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Sphingosine Kinase 1 (SK1) Is Recruited to Nascent Phagosomes in Human Macrophages: Inhibition of SK1 Translocation by Mycobacterium tuberculosis

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*Mycobacterium tuberculosis (M.tb) is a leading cause of global infectious mortality. The pathogenesis of tuberculosis involves inhibition of phagosome maturation, leading to survival of M.tb within human macrophages. A key determinant is M.tb-induced inhibition of macrophage sphingosine kinase (SK) activity, which normally induces Ca2+ signaling and phagosome maturation. Our objective was to determine the spatial localization of SK during phagocytosis and its inhibition by M.tb. Stimulation of SK activity by killed M.tb, live Staphylococcus aureus, or latex beads was associated with translocation of cytosolic SK1 to the phagosome membrane. In contrast, SK1 did not associate with phagosomes containing live M.tb. To characterize the mechanism of phagosomal translocation, live cell confocal microscopy was used to compare the localization of wild-type SK1, catalytically inactive SK1G82D, and a phosphorylation-defective mutant that does not undergo plasma membrane translocation (SK1S225A). The magnitude and kinetics of translocation of SK1G82D and SK1S225A to latex bead phagosomes were indistinguishable from those of wild-type SK1, indicating that novel determinants regulate the association of SK1 with nascent phagosomes. These data are consistent with a model in which M.tb inhibits both the activation and phagosomal translocation of SK1 to block the localized Ca2+ transients required for phagosome maturation. The Journal of Immunology, 2005, 174: 3551−3561.

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modulates critical cellular functions, including migration, proliferation, survival, and cytoskeletal dynamics. Additionally, in a recent study, S-1-P has been shown to modulate interactions between the phagosome and the actin cytoskeleton (13). Two human SK isoforms (SK1 and SK2) have been cloned and partially characterized (14–16). The inhibitor profile of macrophage SK activity supports the hypothesis that SK1 is responsible for phagocytosis-associated elevations of cytosolic Ca\(^{2+}\) and phagosome maturation, and that live \(M\.tb\) inhibits the activity of SK1 (17, 18). Furthermore, human monocyte-derived macrophages (MDM) express SK1, but not SK2 (19).

The mechanism(s) of SK activation is incompletely understood. Recent data indicate that in HEK293T cells stimulated by the phorbol ester, PMA, or by TNF-\(\alpha\), SK1 is activated by phosphorylation of Ser\(^{225}\) (20). The MAPK, ERK2, phosphorylates SK1 at S225 in vitro, and two different MEK inhibitors block this phosphorylation in intact cells stimulated by PMA or TNF-\(\alpha\). PMA-induced phosphorylation of SK1 is also blocked by several protein kinase C (PKC) inhibitors, suggesting the involvement of a kinase cascade in the activation mechanism (21). However, the mechanism(s) by which SK1 is activated in diverse cell types and by physiologic stimuli other than TNF-\(\alpha\) is unknown.

In studies to date, 70–90% of total cellular sphingosine kinase activity is localized to the cytosol (21, 22). Thus, in addition to an increase in catalytic activity, stimulation of SK probably involves a translocation of this cytosolic enzyme to membranes, the site of the substrate, sphingosine. PMA and TNF-\(\alpha\) induce the translocation of SK1 from the cytosol to the plasma membrane, and this response requires phosphorylation of S225 (20, 21). Activation and translocation of SK1 to the plasma membrane of SH-SY5Y neuroblastoma cells in response to methacholine or lysophosphatidic acid requires Ca\(^{2+}\) and Cam (23). Plasma membrane translocation of SK1 has also been demonstrated in FceRI-stimulated mast cells (24), C5a-activated macrophages (19), and PC12 cells stimulated with nerve growth factor (25), although the mechanisms of these phenomena have yet to be elucidated. Together, these studies implicate MAPKs, PKC, and Ca\(^{2+}\)/Cam in the activation and translocation of SK1 to the plasma membrane.

Because Ca\(^{2+}\)-mediated signaling during phagocytosis and phagosome maturation is highly localized (7–9, 26, 27), our hypothesis is that SK1 will exhibit similar spatial restriction. The objectives of this study were to determine 1) if activation of SK1 in human macrophages by phagocytic particles results in its translocation to the phagosome membrane; and 2) whether live, virulent \(M\.tb\) inhibits this specific spatial localization of SK1.

**Materials and Methods**

**Materials**

Unless otherwise stated, materials were obtained from previously reported sources (7, 8, 10). Polystyrene beads were purchased from Polysciences. Glass chamber slides were purchased from Fisher. FuGene 6 was purchased from Roche.

**Antibodies**

Polyclonal rabbit anti-hSK1 Abs were generated as previously described (21). Murine anti-human lysosome-associated membrane protein 1 (LAMP-1; H4A3) Ab was purchased from the Developmental Studies Hybridoma Bank (University of Iowa). Goat anti-rabbit Texas Red and goat anti-mouse Oregon Green secondary Abs were purchased from Molecular Probes.

\(\text{H4A3 (anti-LAMP-1) mAb developed by J. Thomas August and James E. K. Hildreth was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and was maintained by Department of Biological Sciences, University of Iowa (Iowa City, IA).}\)

**Bacteria**

GFP-expressing \(M\.tb\), strain H37Rv, was a gift from V. Deretic (University of New Mexico, Albuquerque, NM) and were cultured and prepared for use in experiments as noted previously (7, 8, 10, 28, 29). Gamma-irradiated (killed) \(M\.tb\) were provided by J. Belisle (Colorado State University, Fort Collins, CO). In select experiments, GFP-\(M\.tb\) were killed by fixation in 10% formalin for 20 min at 25°C. After repeated suspension/sedimentation cycles to remove clumped bacteria, \(M\.tb\) suspensions were counted in a Petroff-Hausser chamber. Final \(M\.tb\) preparations contained \(>95\%\) single bacteria, with \(\approx 75\%\) viability by determination of CFUs.

The effects of various experimental manipulations on the viability of \(M\.tb\) were also determined by analysis of CFUs. GFP-expressing Staphylococcus aureus (ALC1435) was a gift from A. Chung (Dartmouth University, Hanover, NH) and was grown in tryptic soy broth containing 10 \(\mu\)g/ml chloramphenicol. Both \(M\.tb\) and \(S\. aureus\) were opsonized in 50% human serum before use in experiments.

**Macrophage/monocyte cell lines and transfection**

THP-1 human promonocytic cells were purchased from American Type Culture Collection and cultured in RPMI 1640, 10% FBS, 1% penicillin/streptomycin, and 115 \(\mu\)M 2-ME, as previously described (30). THP-1 cells were differentiated to a macrophage-like phenotype by supplementing the medium with 100 nM 1,25-dihydroxyvitamin D3, 1000 U/ml IFN-\(\gamma\), and 1 mM retinoic acid (31). After 3 days, nonadherent cells were washed away with warm RPMI 1640, and adherent cells were replated in RPMI 1640 containing 1% fatty acid-free BSA. The RAW 264.7 murine macrophage cell line was a gift from J. Engelhard (University of Iowa) and was cultured in DMEM containing 10% FBS and 1% antibiotics (100 U/ml penicillin/streptomycin (32). Plasmids encoding enhanced GFP (EGFP) fusion proteins of wild-type, catalytically inactive (SK1\(^{1642D}\)), and phosphorylation-defective (SK1\(^{S225A}\)) SK1 (20, 21) were transfected into RAW 264.7 cells using FuGene 6 (Roche Diagnostics) according to 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 \(\mu\)g of plasmid DNA. Confocal microscopy was performed 18–24 h after transfection.

**Preparation of primary macrophages**

Human MDM were isolated and cultured as previously described (7, 10). Briefly, venous blood was drawn from healthy, adult volunteers in accordance with the human subjects guidelines approved by University of Iowa institutional review board. PBMCs were isolated by density gradient centrifugation on Ficoll-Hypaque and cultured in RPMI 1640 and 20% autologous serum. MDM monolayers were washed twice and then incubated in RPMI 1640 containing 1% fatty acid-free BSA. The RAW 264.7 murine macrophage cell line was a gift from J. Engelhard (University of Iowa) and was cultured in DMEM containing 10% FBS and 1% antibiotics (100 U/ml penicillin/streptomycin (32). Plasmids encoding enhanced GFP (EGFP) fusion proteins of wild-type, catalytically inactive (SK1\(^{1642D}\)), and phosphorylation-defective (SK1\(^{S225A}\)) SK1 (20, 21) were transfected into RAW 264.7 cells using FuGene 6 (Roche Diagnostics) according to 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 \(\mu\)g of plasmid DNA. Confocal microscopy was performed 18–24 h after transfection.

**Determination of SK activity**

Two complementary assays of SK activity were used: 1) phosphorylation of exogenous sphingosine by \(\gamma^\text{32P}\)ATP (10, 33) with both intact macrophages and purified cytosol and membrane fractions; and 2) conversion of \(\text{H}\)sphingosine to \(\text{H}\)sphingosine-1-P was used solely with intact cells (10, 34).

In assay 1, MDMs were incubated with gamma-irradiated (killed) \(M\.tb\) at a multiplicity of infection of 10:1 for 0.5–10 min. Cells were washed twice with ice-cold PBS and scraped into 500 \(\mu\)l of cold lysis buffer (0.2 M Tris, 1 mM EDTA, 0.5 mM deoxyriboside, 15 mM sodium fluoride, 0.01% 2-ME, 1 mM sodium orthovanadate, 10 \(\mu\)g/ml leupeptin, 10 \(\mu\)g/ml aprotinin, 10 \(\mu\)g/ml soybean trypsin inhibitor, 40 mM \(\beta\)-glycerolphosphate, 1 mM PMSF, and 10% glycerol (33). The cells were disrupted by homogenization and centrifuged at 100,000 \(\times\) \(g\) at 4°C. The supernatant (cytosol) were removed, and the membrane fraction was washed and resuspended in lysis buffer. Protein concentrations were determined by the method of Bradford (35). Aliquots of each fraction (5 \(\mu\)g of protein) were reacted with sphingosine substrate (1 \(\mu\)M in 5% Triton X-100; 0.25% Triton X-100 in the final reaction) and \(\gamma^\text{32P}\)ATP for 30 min at 37°C.

In assay 2, Labeled S-1-P was isolated by TLC (butanol/ethanol/acetic acid/water, 80/20/10/20) and quantified by a Typhoon phosphorimager (Amersham Biosciences). In this assay, sphingosine is added in excess to ensure that its level is not rate-limiting in formation of the product, S-1-P. For the
In [32P]ATP method, the sphingosine to S-1-P ratio was 360:1 in resting MDM and 225:1 in cells undergoing phagocytosis of latex beads. In assay 2, to assess S-1-P production in an intact cell system, MDM were analyzed in six-well plates and incubated in the presence of [3H]sphingosine (~410 nCi/sample in RPMI 1640 containing 1% BSA) for 1 min. Phagocytic stimuli were added for the indicated amounts of time, after which the reaction was stopped by removal of the supernatant and addition of 2 ml of ice-cold methanol. The cells were then scraped, and the supernatant was transferred to polypropylene tubes and solubilized by the addition of 1 ml of chloroform. The samples were dried in a Savant Speed-Vac (Global Medical Instrumentation) and resuspended in 60 µl of ethanol. [3H]S-1-P was isolated by TLC (methanol/chloroform/acetic acid/water, experiments).

The ratio of sphingosine to S-1-P in the [3H]sphingosine assay was 90:1 in resting control MDM and 26:1 in cells stimulated with latex beads.

For both assays, SK activity was expressed as the percentage (mean ± range) of control values from paired untreated cells for each experiment to correct for minor variability in the levels of radioactivity between replicate experiments.

Confocal microscopy

For fixed cell imaging, MDM were adhered in eight-chamber glass culture slides at ~50% confluence and incubated with a phagocytic stimulus (live or killed GFP-H37Rv M.tb, GFP-S. aureus, and latex beads (0.3 µm diameter)) at a particle:cell ratio of 5–10:1. Cells were fixed in 3.75% paraformaldehyde for 15 min and permeabilized in ice-cold methanol/acetic acid (1/1) (8). After incubation with blocking buffer (PBS, 5% BSA, and 10% horse serum) for 1 h, rabbit polyclonal anti-hSK1 Ab (1/100 dilution) was added for 1 h, and samples were washed repeatedly and incubated with Texas Red-conjugated goat anti-rabbit IgG secondary Ab for 1 h, all at 25°C. mAb to LAMP-1 was coincubated with the anti-h-SK1 Ab, with subsequent detection with goat anti-murine IgG Oregon Green secondary Ab. In select experiments, cells were preincubated with 100 nM PMA (in 0.1% DMSO) for 30 min or with 1 µM A23187 in buffer containing ~1 µM free Ca2+ (1 mM EGTA and 0.9 mM CaCl2) for 5 min before addition of live M. tuberculosis. The 0.1% DMSO solvent control cells exhibited no difference in viability or SK1 localization compared with cells incubated in buffer alone. Analysis was performed using a Zeiss 510 laser scanning confocal microscope.

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) at 37°C and analyzed at rest and during phagocytosis of latex beads using the Zeiss 510 laser scanning microscope and the associated LSM time series imaging software. Z-series confocal images were acquired every 30–60 s for >30 min. In select experiments, cells were preincubated in 25 µM 1,2-bis-(2-amino-5-methylphenoxy)ethane tetracetic acid tetracetoxyethyl ester (MPTAM) (in 0.1% DMSO) for 20 min before the addition of latex beads.

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. Nonparametric evaluation of other datasets was performed with the Mann-Whitney rank-sum test (36).

Online supplemental material

Three supplemental movies of live cell confocal microscopy are included online. RAW 264.7 cells were transfected with wild-type SK1 (supplemental movie 1), catalytically inactive SK1(183G) (supplemental movie 2), or phosphorylation-defective SK1(212–230A) (supplemental movie 3) and incubated with latex beads as described above. These videos can be viewed at (www.jimmunol.org).

Results

Macrophage phagocytosis is accompanied by stimulation of SK activity

We have previously demonstrated that phagocytosis of killed M.tb by primary human MDMs results in stimulation of SK activity (10). To determine whether this response was stimulus-specific or, rather, shared with other phagocytic particles, we evaluated the effect of 3-µm latex beads on SK activity in human MDM. Latex beads were selected because they are a well-characterized model phagocytic particle that facilitates subsequent localization of proteins by confocal microscopy (4, 37–39). Incubation of [3H]sphingosine-labeled MDM (34) with latex beads, at a particle:cell ratio of 10:1, resulted in significant stimulation of SK activity (Fig. 1A). Within 5 min of particle addition, SK activity increased to 325 ± 22% of the basal value of control, untreated cells, and remained elevated for 60 min. Addition of PMA (100 nM) served as the positive control (21, 40) and resulted in an increase in SK activity to ~180 ± 23% of the control value (Fig. 1A). Macrophage phagocytosis of complement-opsonized zymosan or serum-opsonized S. aureus was also associated with stimulation of SK activity (data not shown).

To determine the appropriateness of macrophage cell lines for analysis of phagocytosis-associated increases in SK activity, THP-1 human promonocytes were differentiated to a macrophage-like phenotype (dTHP-1) by incubation with IFN-γ, 1,25-dihydroxyvitamin D3, and retinoic acid (30, 31). Incubation with killed (gamma-irradiated) M.tb, at a particle:cell ratio of 10:1, resulted in marked activation of SK (Fig. 1B). Increased levels of the SK product, S-1-P, were detected within 2 min, with a maximal increase of ~970% 10 min after addition of bacteria. PMA also stimulated SK activity in dTHP-1 cells (Fig. 1B). Phagocytosis of latex beads or complement-opsonized zymosan by dTHP-1 cells also induced increases in SK activity (not shown). Thus, phagocytosis of multiple particles by both primary macrophages and macrophage-like cell lines was accompanied by stimulation of SK activity.

Phagocytosis is associated with an increase in membrane SK activity

Because phagocytosis and phagosome maturation are regulated by spatially restricted signal transduction cascades, our hypothesis was that stimulation of SK would result in a translocation of the activated enzyme to the phagosome membrane. As a first step in evaluation, we determined the effect of phagocytosis on the subcellular distribution of SK activity between membrane and cytosolic fractions. MDM were incubated with killed M.tb for 0.5–10 min. Nonadherent bacilli were removed by washing, and MDM were disrupted by homogenization. Membrane and cytosolic fractions were separated by centrifugation, and the SK activity of each fraction was determined. In control, resting MDM, 94.4 ± 0.1% of the total cell-associated SK activity was localized to the cytosol, and the remaining 5.6 ± 0.1% was in the membrane fraction (mean ± range of two experiments conducted in triplicate). Phagocytosis of killed M.tb was associated with a marked increase in membrane-associated SK activity, which was maximal at 10 min (180 ± 9% of control; Fig. 1C). In contrast, the cytosolic fraction exhibited a progressive decrease in SK activity. These phagocytosis-associated changes in the subcellular localization of SK activity are consistent with the hypothesis that killed M.tb induces a translocation of SK from cytosol to the membrane.

SK1 is predominantly cytosolic in resting macrophages and translocates to the region of the nascent phagosome during particle ingestion

To further characterize the mechanism of SK activation during phagocytosis, MDM were analyzed by laser scanning confocal microscopy (21). In resting MDM, SK1 was detected in a diffuse cytosolic distribution (Fig. 2A). This cytosolic localization of SK1 is in agreement with data from nonphagocytic cells and is consistent with the predominantly cytosolic distribution of SK activity (20–22). Approximately 90% of MDMs exhibited an exclusively...
cytosolic localization of SK1 by confocal microscopy. In ~10% of cells, this was accompanied by a punctate distribution of SK1 (Fig. 2B). Because these punctate areas may represent intracellular vesicles, cells were counterstained with murine mAb to LAMP-1 (detected by Oregon Green-conjugated secondary Ab). In ~10% of resting MDMs, SK1 was also detected in punctate intracellular structures, a minority of which were positive for LAMP-1. Data are representative images from ≥25 MDMs/sample, from eight independent experiments.
permeabilized, and stained with Abs to SK1 and LAMP-1. Within the first 5 min, SK1 was significantly enriched in the region of the nascent phagosome (Fig. 3A). In fact, the majority of cellular SK1 was localized to the phagosomal region, particularly in the confocal z-slices of the macrophage that contained the latex particle (Fig. 3B). The level of phagosomes enriched for SK1 decreased from ~60% at 5 min to 20–25% at 10 and 15 min (Fig. 4E). By 30 min, no latex bead phagosomes demonstrated enrichment for SK1. These data are consistent with the transient recruitment of SK1 to the region of the newly formed phagosome. Our hypothesis is that SK1 is specifically recruited to the membrane of the nascent phagosome rather than to the periphagosomal cytosol. This hypothesis is based on 1) the biochemical data from this study (Fig. 1C) and from other investigators (20, 23, 43) that membrane-associated SK activity increases upon cellular activation, 2) the membrane localization of the substrate, sphingosine, and 3) previous data that PMA, TNF-α, nerve growth factor, and stimulation of IgE receptors or G protein-coupled receptors increased the plasma membrane localization of SK1 (20, 21, 23–25). The lack of detection of SK1 on the phagosome membrane may be due to the difficulty of resolving transient changes in spatial localization using fixed cell imaging.

**SK1 colocalizes with phagosomes containing live S. aureus or killed M.tb, but not those with live M.tb**

To further test our hypothesis on the induction of SK1 translocation during macrophage phagocytosis, we examined the localization of SK1 during ingestion of serum-opsonized live *S. aureus* or killed *M.tb*. Detection of these organisms by confocal microscopy used plasmid-mediated production of GFP. *M.tb* was killed by formalin-fixation immediately before use, which preserved the intensity of GFP fluorescence for the 30-min duration of the experiment. Phagocytosis of both *S. aureus* and killed *M.tb* was accompanied by transient enrichment of SK1 in the region of the nascent phagosome, which was maximal at 5–10 min (Fig. 4, A and B). In comparison with latex beads, confocal microscopy of fixed samples of live *S. aureus* and killed *M.tb* demonstrated specific localization to the phagosome membrane. The degree of phagosomal localization of SK1 with both *S. aureus* and killed *M.tb* progressively decreased and was undetectable at 30 min, in agreement with data presented above for latex beads (Fig. 4E). Taken together, these data are consistent with a model in which phagocytosis of multiple, chemically diverse particles induces a temporally restricted localization of SK1 to the region of the phagosome. In each case, stimulation of SK activity by polystyrene beads, live *S. aureus*, or killed *M.tb* correlated with the phagosomal translocation of SK1.

Because live, serum-opsonized *M.tb* inhibits SK activity as part of the mechanism of its intracellular persistence (10), we hypothesized that SK1 would not associate with phagosomes containing live tubercle bacilli. In fact, confocal microscopic analysis of MDMs undergoing phagocytosis of live *M.tb* for 0.5, 3, 5, 10, 15, or 30 min demonstrated the absence of significant change in the cytosolic distribution of SK1 (Fig. 4, C and D). At 5 min, there was no significant accumulation of SK1 in the region of the phagosome. At later time points, only a minority of phagosomes had associated SK1 (18% at 15 min and 14% at 30 min). These data and previous work (1) are consistent with the hypothesis that live, virulent *M.tb* inhibits both the activation and phagosomal translocation of SK1.

Because PMA promotes the plasma membrane association of SK1 in nonphagocytic cells (19, 20), we tested whether it could reverse mycobacterial-induced inhibition of SK1 translocation to nascent phagosomes. MDM were preincubated with 100 nM PMA for 30 min before the addition of live *M.tb*. The dose and time of PMA pretreatment were selected based on their effects on SK1 translocation noted in other systems (19, 20). Treatment with PMA did not induce the translocation of SK1 to phagosomes containing live *M.tb* at times ranging from 0.5–30 min after infection (Fig. 4F).

Elevation of macrophage cytosolic Ca $^{2+}$ with the ionophore A23187 reverses mycobacterial inhibition of the phagosomal-specific Cam-CamK II–PI3K–EEA-1 signaling cascade and results in
FIGURE 4. SK1 colocalizes with phagosomes containing live S. aureus or killed M. tb, but not those with live M. tb. Primary human MDM were incubated with S. aureus (A), formalin-fixed killed M. tb (B), or live, virulent M. tb (C and D) for 5 min. Each organism was detected by the expression of transfected GFP, whereas SK1 was detected with polyclonal anti-SK1 Ab and Texas Red-labeled secondary Ab. SK1 was associated with phagosomes containing live S. aureus and killed, but not live, M. tb. The live M. tb marked by the arrow is located within the macrophage, whereas the other bacillus is adherent to the cell surface. E, Summary of the association of SK1 with phagosomes containing polystyrene beads or killed or live M. tb. For each of the indicated times, ≥25 phagosomes were scored for enrichment of SK1 staining around the phagocytosed particle. Data represent the mean number of SK1-associated phagosomes from three identical experiments. Asterisks denote significant differences in the level of SK1-positive phagosomes between the indicated samples and the corresponding latex bead sample at p < 0.05. F, MDM incubated with 100 nM PMA for 30 min before addition of live, GFP-expressing M. tb. G, MDM were preincubated for 5 min with A23187 (1 μM) in buffer containing 1 mM EGTA, 0.9 mM CaCl₂ (free Ca²⁺, ~1 μM). Treatment of MDM with PMA or A23187 failed to induce the localization of SK1 with live M. tb-containing phagosomes at 0.5, 5, 10, 15, or 30 min. The samples shown in F and G are 5 min after addition of live M. tb.
phagosome maturation and increased macrophage antituberculous activity (7–9). Preincubation of MDM with A23187 (1 μM) for 5 min before infection with live *M. tb* did not induce the phagosomal association of SK1, determined at 0.5–30 min (Fig. 4G). Although we cannot exclude potential effects of different doses or durations of incubation with PMA or A23187, these data support the hypothesis that *M. tb*-induced inhibition of the phagosomal translocation of SK1 is not reversible via stimulation of PKC activity or elevation of cytosolic Ca$^{2+}$.

**Live cell confocal microscopy demonstrates translocation of cytosolic SK1 to the phagosome membrane**

Because the enrichment of SK1 in the region of newly formed phagosomes was very transient, imaging of fixed cell preparations did not provide optimal resolution of the kinetics of this process. More specifically, fixed cell imaging could not fully evaluate the hypothesis that SK1 translocates to the phagosome membrane, particularly in the case of latex beads. Therefore, live cell confocal microscopy was used to characterize the subcellular localization of SK1 in macrophages undergoing phagocytosis. For this analysis, the murine macrophage cell line RAW 264.7 was transfected with wild-type EGFP-SK1 or EGFP control vector. Approximately 24 h after transfection, the cells were incubated with latex beads and imaged every 30–45 s for >30 min. In resting cells, EGFP-SK1 localized to the cytosol (Fig. 5A), in agreement with the subcellular distribution of the majority of endogenous SK1 shown in this study and others (20, 22, 23). Phagocytosis of latex beads was associated with transient enrichment of SK1 in the membranes of newly formed phagosomes (Fig. 5B). SK1 was enriched in phagocytic cups, which are the earliest membrane changes detectable during particle ingestion. Maximal levels of SK1 were present in phagosome membranes between 1 and 5 min after contact of polystyrene beads with the macrophage surface (Fig. 5 and supplemental movie 1). SK1 remained detectable on the phagosome membrane for ~10 min, then returned to a diffuse cytosolic distribution. Macrophages transfected with control EGFP

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Phagosomal translocation of SK1 occurs normally in the catalytically inactive and phosphorylation-defective mutants, but is blocked by inhibition of cytosolic Ca$^{2+}$ signaling. RAW 264.7 murine macrophages were transfected with wild-type EGFP-SK1 (A–D), EGFP control vector (E–H), catalytically inactive SK1$^{G82D}$ (I–L), or phosphorylation-defective SK1$^{225A}$ (M–P) via FuGENE6. Twenty-four hours after transfection, the cells were incubated with 3-μm latex beads and imaged by live cell laser scanning confocal microscopy every 40–45 s (see supplemental movies 1–3). Q–S, Macrophages were preincubated with 25 μM MAPTAM for 20 min, followed by addition of latex beads. For each condition, GFP fluorescence is presented in the left column, and the concurrent phase contrast image is shown in the right column. The indicated times are in reference to the point of particle contact with the macrophage surface. The arrows indicate the positions of the latex beads of interest. Data are representative images from at least three identical experiments for each condition. (Figure continues)
vector exhibited the expected cytosolic distribution of GFP in the resting state (Fig. 5E). There was no change in the cytosolic distribution of EGFP during the first 30 min of phagocytosis, and specifically no enrichment of EGFP on the phagosome membrane (Fig. 5, F–H).

The biochemical mechanisms that regulate the transient recruitment of cytosolic SK1 to the membrane of the nascent phagosome are unknown, as are the means by which live \textit{M. tb} inhibits this process. Because \textit{M. tb} also inhibits the catalytic activity of SK (10), we hypothesized that stimulation of the enzymatic activity of SK1 is required for recruitment to the phagosome membrane. To test this hypothesis, RAW 264.7 macrophages were transfected with a catalytically inactive mutant of SK1, EGFP-SK1\textsuperscript{G82D}, which has previously been demonstrated to confer a dominant negative phenotype (21, 44). Despite the absence of enzymatic activity, GFP-SK1G82D localized to the phagosome membrane with kinetics indistinguishable from those of wild-type SK1 (Fig. 5, I–L, and supplemental movie 2). These data are consistent with a model in which stimulation of the catalytic activity of SK1 and the resultant generation of its product, S-1-P, are not required for translocation of the enzyme to the phagosome membrane.

To further examine these hypotheses regarding the regulation of enzymatic activity and membrane translocation of SK1, we transfected RAW 264.7 macrophages with a phosphorylation-defective SK1 mutant, EGFP-SK1\textsuperscript{S225A}. In wild-type SK1, Ser\textsuperscript{225} is phosphorylated in response to PMA or TNF-\alpha, and the SK1\textsuperscript{S225A} mutant does not undergo activation and plasma membrane translocation in HEK cells stimulated by these agonists (20). In RAW macrophages undergoing phagocytosis, GFP-SK1\textsuperscript{S225A} translocated to the membrane of latex bead phagosomes with similar magnitude and kinetics as wild-type SK1 (Fig. 5, M–P, and supplemental movie 3).

Phagocytosis is accompanied by increases in cytosolic Ca\textsuperscript{2+} levels (7–9, 25, 45, 46) and Ca\textsuperscript{2+} is required for the plasma membrane translocation of SK1 in neuroblastoma cells stimulated with ligands for G protein-coupled receptors (23). Therefore, we tested the hypothesis that phagocytosis-induced increases in cytosolic Ca\textsuperscript{2+} levels are required for normal translocation of SK1 to latex bead phagosomes. Addition of the intracellular calcium chelator, MAPTAM (25 \textmu M), under conditions associated with inhibition of phagocytosis-associated increases in cytosolic Ca\textsuperscript{2+} (7), completely blocked SK1 association with phagosomes for 0.5–30 min after particle addition (Fig. 5Q–S). These data support a model in which physiologic translocation of SK1 to nascent phagosomes is regulated by the level of cytosolic Ca\textsuperscript{2+}, but not by the enzyme’s intrinsic catalytic activity or phosphorylation of Ser\textsuperscript{225}. Taken together, these results indicate that \textit{M. tb} exhibits novel inhibition of...
two key features of SK1: its enzymatic activity (10) and its translocation to newly formed phagosomes.

**Discussion**

The maturation of phagosomes to phagolysosomes is critically important to the functions of the innate immune system, including host defense against microbes and tumor cells, clearance of apoptotic cells and inflammatory debris, and processing and presentation of Ags to elicit adaptive immunity (reviewed in Refs. 1–3 and 47–49). Work from multiple laboratories has contributed to our growing knowledge of the biochemical mechanisms and cellular events that regulate phagosome maturation (1–3, 47, 49). Important questions remain concerning the specific spatial and temporal determinants of these signaling pathways, their networks of integration, the intersections with cytoskeletal elements, and the modifications of regulatory mechanisms by physiologic agonists and pathologic microorganisms.

We previously demonstrated that SK activity is necessary for the maturation of phagosomes containing killed *M. tb* in human macrophages (10). SK catalyzes the phosphorylation of sphingosine to S-1-P, which, in turn, causes an efflux of calcium from the endoplasmic reticulum (50, 51). The recent demonstrations of extracellular release of SK-derived S-1-P and the resultant activation of plasma membrane S-1-P receptors suggest that this autocrine pathway of SK signaling could also contribute to phagosome maturation (20, 21, 25, 52). The SK-dependent increase in cytosolic Ca\(^{2+}\) levels is necessary for the transient fusion and fission reactions of phagosome maturation to occur (7–9, 53, 54). In contrast, live *M. tb* inhibits SK activity in human MDMs, thereby blocking S-1-P-induced increases in cytosolic Ca\(^{2+}\) and phagosome maturation (10). This inhibition of macrophage SK activity and the resultant intracellular survival of mycobacteria in immature phagosomes are central to the pathogenesis of tuberculosis. However, the mechanism by which *M. tb* inhibits macrophage SK activity is unknown.

This study presents several novel findings regarding the activation of SK1 in macrophages and its inhibition by *M. tb*. Based on the demonstration of periphagosomal increases in cytosolic Ca\(^{2+}\) (26, 27), we hypothesized that SK1 translocates to the forming phagosome, where it produces S-1-P, to initiate this Ca\(^{2+}\) response. We first addressed this hypothesis by examining SK activity in the cytosol and membrane fractions during phagocytosis. The few studies that have measured the subcellular distribution of SK activity indicate that 70–90% of total SK activity is cytosolic, with the remainder located in the membrane fraction (18–22). Our data indicate that in resting MDMs, ~95% of SK activity is cytosolic, and that phagocytosis is associated with a redistribution of SK activity to the membrane fraction.

To characterize the mechanism of this increase in membrane-associated SK activity, we determined the localization of SK1 by confocal microscopy. In resting MDMs, SK1 is diffusely distributed throughout the cell, consistent with the predominately cytosolic localization demonstrated in other cell types (20, 21, 26). During phagocytosis of multiple diverse particles that stimulate SK activity (latex beads, live *S. aureus*, or killed *M. tb*), SK1 underwent a dramatic relocation to the region of the forming phagosome (Figs. 3–5). In the case of the two organisms, the fixed cell microscopy data were consistent with translocation of SK1 to the phagosomal membrane itself. In all cases, enrichment in the region of the phagosome was very transient (≤10 min; see supplemental movie 1).

To further characterize the kinetics and mechanism of the translocation of SK1 to nascent phagosomes, we conducted live cell confocal microscopy on RAW 264.7 macrophages transiently transfected with SK1-GFP constructs. The localization of SK1 demonstrated by this approach closely approximated that defined by fixed cell imaging of primary macrophages (Fig. 5, A–D, and supplemental movie 1). Specifically, SK1 was enriched in phagocytic cups, pseudopods, and intact phagosomes during the first 10 min after particle contact with the macrophase surface. The distribution of SK1 returned to a diffuse cytosolic pattern within 15–20 min. Catalytically inactive SK1\(^{G82D}\) and the phosphorylation-deficient SK1\(^{S225A}\) mutant translocated normally, whereas preincubation with the intracellular Ca\(^{2+}\) chelator, MAPTAM, blocked the association of SK1 with phagosomes. Based on previous data that phosphorylation of Ser\(^{225}\) is necessary for PMA- or TNF-\(\alpha\)-induced stimulation of SK1 activity and its translocation to the plasma membrane in HEK cells (20), the present studies support a model in which the localization of SK1 to macrophage phagosomes is regulated in a novel manner that is independent of enzymatic activity or phosphorylation of Ser\(^{225}\), but dependent on stimulus-induced increases in cytosolic Ca\(^{2+}\) (Fig. 5, I–S, and supplemental movies 2 and 3).

In striking contrast to latex beads, live *S. aureus*, and killed *M. tb*, SK1 did not associate with early phagosomes containing live virulent *M. tb*. At later time points (15, 30 min), a minority (~20%) of these phagosomes were weakly positive for SK1. Whether this represents the presence of a fraction of dead bacilli in the plate grown preparations (5–10) or a marked delay in delivery of SK1 to a minority of phagosomes containing live tubercle bacilli is unknown. Combined with recent evidence that sphingolipids, including sphingosine and S-1-P, modulate the interactions of latex bead or mycobacterial phagosomes with the actin cytoskeleton (13), these data strongly support the hypothesis that SK1 regulates the...
normal maturation of phagosomes and is a key focal point of maturation disruption by \textit{Mtb}.

\textbf{Mtb} inhibits both the phagosomal translocation of SK1 and the stimulation of its activity \((10)\). The underlying mechanism by which SK1 is normally activated and translocated to nascent phagosomes, and the inhibitory transducers of the mycobacterial effects are unknown. The inhibition of phagocytosis-induced SK activity by dihydrosphingosine \((10)\) and the data presented in this study strongly support the involvement of SK1. Although the potential contribution of the more recently characterized SK2 isoform to these processes has not been directly evaluated, human MDM express SK1, but not SK2 \((19)\). The failure of PMA or A23187 to reverse the \textit{Mtb}-induced inhibition of SK1 translocation also supports the model that novel determinants regulate the association of SK1 with distinct membrane compartments (plasma membrane vs phagosome). Additional characterization of the mechanisms that regulate SK1’s enzymatic activity as well as its localization to specific membranes will contribute to our understanding of the physiologic process of phagosome maturation and its pathogenic disruption during \textit{Mtb} infection.

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\textbf{Disclosures}

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\textbf{References}


