and membrane fractions were prepared by nitrogen cavitation and differential centrifugation. The membrane fractions from resting or PMA-treated cells were lysed and separated into Triton X-100-soluble (TxS) and -insoluble (TxI) fractions, that were analyzed by western blotting with polyclonal Ab to SK1.

In membranes from resting macrophages, the majority of SK1 was detected...
in the TxI fraction (Figure 5A, lane 2). The major protein band (doublet) detected by the polyclonal anti-SK1 Ab had a molecular weight of ~43 kDa, consistent with the primary gene product, as previously demonstrated in diverse cell types (8;10;14;15;31;80). The additional proteins at 45 and 48 kDa likely represent post-translationally-modified SK1 (81), and specific characterization is in progress. Stimulation of cells with PMA, prior to isolation and extraction of membranes, increased the level of SK1 detected in the TxI-insoluble membrane fraction (Fig. 5A, lane 4). Specifically, the levels of each of the proteins in the 43-48 kDa that were detected by the anti-SK1 Ab were increased in the TxI-fraction of membranes isolated from PMA-stimulated macrophages. The level of SK1 in the TxS-fraction of membranes did not change significantly (Fig. 5), whereas cytosolic SK1 declined following PMA stimulation (not shown). These data are consistent with the hypothesis that phorbol esters induce translocation of cytosolic SK1 to the TxI membrane fraction, which also contained the majority of membrane-associated actin and the raft markers, ganglioside GM1 and flotillin (not shown). Since ~95% of SK is cytosolic in resting macrophages, we utilized two cytosolic proteins, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and lactate dehydrogenase (LDH), as additional controls. Stimulation with PMA did not alter the cytosolic localization of GAPDH or LDH (not shown).

To further characterize the physical association of SK1 with actin, we developed a cell-free system utilizing purified membrane and cytosol fractions from resting RAW 264.7 macrophages. Cell-free assays have contributed significant insights into the mechanisms of interfacial catalysis, including the interaction between actin filaments and phospholipases, sphingomyelinases, and lipid kinases/phosphatases (4;48-53;82;83). Our working model is that activation of SK1 is accompanied by its stable association with actin filaments, and we hypothesized that these actin-SK1 interactions can be replicated in a broken cell preparation.

Membrane and cytosolic fractions from resting macrophages were incubated with PMA (or buffer control) for 15 min at 37 ºC. Membranes from PMA- (“Activated”) or buffer-treated (“Control”) incubations were re-isolated by centrifugation, washed, and subjected to detergent extraction in 1% Triton X-100-containing buffer. The TxS- and TxI- fractions were separated and analyzed by western blotting for SK1. “Control” membranes, derived from the incubation with cytosol in buffer, contained a very low level of SK1 in the TxI fraction and no detectable SK1 in the TxS material (Fig. 5B). In contrast, “activated” membranes, derived from the incubation with cytosol + PMA, exhibited a 56-fold increase in the level of TxI-SK1, compared to the corresponding fraction from control membranes (range 49.6 – 61.8-fold, p<0.001, n=4) (Fig. 5B). Similar to Fig. 5A, the major protein detected by the polyclonal Ab to SK1 had an apparent molecular weight of 43 kDa, and lesser amounts of immunoreactive protein migrated in the 45-48 kDa range. No SK1 was detectable in the TxS-fraction derived from activated membranes. The TxI-fractions from both control and PMA-stimulated membranes contained the majority of membrane-associated actin, GM1, and flotillin (not shown). GAPDH and LDH, remained cytosolic in fractions stimulated by PMA (not shown).

The detergent-soluble and-insoluble fractions from this experiment (fig 5B) were subjected to the SK1 activity assay, utilizing equal amounts of protein for each sample. The TxI-fraction from “activated” membranes exhibited 172-fold greater SK activity, compared to the corresponding fraction from control membranes (range 166-179-fold, p<0.001, n=4). No SK activity was detected in the TxS fractions derived from either activated or control membranes.

Taken together, the data from both the intact cell assay (Fig. 5A) and the cell-free system (Fig. 5B) indicate that activation of SK1 markedly enhances its constitutive association with the detergent-resistant membrane fraction, which also contains the majority of membrane-associated actin. (4,49). In intact cells, membrane microdomains (rafts) that are enriched in sphingolipids and GPI-linked proteins are the major sites of nascent actin polymerization, (51-53;60;68;79;84 and Discussion). Thus, the data in Fig. 5 are
concordant with results of confocal microscopy (Figs 2-4) and strongly support a model of basal and activation-induced interaction of SK1 with actin filaments.

To directly evaluate this proposed physical interaction between actin and SK1, a co-immunoprecipitation (co-IP) assay was undertaken. RAW macrophages were incubated with PMA, COZ, or buffer control, washed, and detergent lysates were subjected to IP with anti-SK1 polyclonal Ab or control, irrelevant polyclonal sera. Western blotting with IgM mAb to actin was utilized to determine the level of actin in anti-SK1 IPs from each group of cells (Fig. 5C). No actin was detected in IPs obtained with control, irrelevant Ab. In contrast, each of the anti-SK1 IPs contained actin. Of note, the level of IP’ed actin was greater in PMA- and COZ-treated samples, compared to buffer-treated controls. Further analysis is underway to determine the proportions of G- vs. F-actin in each of the anti-SK1 IPs.

SK1 binds to both actin filaments and monomeric G-actin. The results of the co-immunoprecipitation assay are consistent with the binding of SK1 to filamentous F-actin and/or globular G-actin. The confocal microscopy colocalization studies support the hypothesis that SK1 associates with actin filaments. To further test this hypothesis, we conducted sedimentation velocity centrifugation on sucrose density gradients in the presence and absence of phalloidin. Since phalloidin crosslinks and stabilizes actin filaments, it causes the profile of F-actin and proteins bound to it to “shift” to fractions of greater density (114;126). Membrane and cytosol from RAW macrophages were incubated in the presence of PMA and the “activated membrane” containing increased levels of SK1 was re-isolated, as in Fig. 5B. These membranes were incubated with phalloidin or solvent control, solubilized in 1% Triton X-100, and loaded on a 20-55% linear sucrose gradient. Following centrifugation for 16 hr at 150,000g, 11 fractions were collected and western blotted for SK1 and actin. In the absence of phalloidin, SK1 was detected primarily in fractions 4-6. (Figure 6A) However, in the presence of phalloidin, SK1 exhibited a “density shift” in localizing to the more dense fractions, 7-9 (Fig. 6A) As a control, western blotting for actin revealed that phallodin induced a similar shift in its localization to fractions of greater density.

A second method to biochemically assess the binding of SK1 to actin filaments is co-sedimentation. Cytosol from either murine macrophages (RAW cells) or the human promonocyte line, THP-1, was subjected to high-speed centrifugation to remove pre-existent actin filaments. GTPγS (100 µM) was added to induce actin polymerization (114,123). Jasplakinolide was added to select samples, to stabilize the nascent actin filaments, which were collected by centrifugation. GTPγS alone, resulted in the recovery of a low amount of SK1 in the supernatant, consistent with its cosedimentation with actin filaments (Fig. 6B). Addition of jasplakinolide resulted in a much greater recovery of both actin and SK1 in the pellet fraction, which support the hypothesis that SK1 binds to actin filaments. Positive controls for this assay included the F-actin-binding proteins, gelsolin and profilin, whereas the cytosolic protein lactate dehydrogenase (LDH) served as the negative control (not shown). Of note, since intact cytosol was utilized, the co-sedimentation of SK1 does not indicate direct binding to F-actin. The association of SK1 with actin filaments may be indirect, via actin binding proteins.

In order to determine whether SK1 binds to G-actin, we utilized the property of DNase I that it binds exclusively to monomeric G-actin, but not F-actin. Cytosol from RAW or THP-1 cells was dialyzed extensively against actin monomer buffer and then precleared of actin filaments by ultracentrifugation. Aliquots of clarified cytosol (100 µg) were incubated with or without PMA (100 nM) for 30 min at 37°C and then the ability of each fraction to bind the complex of G-actin and immobilized DNase I was assessed (114). The fraction that bound DNase was pelleted and washed extensively with AMB buffer before probing for SK1 and G-actin.

In the absence of PMA, no SK1 was detected in the G-actin-DNase I complex (Figure 6C). However, in the presence of PMA, SK1 was detected in association with the DNase I pellet, supporting the hypothesis that SK1 bind to G-actin under stimulated, but not basal conditions (Fig 6C). Of note, since treatment of
cytosol with PMA increased the amount of G-actin bound to the fixed level of DNase I, it is not clear at present whether the ability to detect bound SK1 was due to a quantitative or qualitative change in binding properties with G-actin.

Modulation of actin polymerization alters SK1 localization and activity. Based on the physical association of actin and SK1, and previous data that actin filaments can function as spatial and activity determinants of lipid-modifying enzymes (4;48-53;82;83), we hypothesized that modulation of actin polymerization would affect both the subcellular location of SK1 and its activity. To test this hypothesis, we utilized two well-characterized actin toxins: latrunculin B and jasplakinolide. Latrunculin B binds G-actin monomers with high affinity, thus prevents new actin polymerization (85). In addition, since actin filaments “treadmill” (64), sequestration of G-actin results in the progressive depolymerization of previously-formed actin filaments. Macrophages transfected with GFP-WT-SK1 and RFP-actin were incubated with 100 nM PMA prior to addition of latrunculin B (20 µg/mL) or buffer, and then imaged by live cell confocal microscopy.

In the absence of latrunculin B, PMA induced cell spreading and cortical co-localization of GFP-SK1 and RFP-actin (Fig 7A), consistent with the fixed cell microscopy data in Fig. 2. Addition of latrunculin B rapidly (3 min) and dramatically decreased cortical actin structures, as reported previously in diverse cell types (85). In fact, latrunculin B completely inhibited PMA-induced membrane ruffling in macrophages. This depolymerization of actin filaments and decreased cell motility were accompanied by a marked reduction in the level of plasma membrane-localized SK1 in these PMA-stimulated cells (8% of that of control cells, range 5-12%, p<0.01, n=4) (Figure 7B,C). Macrophages incubated with latrunculin B +/- PMA exhibited no change in viability compared to buffer-treated control cells over the 60 min course of the experiment (not shown). Jasplakinolide +/- PMA had no effect on cell viability during the 1 hr course of the experiment. The results of the latrunculin B and jasplakinolide experiments are consistent with the hypothesis that modulation of actin dynamics in macrophages is associated with disruption of basal and stimulus-induced localization of SK1.

In addition to its subcellular localization, the activity of SK1 was also modulated by changes in actin dynamics. Macrophages were incubated with jasplakinolide or latrunculin B for 15 min, as above, and sphingosine kinase activity was determined in cell extracts via incorporation of [32P] into sphingosine, with isolation of [32P]S1P via thin layer chromatography and quantitation with a phosphoimager. The positive control, PMA, increased SK activity by 77% (range 66-88%, p<0.02, n=5). Jasplakinolide caused a 85% increase in SK activity, compared to buffer-treated control cells (range 79-91%, p<0.01) (Figure 8). It was notable that the jasplakinolide-induced increase in SK1 activity was of comparable magnitude to that stimulated by the most efficacious known agonist of macrophage SK1, PMA. Addition of latrunculin B resulted in a more modest increase in SK activity, 38% greater than control (range 25-51%, p<0.02) (Fig. 8). These results are consistent with the hypothesis that actin dynamics and/or the state of actin polymerization modulate SK activity as well as localization. To our knowledge, interactions with actin represent a novel mechanism for the regulation of SK localization and activity.
Inhibition of SK activity results in dysregulation of actin filaments. In light of the strong correlation between the localization of SK1 activity and actin filament dynamics, we tested the converse hypothesis; that inhibition of SK activity will result in alterations in the actin cytoskeleton. SK1 levels in human THP-1 macrophages were reduced via RNA interference (RNAi) with two independent short-interfering (si)RNA oligonucleotides specific to SK1 (83;87) (Figure 9A). Control cells (C-RNAi) were transfected with a point-mutant oligonucleotide that eliminated RNAi-mediated suppression of SK1 levels. Macrophages transfected in the presence of buffer alone (Untransfected, Un, Fig. 8A) served as an additional control. The efficiency of nucleofection of THP-1 macrophages with the SiRNAs was >95%. The two SK1-specific siRNAs resulted in decreases of 72% (range 65-79%) and 91% (range 87-94%) in the level of SK1 protein (p<0.01 for both, n=4), compared to cells transfected with the mutant siRNA or buffer alone (Fig. 9A).

Control macrophages (treated with C-RNAi) exhibited the expected spreading response to PMA (100 nM, 15 min) with enrichment of cortical actin filaments and lamellipodia (Fig. 9B, inset: Z-series). In marked contrast, macrophages in which SK1 was depleted by specific siRNA (SK1-RNAi) demonstrated reduced cell spreading, few lamellipodia, and significantly less cortical actin filaments (Fig. 9C) in the absence of any decrease in cell viability. Specifically, the surface area of SK1-depleted cells was only 73% that of control cells treated with the mutant siRNA (range 69-77%, p<0.05). THP-1 cells with reduced levels of SK1 exhibited only 27% of the level of plasma-membrane-associated actin filaments (range 23-31%, p<0.001, n =4), compared to control cells.

To further test the hypothesis that SK1 activity is required for normal actin dynamics, we utilized D,L-threo-dihydrosphingosine (DHS), a highly-specific inhibitor of SK1, which is the enantiomer of erythro-dihydrosphingosine, the physiologic isomer in the sphingolipid biosynthetic pathway (10;35;88;89). Primary human monocyte-derived macrophages (MDM) were incubated with DHS (25 µM) or 0.1% DMSO solvent control for 15 min prior to stimulation with 100 nM PMA for an additional 15 min.

Primary human MDM exhibited marked PMA-induced spreading, cortical actin filaments and lamellipodia (Fig. 9D). DHS-treated MDM exhibited markedly reduced cell spreading and an absence of lamellipodia or other F-actin-enriched structures, such as filopodia or membrane ruffles (Fig. 8E,F). Specifically, the surface area of DHS-treated macrophages was only 27% that of control cells (range 22-31%, p<0.002). Of note, the magnifications in panels 8E and 8F, are 2-fold greater than that in Fig. 8D. MDMs in which SK1 was inhibited by DHS exhibited only 17% of the level of plasma-membrane-associated actin filaments (range 13-21%, p<0.001, n =4), compared to control cells. SK1-depleted cells also exhibited a prominent central ovoid accumulation of Texas Red-phalloidin staining that was distinct from the plasma membrane or nucleus. Taken together, the experiments utilizing siRNA or DHS to inhibit SK1 support the hypothesis that this lipid kinase is required for optimal actin dynamics and cell spreading in stimulated monocytes/macrophages. Neither DHS nor SK1-siRNA reduced cell viability.

The more severe phenotype of the DHS-treated cells, compared to those in which SK1 inhibition was achieved via siRNA, suggested that the former reagent may inhibit stimulated SK1 activity to a greater degree. Direct determination of SK1 activity and total cellular F-actin content (determined with TRITC-phalloidin (90), confirmed this latter hypothesis (91). DHS exhibited a dose-dependent inhibition of SK1 activity which correlated with concentration-dependent decreases in F-actin levels (not shown). The highest concentration of DHS, (25 µM) resulted in greater inhibition of SK1 activity and lower levels of F-actin, than the SK1-RNAi. Overall, there was a high level of correlation between levels of SK1 activity and F-actin content in PMA-stimulated macrophages (r=0.92, p<0.001).

To gain further information on the effects of SK1 inhibition on actin filaments, scanning electron microscopy (SEM) was performed in primary human MDM that were incubated with DHS or solvent control (exactly as above in Fig. 9D-F, above). Following
stimulation with PMA (100 nM, 15 min), control cells exhibited dense cortical actin filaments with extensive parallel sheets and numerous crosslinks (Fig. 9H), consistent with previous reports (92;93). In contrast, cells in which SK1 was inhibited by DHS (Fig. 9I,J) exhibited thinning and disorganization of cortical actin filaments. As with the confocal microscopy images, the inhibition of cell spreading in SK1-inhibited cells (Fig. 9I,J) necessitated a 2 to 2.5-fold increase in SEM magnification compared to the corresponding images of control MDM (Fig. 8H).

The SEM data confirm the results obtained by confocal microscopy and biochemical assays of SK1 activity and F-actin levels. Inhibition of SK1 is associated with dysregulation of the actin cytoskeleton, specifically, reduction in the level of actin filaments and their spatial disorganization. Although the SEM images do not convey detailed information on the central clusters of fluorescent phalloidin-stained material in SK1-inhibited cells (Figs. 9E,F), the structures visualized on confocal microscopy correspond to the perinuclear clusters of incompletely-extracted membrane-associated actin filaments (“lipid droplets”), seen in the SEM images, Fig. 9I,J. We are attempting to gain more detailed information by increasing the level of detergent extraction (while still preserving actin filaments), as well as via immunoelectron microscopy with anti-SK1 Abs, but have not yet been successful. Taken together, the complementary biochemical and microscopic methods support the hypothesis that SK1 activity modulates actin filaments dynamics.

DISCUSSION

SK1 regulates multiple processes that are critical to the physiologic and pathologic behavior of virtually all cell types, including proliferation (2;94;95), survival and oncogenesis (96-100), motility (20;23;101), cytoskeletal rearrangements (18;30;60;87;102), Ca\(^{2+}\) signaling (2;9;21;25;28;33;35;87;103-110), and secretion (111). These diverse functions, and the ubiquitous distribution of SK1, support the hypothesis that its enzymatic activity is subject to spatial and temporal regulation in mammalian cells (6). We recently demonstrated that specific localization and activation of SK1 in macrophages is central to the mechanism of phagosome maturation (28;35); a key antimicrobial defense of innate immunity in which ingested pathogens are trafficked to lysosomes for degradation and presentation of antigens to the adaptive immune system (38-44). Furthermore, SK1 is a key molecular target of the human pathogen, M. tuberculosis, which inhibits both the activity and phagosomal translocation of macrophage SK1. The resultant block in Ca\(^{2+}\)-signaling and phagosome maturation are required for the bacterium’s intracellular survival and replication (28;35).

The current data support the hypothesis that the mechanisms that regulate the translocation of cytosolic SK1 to its membrane substrate include structural determinants specific to the target membrane. PMA-induced translocation of SK1 to the plasma membrane was impaired in both CI- and PD-SK1, whereas localization of these SK mutants to the phagosome membrane occurred to the same level as the WT-SK1. Our working model is that catalytic activity and phosphorylation of Ser\(^{225}\) are necessary for translocation of SK1 to the plasma membrane but not to the phagosome membrane. A corollary hypothesis is that the CI- and PD- mutants of SK1 exhibit a dominant-negative function at the plasma membrane, but not at the phagosomal membrane. Since a dominant-negative mechanism implies a range of complex phenotypes specific to diverse cell functions, the reductions in PMA-induced membrane ruffling and cortical actin polymerization in CI- and PD-SK1-expressing macrophages are consistent with, but not conclusive of, this secondary hypothesis. Furthermore, preliminary data indicate that these SK1 mutants do not exhibit dominant-negative inhibition of phagosome maturation (not shown). Further study of stimulus-specific, SK1-dependent cellular responses will be instructive in this regard.

Differences between the cytosolic leaflet of membranes at the cell surface vs. the phagosome may account for organelle-specific translocation of SK1, including: (1) distinct protein or lipid components, (including post-translational modifications), (2) specific arrangements of common constituents (eg., membrane domains or rafts), (3) differences in...
membrane-associated actin filaments, or (4) unique intersections with vesicular trafficking pathways along the endocytic and secretory routes. In addition to catalytic activity and phosphorylation of Ser\textsuperscript{225}, potential SK1-specific determinants of routing to different membranes compartments include: (a) distinct post-translational modifications, including acylation and phosphorylation at other sites (25;81), (b) differential activation/recruitment of the SK1 isoforms, SK1a and SK1b (10;81), and compartment-specific recruitment of SK1-binding proteins. Undoubtedly, combinations of these and other unknown mechanisms may fluctuate in importance during specific stages of cellular development and activation.

The localization of SK1 to membrane ruffles suggested the hypothesis that SK1 interacts with actin filaments. In resting macrophages, SK1 was primarily localized to the cytosol, consistent with previous reports (1-6;28). Maximal colocalization of SK1 with cortical actin filaments in PMA-stimulated cells required catalytic activity and the presence of Ser\textsuperscript{225}, paralleling the requirements for translocation to the plasma membrane (Fig. 2). Since nascent phagosomes represent a subset of plasma membrane enriched in cholesterol (112), sphingolipids, PI(4,5)P\textsubscript{2}, phosphatidic acid (PA) (60;65;112), actin filaments (47), and sphingosine kinase (28), we hypothesized that colocalization of SK1 and actin would occur at the early stages of phagosome formation. Live and fixed cell confocal microscopy demonstrated near-simultaneous acquisition of actin and SK1 by phagosomes. Within the temporal resolution of these techniques, the localization of actin filaments at the phagocytic cup preceded that of SK1 (supplemental movies 3 and 4). Live-cell microscopy permits more detailed kinetic analysis than fixed cell studies, but the dependence of the former on expression of a transfected gene (GFP-SK1) make the latter’s detection of endogenous SK1 a valuable complementary approach. In contrast to what was observed at the plasma membrane, both CI-SK1 and PD-SK1 colocalized with actin at the phagosomal membrane with similar kinetics to the wild-type (Figure 3B and C).

It is noteworthy that the phagosomal colocalization of actin and SK1, including similar times of onset and release, occurred for both complement-opsonized and IgG-opsonized particles (Figs. 3A, 4). Further study will be required to determine whether other classes of opsonic ligands and phagocytic receptors (eg., phosphatidylserine and receptors for apoptotic cells, scavenger receptors, etc.), as well as unopsonized particles, demonstrate similar colocalization of SK1 and actin filaments. Of note, PLD, which has been linked to the localization and function of SK1 (6;9;11;15;54), exhibits physical and enzymatic interactions with the actin cytoskeleton (51;53;91;113-115).

To further evaluate the proposed association of SK1 with actin filaments demonstrated by confocal microscopy, we characterized the subcellular distribution of SK1 in both intact macrophages, as well as in a cell-free system derived from resting cells (Figure 5). The TxI fraction is enriched in actin cytoskeletal components as well as several signal transduction components. Consistent with our hypothesis that stimulation of the cell results in translocation of SK1 to actin rich membranes, PMA induced an increase in the level of SK1 in the TxI membrane fraction (Figure 5A). Several distinct proteins bands were detected by the anti-SK1 antibody. These may represent different phosphorylation states of the enzyme, products of acylation, or other covalent modifications. Further study is required to assess these hypotheses, which are likely of broad importance to SK1 function in other cell types.

We also assessed SK1 localization to actin-enriched membrane fractions in a cell-free assay (Figure 5B). PMA induced a significant increase in the amount of SK1 in the TxI membrane fraction, which also contained the majority of filamentous actin. The relationship of these in vitro data to the in vivo co-localization of SK1 and actin filaments revealed by confocal microscopy will be a fruitful area for further investigation. The lipid rafts that are detected in intact cells are specialized membrane microdomains enriched in sphingolipids, cholesterol, and several signaling proteins. Lipid rafts are structurally and functionally linked to the actin cytoskeleton via specific actin-binding protein and integrins (116;125). In stimulated cells, the majority of new actin polymerization is localized to rafts (79). These results are consistent with the hypothesis that SK1
translocates to actin-rich regions of the membrane upon stimulation.

The co-immunoprecipitation data provided further support for physical interactions (direct or indirect) between actin and SK1. Western blotting of anti-SK1 IPs demonstrated that actin levels were greatest in IPs derived from cells treated with PMA or COZ, compared to buffer-treated controls. Since the anti-actin Ab does not discriminate between G- and F-actin, we utilized additional methods to determine the binding specificity of SK1 for actin, under both resting and stimulated conditions. Co-sedimentation analysis and equilibrium velocity sedimentation in the presence of phalloidin indicated that SK1 exhibits constitutive and stimulus-enhanced binding to actin filaments. In contrast, SK1 bound to G-actin (defined by pull-down with immobilized DNase I) only under stimulated conditions. Further analysis will be required to define the molecular requirements for SK1’s association with actin, and to determine whether this is direct or indirect.

This report extends previous studies establishing the critical importance of Ser225 to PMA (or TNF-α)-induced translocation of SK1 to the plasma membrane in HEK293 and NIH3T3 cells (12;29). However, the present data differ from the previous demonstration of PMA-induced plasma membrane translocation of CI-SK1 (13). We hypothesize that both qualitative and quantitative differences in the experimental systems contribute to these divergent findings. First, live cell microscopy (this study) provides a different level of temporal resolution compared with fixed cell microscopy (13). Second, the determinants of SK1 translocation (both those intrinsic to SK1 itself as well as to the target membrane) likely exhibit both general and cell types-specific features. For example, the degree of plasma membrane turnover in macrophages exceeds that for virtually all other cell types examined (117;118). Third, potential differences in the level of translocation (as opposed to positive vs. negative) of WT and mutant SK1 proteins will require direct comparison of these cell types (as well as others) using identical methodologies. Finally, endogenous levels of SK1 may differ between macrophages and HEK cells, thus the phenotype of a specific mutants may be “dominant-negative” in one cell type (HEK), but to a more limited degree in the other (macrophages).

Specific inhibition of SK1 via RNAi-induced knockdown resulted in significant alterations of cell morphology and responses to PMA. Fluorescence microscopy demonstrated dysregulation of actin filaments, and a global decrease in the extent of actin polymerization. DHS, a potent inhibitor of SK1 activity which exhibits 3-fold higher affinity for the enzyme than does its substrate, sphingosine (110), produced similar results. The cortical actin network was thinned and exhibited decreased regularity, compared to control cells. Taken together, the data are consistent with a positive feedback loop in which actin polymerization targets activated SK1 to specific membranes, and SK1 is required for normal actin filament formation. Preliminary immunoelectron microscopy studies have not yielded conclusive data, perhaps due to limitations of the anti-peptide Ab to SK1.

Taken together, the data provide evidence for novel physical and functional interactions between SK1 and the actin cytoskeleton that modulate critical cellular properties. Many of the functions to which SK has been linked require dynamic actin rearrangements, including cell motility, secretion, and apoptosis. We propose that macrophage activation is accompanied by formation of an SK1-actin signaling complex at the surface of the phagosome and/or plasma membrane. Of direct relevance, the molecular determinants of SK1’s binding to calmodulin have recently been established (119). Since calmodulin is a key link in the signaling pathways that regulate phagosome maturation (36), this Ca2+ signal-transducing protein may contribute to the dynamic interactions between SK1 and the actin cytoskeleton at specific membrane compartments. Further study is underway to elucidate the importance of these interactions during phagocytosis, phagosome maturation, membrane ruffling and other aspects of macrophage activation.
REFERENCES

1. Spiegel, S. and Milstien, S. (2002) J. Biol. Chem. 277, 25851-25854
2. Taha, T.A., Hannun, Y.A., and Obeid, L. M. (2006) J. Biochem. Mol. Biol. 39, 113-131.
3. Liu, H., Chakravarty, D., Maceyka, M., Milstien, S., and Spiegel, S. (2002) Prog Nucleic Acid Res Mol Biol 71, 493-511
4. Pyne, S., Long, J. S., Kiistakiss, N. T., and Pyne, N. J. (2005) Biochem Soc.Trans. 33, 1370-1374
5. Maceyka, M., Milstien, S., and Spiegel, S. (2005) Prostaglandins Other Lipid Mediat 77, 15-22
6. Wattenberg, B. W., Pitson, S. M., and Raben, D. M. (2006) Journal of Lipid Research 47, 1128-1139
7. Liu, H., Sugiuara, M., Nava, V. E., Edsall, L. C., Kono, K., Poulton, S., Milstien, S., Kohama, T., and Spiegel, S. (2000) J Biol Chem 275, 19513-19520
8. Melendez, A. J., Carlos-Dias, E., Gosink, M., Allen, J. M., and Takacs, L. (2000) Gene 251, 19-26
9. Melendez, A. J., Floto, R. A., Gillooly, D. J., Harnett, M. M., and Allen, J. M. (1998) J.Biol.Chem. 273, 9393-9402
10. Kohama, T., Olivera, A., Becke, K. P., Mao, C., and Obeid, L. M. (2003) J.Biol.Chem 278, 35257-35262
11. Johnson, K. R., Johnson, K. Y., Becker, K. P., Mao, C., and Obeid, L. M. (2003) J. Biol. Chem. 278, 34541-34547
12. Delon, C., Manifava, M., Wood, E., Thompson, D., Krugmann, S., Pyne, S., and Kiistakiss, N. T. (2004) J. Biol. Chem. 279, 44763-44774
13. Maceyka, M., Sankala, H., Hait, N. C., Le Stunff, H., Liu, H., Toman, R., Collier, C., Zhang, M., Satin, L., Alfred, H., Milstien, S., and Spiegel, S. (2005) J. Biol. Chem. M502207200
14. Olivera, A. and Rivera, J. (2005) J Immunol 174, 1153-1158
15. Hla, T., Lee, M. J., Ancellin, N., Thangada, S., Liu, C. H., Kluk, M., Chae, S. S., and Wu, M. T. (2000) Ann N Y Acad Sci 905, 16-24
16. Hla, T., Lee, M. J., Ancellin, N., Liu, C. H., Wangada, S., Thompson, B. D., and Kluk, M. (1999) Biochem Pharmacol 58, 201-207
17. Hobson, J. P., Rosenfeldt, H. M., Barak, L. S., Olivera, A., Poulton, S., Caron, M. G., Milstien, S., and Spiegel, S. (2001) Science 291, 1800-1803
18. Ignatov, A., Lintzel, J., Kreienkamp, H. J., and Schaller, H. C. (2003) Biochim Biophys Res Commun 311, 329-336
19. Kluk, M. J. and Hla, T. (2002) Biochim.Biophys.Acta 1582, 72-80
20. Wu, W., Mosteller, R. D., and Broek, D. (2004) Mol. Cell. Biol. 24, 7759-7769.
21. Alemany, R., Kleuser, B., Ruwisch, L., Danneberg, K., Lass, H., Hashemi, R., Spiegel, S., Jakobs, K. H., and Meyerzu, H. C. (2001) FASEB J. 2599, 239-244
22. Pitson, S. M., Moretti, P. A. B., Zebol, J. R., Xia, P., Gamble, J. R., Vadas, M. A., D’Andre, R. J., and Wattenberg, B. W. (2000) J. Biol. Chem. 275, 33945-33950.
23. Itagaki, K., Kannan, K. B., and Hauser, C. J. (2005) J.Leukoc.Biol. 77, 181-189
24. Nava, V. E., Lacana, E., Poulton, S., Liu, H., Sugiuara, M., Kono, K., Milstien, S., Kohama, T., and Spiegel, S. (2000) FEBS Lett 473, 81-84
25. Thompson, C. R., Iyer, S. S., Melrose, N., VanOosten, R., Johnson, K., Pitson, S. M., Obeid, L. M., and Kusner, DJ. (2005) J Immunol 174, 3551-3561
26. Pitson, S. M., Xia, P., Leclercq, T. M., Moretti, P. A. B., Zebol, J. R., Lynn, H. E., Wattenberg, B. W., and Vadas, M. A. (2005) Journal of Experimental Medicine 201, 49-54
27. Rosenfeldt, H. M., Hobson, J. P., Maceyka, M., Olivera, A., Nava, V. E., Milstien, S., and Spiegel, S. (2001) FASEB J. 15, 2649-2659
28. Melendez, A. J. and Ibrahim, F. B. M. (2004) J Immunol 173, 1596-1603
32. Pitson, S. M., Moretti, P. A. B., Zebol, J. R., Zareie, R., Derian C. K., Darrow, A. L., Qi, J., D’Andrea, R. J., Bagley, C. J., Vadas, M. A., and Wattenberg, B. W. (2002) *J. Biol. Chem.* 277, 49545-49553.

33. Young, K. W., Willets, J. M., Parkinson, M. J., Bartlett, P., Spiegel, S., Nahorski, S. R., and Challiss, R. A. (2003) *Cell Calcium* 33, 119-128.

34. Young, K. W., Challiss, R. A., Nahorski, S. R., and MacKrirll, J. J. (1999) *Biochem J* 343, 45-52.

35. Malik, Z. A., Thompson, C. R., Hashimi, S., Porter, B., Iyer, S. S., and Kusner, D. J. (2003) *J Immunol.* 170, 2811-2815.

36. Malik, Z. A., Denning, G. M., and Kusner, D. J. (2000) *J. Exp. Med.* 191, 287-303.

37. Malik, Z. A., Iyer, S. S., and Kusner, D. J. (2001) *J Immunol.* 166, 3392-3401.

38. Malik, Z. A., Floto, R. A., Cameron, A. J., Gillooly, D. J., Harnett, M. M., and Allen, J. M. (1998) *Current Biology* 8, 210-221.

39. Yadav, M., Clark, L., and Schorey, J. S. (2006) *J Immunol.* 176, 5494-5503.

40. Panegra, A., Ma, S. X., Zhai, L. W., Wang, X. T., Rhee, S. G., and Khurana, S. (2001) *Am J Physiol Cell Physiol* 281, C1046-C1058.

41. Papakonstanti, E. A., Emmanouel, D. S., Gravanis, A., and Stournaras, C. (2000) *Mol Med* 6, 303-318.

42. Kaneider, N. C., Kaser, A., Dunzendorfer, S., Tilg, H., and Wiedermann, C. J. (2003) *J Virol* 77, 5535-5539.

43. Kaneider, N., Djanani, A., Fischer, C., and Wiedermann, C. (2002) *Biochem Biophys Res Commun* 297, 806-810.

44. Bader, M. F., Doussau, F., Chasserot-Golaz, S., Vitale, N., and Gasman, S. (2004) *Biochim.Biophys.Acta* 1742, 37-49.

45. Alberts, B., Johnson, A., Lewis, J., Martin, R., Roberts, K., and Walter, P. (2004) *Molecular biology of the Cell. Garland Science*, 65.

46. Clemens, D. L. and Horwitz, M. A. (1996) *J.Exp.Med.* 184, 1349-1355.

47. Alberts, B., Johnson, A., Lewis, J., Martin, R., Roberts, K., and Walter, P. (2004) *Molecular biology of the Cell. Garland Science*, 65.

48. Deretic, V. and Fratti, R. A. (1999) *Molecular Microbiology* 31, 1603-1609.

49. Gagnon, E., Duclos, S., Rondeau, C., Chevet, E., Cameron, P. H., Steele-Mortimer, O., Paiement, J., Bergeron, J. J., and Desjardins, M. (2002) *Cell* 110, 793-802.

50. Arora, P. D., Manolson, M. F., Downey, G. P., Sodek, J., and McCulloch, C. A. (2000) *J.Biol.Chem* 275, 35432-35441.

51. Faix, J. and Rottner, K. (2006) *Curr Opin Cell Biol* 18, 18-25.

52. Alberts, B., Johnson, A., Lewis, J., Martin, R., Roberts, K., and Walter, P. (2004) *Molecular biology of the Cell. Garland Science*, 65.

53. Clemens, D. L. and Horwitz, M. A. (1996) *J.Exp.Med.* 184, 1349-1355.

54. Deretic, V. and Fratti, R. A. (1999) *Molecular Microbiology* 31, 1603-1609.

55. Gagnon, E., Duclos, S., Rondeau, C., Chevet, E., Cameron, P. H., Steele-Mortimer, O., Paiement, J., Bergeron, J. J., and Desjardins, M. (2002) *Cell* 110, 119-131.

56. Li, N., Mak, A., Richards, D. P., Naber, C., Keller, B. O., Li, L., and Shaw, A. R. (2003) *Proteomics* 3, 536-548.

57. Desjardins, M., Huber, L. A., Parton, R. G., and Griffiths, G. (1994) *J.Cell Biol.* 124, 677-688.

58. Touret, N., Paroutis, P., Terebiznik, M., Harrison, R. E., Trombetta, S., Pypaert, M., Chow, A., Jiang, A., Shaw, J., Yip, C., Moore, H. P., van der Wel, N., Houben, D., Peters, P. J., de Chastellier, C., Mellman, I., and Grinstein, S. (2005) *Cell* 123, 157-170.
71. Touret, N., Paroutis, P., and Grinstein, S. (2005) *J.Leukoc.Biol.* 77, 878-885
72. Garin, J., Diez, R., Kiefker, S., Dermine, J. F., Duclos, S., Gagnon, E., Sadoul, R., Rondeau, C., and Desjardins, M. (2001) *Journal of Cell Biology* 152, 165
73. de Melker, A. A., van der Horst, G., and Borst, J. (2004) *J.Cell Sci.* 117, 5001-5012
74. Roy, C. R. and Tilney, L. G. (2002) *J Cell Biol* 158, 415-419
75. Scott, C. C., Dobson, W., Botelho, R. J., Coady-Osberg, N., Chavrier, P., Knecht, D. A., Heath, C., Stahl, P., and Grinstein, S. (2005) *Journal of Cell Biology* 169, 139-149
76. Henry, R. M., Hoppe, A. D., Joshi, N., and Swanson, J. A. (2004) *J.Cell Biol.* 164, 185-194
77. Vasioukhin, V. and Fuchs, E. (2001) *Curr Opin Cell Biol* 13, 76-84
78. Funatsu, N., Kumanogoh, H., Soka, Y., and Maekawa, S. (2000) *Neurosci Res* 36, 311-317
79. Rozelle, A. L., Machesky, L. M., Yamamoto, M., Driessens, M. H., Insall, R. H., Roth, MG, Luby-Phelps, K., Marriott, G., and Yin, H. L. (2000) *Current Biology* 10, 311-320
80. Pitson, S. M., D'Andrea, R. J., Yamamoto, M., Driessens, M. H., Insall, R. H., Roth, MG, Luby-Phelps, K., Marriott, G., and Yin, H. L. (2000) *Biochem J* 350 Pt 2, 429-441
81. Kihara, A., Anada, Y., and Igarashi, Y. (2006) *J.Biol.Chem* 281, 4532-4539
82. Pyne, S. and Pyne, N. J. (2000) *Biochem J.* 349, 385-402
83. Pettus, B. J., Bielawski, J., Porcelli, A. M., Reames, D. L., Johnson, K. R., Morrow, J., Chalfant, C. E., Obeid, L. M., and Hannun, Y. A. (2003) *FASEB J* 17, 1411-1421
84. Kenworthy, A. (2002) *Trends in Biochemical Sciences* 27, 435-438
85. Wakatsuki, T., Schwab, B., Thompson, N. C., and Elson, E. L. (2001) *J.Cell Sci.* 114, 1025-1036
86. Holzinger, A. (2001) *Methods Mol Biol* 161, 109-120
87. Ancellin, N., Colmont, C., Su, J., Li, Q., Mittereder, N., Chae, S. S., Stefansson, S., Liu, G., and Hla, T. (2002) *J Biol Chem* 277, 6667-6675
88. Yatomi, Y., Ruan, F., Megidish, T., Toyokuni, T., Hakomori, S., and Igarashi, Y. (1996) *Biochemistry* 35, 626-633
89. Rench, K. J., Schrecengost, R. S., Lee, B. D., Zhuang, Y., and Smith, S. N. (2003) *Cancer Res.* 63, 5962-5969
90. Cano, M. L., Cassimeris, L., Joyce, M., and Zimgond, S. H. (1992) *Cell Motility & the Cytoskeleton* 21, 147-158
91. Iyer, S. S., Agrawal, R. S., Thompson, C. R., Thompson, S., Barton, J. A., and Kusner, D. J. (2006) *J Immunol* 176, 3686-3696
92. Ohta, Y., Hartwig, J. H., and Stossel, T. P. (2006) *Nat Cell Biol* 8, 803-814
93. Stossel, T. P., Fenteany, G., and Hartwig, J. H. (2006) *J.Cell Sci.* 119, 3261-3264
94. Katsuma, S., Hada, Y., eda, T., Shiojima, S., Hirasawa, A., Tanoue, A., Takagaki, K., Ohgi, T., Yano, J., and Tsujimoto, G. (2002) *Genes Cells* 7, 1217-1230
95. Xu, C. B., Zhang, Y., Stenman, E., and Edvinsson, L. (2002) *Atherosclerosis* 164, 237-243
96. Cuvillier, O. (2002) *Biochim Biophys Acta* 1585, 153-162
97. Cuvillier, O. and Levade, T. (2001) *Blood* 98, 2828-2836
98. Cuvillier, O., Rosenthal, D. S., Smulson, M. E., and Spiegel, S. (1998) *J.Biol.Chem* 273, 2910-2916
99. Cuvillier, O., Pirianov, G., Kaufer, B., Vanek, P. G., Coso, O. A., Gutkind, S., and Spiegel, S. (1996) *Nature* 381, 800-803
100. EDSALL, L. C., Cuvillier, O., Twitty, S., Spiegel, S., and Milstien, S. (2001) *J Neurochem* 76, 1573-1584
101. Wang, F., Van Brocklyn, J. R., EDSALL, L., Nava, V. E., and Spiegel, S. (1999) *Cancer Res.* 59, 6185-6191
102. HANNA, A. N., Berthiaume, L. G., Kikuchi, Y., Begg, D., Bourgoin, S., and Brindley, D. N. (2001) *Molecular Biology of the Cell* 12, 3618-3630
103. Aas, V., Algeroy, S., Sand, K. L., and Iversen, J. G. (2001) *Cell Commun Adhes* 8, 125-138
104. Alemany, R., Sichelschmidt, B., zu Heringdorf, D. M., Lass, H., van Koppen, C. J., and Jakobs, K. H. (2000) *Mol Pharmacol* 58, 491-497
105. Beaven, M. A. (1996) *Current Biology* 6, 798-801
106. Chao, C. P., Laulderkerk, S. J., and Ballou, L. R. (1994) *J.Biol.Chem* 269, 5849-5856
107. Choi, O. H., Kim, J. H., and Kinet, J. P. (1996) *Nature* 380, 634-636
108. Mayerzu, H. D., Lass, H., Kuchar, I., Alemany, R., Guo, Y., Schmidt, M., and Jakobs, K. H. (1999) *FEBS Lett* 461, 217-222
FOOTNOTES

1 The abbreviations used are: SK, sphingosine kinase; WT, wild-type; CI, catalytically-inactive; PD, phosphorylation-defective; PM, plasma membrane; DHS, D, L-threo-dihydrosphingosine; PMA, phorbol myristate acetate; PKC, protein kinase C; PLD, phospholipase D; S1P, sphingosine-1-phosphate; IP3, inositol-(1,4,5)-trisphosphate; TNF-α, tumor necrosis factor-α; PI-PLC, phosphatidylinositol phospholipase C; COZ, complement-opsonized zymosan; FcyRs, Fcy receptors; TxS, Triton X-100- soluble; TxI, Triton X-100- insoluble; IP, immunoprecipitation; GFP, green fluorescent protein; RFP, red fluorescent protein; MDM, monocyte-derived macrophages; RNAi, RNA interference; siRNA, short-interfering RNA

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4 No author had any conflicts of interest.

FIGURE LEGENDS

Figure 1. Stimulation of RAW murine macrophages with PMA induces a strong, transient localization of SK1 at membrane ruffles. RAW 264.7 murine macrophages were transfected with GFP-labeled WT-SK1 (A-D), CI-SK1 (E-H), or PD-SK1 (I-L) as described in Materials and Methods and allowed to adhere to glass coverslips. 18-24 hours after transfection, the cells were stimulated with PMA and imaged every 30-45 seconds. WT-SK1
locates at plasma membrane ruffles while CI- and PD-SK1 show dramatically decreased enrichment. A representative sample of images is shown, with GFP fluorescence on the left and the coincident phase contrast displayed in the right panel. Arrows indicate membrane ruffles. There is significantly less ruffling in cells transfected with CI-SK1 or PD-SK1, and these SK1 mutants exhibit decreased localization to these ruffles.

**Figure 2.** Wild-type SK1 colocalizes with cortical actin after stimulation with PMA, while CI- and PD-SK1 have significantly less colocalization. RAW 264.7 murine macrophages were transfected with GFP-labeled WT-SK1 (A and B), CI-SK1 (C and D), or PD-SK1 (E and F) as described in Materials and Methods and allowed to adhere to chambered slides. 18-24 hours after transfection, the cells were stimulated, fixed, stained with Texas Red phalloidin, and imaged. GFP indicates the location of WT-SK1 (A and B), CI-SK1 (C and D), and PD-SK1 (E and F) and red indicates F-actin in resting (A, C, E) and PMA-stimulated (B, D, F) RAW cells.

**Figure 3.** SK1 colocalization with actin at the phagosome during phagocytosis is independent of catalytic activity and phosphorylation state. RAW 264.7 cells were transfected with WT-SK1 (A), CI-SK1 (B), or PD-SK1 (C). At 24 hr, macrophages were incubated with COZ (particle: cell ratio 10:1) for 15 min at 37 C. Cells were fixed in formalin, permeabilized with acetone, and stained for actin filaments with Texas Red-phalloidin. Colocalization of SK1 (green) and actin (red) at the phagosome was evident in WT-SK1 as well as the two mutant SK1s. Images are representative of >25 samples from one of 5 identical experiments.

**Figure 4.** Both complement receptor- and Fcγ receptor-mediated phagocytosis induce colocalization of SK1 and actin filaments. Live cell confocal microscopy was performed as described in Materials and Methods. 24 hours after transfection, RAW 264.7 cells were allowed to phagocytose COZ (A-D) or IgG-coated beads (E-H) for >45 minutes while Z-series images were taken every 60 seconds. Actin (red) and WT-SK1 (green) localize on the phagosome very early and with similar kinetics. Arrows indicate particles being phagocytosed at the various time points. Images are representative of 3 separate experiments.

**Figure 5.** SK1 exhibits constitutive and PMA-stimulated association with detergent-resistant membrane domains. (A) Intact Cell Assay: 3 x 10⁶ RAW 264.7 cells were incubated in the presence of PMA (2µM) or buffer for 30 min at 37 ºC. Cells were disrupted by N₂ cavitation, and membrane and cytosol fractions were separated by differential centrifugation. Membranes were extracted with Lysis Buffer containing 1% Triton X-100, and the detergent-insoluble membrane fraction was re-isolated and washed. Membrane-derived fractions were subjected to SDS-PAGE followed by immunoblotting for SK1. Lanes are as follows: 1) Control, Triton X-100 soluble (C-TxS); 2) Control, Triton X-100 insoluble (C-TxI); 3) PMA-stimulated, TxS; 4) PMA-stimulated, TxI. Positive controls included: (5) Cytosol from U937 cells, (6) Hela cell lysate. (B) Cell-Free Assay: Membrane and cytosol fractions from resting RAW cells were incubated with PMA (2 µM) for 15 min at 37 ºC. The membrane fraction was re-isolated, by centrifugation, washed, and extracted with 1% Triton X-100. Lanes designations follows: 1) Control (C-TxS); 2) C-TxI; 3) PMA(P)-TxS; 4) P-TxI. Data are representative of four identical experiments. (C) Lysates from resting, PMA-stimulated, or COZ-stimulated RAW cells were prepared by nitrogen cavitation and immunoprecipitation was performed with the indicated antibodies. HeLa lysates were used as a control cell line. Gels were then blotted using an anti-actin antibody.

**Figure 6.** SK1 binds to both actin filaments and monomeric G-actin. (A) Effect of phalloidin on velocity sedimentation on sucrose density gradients. Membranes and cytosol from 2 x 10⁸ RAW cells or THP-1 cells were incubated with PMA (100 nM) at 37ºC for 30 min. Membranes were re-isolated by centrifugation at 17,500g, washed, and phalloidin (10 µM) was added to select samples for the final 15 min of the incubation. Membranes were washed and solubilized in 1% Triton X-100 buffer and loaded on top of a 4-ml linear sucrose gradient (20-55%). Samples were centrifuged at 150,000g for 16 h at 8 ºC, and fractions (400 µl) collected from the top of the gradient were subjected to SDS-PAGE and probed for SK-1 and actin. (B) Co-sedimentation assay: Cytosol from 10⁷ THP-1 cells was precleared by ultracentrifugation (150,000g, 30 min, 4ºC). Aliquots (100 µg) were incubated with GTPγS (100 µM) to induce actin polymerization, and jasplakinolide was added at a final concentration of 1 µM to stabilize actin filaments. Actin filaments were pelleted by centrifugation at 17,500g, for 30 min, at 4ºC, washed extensively, and then supernatant and pellet fractions were analyzed by 10% SDS-PAGE gels and probed.
for sphingosine kinase 1 and actin by immunoblotting. (C) Co-isolation of SK1 with G-actin by binding to immobilized DNase 1. Cytosol from 10^9 THP-1 cells was dialyzed against actin monomer buffer, precleared by ultracentrifugation, and then incubated with or without PMA (100 nM) for 30 min at 37°C. Immobilized DNase 1 was added to each fraction and incubated for 4 hr at 4°C (114). The DNase-bound material was pelleted and washed extensively with AMB buffer before subjecting to SDS-PAGE and immunoblotting for SK1 and actin.

**Figure 7.** Actin polymerization is a determinant of SK localization to the plasma membrane. (A-C) RAW cells were transfected with GFP-tagged WT-SK1 and RFP-labeled actin. 24 hr later, PMA (100 nM) was added for 15 min, followed by latrunculin B for the indicated times. Live cell confocal microscopy was performed, as described in the legend to Fig. 1. (D-F) Restoring macrophages were incubated with jasplakinolide (1 µM) for the indicated times and analyzed by live cell confocal microscopy. Images are representative of 4 identical experiments.

**Figure 8.** Modulation of actin polymerization influences SK activity. SK activity in RAW cells was determined as described in Materials and Methods. RAW cells were preincubated with jasplakinolide (1 µM) for 15 minutes, latrunculin B (20 µg/mL), or buffer for 15 min. After the preincubation period, 100 µM PMA or buffer was added for 30 min. Macrophages were disrupted by sonication and sphingosine kinase activity determined in the cell extract via incorporation of [^32P] into [^32P]S1P. Data represent mean +/- SEM of four identical experiments repeated in duplicate.

**Figure 9.** Inhibition of SK activity results in dysregulated actin structures. (A) Human promonocytoid THP-1 cells were transfected with two distinct siRNA to SK1 (SK1-RNAi 1 and 2), control mutant oligonucleotide (C-RNAi) or buffer(Un, untransfected), as described in Methods. At 72 hr, samples were subjected to SDS-PAGE and western blotting with polyclonal Ab to SK1. Confocal microscopy was performed on THP-1 cells at 72 hr after transfection with (B) C-RNAi, (C) SK1-RNAi, following stimulation with PMA (100 nM) for 15 min and staining with Texas Red phalloidin to detect actin filaments. (D-F) Primary human MDM were treated with buffer control (D) or the SK-inhibitor, DHS (25 µM) (E,F) for 15 min, prior to stimulation with PMA (100 nM) for 15 min. Cells were fixed, permeabilized, and stained with Texas Red-phalloidin. (I) Scanning electron microscopy (SEM): MDM were incubated with (H) buffer control (Ctr), or (I,J) 25 µM DHS for 15 min. Images are representative of 25 cells visualized from three 3 identical experiments performed in duplicate utilizing MDM from different donors.

**Video Legends:**

movie1.avi - RAW 264.7 cells were transfected with GFP-WT-SK1 as described in the legend to Fig. 1. PMA (100 nM) was added to cells for 1 min, and live cell confocal microscopy was performed on a Zeiss 510 laser scanning microscope. Images were acquired every 45 sec. Fig. 1A-D contains still images from this movie.

movie2.avi - RAW 264.7 macrophages were transfected with GFP-C1-SK1^{G82D} as described above. Cells were treated with PMA for 1 min, and images acquired every 45 sec by live cell confocal microscopy. Fig. 1E-H contains still images from this movie.

movie3.avi - RAW 264.7 macrophages were transfected with GFP-WT-SK1 and RFP-actin, as described in the legend to Fig. 4. COZ was added at a particle:cell ratio of 10:1 for 15 min prior to acquiring confocal images every 45 sec. Fig. 4A-D contains still images from this movie.

movie4.avi - RAW 264.7 macrophages were transfected with GFP-WT-SK1 and RFP-actin as described above. IgG-beads were added at a particle: cell ratio of 10:1 for 15 min prior to acquiring confocal images every 45 sec. Fig. 4E-H contains still images from this movie.
FIGURES

A. 0 s

B. 600 s

C. 1080 s

Figure 1
Figure 1

D. 1500 s

E. 60 s

F. 600 s

G. 1080 s
Figure 1

H. 1500 s

I. 0 s

J. 660 s

K. 980 s

L. 1080 s
Figure 4

A. 0s

B. 480s

C. 720s

D. 1020s
Figure 4

E. 0s

F. 360s

G. 480s

H. 900s
A

Condition: PMA, COZ, Resting, HeLa

C-TxS  C-TxI  P-TxS  P-Tx  Cvt  Lys

kDa

-48.8
-37.1

SK1

B

C-TxS  C-TxI  P-TxS  P-TxI

kDa

-48.8
-37.1

SK1

C

IP ab: Ctr  SK1  Ctr  SK1  Ctr  SK1

Condition: PMA  COZ  Resting  HeLa

Actin
Figure 7

D.

E.

F.
Figure 8

SK Activity in RAW Cells

Percent of Control

Condition

Resting  PMA  Jasplakinolide  Latrunculin B

0.00%  50.00%  100.00%  150.00%  200.00%  250.00%
Figure 9

A

Un C-RNAi 1 2
SK1-RNAi

B

C

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Figure 9
The localization and activity of sphingosine kinase 1 are coordinately-regulated with actin cytoskeletal dynamics in macrophages
David J. Kusner, Christopher R. Thompson, Natalie A. Melrose, Stuart M. Pitson, Lina M. Obeid and Shankar S. Iyer

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ADDITIONS AND CORRECTIONS

VOLUME 282 (2007) PAGES 37738–37746

Poly(ADP-ribose) polymerase-2 controls adipocyte differentiation and adipose tissue function through the regulation of the activity of the retinoid X receptor/peroxisome proliferator-activated receptor-γ heterodimer.

Péter Bai, Sander M. Houten, Aline Huber, Valérie Schreiber, Mitsuhiro Watanabe, Borbála Kiss, Gilbert de Murcia, Johan Auwerx, and Josiane Ménissier-de Murcia

There was an error in the title of the article. The correct title is shown above.

VOLUME 283 (2008) PAGES 1653–1659

Novel binding site for Src Homology 2-containing protein-tyrosine phosphatase-1 in CD22 activated by B lymphocyte stimulation with antigen.

Chenghua Zhu, Motohiko Sato, Teruhiko Yanagisawa, Manabu Fujimoto, Takahiro Adachi, and Takeshi Tsubata

Dr. Adachi was inadvertently omitted as an author of this article. The correct authors are listed above. Dr. Adachi’s affiliation is the Laboratory of Immunology, School of Biomedical Science, and the Department of Immunology, Medical Research Institute, Tokyo Medical and Dental University and Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, 113-8510 Tokyo, Japan.

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The localization and activity of sphingosine kinase 1 are coordinately regulated with actin cytoskeletal dynamics in macrophages.

David J. Kusner, Christopher R. Thompson, Natalie A. Melrose, Stuart M. Pitson, Lina M. Obeid, and Shankar S. Iyer

On Page 23157, the final sentence of the legend to Fig. 8 should read as follows: Data represent the mean ± S.D. of duplicate determinations from a single representative experiment of a total of four identical experiments. On Page 23158, there is an error in the data in Fig. 9 (A–C), and these three panels should be retracted.

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