Bacterial invasion, like the process of phagocytosis, involves extensive and localized protrusion of the host cell plasma membrane. To examine the molecular mechanisms of the membrane remodeling that accompanies bacterial invasion, soluble NSF attachment protein receptor (SNARE)-mediated membrane traffic was studied in cultured cells during infection by Salmonella typhimurium. A green fluorescent protein-tagged chimera of VAMP3, a SNARE characteristic of recycling endosomes, was found to accumulate at sites of Salmonella invasion. To analyze the possible role of SNARE-mediated membrane traffic in bacterial infection, invasion was measured in cells expressing a dominant-negative form of N-ethylmaleimide-sensitive factor (NSF), an essential regulator of membrane fusion. Inhibition of NSF activity did not affect cellular invasion by S. typhimurium nor the associated membrane remodeling. By contrast, Fcγ receptor-mediated phagocytosis was greatly reduced in the presence of the mutant NSF. Most important, dominant-negative NSF significantly impaired the fusion of Salmonella-containing vacuoles with endomembranes. These observations indicate that the membrane protrusions elicited by Salmonella invasion, unlike those involved in phagocytosis, occur via an NSF-independent mechanism, whereas maturation of Salmonella-containing vacuoles is NSF-dependent.

Invasive bacterial species gain access to intracellular compartments, where they survive and replicate by subverting host cell function. Although it is not yet clear how a variety of bacterial species induce the formation of the vacuoles that engulf them, it has been established that the process of invasion has much in common with that of phagocytosis (1–3). Both processes typically involve membrane remodeling events, namely pseudopodial extension during phagocytosis and ruffling during bacterial invasion, and both phenomena are associated with changes in the actin cytoskeleton of the host cell (1). As well, like phagosomes, bacteria-containing vacuoles can undergo a maturation process (2, 4). However, whereas phagocytosis and invasion are superficially similar, it is not clear whether the molecular mechanisms of internalization are shared.

Current models of phagocytosis suggest that phagosomes are generated by receptor-mediated “zippering” of the host cell plasma membrane against the surface of opsonized particles, accompanied by the active extension of pseudopodia (5–8). A source of intracellular membrane is required for the membrane remodeling that allows for pseudopod formation, and recent evidence suggests that specific endosomal compartments are involved in particle engulfment (9–11). It is now apparent that the soluble NSF attachment protein receptor (SNARE)1 protein VAMP3, an integral component of the recycling endosome, plays an important role in phagocytosis, possibly participating in localized fusion events at the site of phagosome formation and thus driving pseudopodial extension (12).

It is not known if endosomal compartments have a central role in the process of bacterial invasion, but the extensive membrane remodeling that accompanies cellular infection by Salmonella typhimurium suggests that invasion by this bacterial pathogen may mechanistically parallel phagocytosis. In the current study, we examined the role of SNARE-mediated membrane fusion in S. typhimurium invasion. Because of the established similarities with phagocytosis, the role of VAMP3 in invasion was analyzed first. However, because the exact intracellular compartments and the repertoire of SNARE proteins that may be involved in bacterial invasion are not known, the role of membrane fusion was also investigated through the general inhibition of SNARE-mediated membrane traffic, specifically by blocking the activity of N-ethylmaleimide-sensitive factor (NSF). NSF is an ATPase/chaperone that is responsible for the disassembly of SNARE complexes and is therefore an essential regulator of intracellular membrane fusion events.

1 The abbreviations used are: SNARE, soluble NSF attachment protein receptor; NSF, N-ethylmaleimide-sensitive factor; SCVs, Salmonella-containing vacuoles; NEM, N-ethylmaleimide; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; Tfα, transferrin; pNPP, p-nitrophenyl phosphate; TRITC, tetramethylrhodamine isothiocyanate; PTDIC, Fluorescein isothiocyanate; WT, wild type; GFP, green fluorescent protein; EGFP, enhanced GFP; PBS, phosphate-buffered saline; SRBCs, sheep red blood cells; PE, phycoerythrin; TeTx, tetanus toxin; LPS, lipopolysaccharide.
We generated a mutant form of NSF (E329Q-NSF) that is deficient in ATP hydrolysis and therefore inhibits the function of endogenous NSF, resulting in a dominant-negative effect (14, 15). We found that acute expression of E329Q-NSF inhibits phagocytosis in a macrophage cell line. By contrast, invasion of *S. typhimurium* into epithelial cells was not impaired by the dominant-negative NSF; however, maturation of *Salmonella*-containing vacuoles (SCVs) was inhibited.

**Experimental Procedures**

**Reagents and Antibodies**—Sheep red blood cells (SRBC), goat anti-SRBC IgG, and N-ethylmaleimide (NEM) were purchased from ICN. Human IgG was purchased from Baxter Healthcare Corp. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were obtained from Wisent (Montreal, Quebec, Canada). Brefeldin A and G418 were obtained from Calbiochem and Fugene 6 from Roche Molecular Biochemicals. Tetrathymahydrodihydri isoincoyate-transferrin (Tfn) and tetrathymahydrodihydri phallodin were from Molecular Probes. Restriction enzymes were obtained from New England Biolabs. Latex beads (3 μm diameter), ATP, and p-nitrophenyl phosphate (pNPP) and all other reagents were purchased from Sigma.

Polyclonal antibody to o-mannosidase II was a generous gift of Dr. Marilyn Farquhar (University of California, San Diego). The phycoerythrin (PE)-labeled antibody to mouse Fc \(_E\) receptors (CD16/CD32) was purchased from PharMingen. Rabbit polyclonal antiserum to \(\alpha\)-mannosidase II was purchased from the American Tissue Culture Collection, and Fc \(_E\) receptor II/III receptors (CD16/CD32) was purchased from PharMingen. Human IgG was purchased from Baxter Healthcare Corp. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were obtained from Wisent (Montreal, Quebec, Canada). Brefeldin A and G418 were obtained from Calbiochem and Fugene 6 from Roche Molecular Biochemicals. Tetrathymahydrodihydri isoincoyate-transferrin (Tfn) and tetrathymahydrodihydri phallodin were from Molecular Probes. Restriction enzymes were obtained from New England Biolabs. Latex beads (3 μm diameter), ATP, and p-nitrophenyl phosphate (pNPP) and all other reagents were purchased from Sigma.

Polyclonal antibody to o-mannosidase II was a generous gift of Dr. Marilyn Farquhar (University of California, San Diego). The phycoerythrin (PE)-labeled antibody to mouse Fc \(_E\) receptors (CD16/CD32) was purchased from PharMingen. Rabbit polyclonal antiserum to \(\alpha\)-mannosidase II was purchased from the American Tissue Culture Collection, and Fc \(_E\) receptor II/III receptors (CD16/CD32) was purchased from PharMingen. Human IgG was purchased from Baxter Healthcare Corp. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were obtained from Wisent (Montreal, Quebec, Canada). Brefeldin A and G418 were obtained from Calbiochem and Fugene 6 from Roche Molecular Biochemicals. Tetrathymahydrodihydri isoincoyate-transferrin (Tfn) and tetrathymahydrodihydri phallodin were from Molecular Probes. Restriction enzymes were obtained from New England Biolabs. Latex beads (3 μm diameter), ATP, and p-nitrophenyl phosphate (pNPP) and all other reagents were purchased from Sigma.
SRBCs were added to COS-2A cells plated in complete medium and incubated for 30 min or 1 h at 37 °C. Extracellular SRBCs were removed by hypotonic lysis (water, 30 s), and internalized SRBCs were quantified as above. When assessing phagocytosis in cells transduced with EGFP, it was possible to quantify internalized particles using fluorescence microscopy due to the exclusion of GFP from the interior of the phagosomes. In this way, cells could be fixed after phagocytosis and counter-stained, for example with NSF antibody in experiments where cells had been cotransfected with NSF cDNA.

To assess particle binding efficiency, transfected RAW 264.7 cells were incubated with opsonized SRBCs for 30 min at 4 °C. Nonadherent SRBCs were washed away with PBS, and the number of bound SRBCs/cell was quantified under light microscopy.

**Bacterial Infection**—The *S. typhimurium* strain 1344 was used and has been described previously (20). Overnight bacterial cultures were diluted 1:30 into fresh Luria-Burtani broth and incubated at 37 °C, shaking for 3 h. Bacteria were pelleted by centrifugation at 10,000 rpm for 2 min and then resuspended in PBS. The bacteria were diluted in Earle's buffered salt solution (EBSS), pH 7.4, and added to COS-1 cells that had been plated on coverslips at a density of ~50 bacteria per cell. Infection was carried out at 37 °C for 15 min. Excess bacteria were washed away with PBS, and the cell monolayers were then incubated in growth medium containing 100 μg/ml gentamicin for 15 min at 37 °C. The cells were processed for immunofluorescence by fixing in 2.5% paraformaldehyde/PBS for 10 min, washing, and blocking with 1% BSA/2% donkey serum/PBS. Samples were stained for external bacteria by incubating with rabbit anti- S. *typhimurium* LPS antibody followed by Alexa 594-conjugated donkey anti-rabbit antibody. Subsequent to this, the samples were again incubated in blocking buffer, this time containing 0.1% saponin. The samples were then stained for total bacteria (external and internalized) with the same primary antibody followed by Alexa 594-conjugated donkey anti-rabbit antibody. For examination of vacuole maturation, fixed and permeabilized samples were stained for total bacteria with the anti-LPS antibody and Alexa 594-conjugated donkey anti-rabbit antibody and counterstained for LAMP-1 with the monoclonal anti-human LAMP-1 antibody and FITC-conjugated goat anti-mouse IgG.

**RESULTS**

**VAMP3-GFP Localizes to Sites of Salmonella Invasion**—Previous studies from our laboratory demonstrated the focal exocytosis of VAMP3-containing vesicles at sites of particle internalization during phagocytosis (12). Here, we examined the possibility that VAMP3-containing vesicles may play a role in the membrane remodeling that accompanies cellular invasion by *S. typhimurium*. Cells expressing a chimeric VAMP3-GFP construct (12) were subjected to infection by *S. typhimurium* as described under "Experimental Procedures." Shortly after infection (6–8 min), the distribution of VAMP3-GFP was monitored by fluorescence microscopy, whereas the localization of bacteria was detected by indirect immunofluorescence using an antibody to *S. typhimurium* lipopolysaccharide (anti-LPS). In resting COS-1 cells VAMP3-GFP was present in vesicles that were observable throughout the cytoplasm but accumulated particularly in a juxta-nuclear cluster, typical of recycling endosomes (Fig. 1a), consistent with previously published observations (21). Comparatively little VAMP3 was present on the cell surface in otherwise untreated cells. In cells undergoing invasion by *S. typhimurium*, in contrast, VAMP3-GFP was seen to accumulate at or near the plasma membrane at sites of bacterial attachment (Fig. 1, b and c).

Accumulation of VAMP3-GFP occurred both at sites where *Salmonella* bacteria were external and at sites where the bacteria were internalized. However, although VAMP3-GFP accumulated at these sites, it was not clear if this represented only intracellular movement of the fluorescent protein or mobilization and exocytosis of this SNARE at sites of invasion. To examine this possibility, we tested for the appearance of the COOH terminus of VAMP3-GFP (bearing the GFP moiety) on the cell surface by staining the cells with an antibody against GFP. Following invasion, COS-1 cells transfected with VAMP3-GFP were gently fixed (using 2.5% paraformaldehyde) but not permeabilized. Staining of the cells then revealed external GFP (Fig. 1d) at sites of VAMP3-GFP accumulation (Fig. 1e) that corresponded with sites of attachment of external bacteria (Fig. 1f). These observations indicate that accumulation of VAMP3-GFP can occur prior to internalization of *S. typhimurium* and that the accumulation of this chimera is accompanied by its insertion into the plasma membrane.

**Tetanus Toxin Blocks Recruitment of VAMP3-GFP to Sites of Salmonella Internalization but Does Not Inhibit Invasion**—The localization and plasmalemmal insertion of VAMP3-GFP at sites of bacterial invasion suggest that membrane fusion events mediated by this SNARE may contribute to the membrane remodeling that is involved in bacterial invasion. To test this possibility, a cDNA encoding the light chain of the tetanus toxin (TeTx) was cotransfected into COS-1 cells along with VAMP3-GFP. To confirm that cotransfection of these two vectors resulted in significant cleavage of VAMP3-GFP, transfected cell populations were harvested and examined by Western blotting with an anti-GFP antibody. Fig. 2 shows an anti-GFP Western blot of extracts from mock-transfected COS-1 cells (lane 1) and COS-1 cells transfected with EGFP alone (lane 2), VAMP3-GFP alone (lane 3), or VAMP3-GFP plus TeTx (lane 4). Comparison of lanes 3 and 4 reveals that coexpression of TeTx and VAMP3-GFP results in essentially the complete cleavage of the VAMP3-GFP chimera. The two lower bands in lanes 3 and 4 are protein species, possibly products of degradation, that are not influenced by coexpression of tetanus toxin.

To study the effect of TeTx on the mobilization of VAMP3-GFP that accompanies *Salmonella* invasion, COS-1 cells were transfected with both VAMP3-GFP and TeTx prior to invasion assays with *S. typhimurium*. As depicted in Fig. 3b, the expression of TeTx altered the distribution of VAMP3-GFP within COS-1 cells. Tetanus toxin is known to cleave the cytoplasmic portion of VAMP3, and the observed redistribution of this SNARE is indicative of the accumulation of the transmembrane/fusional domain-GFP moiety of the chimera in the Golgi apparatus (22). Most important, in COS cells coexpressing VAMP3-GFP and TeTx, accumulation of VAMP3-GFP at sites of *S. typhimurium* invasion was markedly reduced (Fig. 3, b and c).

The observation that TeTx blocked the accumulation of VAMP3 near sites of *Salmonella* invasion prompted us to test...
whether TeTx would also block *Salmonella* invasion, in a manner analogous to its known effect on phagocytosis (10). To quantify the effect of TeTx on invasion, transfected cells were exposed to *S. typhimurium*, and the fraction of cells harboring intracellular bacteria was estimated as described under “Experimental Procedures” (see also Fig. 3, d-f). Surprisingly, whereas expression of TeTx altered the basal distribution of VAMP3 and prevented its accumulation at sites of bacterial internalization, it had no discernible effect on the number of cells invaded by *Salmonella* (Fig. 4). As well, the number of bacteria that invaded individual COS-1 cells was not affected by the expression of tetanus toxin (data not shown). Furthermore, expressing EGFP, alone, or EGFP and TeTx in COS-1 cells had no effect on *Salmonella* invasion (not shown). These results suggest that, although VAMP3 accumulates at sites of invasion, accumulation and function of this protein are not required for *S. typhimurium* entry into the cells.

**Characterization and Expression of Dominant-negative NSF**—The finding that TeTx had no effect on *Salmonella* invasion contrasts with previous studies that demonstrated a role of VAMP proteins in phagocytosis (10). These results are not necessarily incompatible because, although VAMP3 may be required for phagocytosis, other SNARE proteins, possibly including TeTx-insensitive isoforms of VAMP, may be essential for *S. typhimurium* invasion. It is impractical and in some cases impossible to use specific toxins to test the function of all the SNARE proteins expressed in COS-1 cells that may be involved in *Salmonella* invasion. Instead, a general inhibitor of SNARE-mediated membrane traffic was sought. A logical target for this purpose was NSF, an ATPase/chaperone protein responsible for the disassembly of SNARE complexes and an essential regulator of membrane fusion events. To inhibit NSF activity, we introduced a point mutation in the Chinese hamster NSF gene which resulted in the substitution of glutamine for glutamic acid at residue 329 (E329Q) within the catalytic site of the D1 domain of NSF. E329Q-NSF has been described elsewhere and shown to form hexamers that bind ATP but do not hydrolyze it (14, 15). When mixed with wild-type protein, E329Q-NSF forms mixed hexamers that also lack activity, leading to a dominant-negative effect. To verify the effectiveness of the mutation, purified, recombinant wild-type, and mutant NSFs were assayed for nucleotidase activity *in vitro*, as described under “Experimental Procedures.” Wild-type NSF had an activity of 0.382 ± 0.019 μmol pNPP/mg/h (means ± S.E.). Wild-type NSF, incubated with 2.5 mM NEM, had an activity of 0.05 ± 0.023 μmol of pNPP/mg/h, and the activity of E329Q-NSF was 0.142 ± 0.011 μmol of pNPP/mg/h. Consistent with the findings of others (15), the intrinsic activity of E329Q NSF was ~30% that of wild-type NSF. The remaining nucleotidase activity of E329Q-NSF is attributable to the second catalytic site in the D2 domain of the protein (15).

To characterize the function of E329Q-NSF in intact cells, we chose to express the mutant protein in a macrophage cell line (RAW 264.7). This provided us with the opportunity to compare NSF function in phagocytosis, a receptor-mediated event initiated by the macrophage, and in invasion, an opsonin receptor-independent process triggered by *Salmonella*. Wild-type and mutant NSF genes in pcDNA 3.1 were expressed in mouse macrophages by transient transfection along with EGFP. As illustrated in Fig. 5, b and d, the endogenous levels of NSF (see EGFP-negative cells) were barely detectable by immunofluorescence, but the ectopic expression of either wild-type (WT) or mutant NSF was clearly observable. Quantitation of the expression of E329Q-NSF (Fig. 6) revealed that 5 h after transfection the amount of the dominant-negative NSF was slightly increased over that of the endogenous protein. By 8 h post-transfection, the level of ectopic expression of NSF was ~3 times the endogenous NSF (Fig. 6).

We next assessed the biological effects of the dominant-negative NSF *in vivo* by monitoring transferrin uptake. Transferrin is internalized by a receptor-mediated, clathrin-depend-
ent process and cycles through endosomal compartments before returning to the cell surface. By incubating cells in the presence of rhodamine-labeled transferrin, we were able to determine that RAW cells overexpressing WT NSF internalized transferrin in a manner that was indistinguishable from untransfected cells (Fig. 5, e and f). In contrast, transferrin uptake was strikingly decreased in cells expressing E329Q-NSF (Fig. 5, g and h). A detailed quantitation of this effect is presented in Fig. 6, as a function of time after transfection. The level of transferrin uptake (as a percentage of control cells) was reduced by ~60% 5 h after transfection and by ~95% after 8 h. Expression of E329Q-NSF also inhibited the lysosomal accumulation of material normally internalized by fluid phase and altered the morphology of the Golgi complex in a manner resembling the effects of brefeldin A (not illustrated). Comparable levels of expression of WT NSF were without effect on the uptake of transferrin (Fig. 6) or fluid phase markers or on Golgi morphology. These experiments indicate that ectopic expression of NSF to levels that can cause altered SNARE function can be attained shortly after transfection (within 8 h). Importantly, such brief periods of expression preserved cellular viability and had no discernible effect on several aspects of cell function. Specifically, the acidity of lysosomes, assessed by partition of fluorescent weak bases, was preserved, as was the electrical potential across the mitochondrial inner membrane, estimated using rhodamine 143 (not shown). Unless indicated otherwise, an 8-h transfection protocol was used thereafter.

**FIG. 5.** Expression of WT and mutant NSF in RAW macrophages. RAW 264.7 cells were transfected with a combination of EGFP and either WT-NSF (a and b) or E329Q-NSF (c and d). Alternatively, cells were transfected with WT-NSF (e and f) or E329Q-NSF (g and h) constructs alone. a–d, cells were fixed after 8-h transfections and stained with anti-NSF antiserum and a TRITC-conjugated secondary antibody. EGFP (a and c) and NSF (b and d) images from the same fields of view are shown. e–h, at 8 h post-transfection, serum-starved cells were labeled with TRITC-transferrin and then fixed and stained for NSF using a FITC-conjugated secondary antibody. NSF (e and g) and transferrin (f and h) distributions in corresponding fields of view are shown. Arrows indicate transfected cells. Scale bar represents 10 μm.

**FIG. 6.** Transient, acute expression of E329Q-NSF in RAW cells impairs transport of transferrin. Untransfected RAW cells or cells at 5 and 8 h after transfection with E329Q-NSF were labeled with TRITC-transferrin and stained for NSF as above. The levels of NSF expression (open squares) and the transferrin content (closed circles) in the cells were then determined, relative to control cells. The 8-h time point for transferrin uptake in cells transfected with WT-NSF is also shown (open circle). Data points are means ± S.E. from three independent experiments representing more than 40 randomly chosen cells measured for each point plotted.

**FIG. 7.** Expression of dominant-negative NSF inhibits phagocytosis. a, RAW 264.7 cells were transiently transfected with a combination of EGFP and either WT or E329Q-NSF. After 8 h, cells were subjected to phagocytosis assays using opsonized latex beads. Corresponding fields of view, using fluorescent and DIC microscopy, are shown. Arrows indicate internalized beads, and arrowheads indicate external beads. Scale bar, 10 μm. b, quantitation of phagocytosis in transfected RAW cells. Phagocytic index represents average number of beads ingested per 100 cells. Control samples were transfected with pcDNA 3.1 vector alone along with EGFP and treated with or without 5 μg/ml brefeldin A for 3 h. Means ± S.E. are taken from at least three experiments for each treatment, and the number of cells examined is indicated in parentheses.
transfected with EGFP and pcDNA 3.1 vector alone (see quantitation in Fig. 7b). In contrast, RAW cells transfected with E329Q-NSF ingested significantly fewer beads (Fig. 7a and b). The phagocytic indices for WT-NSF and E329Q-NSF transfec- tants were 205 ± 26 and 66 ± 11, respectively (Fig. 7b), equivalent to 68% inhibition. Similar results were obtained using IgG-opsonized red blood cells.

It was conceivable that inhibition of phagocytosis by E329Q-NSF resulted from impairment of constitutive secretion, causing depletion of proteins that turn over rapidly. This possibility was tested by exposing the cells to brefeldin A, a potent inhibitor of ARF GTPase activity and COP I function (23), for 3 h prior to phagocytosis assays. This time is comparable to the period of expression of inhibitory concentrations of E329Q-NSF (see Fig. 6). Incubation with brefeldin A for 3 h had no detectable effects on phagocytosis (Fig. 7b), despite inducing very rapid dispersal of the Golgi complex. Thus, the impairment of phagocytosis in RAW cells caused by dominant-negative NSF is not due to the inhibition of constitutive secretion.

Dominant-negative NSF Does Not Block Fcγ Receptor Function—To define further the nature of the phagocytic defect in RAW cells expressing mutant NSF, we examined the level of Fcγ receptor expression on the surface of transfected RAW cells. Flow cytometry of RAW cells expressing WT or E329Q-NSF revealed that transfected cells had levels of FcγII/III receptors on their surface that were similar to those of untransfected cells (Fig. 8a). The presence of receptors and their ability to engage opsonized particles was also assessed by particle binding assays using both latex beads and red blood cells. As seen in Fig. 7a and quantified in Fig. 8b, RAW cells expressing WT or E329Q-NSF bound opsonized particles with equal efficiency.

Actin cup formation also proceeded normally in cells transfected with E329Q-NSF. Actin remodeling is a well characterized post-receptor event that occurs early and transiently in the process of phagocytosis (24–26). Staining of F-actin in RAW cells exposed to opsonized particles for 5 min showed that the reorganization of actin fibers in nascent phagosomal cups was indistinguishable in WT or mutant NSF transfectants (Fig. 8c).
Collectively, these results indicate that impairment of phagocytosis by the dominant-negative NSF occurred at a step distal to Fc receptor activation and is independent of actin reorganization.

**Dominant-negative NSF Inhibits VAMP3-GFP Recruitment to Sites of Salmonella Internalization but Does Not Block Membrane Ruffling**—Based on the inhibitory effect of dominant-negative NSF on phagocytosis reported above, we anticipated that inhibition of NSF activity would also have adverse effects on *Salmonella* invasion. To ensure that the dominant-negative NSF construct was having inhibitory effects in the cells used for invasion assays, COS-1 cells were cotransfected with EGFP and either WT-NSF or E329Q-NSF for 8 h, and transferrin uptake was determined. As in RAW cells, E329Q-NSF impaired the intracellular accumulation of transferrin in COS-1 cells (data not shown). Also, immunofluorescent staining of α-mannosidase II in cells expressing E329Q-NSF indicated that Golgi morphology had been altered by expression of the dominant-negative mutant (not illustrated). Ectopic expression of WT-NSF had no apparent effect in either of these assays.

We next cotransfected COS-1 cells with VAMP3-GFP and either WT-NSF or E329Q-NSF and performed *Salmonella* invasion assays as described above. As seen in Fig. 9b, coexpression of E329Q-NSF influenced the intracellular distribution of VAMP3-GFP (compare Fig. 9b with Fig. 1a) and prevented the localization of VAMP3-GFP to sites of *Salmonella* attachment (Fig. 9, b and c). Coexpression of WT-NSF did not affect the expression pattern of VAMP3-GFP nor did it influence its redistribution to *Salmonella*-associated ruffles (not shown).

To determine whether membrane ruffling had been blocked by expression of E329Q-NSF, we next expressed either the WT or mutant NSF together with a CDNA encoding a chimera of GFP and the acylation motif of the membrane-associated kinase Lyn (PM-GFP). This construct has been described elsewhere and has been demonstrated to label preferentially the plasma membrane, making it a useful marker of plasmalemmal redistribution (27). The basal pattern of PM-GFP is shown in Fig. 9d. This relatively homogeneous plasmalemmal distribution was not altered by coexpression of WT-NSF. PM-GFP was found to accumulate at sites of *Salmonella* invasion (not illustrated), in accordance with the reported formation of membrane ruffles. Coexpression with E329Q-NSF had subtle effects on the resting distribution of PM-GFP in COS-1 cells but, importantly, did not alter the redistribution of PM-GFP to sites of *Salmonella* invasion (Fig. 9, e and f). Together, the results presented in Fig. 9 indicate that dominant-negative NSF blocked the participation of SNARE-mediated membrane traffic in the membrane redistribution that accompanies *Salmonella* invasion but did not eliminate membrane ruffling.

**NSF Activity Is Not Required for Invasion of COS-1 cells by *S. typhimurium* but Is Necessary for Particle Ingestion by Phagocytically Competent COS-1 Cells**—The combined observations that dominant-negative NSF inhibited the phagocytosis of opsonized particles but did not block *Salmonella*-induced membrane ruffling prompted us to quantify *Salmonella* invasion in COS-1 cells expressing E329Q-NSF. COS-1 cells were cotransfected with EGFP and either WT or E329Q-NSF prior to assaying *Salmonella* invasion as described in Fig. 1. As seen in Fig. 10a, expression of either WT or dominant-negative NSF had no effect on the invasion of *S. typhimurium* into COS-1 cells. The percentage of cells invaded was not affected by expression of mutant NSF (Fig. 10a) nor was the number of *Salmonella* bacteria per invaded cell altered (not shown). Similar results were obtained when HeLa cells were used, when NSF constructs were coexpressed with VAMP3-GFP or PM-GFP, and when transient expression of E329Q-NSF was extended to 16-h periods.

The differential effects of E329Q-NSF on phagocytosis and bacterial invasion may reflect differences in the cell types used, rather than in the mechanisms underlying the two processes. To address this possibility, we conferred phagocytic capability to COS-1 cells by stable transfection of Fc receptor IIA. These cells, designated COS-2A, have been characterized previously and shown to support phagocytosis in a manner that closely resembles the behavior of “professional” phagocytes such as macrophages (8, 17–19). COS-2A cells were transiently co-transfected with EGFP and NSF constructs for use in phagocytosis assays as described under “Experimental Procedures.” Cells transfected with WT-NSF ingested opsonized latex beads efficiently, whereas their E329Q-NSF-transfected counterparts showed significantly lower phagocytic indices (Fig. 10b). Cells transfected with WT-NSF ingested particles as efficiently as untransfected or mock-transfected COS-2A (data not shown). As was the case for RAW cells, inhibition of phagocytosis was not attributable to changes in receptor expression. Quantification of surface Fc receptor IIA expression on transfected COS-2A cells, using indirect immunofluorescence and NIH Image software, revealed no differences between WT and E329Q-NSF-transfected samples (Fig. 10c).

**Dominant-negative NSF Impairs SCV Maturation**—SCVs are known to undergo a maturation process resulting in the insertion of specific endomembrane components into the SCV membrane along with changes in vacuolar contents (2, 28). The appearance of lysosomal-associated membrane protein 1 (LAMP-1) in the vacuolar membrane is a marker of such maturation. To examine the possible role of NSF in vacuolar maturation, we examined the distribution of LAMP-1 in infected cells using indirect immunofluorescence microscopy. Fig. 11 shows COS-1 cells cotransfected with EGFP and WT or E329Q-NSF that have been infected with *S. typhimurium*. The percentage of *S. typhimurium*-containing vacuoles that were positively stained for LAMP-1 was significantly reduced in cells expressing dominant-negative NSF (Fig. 11e). The expression of E329Q NSF had no effect on the level of expression of LAMP-1, as determined by Western blot, or on the basal distribution of this lysosomal marker (data not shown). It is im-

---

**Fig. 9.** E329Q-NSF blocks the accumulation of VAMP3-GFP in *Salmonella*-induced membrane ruffles. COS-1 cells were transfected with E329Q NSF together with either VAMP3-GFP (a–c) or PM-GFP (e and f) and then subjected to *Salmonella* infection followed by immunofluorescent staining for LPS. a, DIC image corresponding to the fluorescent micrographs shown in b and c. VAMP3-GFP distribution in cells transfected with E329Q and invaded by *Salmonella* is shown in b, and the bacteria are shown in c. Arrowheads indicate lack of VAMP3-GFP accumulation and corresponding sites of *Salmonella* localization. d, cell transfected with PM-GFP alone, showing resting distribution of PM-GFP. PM-GFP distribution in E329Q-transfected cell during invasion is shown in e and corresponding bacteria in f. Arrowheads in e and f indicate overlapping bacteria and PM-GFP accumulation. Scale bar represents 10 μm.
important to note that the inhibition of LAMP-1 acquisition is likely to be underestimated in these experiments because scoring was done on an all-or-none basis, not taking relative degrees of staining into account. Thus, while internalization of S. typhimurium into COS-1 cells was not impeded by inhibition of NSF function, expression of dominant-negative NSF in these cells did perturb the maturation of the resultant SCVs.

DISCUSSION

Our findings indicate that NSF-regulated membrane fusion is required to support Fc receptor-mediated phagocytosis but not bacterial invasion. Specifically, whereas expression of dominant-negative NSF inhibited particle engulfment both in macrophages and phagocytically competent fibroblastic cells, invasive bacterial internalization was not significantly altered. This does not rule out the possibility that other AAA proteins related to NSF, such as p97 (29), are active in response to Salmonella invasion, and this alternative is currently being investigated. Importantly, however, inactive NSF did impair the maturation of Salmonella-containing vacuoles at a stage after bacterial internalization was complete. These results suggest that membrane traffic has different roles during these two processes.

The finding that NSF function is required for optimal phagocytosis is consistent with an emerging body of evidence that suggests that regulated fusion of endomembranes with the plasmalemma is essential for phagocytosis (10, 11, 30–32).

**FIG. 10.** Dominant-negative NSF does not inhibit invasion by S. typhimurium. **a,** quantitation of bacterial invasion in transfected COS-1 cells. In cells transfected with EGFP alone (EGFP) or in combination with WT or E329Q-NSF, the percentage of cells containing internalized bacteria was determined. Means ± S.E. from four experiments are shown. **b,** dominant-negative NSF inhibits phagocytosis in Fc receptor-expressing COS-1 cells. Phagocytic indices (particles/100 cells) of COS-1 cells stably expressing Fc receptor IIA were determined, and the means ± S.E. of more than three experiments are shown. The number of cells examined is indicated for each condition. **c,** quantitation of cell surface Fc receptor IIA expression in NSF-expressing COS-2A cells. In parallel with analyses of phagocytosis, transfected COS-2A cells were labeled with monoclonal anti-human Fc receptor antibody. Experiments were done at 4 °C to prevent receptor internalization. Cells were then fixed and counterstained with NSF antibody. Surface receptor levels in transfected cells were then quantified using NIH Image. Shown are the means ± S.E. from three experiments representing >100 cells.

**FIG. 11.** Maturation of S. typhimurium-containing vacuoles is impaired in COS-1 cells expressing dominant-negative NSF. Bacterial invasion assays were performed in COS-1 cells transfected with EGFP along with either WT-NSF (a, b) or E329Q-NSF (c, d). After invasion, cells were fixed and stained for Salmonella LPS and endogenous LAMP-1. GFP expression revealed transfected cells in which the distributions of SCVs and LAMP-1 were examined. Arrowheads indicate SCVs in a and c and corresponding locations in b and d, respectively, showing LAMP-1 distributions. Scale bar is 10 μm. e, quantitation of LAMP-1-positive SCVs. In COS-1 cells transfected with EGFP alone or with EGFP together with WT or dominant-negative NSF constructs, the percentage of SCVs that were labeled with LAMP-1 antibody was determined. Means ± S.E. from three independent experiments representing >100 cells for each condition are shown (*, p < 0.0025).
Recent work from our laboratory indicates that VAMP3-containing vesicles participate in the focal exocytosis that occurs at sites of particle engulfment during phagocytosis (12), and together with the results presented here, these data are compatible with a model wherein NSF-regulated, SNARE-mediated membrane fusion drives exocytic events that are essential for the completion of particle engulfment. This requirement is independent of particle size, as phagocytosis of IgG-opsonized, invasion-null Salmonella bacteria (~1 μm long) was also blocked by expression of dominant-negative NSF. Fusion of intracellular vesicles with the plasma membrane may be required primarily to provide additional bilayer area required for pseudopod extension. Alternatively, it is possible that fusion of endomembranes is needed to deliver components of the molecular machinery or signaling complexes necessary for completion of the particle engulfment process. The examination of phagocytosis-associated downstream signaling events in the presence of dominant-negative NSF will be informative in this regard.

Whereas mechanisms of bacterial invasion are currently the subject of intensive study, it is not yet clear how a variety of bacterial species induce the formation of the vacuoles that engulf them. In the case of Salmonella species, it is known to involve the type III secretion system encoded for by the genes of pathogenicity island-1. However, the functionality of many of the gene products in this system is still poorly understood (2, 33, 34). It has been established that Salmonella invasion can be accompanied by localized membrane ruffling and membrane protrusions that resemble the pseudopods observed during phagocytosis. The current study suggests that, although VAMP3 does localize to Salmonella-induced ruffles, this localization is not required for the formation of ruffles (see results with PM-GFP, Fig. 8). Furthermore, internalization of bacteria during the invasion of cells by S. typhimurium was not impaired by TeTx or by inhibition of NSF, revealing an important contrast between phagocytosis and Salmonella invasion.

Collectively, these findings suggest that the ruffling noted during invasion may be largely driven by rearrangement of the underlying cytoskeleton and does not require membrane fusion. This model is in agreement with previous observations that indicate that invasion by S. typhimurium occurs independently of the phosphatidylinositol 3-kinase activity (PI3K) of the host (1, 35). This enzyme is an acknowledged mediator of membrane traffic in animal cells. Remarkably, inhibition of PI3K effectively precludes phagocytosis (31, 36), highlighting the differences between the latter process and bacterial invasion. It is also possible that membrane fusion events are involved in vacuole formation but that these are triggered by bacterial proteins that are independent of host NSF and PI3K and therefore insensitive to E259Q-NSF and to wortmannin, respectively. Although it has been shown that bacterial proteins are required for the fusion of Chlamydia trachomatis-containing inclusions (37), no fusogenic bacterial products have been described in Salmonella species.

After internalization of some invasive bacterial species, the vacuoles formed can undergo a series of fission and fusion events involving endomembrane compartments such as endosomes and lysosomes (2). S. typhimurium is known to reside initially in vacuoles that transiently bear the markers EEA1 and transferrin receptor and subsequently become enriched in markers of later compartments such as the vacuolar H^+−ATPase and LAMP-1. We found that the percentage of

References

We are thankful to Dr. Xiao-Rong Peng for providing VAMP3-GFP.

REFERENCES

1. Brumell, J. H., Steele-Mortimer, O., and Finlay, B. B. (1999) *Curr. Biol.* 9, R277–R280
2. Maresse, S., Steele-Mortimer, O., Moreno, E., Desjardins, M., Finlay, B., and Gervel, J. P. (1999) *Nat. Cell Biol.* 1, E183–E188
3. Sinai, A. P., and Joiner, K. A. (1997) *Annu. Rev. Microbiol.* 51, 415–462
4. Garcia-del Portillo, F., and Finlay, B. B. (1995) *Trends Microbiol.* 3, 373–380
5. Griffin, F. M., Jr., Griffin, J. A., and Silverstein, S. C. (1976) *J. Exp. Med.* 144, 1788–1809
6. Brown, R. J. (1995) *BioEssays* 17, 109–117
7. Allen, L. A., and Aderem, A. (1996) *Curr. Opin. Immunol.* 8, 36–40
8. Greenberg, S., Chang, P., Wang, D. C., Xaver, R., and Seed, B. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 1103–1107
9. Lennart, M. R., Yuen, A. F., Masi, S. M., Russell, D. G., Buttle, K. F., and Smith, J. J. (1997) *J. Cell Sci.* 110, 2041–2052
10. Hackam, D. J., Rotstein, O. D., Spilin, C., Schreiber, A. D., Trimble, W. S., and Grinstein, S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 11691–11696
11. Cox, D., Lee, D. J., Dale, B. M., Calafat, J., and Greenberg, S. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 680–685
12. Bajno, L., Peng, X.-R., Schreiber, A. D., Moore, H.-P., Trimble, W. S., and Grinstein, S. (2000) *J. Cell Biol.* 149, 697–705
13. Jahn, R., and Sudhof, T. C. (1999) *Annu. Rev. Biochem.* 68, 863–911
14. Ward, D. M., Leslie, J. D., and Kaplan, J. (1997) *J. Cell Biol.* 139, 665–673
15. Whiteheart, S. W., Rossnagel, K., Buhrow, S. A., Brunner, M., Jaenicke, R., and Rothman, J. E. (1994) *J. Cell Biol.* 126, 945–954
16. Mignaco, J. A., Barrahin, H., and Sedinco, H. M. (1997) *Biochem. Biophys. Acta* 1321, 252–258
17. Indik, Z. K., Park, J. G., Hunter, S., and Schreiber, A. D. (1995) *Blood* 86, 4389–4399
18. Lowry, M. B., Duchemin, A. M., Coggeshall, K. M., Robinson, J. M., and Anderson, C. L. (1998) *J. Biol. Chem.* 273, 24515–24520
19. Diekmann, P. G., Botelho, R. J., Maltzaller, Y. C., Chien, P., Schreiber, A. D., and Grinstein, S. (1999) *J. Biol. Chem.* 274, 28436–28444
20. Francis, C. L., Starnbach, M. N., and Falkow, S. (1992) *Mol. Microbiol.* 6, 3077–3087
21. McPhaden, H. T., Ushkaryov, Y. A., Edelmann, L., Link, E., Bina, T., Niemann, H., Jahn, R., and Sudhof, T. C. (1993) *Nature* 364, 348–349
22. Randhawa, V. K., Bilan, P. J., Khayat, Z. A., Daneman, N., Liu, Z., Ramlat, T., Volchuk, A., Peng, X. R., Coppola, T., Ragazzi, R., Trimble, W. S., and Klipp, A. (2000) *Mol. Biol. Cell* 11, 2403–2417
23. Chardin, P., and McCormick, F. (1999) *Cell* 97, 153–155
24. Maniak, M., Bouchenerber, R., Albrecht, R., Murphy, J., and Gerisch, G. (1995) *Cell* 81, 915–924
25. Furukawa, R., and Fechheimer, M. (1994) *Cell Motil. Cytoskeleton* 29, 46–56
26. Greenberg, S., Burridge, K., and Silverstein, S. C. (1999) *Cell Motil. Cytoskeleton* 41, 153–155
27. Teruel, M. N., Blanpied, T. A., Shen, K., Hendriks, R., Gushue, J., Fazel, A., Pelletter, A., Moore, D. J., Subramaniam, V. N., Hong, W., and Paiement, J. (2000) *Mol. Biol. Cell* 11, 2529–2542
28. Suzuki, K., Kohayashi, H., Kodama, Y., Masuijima, T., and Terakawa, S. (1997) *Cell Motil. Cytoskeleton* 38, 215–228
29. Holevinsky, K. O., and Nelson, D. J. (1998) *Biophys. J.* 78, 863–911
30. Cox, D., Tseng, C. C., Bjekic, G., and Greenberg, S. (1999) *J. Cell Biol.* 142, 1083–1088
31. Holevinsky, K. O., and Nelson, D. J. (1998) *Infect. Immun.* 66, 530–536
32. Jones, B. D., and Falkow, S. (1996) *Mol. Microbiol.* 21, 1240–1247
33. Collazo, C. M., and Galan, J. E. (1997) *Gene* (Amst.) 192, 51–59
34. Jones, B. D., and Falkow, S. (1996) *Annu. Rev. Immunol.* 14, 533–561
35. Mecas, J., Raubach, B., and Falkow, S. (1996) *Mol. Microbiol.* 22, 1269–1281
36. Araki, N., Johnson, M. T., and Swanson, J. A. (1996) *J. Cell Biol.* 135, 1249–1260
37. Van Ooij, C., Homola, E., Kincaid, E., and Engel, J. (1998) *Infect. Immun.* 66, 5364–5371
38. Mukherjee, K., Siddiqui, S. A., Hashim, S., Raje, M., Basu, S. K., and Mukhopadhyay, A. (2000) *J. Cell Biol.* 148, 741–753