SopE Acts as an Rab5-specific Nucleotide Exchange Factor and Recruits Non-prenylated Rab5 on Salmonella-containing Phagosomes to Promote Fusion with Early Endosomes*

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Rab-GTPase regulates the fusion between two biological vesicles. It is well documented that, for their biological function, Rab proteins need to be prenylated for attachment to the vesicle membrane. In contrast, we showed in the present investigation that SopE, a type III secretory protein of Salmonella, translocates onto Salmonella-containing phagosomes (LSP) and mediates the recruitment of non-prenylated Rab5 (Rab5:ΔC4) on LSP in GTP form. Simultaneously, SopE present in infected cell cytosol acts as an Rab5-specific exchange factor and converts the inactive Rab-GDP to the GTP form. The non-prenylated Rab5 subsequently promoted efficient fusion of LSP with early endosomes. This is the first demonstration that a prenylation-deficient Rab protein retains biological activity and can promote vesicle fusion, if it is recruited on the membrane by some other method.

Small GTP binding proteins of the Rab family regulate transport of intravesicular material from one specific compartment to other compartments (1, 2). C-terminal prenyl lipid moieties on Rab proteins serve to anchor them specifically to the cytoplasmic surface of the intracellular compartment (3). Unlike membrane insertion via transmembrane domains, membrane association of the Rab proteins via prenylation can be transient, which allows efficient regulation of intracellular flow of the membrane compartment (4). Thus, prenylation of Rab proteins is believed to be indispensable for membrane attachment and subsequent biological function (5).

Similarly, maturation of phagosomes has also been found to be regulated by the fusion of endocytic vesicles, which is controlled by Rab-GTPases (6, 7). Among the different Rab proteins, Rab5 and Rab7 are associated with the endocytic pathway (8). Rab5 mediates homotypic fusion among early compartments (9), whereas Rab7 serves as a targeting signal for transport from the early to late lysosomal compartment (10, 11). Subsequently, the low pH of the lysosomal compartment mediates the killing of invading microorganisms by lysosomal hydrolases. Thus, live bacteria appear to regulate maturation of the phagosomes by modulating the recruitment of endocytic Rab5 on phagosomes (12–14). We have recently demonstrated that LSP specifically recruits Rab5 on the phagosomes and promotes fusion with early endosomes, thereby inhibiting transport to the lysosomes (15, 16). The recruitment of Rab5 on the LSP depends on the presence of viable bacteria in the phagosomes, suggesting that a signal from the bacteria is required for Rab5 recruitment on the phagosomes. However, the mechanism of recruitment of a particular Rab protein by the respective bacteria-containing phagosomes is not clearly understood. In the present investigation, we have shown that SopE, a type III secretory protein of Salmonella, acts as a nucleotide exchange factor for Rab5 and converts the Rab5 into the GTP form, which specifically binds with SopE transported onto the Salmonella-containing phagosomes. Our results have also demonstrated that LSP can recruit prenylation-defective Rab5 (Rab5:ΔC4) and promote efficient fusion of LSP with early endosomes, indicating that prenylation of Rab protein is not essential for the biological activity of Rab protein.

**EXPERIMENTAL PROCEDURES**

Reagents—Unless otherwise stated, all reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Tissue culture supplies were obtained from the Life Technologies, Inc. (Grand Island, NY). N-Hydroxysuccinimidobiotin (NHS-biotin), avidin-horseradish peroxidase (avidin-HRP), avidin, biocinonic acid (BCA) reagents were purchased from Pierce Biocemicals, Rockford, IL. Goat anti-rabbit IgG conjugated with 18-nm colloidal gold and goat anti-mouse IgG conjugated with 12-nm colloidal gold were purchased from Jackson ImmunoResearch Laboratory, West Grove, PA. ECL reagents were procured from Amersham Pharmacia Biotech (UK). Other reagents used were of analytical grade.

Antibodies and Recombinant Proteins—Affinity-purified rabbit polyclonal anti-Rab5 and anti-Rab7 antibodies were generously provided by Dr. J. Gruenberg (EMBL, Heidelberg, Germany) and Dr. A. Wandinger-Ness (Northwestern University, Evanston, IL), respectively. Recombinant GDI and different constructs of Rab5 were kindly provided by Dr. Philip Stahl (Washington University School of Medicine, St. Louis, MO). A GST-SopE (~78–240) (pSB 1188) construct, to prepare recombimant SopE fusion protein, was received as a kind gift from Dr. J. E. Galan (Yale University School of Medicine, New Haven, CT). Anti-Salmonella antibodies (anti-SopE, anti-SopB, and anti-SipC) were kindly provided by Dr. E. E. Galov from the Institute for Animal Health, Berkshire, UK. Mouse anti-actin antibody was purchased from Calbiochem (La Jolla, CA). All the second antibodies labeled with HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Bacterial Strains—The virulent wild type (WT) Salmonella typhi-

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¹ The abbreviations used are: LSP, live Salmonella-containing phagosomes; DSP, dead Salmonella-containing phagosomes; HRP, horseradish peroxidase; GDI, GDP dissociation inhibitor; GST, glutathione S-transferase; WT, wild type; LB, Luria broth; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; SNAP, soluble NSF accessory protein; GEF, guanine nucleotide exchange factor; NHS-biotin, N-hydroxysuccinimidobiotin.
Non-prenylated Rab5 Promotes Fusion between Endosomes and Phagosomes

*murium* (a clinical isolate from Lady Harding Medical College, New Delhi, India) was obtained from Dr. Vineeta Bal of the National Institute of Immunology, New Delhi, India. *Salmonella dublin* wild type strain (2229) and SopE knockout mutant *S. dublin* (SE1 CMR) were kindly provided by Dr. E. E. Galyov. Bacteria were grown overnight in LB medium containing 100 μg/ml of ampicillin and then resuspended in ice-cold PBS containing 0.1% sodium cacodylate buffer, pH 7.2, washed, and postfixed with 1% OsO₄ in the same buffer. The phagosomes were rinsed and dehydrated in ethanol and embedded in araldite (16). Thin sections were double-stained with uranyl acetate and lead citrate and examined in a transmission electron microscope (JEOl 1200 EX11).

Detection of Rab5 Binding Protein from Salmonella—To detect the Rab5 binding protein from *Salmonella*, Salmonella were grown over-night in LB and metabolically labeled with [35S]methionine (18). Briefly, cells were washed three times with PBS and grown in methionine-free RPMI 1640 medium containing 1 mCi of [35S]methionine with constant shaking (300 rpm) for 9 h at 37 °C. The cells were washed five times with PBS to remove unincorporated radioactivity. Bacteria were lysed by sonication followed by incubation in 1% Triton X in PBS at 4 °C for 30 min. Lysate was centrifuged at 12,000 rpm to get rid of unbroken cells and other debris. GST-Rab5 (200 μg) was immobilized with glutathione beads as described previously (15) and incubated in the presence of the *Salmonella* lysate for 1 h at 4 °C. Beads were washed (10,000 × g for 5 min) three times to remove unbound proteins. Subsequently, the proteins were separated by 12% SDS-PAGE. The gel was dried and autoradiographed at −70 °C. A similar experiment was carried out with unlabelled *Salmonella* lysate, and the indicated protein was identified using antibodies against SopE by Western blot analysis. Proteins were visualized using an appropriate HRP-labeled second antibody and ECL.

Specificity of Rab5 Binding with Salmonella—To determine the specificity of Rab5 binding with *Salmonella*, first the GST-Rab5 was biotinylated using the procedure as described previously (17). Biotinylated GST-Rab5 (10 μg/ml) was incubated in PBS (pH 7.2) containing 1 × 10⁷ *Salmonella* at 4 °C for 1 h in the presence or absence of a 100-fold excess of non-biotinylated Rab5 or SopE. Subsequently, cells were washed five times with PBS and lysed in SDS sample buffer. Proteins were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane. The biotinylated macrophage proteins bound to *Salmonella* were detected by Western blot analysis using avidin-HRP. Finally, cytosolic proteins were recognized by the presence or absence of competitors were detected by Western blot analysis using specific antibodies against endocytic Rabs and actin.

Removing the Rab Protein from Phagosomes by GDI Treatment—To strip off the Rab protein from each phagosome, the phagosomes were treated with Rab-GDI dissociation inhibitor (GDI) as described previously (15). Briefly, respective phagosomes were purified and incubated in fusion buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 20 μg/ml aprotinin) for 20 min at room temperature in the presence of 1 mM GDP. Subsequently, 6 μg of the purified GDI was added to one set of phagosomes in fusion buffer. The other set was carried at 4 °C at room temperature. Phagosomes were sedimented by centrifugation (10,000 × g for 5 min), and these Rab-stripped phagosomes were washed with PBS and used for indicated experiments.

Recruitment of Rab5 and Its Mutant Proteins on Salmonella-containing Phagosomes—To determine the recruitment of different forms of Rab5 proteins, purified phagosomes containing respective *Salmonella* were incubated with Rab-GDI. Proteins were isolated from the phagosomes (15). Respective Rab fusion proteins were preincubated in the presence of J774E cytosol (3.5 mg/ml) for 30 min at 37 °C in fusion buffer (250 mM sucrose, 0.5 mM EGTA, 20 mM Hepes-KOH, pH 7.2, 1.0 mM dithiothreitol, 1.5 mM MgCl₂, 100 mM KCl, including an ATP-regenerating system, 1 mM ATP, 8 mM creatine phosphate, 31 units/ml creatine phosphokinase) for 5 min prior to i.v. injection (19). Subsequently, recruitment of the different forms of Rab5 by the respective phagosomes was carried out by incubating the phagosomes at 37 °C for 10 min in fusion buffer containing 1 mg/ml cytosol supplemented with 30 ng of the indicated purified GST-Rab5 proteins. The presence of Rab5 (50 kDa of GST-Rab5) on the phagosomes was determined by Western blot analysis using specific antibodies against Rab5. Similar studies were carried out to determine the recruitment of the Rab7 by respective phagosomes.

Detection of Different Forms of Rab5 with SopE—To determine the direct interaction of different forms of Rab5 mutant proteins with SopE, we developed an in vitro assay to study the protein-protein interaction. First, the recombinant SopE-(78-240) (10 μg/ml) was incubated in an ELISA plate for 2 h at 37 °C in coating buffer (0.1 × phosphate buffered saline, PBS) at a concentration of 0.1 μg/ml. Subsequently, wells were washed thrice and incubated for 1 h at 37 °C in blocking buffer (PBS containing 0.1% of Tween 20 and 2% of bovine serum albumin). Wells were washed three times with PBS-Tween, and 0.2 mg/ml of different constructs of GST-Rab5WT or mutant proteins was incubated in PBS for 1 h at 37 °C to allow binding. To determine the binding of Rab5 with SopE, wells were incubated with Rab5-specific antibodies and detected using ECL.
polycanal antibody (1:2000 dilution) in PBS-Tween for 1 h at 37 °C. Excess antibodies were removed by washing the wells three times with PBS-Tween followed by three washes with PBS. Subsequently, wells were incubated with secondary antibodies labeled with HRP (1:10,000 dilution) for 1 h at 37 °C, washed five times, and finally the HRP activity present in each well was measured as described previously (15) to measure the binding of different preparation of Rab5 with SopE. 

Binding of SopE with Rab5—To determine the specificity of Rab5 binding with SopE, similar experiments were carried out using biotinylated Rab5 subsequently probed with avidin-HRP. First, the recombiant SopE-(78–240) (10 μg/ml) was carried out in the presence of different concentrations of biotinylated GST-Rab5-WT in PBS for 1 h at 37 °C to allow binding. To determine the binding of biotinylated Rab5 with SopE, wells were washed and incubated with avidin-HRP (1 μg/ml) for 30 min at 37 °C. Unbound avidin-HRP were removed by washing the wells three times with PBS-Tween followed by three washes with PBS. Subsequently, HRP activity present in each well was measured as described previously (15) to measure the binding of Rab5 with SopE. Binding of biotinylated Rab5 (10 μg/ml) with immobilized SopE (10 μg/ml) was carried out in the presence of different concentrations of non-biotinylated Rab5 or SopE to determine the specificity. 

Removal of Rab5 from the Cytosol—Immunodepletion of Rab5 from the cytosol was carried out using the procedure described previously (15). Briefly, 100 μl of protein A/G plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with 10 μl of anti-Rab5 antibody in PBS overnight at 4 °C. The antibody-protein A/G agarose complex was washed and centrifuged at 10,000 × g for 5 min at 4 °C. Subsequently, 100 μl of J774E cytosol (600 μg) was added to the protein A/G agarose-antiRab5 complex and incubated for 2 h at 4 °C to deplete the Rab5 from the cytosol. Subsequently, Rab5-depleted cytosol was separated from the agarose beads by centrifugation. Immunodepletion of Rab5 from the cytosol was confirmed by Western blot analysis using an anti-Rab5 antibody. Rab5-depleted cytosol was used for the in vitro fusion assay. 

SopE-mediated Nucleotide Exchange of Rab5—The SopE-mediated nucleotide exchange of Rab5 was determined using an assay previously described (20), with some modifications. First, the respective Rab proteins were loaded with GDP and subsequently, the exchange of GDP to [32P]GTP was determined in the presence or absence of GST-SopE-(78–240). Briefly, different mutants of Rab5 (60 pmol of each protein) was incubated at room temperature for 30 min in loading buffer (20 mM Tris-HCl (pH 7.5), 5 mM GDP, 50 mM NaCl, 3 mM MgCl2, 0.1 mM dithiothreitol, 0.1 mM EDTA). Exchange reaction was carried out in 200 μl of exchange buffer (20 mM Tris-HCl (pH 7.5), 5 μM [32P]GTP, 100 mM NaCl, 10 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM bovine serum albumin) containing 15 pmol of GDP-bound Rab proteins with/without an equimolar concentration of GST-SopE-(78–240) for 30 min at room temperature. Aliquots from the reaction were blotted onto a nitrocellulose membrane, and the membranes were extensively washed in ice-cold solution containing 20 mM Tris-HCl (pH 8), 100 mM NaCl, and 10 mM EDTA to remove free radioactivity. The membranes were then transferred to scintillation vials, and counts were determined in the presence of scintillation fluid. A similar exchange reaction of Rab5 was carried out in the presence of different concentrations of SopE as indicated.

Role of Different Constructs of Rab5 in Fusion of Early Endosome with LSP in Vitro—In vitro fusion of endosomes containing the biotinylated Salmonella with early endosomes containing avidin-HRP were carried out using a procedure similar to that described previously (15). Briefly, phagosomes were purified from macrophages, and endogenous Rab proteins were stripped off from the phagosomes by GDI-GDP treatment. To determine the role of different mutant constructs of Rab5, fusion of Rab-stripped LSP with early endosomes was carried out in the presence or absence of different factors from the host cell cytoplasm. The results are shown that live Salmonella, Salmonella were incubated in the presence of biotinylated cytosol prepared from the macrophages as described under “Experimental Procedures.” Subsequently, biotinylated protein from the macrophage cytosol bound to Salmonella was detected by Western blot analysis using avidin-HRP (lane 1). To identify the macrophage protein bound to Salmonella, Western blot analysis was carried out using specific antibodies against Rab5 (lane 2), Rab7 (lane 3), and actin (lane 4). Proteins were visualized using HRP-conjugated secondary antibody by ECL. Results from the Western blots are representative of three independent preparation. B, detection of Rab5 binding protein from Salmonella. To detect the Rab5 binding protein from the Salmonella, a GST pullout assay was carried out with Rab5-GST from a metabolically labeled Salmonella lysate as described under “Experimental Procedures.” The Salmonella proteins bound to the beads were analyzed by SDS-PAGE followed by autoradiography (lane 1). To identify the Salmonella protein bound to Rab5, Western blot analysis was carried out using specific antibodies against Salmonella secretary proteins like SopE (lane 2) and SipC (lane 3). Proteins were visualized using HRP-conjugated secondary antibody by ECL. Results from the Western blots are representative of three independent preparations. C, specificity of Rab5 binding with Salmonella. To determine the specificity of Rab5 binding with Salmonella, biotinylated Rab5 was incubated with Salmonella in the absence (Control) or the presence of excess non-biotinylated Rab5 (Rab5) or SopE (SopE) as described under “Experimental Procedures.” The biotinylated Rab5 bound to Salmonella in the presence or absence of competitors was detected by Western blot analysis using avidin-HRP by ECL. Results from the Western blots are representative of three independent preparations. 

SopE Specifically Binds with Rab5—We have previously shown that live Salmonella-containing phagosomes specifically recruited Rab5 on the phagosomes and promote fusion with the early endosomes (15). We therefore investigated the possibility of bacterial proteins that might be involved in the recruitment of different factors from the host cell cytoplasm. The results observed at 0.5 mg/ml normal cytosol concentration, which was expressed as 1 unit of relative fusion. The HRP activity, corresponding to 1 unit, is mentioned in the legend to Fig. 6.
FIG. 2. A, electron microscopic observations of the purified phagosomes. To check the purity of the phagosomes, purified phagosomes containing S. typhimurium were washed and processed for electron microscopy as described under “Experimental Procedures.” The arrow indicates the presence of bacteria in the purified phagosomes at low magnification (a and c) and high magnification (b and d). Bars, 200 nm. B, immunolocalization of Rab5 and SopE on Salmonella-containing phagosomes. Phagosomes containing live S. typhimurium (a), dead S. typhimurium (b), S. dublin:WT (c), or S. dublin SopE-negative mutant (f) were incubated with specific mouse anti-SopE antibody for 2 h at room temperature followed by determination of the primary antibody binding site with goat anti-mouse antibody conjugated with 12-nm gold particles as described under “Experimental Procedures.” Similarly, localization of the Rab5 on phagosomes containing S. dublin:WT (c) or S. dublin SopE-negative mutant (d) was determined using specific rabbit anti-Rab5 antibody subsequently probed with goat anti-rabbit antibody conjugated with 18-nm gold particles. Bars, 200 nm.
presented in Fig. 1A show that Salmonella specifically binds two proteins from the macrophage cytosol of apparent molecular mass of 25 and 42 kDa (lane 1). To identify these proteins, Western blot analyses were carried out with antibodies against different endocytic Rab proteins, which are of about 25 kDa and regulate vesicular trafficking. The anti-Rab5 antibody specifically detected the 25-kDa protein (lane 2) bound to Salmonella, while the anti-Rab7 antibody (lane 3) did not, demonstrating that Salmonella specifically recognized Rab5 from the host cells. Similarly, the 42-kDa protein bound to Salmonella was identified as actin (lane 4), which is consistent with the previous report that SipA, a protein from Salmonella, binds with actin to induce membrane ruffling, which facilitates the entry of the bacteria (21). To search for the bacterial surface protein that interacts with Rab5, GST-Rab5 was incubated with a Salmonella lysate obtained after growing the cells in the presence of [35S]methionine. The data presented in Fig. 1B show that GST-Rab5 specifically picked up two proteins of apparent molecular masses of 30 and 50 kDa from the Salmonella lysate. In contrast, no proteins were detected when GST or GST-Rab7 was used under similar conditions. Western blot analysis with anti-SopE antibody revealed the 30-kDa protein as SopE. However, we have not yet identified the 50-kDa proteins, which also bind to Rab5. Similar results were also obtained using WT S. dublin (data not shown) indicating that SopE, a type III secretion protein of Salmonella, specifically binds with Rab5. To determine the specificity of SopE-mediated binding of Rab5, S. typhimurium was incubated with biotinylated GST-Rab5 in the presence and absence of non-biotinylated Rab5 or SopE. Western blot analysis presented in Fig. 1C shows that binding of biotinylated Rab5 with Salmonella is effectively competed by both Rab5 and SopE.

**Live Salmonella Transport SopE onto Phagosomes and Recruit Rab5**—To determine whether the SopE produced by Salmonella is transported onto the phagosomes, we purified phagosomes containing respective wild type and mutant Salmonella. The electron micrograph presented in Fig. 2A shows that the purified Salmonella-containing phagosomes are relatively pure without any contamination with other intracellular organelles. Moreover, biochemical characterization demonstrated that these phagosomes are free of endosome, lysosome, Golgi, and endoplasmic reticulum contamination (16). Subsequently, purified live Salmonella-containing phagosomes were probed with anti-SopE antibody followed by a secondary antibody conjugated with colloidal gold particles to determine the presence of SopE on the phagosomes. The immunolocalization studies presented in Fig. 2B demonstrate that SopE is present on LSP (panel a). In contrast, phagosomes containing dead Salmonella (DSP) do not show the presence of SopE (panel b). The results presented in Fig. 2B show that phagosomes containing live, SopE knockout mutant Salmonella (S. dublin, strain SE1) were unable to recruit Rab5 on LSP (panel d). However, phagosomes containing wild type S. dublin (strain 2229) recruited significant amounts of Rab5 (panel e) on LSP, comparable to that observed with phagosomes containing wild type S. typhimurium (15). The data presented in Fig. 2B (panel e) show that SopE is also localized on LSP harboring wild type S. dublin. It is pertinent to mention that mean numbers of gold particles are significantly higher in panels a, c, and e than in panels b, d, and f (Fig. 2b) as observed from 100 respective phagosomes, indicating that SopE is transported onto LSP and thereby possibly recruit Rab5 on Salmonella-containing phagosomes.

**LSP Efficiently Recruits Prenylation-defective Rab5**—To understand the mechanism of Rab5 recruitment by LSP, we studied the binding of LSP with various mutant forms of Rab5, namely Rab5:Q79L, a GTPase-defective mutant (22, 23); Rab5:S34N, a dominant-negative mutant locked in GDP conformation (9, 24); Rab5:C4, where the isoprenylation motif is deleted (24); and Rab5:WT. Respective Rab proteins were preincubated with macrophage cytosol in the presence of the ATG-regenerating system to allow in vitro prenylation (19). Phagosomes containing either S. typhimurium (WT), or S. dublin (WT) or SopE knockout mutant of S. dublin were treated with Rab-GDI to deplete the endogenous Rab and incubated in the presence of indicated GST-Rab5 mutant protein in fusion buffer containing cytosol as described under “Experimental Procedures.” To determine the binding of indicated GST-Rab5 (50 kDa) with phagosomes, respective phagosomal proteins (40 μg of protein each per lane) were analyzed by 12% SDS-PAGE, transferred onto the nitrocellulose membrane, and subsequently probed with specific anti-Rab5 antibody. Proteins were visualized using appropriate HRP-labeled secondary antibody and ECL. Similarly, Rab7 was used as control. Western blots are representative of three independent preparations. B, interaction of different forms of Rab5 with SopE. To determine the direct interaction of different forms of Rab5 mutant proteins with SopE, the recombinant SopE-(78–240) (10 μg/ml) was coated in an ELISA plate, washed, and incubated with different forms of GST-Rab5:WT or mutant proteins (0.2 mg/ml). Finally, Rab5 binding with SopE was detected using Rab5-specific polyclonal antibody, subsequence probed with secondary antibodies labeled with HRP. The HRP activity present in each well was measured to quantitate binding, and the results are expressed as relative binding of three independent experiments ± S.D.
in the GTP form is recognized by LSP. Significant binding of Rab5:ΔC4, a prenylation-defective Rab5 mutant, suggested that prenylation of Rab5 is not required for the binding of Rab5 with LSP. No significant binding of Rab7 WT with LSP was observed under similar conditions, indicating that LSP specifically binds with Rab5. It is pertinent to mention that all the forms of Rab5 excepting Rab5:ΔC4 were prenylated when pre-incubated in the presence of cytosol-containing labeled substrate (data not shown). Furthermore, phagosomes containing SopE knockout mutant *Salmonella* were unable to bind any form of Rab5 under similar conditions, demonstrating that SopE present on LSP is responsible for the recruitment of Rab5 (Fig. 3A).

We further characterized the interaction of Rab5 with SopE in an in vitro assay in which the recombinant SopE immobilized on ELISA plates was incubated with different GST-Rab5 mutant proteins. Binding of Rab5 with SopE was detected using a Rab5-specific primary antibody and HRP-labeled secondary antibody. Results presented in Fig. 3B show that Rab5: S34N, which is locked in the GDP form, does not bind with SopE. In contrast, SopE specifically binds with Rab5 WT, Rab5:Q79L, and Rab5:ΔC4 demonstrating that SopE can specifically bind only with the GTP form of Rab5. The fact that Rab5:ΔC4, where the prenylation site was deleted, still bound SopE further indicated that prenylation is not required for the binding of Rab5 with SopE.

**Binding of SopE with Rab5**—The data presented in Fig. 4A show that biotinylated Rab5 binds with immobilized SopE with saturation kinetics. Half-maximal binding of biotinylated Rab5 with SopE occurred at a concentration of about 0.5 μg/ml, and maximum binding was observed at 5 μg/ml biotinylated Rab5. The binding of biotinylated Rab5 to immobilized SopE was effectively inhibited by both unlabeled Rab5 and SopE (Fig. 4B) with 50% inhibition achieved at about 50 μg/ml Rab5 or SopE, indicating the specificity of SopE binding with Rab5.

**SopE Acts as a GDP/GTP Nucleotide Exchange Factor of Rab5**—Our results (Fig. 3) demonstrated that *Salmonella*-containing phagosomes specifically bind Rab5 in its GTP form, the active form of the protein, which promotes endosome-endosome fusion. Furthermore, SopE can induce the GDP to GTP exchange of Rab5:WT in an in vitro assay in which the recombinant SopE immobilized on ELISA plates was incubated with different GST-Rab5 mutant proteins. Binding of Rab5 with SopE was detected using a Rab5-specific primary antibody and HRP-labeled secondary antibody. Results presented in Fig. 4A show that biotinylated Rab5 binds with immobilized SopE with saturation kinetics. Half-maximal binding of biotinylated Rab5 with SopE occurred at a concentration of about 0.5 μg/ml, and maximum binding was observed at 5 μg/ml biotinylated Rab5. The binding of biotinylated Rab5 to immobilized SopE was effectively inhibited by both unlabeled Rab5 and SopE (Fig. 4B) with 50% inhibition achieved at about 50 μg/ml Rab5 or SopE, indicating the specificity of SopE binding with Rab5.

**Prenylation-defective Rab5 Mutant Is Functionally Active**—To determine whether non-prenylated Rab5, e.g. Rab5: ΔC4, recruited on the phagosomes is functionally active, we used an in vitro fusion assay. LSP (containing *S. typhimurium* WT) were incubated for 10 min with endosomes loaded with avidin-HRP at 37 °C in the presence of cytosol and an ATP-regenerating system. Results presented in Fig. 6 show that LSP efficiently fuse with early endosomes in 10 min (Control). To establish the role of different forms of Rab5 in this fusion event, the endogenous Rab5 from the phagosomes were stripped off by Rab-GDI treatment, and fusion was carried out in Rab5-immunodepleted cytosol in the presence of indicated Rab5 mutant proteins. Data presented in Fig. 6 show that fusion of phagosomes with endosomes is inhibited in the Rab5-depleted condition. Addition of Rab5 WT and Rab5:Q79L re-stored the fusion of the phagosomes with endosomes by more than 90%, whereas Rab5: S34N, which is locked in the GDP form did not stimulate the fusion (Fig. 6). Interestingly, Rab5: ΔC4 stimulated the fusion of phagosomes with endosomes by more than 70% (Fig. 6). Our finding that Rab5:ΔC4, which is identical with Rab5:WT excepting for the deletion of the C terminus cysteine motif that is essential for prenylation, promotes fusion suggests that non-prenylated Rab5 is functionally active. However, when Rab-stripped LSP were pretreated with...
equimolar concentrations of GST-SopE-(78–240) in the presence of 5 pmol of GDP-load-indicated Rab5 and their mutants were incubated either in the presence (closed box) or in the absence (open box) of equimolar concentrations of GST-SopE-(78–240) in the presence of 5 μM [32P]GTP for 30 min. Aliquots from the reaction were blotted onto a nitrocellulose membrane to evaluate the binding of [32P]GTP. Results are expressed as an average of GTP bound per pmol of Rab5 of three independent experiments ± S.D. One unit corresponds to 13.7 ng of HRP activity/mg of protein in control fusion.

Intracellular trafficking of phagosomes depends on vesicular membrane composition as well as intravesicular content and involves dynamic modulations of the phagosomal membrane (6, 13) brought about by fusion with other endocytic vesicles and recruitment of various proteins from the cytosol. Recent studies have shown that small GTP binding proteins of the Rab family regulate intercompartmental transport (1, 2). Intracellular pathogens modulate the recruitment of these proteins on phagosomes for their survival by avoiding or inducing specific interactions of phagosomes with other vacuolar compartments

**DISCUSSION**

Intracellular trafficking of phagosomes depends on vesicular membrane composition as well as intravesicular content and involves dynamic modulations of the phagosomal membrane (6, 13) brought about by fusion with other endocytic vesicles and recruitment of various proteins from the cytosol. Recent studies have shown that small GTP binding proteins of the Rab family regulate intercompartmental transport (1, 2). Intracellular pathogens modulate the recruitment of these proteins on phagosomes for their survival by avoiding or inducing specific interactions of phagosomes with other vacuolar compartments.

**FIG. 5.** A, determination of SopE-mediated nucleotide exchange of Rab5. To determine the SopE-mediated nucleotide exchange of Rab5, 15 pmol of GDP-load-induced Rab5 and their mutants were incubated in the presence (closed box) or in the absence (open box) of equimolar concentrations of GST-SopE-(78–240) in the presence of 5 μM [32P]GTP for 30 min. Aliquots from the reaction were blotted onto a nitrocellulose membrane to evaluate the binding of [32P]GTP. Results are expressed as an average of GTP bound per pmol of Rab5 of three independent experiments ± S.D. One unit corresponds to 13.7 ng of HRP activity/mg of protein in control fusion.

**FIG. 6.** Role of different mutants of Rab5 in *in vitro* fusion of early endosome with LSP. In *in vitro* fusion of Rab-stripped (GDI-GDP-treated) phagosomes containing the biotinylated Salmonella with early endosomes containing avidin-HRP were carried out in the presence of an ATP-regenerating system containing Rab5-immunoedeploated cytosol (0.5 mg/ml) supplemented with different mutants of Rab5 (10 μg of each GST-Rab 5) as described under “Experimental Procedures.” The maximum fusion between endosomes and phagosomes (Control, LSP without GDI-GDP treatment) was observed at 0.5 mg/ml normal cytosol concentration, which was chosen as 1 unit; the results are expressed as the relative fusion of three independent experiments ± S.D. One unit corresponds to 13.7 ng of HRP activity/mg of protein in control fusion.

Recently, we have shown that live Salmonella-containing phagosomes (LSP) recruit the early-acting Rab5, and the fusion factors, NSF and α-SNAP, to promote fusion with early endosomes (15) thus avoiding transport to the lysosomes so that live Salmonella could persist in a low acidity compartment lacking active lysosomal enzymes (16). In the present study, we sought to delineate how Salmonella-containing phagosomes specifically recruit Rab5 to modulate the maturation of the phagosomes.

Salmonella have evolved a complex protein secretion system termed type III to deliver bacterial effector proteins into host cells, which serve to modulate host cellular function (21, 26). Hardt et al. (20) showed that SopE, also a type III secretory protein of Salmonella, stimulates the GDP to GTP nucleotide exchange of several Rho-GTPases, which modulate the cytoskeletal architecture to facilitate entry of Salmonella into epithelial cells. However, uptake of Salmonella, even the noninvasive mutant organism, in macrophages is mediated through lectinophagocytosis (27). We have previously shown that uptake of metabolically labeled live or dead Salmonella by macrophages was essentially the same (16), supporting the previous observation that Salmonella enters into macrophages through a host cell-mediated mechanism such as phagocytosis as opposed to pathogen-induced membrane ruffling. Our previous studies (e.g. Ref. 15) have shown that live Salmonella-containing phagosomes specifically recruit Rab5 on the phagosomes and promote fusion with the early endosomes. We therefore investigated the possibility of bacterial proteins that might be involved in the recruitment of different factors from the host cell cytoplasm, and our results demonstrated that SopE specifically binds with Rab5 (Fig. 1). In light of this finding, we inferred that SopE from the bacteria should be transported onto the phagosomes where they participate in the recruitment of Rab5 on the phagosomes to promote fusion with the early endosomal compartments. Thus, we looked for the presence of SopE on the surface of the phagosomes containing wild type and mutant Salmonella. The immunoelectron micrograph presented in Fig. 2B demonstrates that SopE is indeed
present on LSP containing wild type *S. typhimurium* or *S. dublin* (panels a and c), and this facilitates the binding of Rab5 on LSP (panel c). However, live SopE knockout mutant *Salmonella*-containing phagosomes (S. *dublin*, strain SE1) lacked SopE on the phagosomes (panel f) and were unable to recruit Rab5 on LSP (Panel d). Taken together, these results demonstrate that SopE is transported onto LSP and thereby helps recruit Rab5 on *Salmonella*-containing phagosomes.

Membrane association and subsequent biological functions of Rab proteins have been attributed to the C-terminal isoprenylation, which is characteristic of these proteins (28–30). Isoprenylation of Rab proteins occurs at the C-terminal motifs, which include CC (Rab1, Rab2, Rab9, and Rab10), CXC (Rab3, Rab4, Rab6, Rab7, Rab13, and Rab14), CCXX (Rab11), CXX (Rab8, Rab12), and CCXX (Rab5) (X can be any amino acid residue) (31–36). Deletion of the C-terminal tetrapeptide motif (CCSN) of Rab5 abolishes post-translation isoprenylation, membrane association, and homotypic fusion between endosomes (24). Similar studies (8, 9, 19, 22, 24, 37–39) have shown that deletion of the C-terminal motif of other Ras/Rab proteins results in failure to attach with the target membrane and inhibition of the specific transport process. In contrast, we have demonstrated that Rab5ΔC4 is recruited on LSP (Fig. 3A), which indicates that binding of Rab5 with LSP is independent of Rab prenylation. However, Rab5 locked in GDP conformation (Rab5:S34N) is unable to bind LSP, indicating that SopE present on LSP specifically recognize Rab5 only in the GTP form. These results are further supported by the fact that immobilized SopE specifically binds Rab5:WT, Rab5:Q79L, and Rab5:ΔC4 but not Rab5:S34N (Fig. 3B). Moreover, binding of biotinylated Rab5 with SopE is competed by unlabeled Rab5 (Fig. 4B). Essentially, results presented in Figs. 3 and 4 demonstrate that SopE acts as the Rab5-specific determinant and mediates the binding of Rab5 in GTP form on LSP. The major functional significance of our observations on SopE-mediated recruitment of non-prenylated Rab5 is that there could be a region of Rab5 outside the prenylation motif, which is specifically recognized by SopE.

Small molecular weight GTP binding proteins of the Rab family regulate vesicular transport. Rab proteins cycle between an active GTP-bound form and an inactive GDP-bound form, the latter being mainly present in the cytosol. Rab-specific guanine nucleotide exchange factor (40) (GEF) catalyzes the conversion of Rab-GDP to Rab-GTP and mediates the particulate transport event through other accessory proteins. After the membrane fusion, GTPase-activating protein increases the GTPase rate of Rab and converts them into their GDP-bound state, which is, finally, retrieved by a cytosolic protein termed GDI. GDI delivers the GDP-bound Rab to the membrane and is subsequently reactivated by GEF. Recent studies have shown that SopE acts as a specific GEF on Rho-GTPase proteins such as Cdc42 and Rac to induce membrane ruffling to facilitate *Salmonella* invasion (20). The results presented in Fig. 5, A and B, clearly demonstrated that SopE also acts as a GEF for Rab5 but not for Rab7. We (15) and others (20, 25, 26) have shown in vitro that prenylation-defective Rab5 mutant is due to the fact that prenylation-defective Rab proteins do not bind to the target membrane (8, 9, 19, 22, 24, 39) and thus fail to trigger downstream events in vesicle fusion. Rab5:S34N, which is locked in the GDP form, does not promote the fusion between LSP and early endosomes, which is consistent with previous demonstrations that this mutant protein does not support homotypic fusion between early endosomes (9, 23, 24, 28). Furthermore, treatment of Rab-stripped LSP with anti-SopE antibody inhibits the Rab5-mediated fusion between LSP and early endosomes (Fig. 6), indicating that SopE-mediated recruitment of Rab5 on LSP promotes the fusion. Therefore, our data indicate that prenylation-defective Rab5 protein is functionally active when it is recruited on LSP through SopE. Thus, it appears that prenylation of Rab proteins in general is only required for their attachment with the membrane.

In conclusion, our results demonstrate that SopE acts as a nucleotide exchange factor for Rab5 and mediates the specific recruitment of Rab5 in the GTP form on LSP, irrespective of prenylation, thus promoting fusion of LSP with early endosomes. In contrast to the current concept of Rab function, that prenylation of Rab protein is required for membrane attachment and biological function, this is the first demonstration that a non-prenylated Rab protein can sustain its biological activity of promoting fusion when recruited on the target membrane. Thus, these results indicate that prenylation of Rab proteins is not essential for their biological function; it is simply required for membrane attachment. The physiological significance of this finding derives from the fact that SopE acts as an Rab5-specific exchange factor and thereby mediates the recruitment of Rab5 in the GTP form on phagosomes containing live *Salmonella*. This constitutes a salvage mechanism that ensures the sustained fusion of LSP with early endosomes, independent of Rab5 prenylation, thereby inhibiting targeting of live *Salmonella* to the lysosomes and their eventual destruction.

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