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Simvastatin Inhibits *Staphylococcus aureus* Host Cell Invasion through Modulation of Isoprenoid Intermediates

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ABSTRACT

Patients on a statin regimen have a decreased risk of death due to bacterial sepsis. We have found that protection by simvastatin includes the inhibition of host cell invasion by *Staphylococcus aureus*, the most common etiologic agent of sepsis. Inhibition was due in part to depletion of isoprenoid intermediates within the cholesterol biosynthesis pathway and led to the cytosolic accumulation of the small GTPases CDC42, Rac, and RhoB. Actin stress fiber disassembly required for host invasion was attenuated by simvastatin and by the inhibition of phosphoinositide 3-kinase (PI3K) activity. PI3K relies on coupling to prenylated proteins, such as this subset of small GTPases, for access to membrane-bound phosphoinositide to mediate stress fiber disassembly. Therefore, we examined whether simvastatin restricts PI3K cellular localization. In response to simvastatin, the PI3K isoform p85, coupled to these small-GTPases, was sequestered within the cytosol. From these findings, we propose a mechanism whereby simvastatin restricts p85 localization, inhibiting the actin dynamics required for bacterial endocytosis. This approach may provide the basis for protection at the level of the host in invasive infections by *S. aureus*.

Each year in the United States, death due to sepsis is as common as death due to myocardial infarction (Angus et al., 2001). The most prevalent etiologic agent, *Staphylococcus aureus* (Diekema et al., 2001), is a leading hospital-acquired pathogen, and community-acquired infection is becoming increasingly widespread (Lowy, 1998). The incidence of complicated infection has increased with the development of invasive procedures and with increased aging, immunocompromised patient populations. Bacterial sepsis is complicated further by invasive endocarditis and osteomyelitis, with persistent infections commonly requiring lengthy hospital stays and treatment. The emergence of antibiotic-resistant strains, including methicillin-resistant *S. aureus* (MRSA), has limited treatment options. We were surprised to find that statins, a class of drugs generally prescribed for lowering cholesterol, may provide a new adjunctive therapy for bacterial sepsis (Liappis et al., 2001; Krüger and Merx, 2007).

The pharmacology of statins includes effects caused by the lowering of plasma cholesterol and the depletion of intermediates within the cholesterol biosynthesis pathway. Statins lower cholesterol through inhibition of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis (Tobert, 2003). In addition to the reduction in cholesterol levels, inhibition diminishes the synthesis of intermediates within the pathway, including mevalonate and the hydrophobic isoprenoids geranylgeranyl pyrophosphate (GGpp) and farnesyl pyrophosphate (Fpp) (Goldstein and Brown, 1990). Pleiotropic benefits independent of cholesterol lowering have been associated with this depletion of isoprenoid intermediates. Isoprenoids function as membrane anchors and in protein-protein interactions. During post-translational prenylation, 10-carbon GGpp or 15-carbon Fpp is covalently added at the

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ABBREVIATIONS: MRSA, methicillin-resistant *S. aureus*; GGpp, geranylgeranyl pyrophosphate; Fpp, farnesyl pyrophosphate; PI3K, phosphoinositide 3-kinase; GAP, GTPase-activating protein; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; HUVEC, human umbilical vein endothelial cells; FBS, fetal bovine serum; HEK, human embryonic kidney; ANOVA, analysis of variance; GGOH, dephosphorylated form of GGpp; PAGE, polyacrylamide gel electrophoresis; GGTI-2147, 4-{[*N*-(imidazol-4-yl)methyleneamino]-2-(1-naphthyl)benzoyl}leucine methyl ester; FTI-277, methyl {*N*-[2-phenyl-4-*N*[2(*R*)-amino-3-mercaptopropylamino]benzoyl]}-methionate.

cysteine residue of the conserved carboxyl-terminal motif CaaX, where "C" designates the prenylated cysteine residue, "a" designates aliphatic residues, and "X" determines whether the target is recognized by geranylgeranyl or farnesyl transferases (Zhang and Casey, 1996). With statin treatment, the pool of GGpp and Fpp is reduced, diminishing prenylation, sequestering CaaX-containing proteins within the cytosol, and thereby impairing functions that require membrane localization.

At the cell membrane, the interaction between the CaaX-containing proteins Rac and CDC42 and the phosphoinositide 3-kinase (PI3K) isoform p85 (Zheng et al., 1994; Bokoch et al., 1996) may facilitate actin dynamics and endocytic trafficking. p85 is a regulatory subunit within the PI3K family of proteins (Vanhaesebroeck and Waterfield, 1999). The regulatory subunits, which include p85α and -β, p55α and -γ, and p50α, possess domains for interaction with membrane-bound proteins and for heterodimerization with catalytic subunits p110α, -β, and -δ. Regulatory subunits provide a linkage between membrane-bound proteins and the catalytic subunits. These catalytic subunits phosphorylate membrane-bound phosphoinositides, including phosphoinositide 4,5-bisphosphate, forming phosphoinositide 3,4,5-trisphosphate. By coupling through the Rho-GTPase-activating protein (GAP) domain of p85, a domain missing in the truncated p55 and p50 isoforms, prenylated small GTPases would be well positioned to bring the catalytic p110 domains in proximity with membrane-bound phosphoinositides. PI3K may function in this manner as an intermediary to regulate actin dynamics and initiate endocytosis (Johnson, 1999) as membrane-bound phosphoinositide 3,4,5-trisphosphate binds to α-actinin, dislodging this bundling protein from actin filaments and disassembling actin stress fibers (Fraley et al., 2005). Thus, prenylation of a subset of CaaX-containing proteins coupled to p85 possibly localizes PI3K at the cell membrane to facilitate the reordering of actin stress fibers.

Actin dynamics can drive both clathrin- and nonclathrin-mediated endocytosis, cellular processes that are exploited by pathogenic bacteria for host invasion (Sinha and Herrmann, 2005; Nitsche-Schmitz et al., 2007). To initiate invasion, surface adhesins of pathogenic *S. aureus* bind to extracellular matrix proteins. As matrix-bound *S. aureus* engages host receptors, the pathogen enters the cell during endocytosis of matrix/receptor complexes. Although *S. aureus* has been primarily considered as an extracellular pathogen, it is increasingly clear that invasiveness contributes to pathogenesis (Lowy, 1998; Alexander and Hudson, 2001; Foster, 2005; Que et al., 2005; Sinha and Herrmann, 2005; Hauck and Ohlsen, 2006; Proctor et al., 2006), and blocking receptor engagement is an emerging immunotherapeutic target (Rivas et al., 2004).

We explored whether statins inhibit endocytic invasion by *S. aureus.* Our hypothesis is that by diminishing protein prenylation, simvastatin sequesters CaaX-containing proteins coupled to PI3K within the cytosol, inhibiting actin dynamics required for *S. aureus* endocytic invasion. We have examined whether pretreatment of host cells with simvastatin at therapeutic concentrations prevents *S. aureus* invasion and the mechanism of this inhibition.

Materials and Methods

Chemicals. The following were used at the concentrations and durations indicated within each figure or method described below: simvastatin, geranylgeranyl transferase inhibitor GGTI-2147, and farnesyl transferase inhibitor FTI-277 (Calbiochem, San Diego, CA); dimethyl sulfoxide (DMSO) and NaCl (Thermo Fisher Scientific, Waltham, MA); tryptic soy agar, saponin, cholesterol, mevalonate, geranylgeraniol, farnesyl pyrophosphate, lysostaphin, gentamicin, PIPES, EGTA, KCl, Triton X-100, Tween 20, bovine serum albumin, and LiCl (Sigma-Aldrich, St. Louis, MO); LY294002 (Cell Signaling Technology Inc., Danvers, MA); Mini-Tab (Roche, Indianapolis, IN); $MgCl_2$ (VWR, West Chester, PA), phosphate-buffered saline (PBS), and Tris-HCl (Invitrogen, Carlsbad, CA); and secramine A (Xu et al., 2006).

Endothelial Cell Culture. Human umbilical vein endothelial cells (HUVEC; Cascade Biologics, Portland, OR) were grown in M200 medium supplemented with low serum growth supplement (Cascade Biologics; 5% CO_2, 37°C, 75-cm^2-vented cap flasks) before plating. For invasion assays and flow cytometry, 10^5 cells were plated on 35-mm tissue culture dishes (Thermo Fisher Scientific) coated with Attachment Factor (a gelatin that contains no extracellular matrix proteins; Cascade Biologics). For confocal imaging, cells were plated on 35-mm glass-bottom dishes (MatTek, Ashland, MA) and coated, as described above. Simvastatin treatments were initiated on day 3 of plating. LY294002 and secramine A treatments were performed on day 4.

Invasion Assay. *S. aureus* (29213; American Type Culture Collection, Manassas, VA) were subcultured daily in tryptic soy broth (200 rpm, 37°C). Bacteria were harvested by centrifugation (10,000 rpm, 3 min, 37°C), washed once, and resuspended at 3×10^8 cells/ml in saline. HUVEC were washed once with 1× PBS and incubated with *S. aureus* (1.2×10^8) in 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA)/PBS (1 h, 5% CO_2, 37°C). Invasion was terminated by incubation with lysostaphin (20 μg/ml) and gentamicin (50 μg/ml) in 10% FBS/PBS (45 min, 5% CO_2, 37°C). Intracellular bacteria were released into the medium using 1% saponin/PBS (20 min, 5% CO_2, 37°C). Serial dilutions of the medium were plated on tryptic soy agar, and colony counts were performed (16 h, 37°C). All invasion assays were performed on day 4 of plating. To assess whether compounds were bactericidal, *S. aureus* (1.2×10^8 cfu) were incubated with compounds at the concentrations indicated in 10% FBS (1 h), and serial dilutions were plated on tryptic soy agar.

Generation of CDC42 C507V/V5. Site-directed mutagenesis (QuikChange; Agilent Technologies, Santa Clara, CA) of human CDC42 cDNA in pUSE (Millipore, Billerica, MA) was performed to substitute the cysteine required for prenylation at position 507 with a valine, remove the stop codon, and fuse to the V5 tag (Invitrogen). The following primer sequence was used: ccg aag aag agc cgc agg gta gtg ctg cta cta ggt aag cct atc cct aac cct ctc ctc ggt cta gat tct acg tga ctc gag tct aga ggg ccc g. Human embryonic kidney (HEK) 293A cells (Invitrogen) were transfected with CDC42 C507V/V5/pUSE using Lipofectamine (Invitrogen), and a stable cell line was created from a geneticin-resistant clone. Invasion assays were performed as described above, with the exception that after the gentamicin/lysostaphin treatment, cells were suspended using trypsin and pelleted before the saponin addition.

Cytotoxicity Assay. HUVEC or HEK 293A were pretreated with compounds at the concentrations indicated in the figure legends, and cytotoxicity was assessed using flow cytometry. Propidium iodide uptake was measured in 1×10^4 cells/treatment.

Immunofluorescence. After infection, HUVEC were washed with 1× PBS, fixed (4% paraformaldehyde/PBS, 30 min; Electron Microscopy Sciences, Hatfield, PA), permeabilized, blocked (0.1% Triton X-100, 1% bovine serum albumin, 30 min), and incubated with Alexa Fluor 488 phalloidin for actin (1:40; Invitrogen). Confocal images were acquired using an inverted Zeiss Axiovert200 microscope equipped with a plan-apochromat 40×, 1.2 NA water immer-

sion lens with correction collar and LSM 5 pascal scan head. Alexa 488 was excited by the 488-nm argon laser line and detected by using a 505- to 530-nm bandpass filter. Z-sectioning and frame size were set to Nyquist sampling. Maximal pixel projections from the Z-stacks were generated and analyzed for actin morphology. Differential interference contrast images were acquired simultaneously with fluorescence images.

Adenoviral Constructs. Wild-type human PI3Kp110α in pDONR201 was kindly provided by Dr. Jim Thomas (Eli Lilly and Co.). By site-directed mutagenesis (QuikChange), the catalytic domain was truncated at amino acid 915 to remove the region that putatively interacts with the phosphoinositide polar head group for lipid kinase activity (Yart et al., 2002). A point mutation was generated to substitute lysine with arginine at amino acid 805, the site required for protein kinase activity (Bondeva et al., 1998). Mutated p110α was cloned into pAD/CMV/V5 through LR recombination (Gateway System; Invitrogen). Kinase-dead p110α/pAD/CMV/V5 and LacZ/pAD/CMV/V5 (Invitrogen) were transfected into HEK 293A using Lipofectamine, viral particles were harvested and purified (BD Biosciences, Franklin Lakes, NJ), and titer was determined. Optimal multiplicity of infection and duration of infection were determined for both constructs by infecting HEK 293A and assessing expression by Western blot analysis. Invasion assays were performed as described above for assessing CDC42 C507V/V5.

Cell Fractionation. HEK 293A cells were plated in Dulbecco's modified Eagle's medium/10% FBS at a density of 7.5×10^5 cells on 100-mm tissue culture dishes (Thermo Fisher Scientific) coated with Attachment Factor. At day 3, cells were pretreated with DMSO or simvastatin. On day 4, cells were washed once with ice-cold 1× PBS, incubated on ice in buffer (15 min; 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1.25 mM EGTA, and 10 mM PIPES, pH 7.3; 1× Mini-Tab), and harvested using cell scrapers. After sonication (10 s, 40TL probe; Ultrasonic Power Corporation, Freeport, Illinois), lysates were pelleted (500*g*, 5 min, 4°C), and supernatants were centrifuged (1 h, 110,000*g*, 4°C, Beckman SW41). The supernatant was designated as the cytosolic fraction. Total protein concentration was determined using the Bio-Rad Protein Assay (Hercules, CA).

Western Blot Analysis. Cytosolic and membrane fractions were subjected to electrophoresis using NU-PAGE (1× MES buffer) and transferred to polyvinylidene difluoride (Invitrogen). Membranes were blocked using Odyssey Blocking Buffer (LI-COR, Lincoln, NE; room temperature, 1 h) and then incubated in blocking buffer/0.1% Tween 20 (4°C, 16 h) with anti-CDC42 (no. 610928; BD Biosciences), with anti-Rac (no. 05-389; Millipore), or anti-RhoB (sc-180; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and subsequently with anti-flotillin-1 (no. 610820; BD Biosciences) and anti-actin (Millipore Bioscience Research Reagents, Temecula, CA). Membranes were washed in 1× PBS/0.1% Tween 20 and incubated with the following: anti-mouse IRDye 800CW (Rockland Immunochemicals, Gilbertsville, PA) for CDC42, Rac, and actin; anti-rabbit IRDye 800CW (Rockland Immunochemicals) for RhoB; and anti-mouse Alexa Fluor 680 for flotillin and/or actin (1:40,000, room temperature, 1 h, in blocking buffer/0.1% Tween 20). Membranes were washed in 1× PBS/0.1% Tween 20, and fluorescent signals (680 and 800) were detected using the Odyssey Infrared Imaging System (LI-COR). Integrated intensity values were acquired using Odyssey software (LI-COR).

Immunoprecipitation. To assess coupling between PI3K p85 and CDC42, Rac, or RhoB, cytosolic fractions (500–1000 μg) were incubated overnight with anti-CDC42, anti-Rac, or anti-RhoB, and immunocomplexes were recovered using Protein A Agarose (Invitrogen). After extensive washing with ice-cold 1× PBS and subsequently with ice-cold LiCl buffer (500 mM LiCl/100 mM Tris-HCl, pH 7.5), immunocomplexes were retrieved by boiling in LDS-Sample buffer containing reducing agent (Invitrogen). Immunocomplexes were assessed by Western blot analysis as described above, probed with anti-PI3K p85 (no. 06-497; Millipore; 16 h, 4°C) followed by anti-rabbit IRDye 800CW (1:40,000, room temperature, 1 h), and

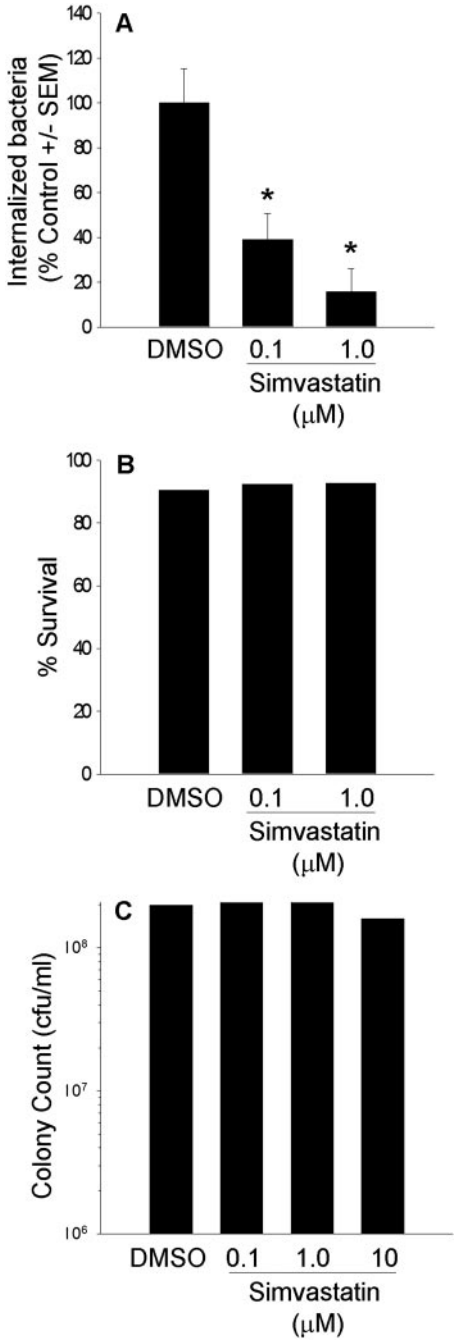

Fig. 1. Simvastatin inhibits *S. aureus* host cell invasion. A, HUVEC were pretreated with simvastatin (0.1 or 1.0 μM, 20 h) or vehicle control (DMSO, 20 h) followed by *S. aureus* infection (1 h). Extracellular bacteria were removed by the bactericides gentamicin and lysostaphin. Intracellular bacteria were released from HUVEC into the medium by the detergent saponin, and serial dilutions of the medium were plated on tryptic soy agar for colony counts (16 h). Data are percentage control ± S.E.M. and are representative of three separate experiments (*, $p \leq 0.05$ compared with control, $n = 3$/treatment by one-way ANOVA and Student's Newman-Keuls post hoc analysis). B, simvastatin treatment is noncytotoxic. HUVEC were pretreated with simvastatin (0.1 or 1.0 μM) or DMSO (20 h), and cytotoxicity was assessed using flow cytometry to detect the uptake of propidium iodide. A total of 1×10^4 cells was counted per treatment. C, simvastatin treatment is nonbactericidal. *S. aureus* (1.2×10^8 colony-forming units) were treated with simvastatin (0.1, 1.0, or 10 μM) or DMSO (1 h), and serial dilutions were plated on tryptic soy agar. Data are colony counts ± S.E.M.

detected using the Odyssey Infrared Imaging System. Integrated intensity values were acquired for p85 bands and background using Odyssey software.

Statistical Analyses. Normally distributed data from invasion assays were analyzed using one-way analysis of variance (ANOVA) followed by Student's Newman-Keuls post hoc analysis (SigmaStat; Systat Software, Inc., Point Richmond, CA). Actin stress fibers were evaluated in 200 cells/treatment from randomly selected fields using an inverted fluorescent microscope, and data were assessed using a χ^2 test of association (SPSS, Inc., Chicago, IL). Differences between groups were considered statistically significant at $p \leq 0.05$.

Results

Simvastatin Prevents *S. aureus* Host Cell Invasion through Inhibition of Isoprenoid Production. Nanomolar amounts of simvastatin inhibited *S. aureus* invasion (Fig. 1). The doses used were neither cytotoxic to HUVEC nor bactericidal (Fig. 1, B and C). We examined whether the inhibition by simvastatin was caused by diminished availability of intermediates within the cholesterol biosynthesis pathway or by diminished levels of cholesterol. Replenishing mevalonate, the product of HMG-CoA reduction, restored *S. aureus* invasiveness (Fig. 2A). In contrast, replenishing cholesterol failed to restore *S. aureus* invasion (Fig. 2B). Host cell invasion was restored with replenishment of the dephosphorylated form of GGpp, GGOH, and partially restored with Fpp (Fig. 2, C and D). Inhibition of geranylgeranylation and farnesylation decreased invasiveness (Fig. 2, E and F).

Inhibiting the Membrane Insertion of CDC42 Decreases Host Cell Invasion. CDC42 is a CaaX-containing protein known to be activated early during *S. aureus* invasion (Arbibe et al., 2000) and to mediate actin dynamics (Johnson, 1999). To determine whether membrane localization by prenylated CDC42 is required for *S. aureus* invasion,

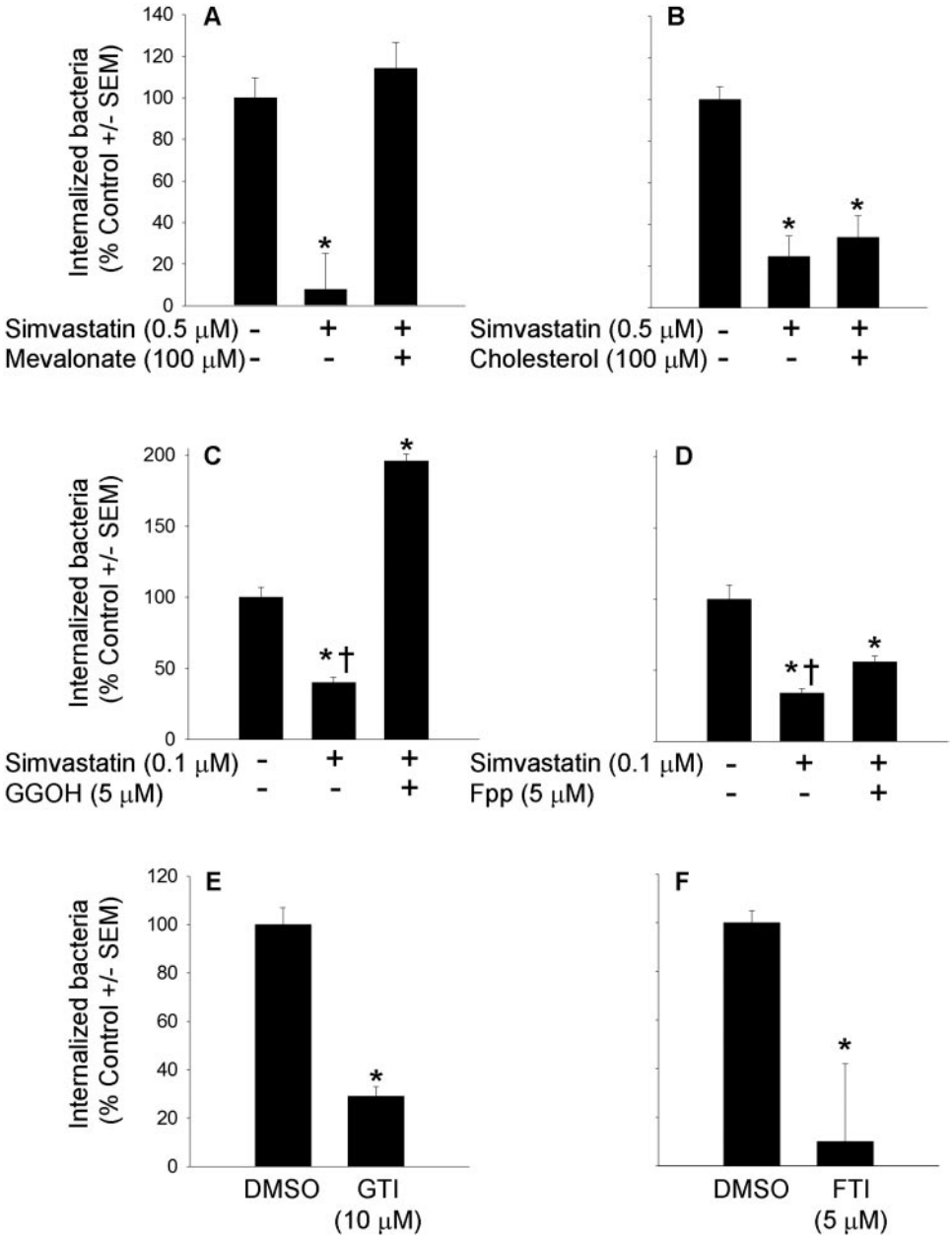

Fig. 2. Decreased isoprenoid production inhibits *S. aureus* host cell invasion. A, mevalonate reverses simvastatin inhibition of *S. aureus* host cell invasion. HUVEC were pretreated (20 h) with the vehicle control DMSO, simvastatin (0.5 μM), or simvastatin supplemented with mevalonate (100 μM), the product of HMG-CoA reduction. Cells were incubated with *S. aureus* for 1 h. After infection, extracellular bacteria were removed by the bactericides gentamicin and lysostaphin. Intracellular bacteria were released from HUVEC into the medium by the detergent saponin, and serial dilutions of the medium were plated on tryptic soy agar for colony counts. B, cholesterol fails to reverse simvastatin inhibition of *S. aureus* invasion. HUVEC were pretreated (20 h) with DMSO, simvastatin (0.5 μM) alone or in the presence of cholesterol (100 μM). Cells were infected with *S. aureus* (1 h), intracellular bacteria were recovered, and colony counts were performed as described above. C, geranylgeraniol (GGOH) restores host cell invasion. HUVEC were pretreated with simvastatin alone (0.1 μM, 20 h) or in the presence of GGOH, an isoprenoid intermediate (5 μM, last 6 h). Cells were infected (1 h), and invasiveness was assessed as described above. D, Fpp partially restores invasiveness. HUVEC were pretreated with simvastatin alone (0.1 μM, 20 h) or in the presence of the isoprenoid intermediate Fpp (5 μM, last 6 h). Cells were infected (1 h), and invasiveness was assessed as described above. E and F, inhibition of geranylgeranyl transferase or farnesyl transferase decreases *S. aureus* host cell invasion. HUVEC were pretreated (6 h) with geranylgeranyl transferase inhibitors (GGTI; 10 μM) or farnesyl transferase inhibitors (FTI; 5 μM) or DMSO and infected (1 h), and colony counts were performed as described above. Data are representative of replicate experiments and are presented as percentage control. One-way ANOVA and Student's Newman-Keuls post hoc analysis were performed, n = 3–5/treatment (*, different from DMSO control; †, less than GGOH/Fpp in the presence of simvastatin; $p \leq 0.05$).

HEK 293A cells were pretreated with secramine A (1 h), an antagonist of prenylated CDC42 membrane association (Xu et al., 2006). Secramine A inhibited *S. aureus* invasion (Fig. 3A) at concentrations that were neither cytotoxic (Fig. 3B) nor bactericidal (data not shown). To directly assess the role of prenylated CDC42 in *S. aureus* invasion, a mutated construct was generated in which the cysteine residue required for prenylation was substituted with a valine and expressed as a stable clone. Expression of CDC42 C507V/V5 inhibited *S. aureus* invasion (Fig. 3C).

Pretreatment with Simvastatin (20 h) Inhibited Actin Stress Fiber Disassembly during *S. aureus* Invasion. At day 4 of plating, HUVEC displayed extensive stress fiber formations (Fig. 4A). During invasion, stress fibers disassembled (Fig. 4A), and disassembly persisted throughout 2 h of invasion (Fig. 4B). In contrast, stress fibers remained intact in control cells over time (Fig. 4B). Stress fibers depolymerized in the presence of heat-killed *S. aureus* (data not shown). Simvastatin reduced actin stress fiber disassembly (Fig. 4C).

Inhibition of PI3K Decreases Actin Stress Fiber Disassembly and *S. aureus* Invasiveness. The inhibition of PI3K lipid-kinase activity using LY294002 restricted the disassembly of actin stress fibers during invasion (Fig. 5A). In a dose-dependent manner, LY294002 inhibited *S. aureus* host cell invasion (Fig. 5B). Adenoviral expression of a kinase-dead form of the PI3K isoform p110α inhibited *S. aureus* invasion by 70%, more than the 45% decrease in response to adenoviral expression of the control LacZ (Fig. 5C).

Simvastatin Increases the Accumulation of Small GTPases Coupled to PI3K within the Cytosol. Cell fractionation was performed to assess the localization of CDC42. In the absence of simvastatin, cytosolic CDC42 is minimal (Fig. 6A). In response to simvastatin pretreatment, CDC42 accumulated in the cytosol (Fig. 6A). Constitutively, Rac is present at the membrane and within the cytosol. In response to simvastatin, Rac localized primarily within the cytosol (Fig. 6B). RhoB is primarily membrane-associated in the absence of simvastatin, accumulating in the cytosol following pretreatment (Fig. 6B). The membrane marker, flotillin, was detected only in the membrane samples (Fig. 6B). Actin was detected in both cytosolic and membrane fractions, diminishing in the membrane fraction and accumulating in the cytosol in response to simvastatin (Fig. 6, A and B). Because CDC42 activation is immediate and transient in response to *S. aureus* (Arbibe et al., 2000), membrane and cytosolic fractions from cells pretreated with simvastatin or DMSO were assessed upon initial infection (2 min). In the presence of simvastatin, CDC42, Rac, and RhoB remained sequestered in the cytosol with infection (Fig. 6B).

To evaluate whether the cytosolic accumulation of these small GTPases led to sequestration of PI3K p85 within the cytosol, each small GTPase was immunoprecipitated from the cytosolic fraction, immunocomplexes were subjected to polyacrylamide gel electrophoresis (PAGE), and blots were probed for associated p85. In response to simvastatin, p85 coupled to CDC42, Rac, and RhoB was more abundant within the cytosolic fraction (Fig. 6C).

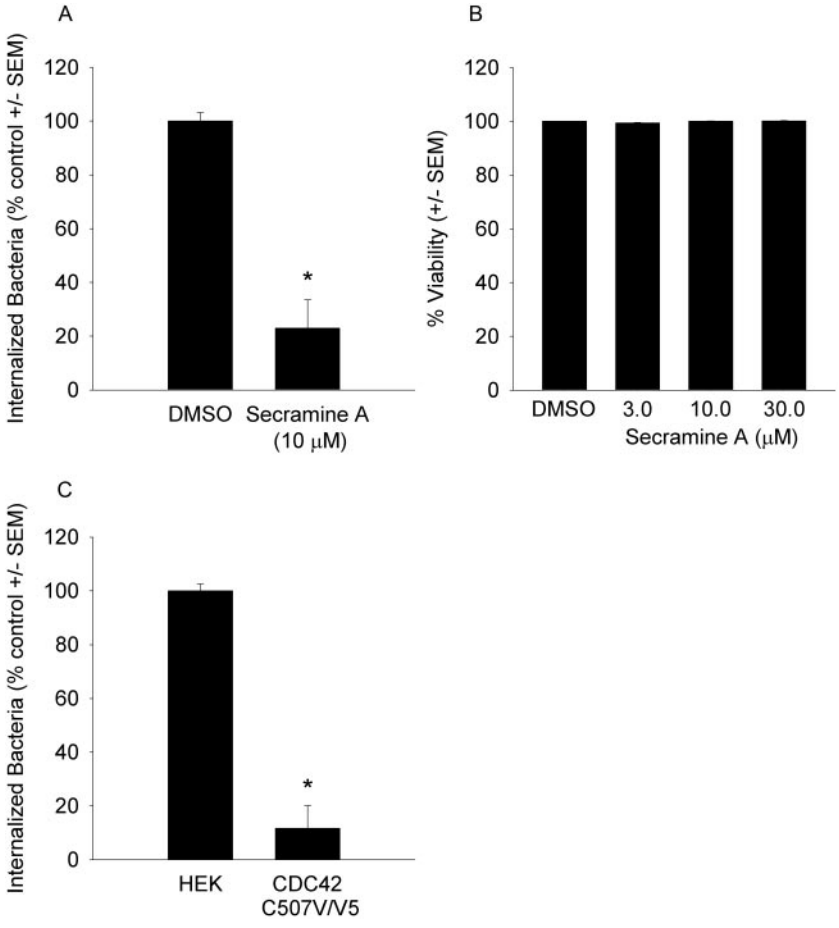

Fig. 3. Antagonism of prenylated CDC42 membrane association inhibits host cell invasion. A, HEK 293A were pretreated with the vehicle control dimethyl sulfoxide (1% DMSO) or secramine A (10 μM, 1 h), a compound that inhibits membrane association by prenylated CDC42 (Xu et al., 2006). Cells were incubated with *S. aureus* for 1 h, extracellular bacteria were removed by the bactericides gentamicin and lysostaphin, and intracellular bacteria were released from the host cells into the medium by the detergent saponin. Serial dilutions of the medium were plated on tryptic soy agar for colony counts. B, Inhibitory concentrations of secramine A are noncytotoxic. HEK 293A were pretreated with 1% DMSO or secramine A (3, 10, or 30 μM,1 h), and cytotoxicity was assessed by propidium iodide uptake in 1×10^4 cells per treatment. C, expression of CDC42 C507V/V5, a mutated construct lacking the prenylation site, inhibits invasion. Stably transfected HEK 293A-expressing CDC42 C507V/V5 were incubated with *S. aureus* (1 h), and invasion was assessed as described in A. Data are representative of replicate experiments. One-way ANOVA followed by Student's Newman-Keuls post hoc analysis were performed, n = 3–5/condition (*, less than control, $p \leq 0.05$).

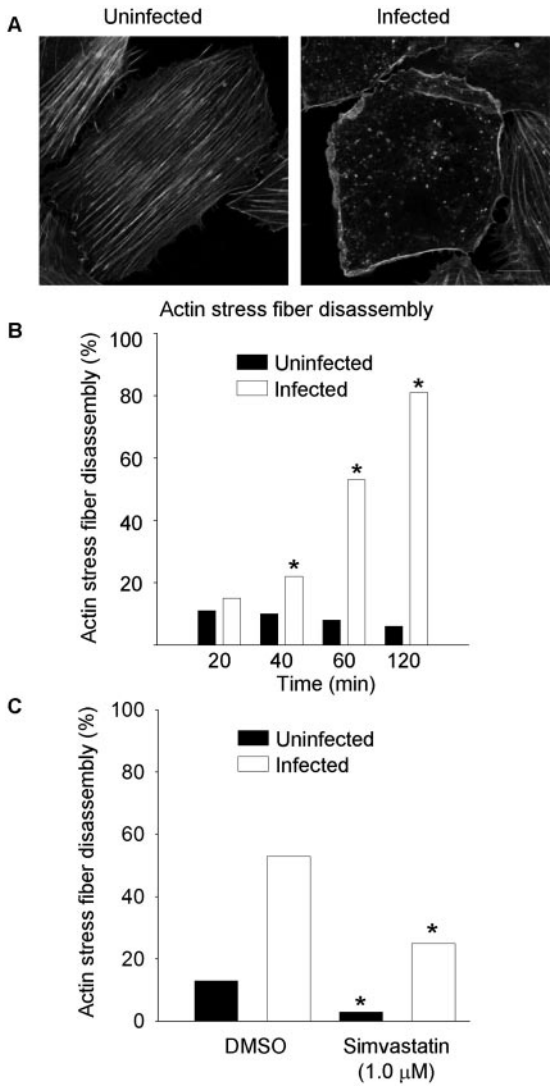

Fig. 4. Simvastatin limits disruption of actin stress fibers during *S. aureus* host cell invasion. A, actin stress fibers disassemble during infection. HUVEC were infected with *S. aureus* (1 h). After invasion, extracellular bacteria were removed using the bactericides lysostaphin and gentamicin. HUVEC were fixed, permeabilized, and blocked. Actin was detected using Alexa Fluor 488 phalloidin. The scale bar is 10 μM. B, stress fiber disassembly continues throughout infection. HUVEC were infected with *S. aureus* for times indicated. Actin stress fibers were evaluated in 200 cells in uninfected or infected samples from randomly selected fields. C, simvastatin attenuates actin stress fiber disassembly. HUVEC were pretreated (20 h) with simvastatin (1.0 μM) or vehicle control (DMSO) and infected with *S. aureus* (1 h), and actin stress fibers were evaluated as described in B. Data are presented as the percentage of cells displaying actin stress fiber disassembly (*, different than control; $p \leq 0.05$ by χ^2 test of association).

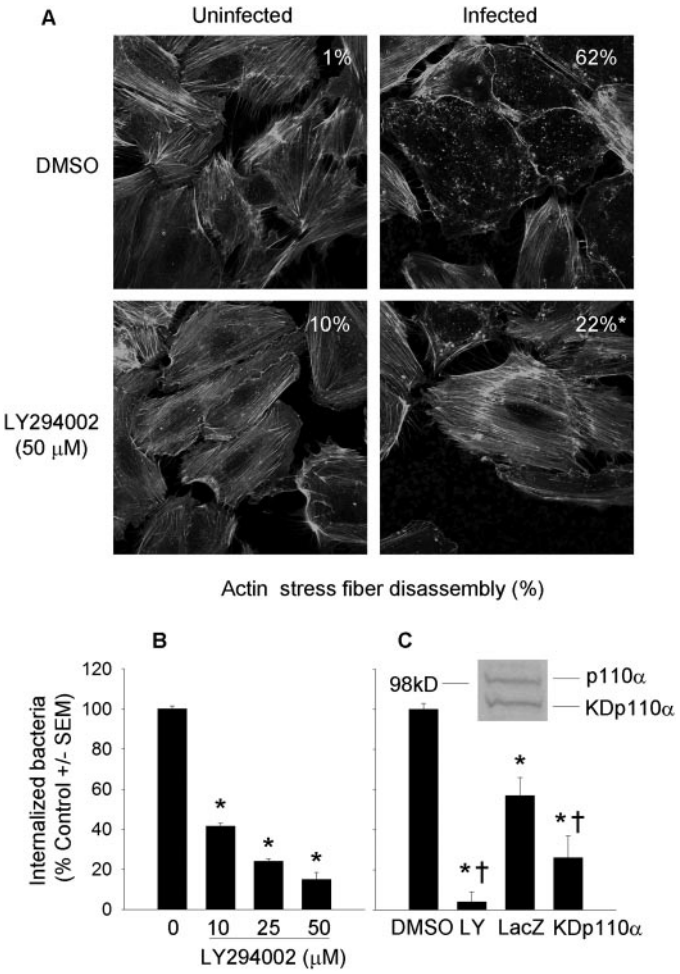

Fig. 5. Inhibition of PI3K attenuates actin stress fiber disassembly and *S. aureus* host cell invasion. A, HUVEC were pretreated (1 h) with vehicle control (DMSO) or with LY294002 (50 μM) and infected with *S. aureus* (1 h), and extracellular bacteria were removed using gentamicin and lysostaphin. HUVEC were fixed and permeabilized, and actin was detected using phalloidin 488. Stress fibers were evaluated in 200 cells from randomly selected fields. Data are presented as the percentage of cells displaying actin stress fiber disassembly (*, less than infected control; $p \leq 0.05$ by χ^2 test of association). B, LY294002 decreases host cell invasion in a dose-dependent manner. HUVEC were pretreated with DMSO or LY294002 (10, 25, or 50 μM, 1 h), and infected with *S. aureus* (1 h). After infection, extracellular bacteria were removed by the bactericides gentamicin and lysostaphin. Intracellular bacteria were released from HUVEC into the medium by the detergent saponin, serial dilutions of the medium were plated on tryptic soy agar, and colonies were counted. C, adenoviral expression of kinase-dead PI3Kp110α inhibits invasion. HEK 293A were pretreated with DMSO or LY294002 (50 μM, 1 h), or exposed to LacZ or kinase-dead p110α (KDp110α) adenovirus (24 h), infected with *S. aureus* (1 h), and invasion was assessed as described in B. Inset is an immunoblot of lysate from HEK 293A after exposure to KDp110α adenovirus (24 h), probed with anti-p110α, showing endogenous p110α compared with truncated mutant expression. Data are representative of replicate experiments, $n = 3$/treatment (*, less than infected DMSO control; †, less than LacZ control; $p \leq 0.05$ by one-way ANOVA with Student's Newman-Keuls post hoc analysis).

Discussion

In this study, we have addressed the question of whether the pharmacology of statins extends to the inhibition of host cell invasion by *S. aureus*. Our findings indicate that at nanomolar concentrations within the therapeutic range, simvastatin inhibits invasion by a mechanism that is neither cytotoxic nor bactericidal (Fig. 1). Inhibition is dependent upon mevalonate, an early product within the cholesterol biosynthesis pathway, and is due in part to depletion of the isoprenoid intermediates GGpp and Fpp, because replenishment of each restored invasion and inhibition of geranylgeranyl or farnesyl transferase decreased invasiveness (Fig. 2). Secramine A, which blocks membrane association by prenylated CDC42, inhibited invasion, as did expression of CDC42 C507V/V5, a mutated construct deficient in the site for prenylation (Fig. 3). Taken together, these findings support the concept that inhibition of the prenylation-dependent localization of this CaaX-containing protein is central to inhibition of invasion. The mechanism of inhibition by simvastatin in-

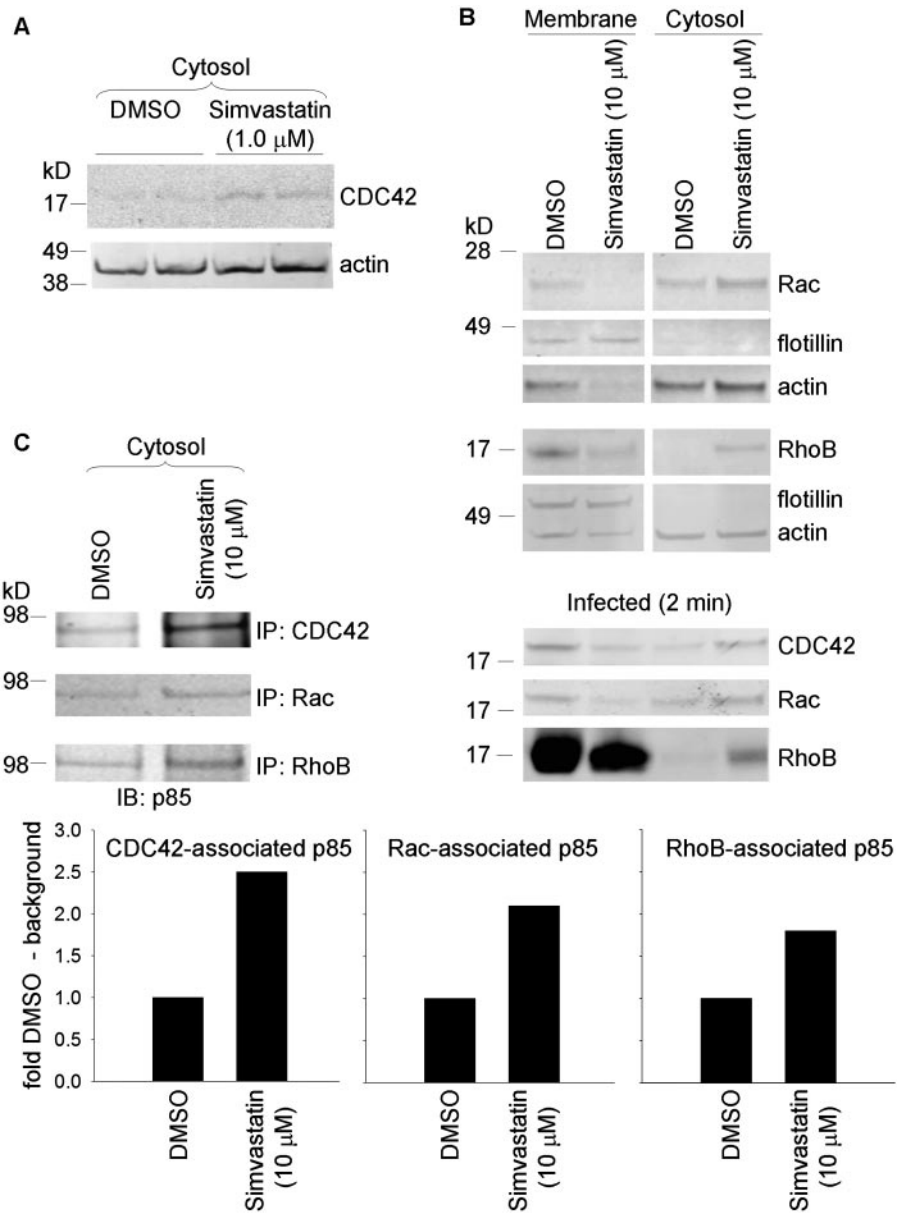

Fig. 6. Simvastatin leads to the accumulation of CDC42, Rac, and RhoB, coupled to PI3K p85 within the cytosol. A, HEK 293A were pretreated (20 h) with the vehicle control DMSO or with simvastatin (1.0 μM), cytosolic fractions were isolated, and Western blot analysis was performed loading 15 μg of total protein per sample. The immunoblot was probed with anti-CDC42 and anti-mouse IRDye 800CW followed by anti-actin and anti-mouse Alexa Fluor 680. B, membrane and cytosolic fractions from cells pretreated with simvastatin (10 μM) or DMSO in the absence or presence of infection (2 min) were subjected to SDS-PAGE and probed with antibodies to Rac, RhoB, actin, and the membrane marker flotillin, followed by fluorophore-conjugated secondary antibodies. C, cytosolic lysates were immunoprecipitated (IP) using anti-CDC42, anti-Rac, or anti-RhoB, and immunocomplexes were subjected to SDS-PAGE. Immunoblots (IB) were probed with anti-PI3K p85 followed by Alexa Fluor 680 goat anti-rabbit. Fluorescence was detected using the Odyssey Infrared Imaging System. Integrated intensities and background correction were performed using Odyssey software. Data are presented as -fold control and are representative of replicate experiments.

cluded the attenuation of actin stress fiber disassembly required for endocytosis of the bacterium (Fig. 4). Because PI3K mediates stress fiber disassembly and invasion (Fig. 5), we examined whether simvastatin was affecting PI3K cellular localization. PI3K lacks prenylation domains but can rely on coupling to prenylated proteins, such as a subset of small GTPases, for access to membrane-bound phosphoinositide. Simvastatin led to the cytosolic accumulation of the small GTPases CDC42, Rac, and RhoB, coupled to the PI3K isoform p85 (Fig. 6). From these findings, we propose a mechanism whereby simvastatin restricts p85 localization, inhibiting actin dynamics required for caveolar endocytosis. This may provide the basis for protection at the level of the host in invasive infections such as endocarditis and osteomyelitis as well as in the treatment of bacterial sepsis.

Complex actin dynamics mediate the endocytosis of *S. aureus* (Alexander and Hudson, 2001; Schröder et al., 2006), possibly generating the pulling forces necessary for inward movement and vesicular trafficking (Agerer et al., 2005). Inhibition of actin stress fiber depolymerization by LY294002 (Fig. 5A) indicated that these actin dynamics are in part regulated via a PI3K p110 catalytic domain, because the inhibitory action of this compound is within the ATP-binding site of the p110 domain (Walker et al., 2000). Sequestering the regulatory subunit p85 within the cytosol could impair p110 catalytic activity by restricting access to membrane-bound phosphoinositide. p85 could also affect actin dynamics independently of the p110 subunits because p85 is functional as an unbound monomer (Brachmann et al., 2005). Our data indicate a continuous loss of fibers during the invasion by *S. aureus* (Fig. 4B), which corresponds temporally with the recent report that bridging between *S. aureus* and the integrin receptor α5β1 results in sustained actin dynamics (Schröder et al., 2006). Bacteria were found to spend, on average, 45 min on the endothelial cell surface with stimulation of actin waves, formation of actin cups, and generation of comet tails before endocytosis of the bacterium. The current findings indicate that depletion of isoprenoid intermediates by simva-

statin and inhibition of PI3K catalytic activity attenuates the cortical actin dynamics required for invasion by the bacterium.

An early event in *S. aureus* invasion is the activation of CDC42 (Arbibe et al., 2000), a CaaX-containing small GTPase known to facilitate actin dynamics (Johnson, 1999). CDC42 stimulates actin dynamics through a number of effectors. One potential effector is PI3K. CDC42 couples to PI3K through the Rho-GAP domain of PI3Kp85, and it has been postulated that the interaction determines appropriate cellular localization for the lipid kinase (Zheng et al., 1994). Therefore, we examined the possibility that simvastatin inhibits *S. aureus* invasion by impeding the access of PI3K to membrane-bound phosphoinositide. Nanomolar concentrations of simvastatin were sufficient to significantly inhibit invasion, suggesting that the depletion of isoprenoid intermediates impairs the translocation of multiple prenylated proteins, possibly coupled to distinct PI3K isoforms. In addition to CDC42, Rac had previously been found to couple with PI3K within the Rho-GAP domain of p85 (Bokoch et al., 1996), and, more recently, both Rac and RhoB were found to localize within the cytosol in response to simvastatin (Stamatakis et al., 2002; Cordle et al., 2005). Interaction between p85 and Rac or CDC42 requires GTP loading of these small GTPases. It is interesting to note that Cordle et al. (2005) found that simvastatin stimulates GTP loading of Rac, whereas the small GTPase remained functionally inactivated and translocation to the cell membrane was inhibited. Likewise, *S. aureus* stimulates an immediate and transient GTP loading of CDC42 (Arbibe et al., 2000). Therefore, in response to either simvastatin or *S. aureus*, the requirement for GTP loading of small GTPases for interaction with p85 seems to be met, and our findings indicate that this association sequesters p85 within the cytosol in response to simvastatin.

In addition to the small GTPases examined in the current study, additional candidates that may be mediating inhibition include the γ-subunit of heterotrimeric G proteins that couple to the PI3K isoforms p110γ and -β and which require prenylation for membrane insertion (Zhang and Casey, 1996) and small GTPases capable of coupling with p85 through the Rho-GAP domain that have yet to be identified. Our findings also reveal a role for the p110 catalytic domain because treatment with LY294002 or adenoviral expression of a kinase-dead form of p110α decreased invasion more than DMSO or LacZ controls, respectively (Fig. 5B). Adenoviral expression of LacZ diminished infection relative to DMSO control by 45% (Fig. 5B), supporting the concept that the endocytic pathway used for adenoviral uptake is shared by *S. aureus* for invasion (Sinha and Herrmann, 2005). Previous work has clearly demonstrated that the acute effect of simvastatin on the PI3K signaling pathway is the activation of the downstream mediator Akt (Kureishi et al., 2000). Our study reveals that the pharmacology of long-term (20 h) statin treatment includes sequestration of PI3K through coupling with CaaX-containing proteins.

Although a number of clinical studies indicate an improved prognosis for individuals on a statin regimen, the safety and efficacy of statin therapy during sepsis has been questioned (Mahboobi et al., 2006; Vincent and Miller, 2006; Bromilow and Schuster Bruce, 2007). Concerns include the altered pharmacokinetics of statins in critically ill patients that result in high levels of circulating, nonmetabolized drug, predisposing this patient population to myopathy and rhabdomyolysis. Large, clinical studies will begin to address these concerns. In addition, developing an understanding of the underlying mechanism of statin pharmacology will aid in determining whether statins are an efficacious adjunctive therapy in infectious disease and may provide the rationale for the development of more targeted therapeutics.

Invasion by *S. aureus* facilitates persistent, relapsing infection (Clement et al., 2005; Proctor et al., 2006) and, in the case of endocarditis, is central to pathogenesis (Yeaman and Bayer, 2000; Que et al., 2005). Immunotherapeutics directed against bacterial adhesin molecules have shown clinical promise, and it is postulated that the protective effect is due in part to impaired endothelial invasion (Rivas et al., 2004). The current study raises the possibility that inhibition of invasion by simvastatin could improve extracellular antibiotic efficacy, reduce hematogenous spread, and impede intracellular pathogenicity including persistent infection. Previous work has shown that statin treatment during sepsis also improves cardiovascular function and modulates the immune response (Kwak et al., 2000; Merx et al., 2004; Jacobson et al., 2005). Taken together, these findings reveal that the pharmacology of simvastatin extends to the inhibition of host invasion and provide a basis for further studies into the usefulness and efficacy of simvastatin, as well as therapeutics targeting distinct PI3K isoforms, as adjunctive approaches in the treatment of invasive infection.

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