Reconstitution of Phagosome-Lysosome Fusion in Streptolysin O-permeabilized Cells

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We have reconstituted fusion between phagosomes and lysosomes in streptolysin O-permeabilized J774-E macrophages. Fusion was assessed by measuring the delivery of avidin-conjugated horseradish peroxidase pre-internalized into lysosomes to phagosomes containing biotinylated β-glucuronidase-conjugated paramagnetic beads (1–2 μm). Fusion was dependent on energy and exogenously supplied cytosol. Phagosome-lysosome fusion was greatly inhibited when microtubules were depolymerized by nocodazole treatment, suggesting that fusion occurs via microtubule-dependent transport. Furthermore, fusion was inhibited by GTPγS and Rab GDP dissociation inhibitor. These results suggest that Rab proteins are involved in the regulation of fusion. Lastly, anti-NEM-sensitive factor (NSF) antibodies inhibited fusion, and addition of recombinant NSF wild type partially restored the fusogenic activity, indicating that NSF is required for fusion between phagosomes and lysosomes.

Phagocytosis is mediated by binding of organisms or large particles to plasma membrane receptors on phagocytes followed by internalization. After internalization (1), newly formed phagosomes containing microorganisms or particles mature into acidic and protease-rich phagolysosomes, where microorganisms or materials phagocytosed are killed and/or degraded. Maturation of phagosomes involves multiple membrane fusion and budding events and the apparent recruitment of multiple proteins from the cytosol (2–3).

Recently, in vitro reconstitution assays have been used to develop an understanding of the mechanisms that regulate fusion during vesicular transport. We have shown that early phagosomes can fuse with early endosomes in a cell-free system (4). Early phagosome-early endosome fusion requires cytosol, ATP, and NEM-sensitive1 fusion protein (NSF). Phagosome-lysosome fusion is also sensitive to GTPγS, the non-hydrolyzable GTP analogue, suggesting a role for GTP binding proteins in the fusion process. Desjardins et al. have reported that Rab5 is present on early phagosomes isolated following phagocytosis of latex beads while Rab7 has been detected on late phagosomes (5). Alvarez-Dominguez et al. (6) have shown that Rab5 is required for phagosome-lysosome fusion. Several heterotrimeric G proteins have also been found to localize to early phagosomes containing IgG-coated Staphylococcus aureus or latex beads (7–8). In vitro reconstitution assays, both Rab5 and Gαs, a subunit of trimeric G proteins, have been shown to regulate early phagosome-early endosome fusion (6–7), and Rab5 has been shown to be regulated by live intraphagosomal Listeria monocytogenes (6). However, the molecular mechanisms underlying membrane fusion in late steps of phagocytic pathway, viz. phagosome-lysosome fusion, are poorly studied.

To dissect the molecular mechanisms involved in the process of phagosome-lysosome fusion, we developed an in vitro reconstitution assay using SL-O permeabilized J774-E macrophages. The assay is based on content mixing between phagosomes and lysosomes. Biotinylated β-glucuronidase-conjugated particles and avidin-HRP or mannoside-BSA colloidal gold were used as probes for phagosomes and lysosomes, respectively. Formation of avidin-HRP-biotin complexes on the phagosomal particles or localization of colloidal gold in compartments containing phagocytic particles results from fusion between phagosomes and lysosomes. The data indicate that fusion is dependent on energy and exogenously supplied cytosol and that fusion is inhibited by nocodazole, indicating a role for microtubules. In addition, our data indicate that Rab proteins and NSF are required for fusion between phagosomes and lysosomes.

EXPERIMENTAL PROCEDURES

Cells and Materials—J774-E, a murine macrophage cell line, was grown in minimal essential medium containing Earle’s salts and 10% fetal calf serum. β-Glucuronidase was isolated from rat preputial glands as described (9). His6-tagged Rab3A-GDI was purified on Ni2+-agarose according to the manufacturer instructions (Qiagen, Chatsworth, CA). Purified SL-O was obtained from S. Bhakdi (University of Mainz, Mainz, Germany) (10). Anti-NSF, 4A6, a mouse IgM monoclonal antibody, was a generous gift from James E. Rothman (Sloan-Kettering Memorial Cancer Center, New York), and the recombinant NSF wild type was kindly provided by Sidney W. Whiteheart (University of Kentucky, Lexington, Kentucky). Horseradish peroxidase (HRP) conjugated adenosine 5’-O-(thio)triphosphate; NSF, NEM-sensitive factor; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; wt, wild type; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HBSA, Hanks’ balanced salt solution with 0.1% BSA; MES, 4-morpholineethanesulfonic acid.
with avidin (avidin-HRP) and avidin were obtained from Pierce. All other chemicals were from Sigma.

Phagocytic Probe—β-Glucuronidase was coupled to carboxylated paramagnetic polystyrene particles (particle diameter of 1–2 μm; Polysciences, Inc., Warrington, PA) using a water-soluble carboxidime (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, EDAC). Particles were washed with MES buffer (pH 6.0, 50 mM), and after incubating particles (1 ml; 2.5% solids) with β-glucuronidase (1 ml; 1 mg/ml) in MES buffer (pH 6.0, 50 mM) for 15 min at room temperature, EDAC (15 mg) was added very slowly. The pH was adjusted to 6.5 ± 0.2 with dilute NaOH, and the mixture was incubated for 15 h at room temperature. The reaction was stopped by incubation for 30 min at room temperature in MES buffer, pH 6.5, containing glycine (100 mM).

Particles were washed three times with phosphate-buffered saline (PBS, pH 7.4) and resuspended in PBS. Subsequently, β-glucuronidase-conjugated magnetic particles were biotinylated with NHS-LC-biotin (Pierce) according to the manufacturer recommendations. Particles (5% solids) were washed in 50 mM sodium bicarbonate buffer, pH 8.5, resuspended in the same buffer, and incubated with NHS-LC-biotin (0.27 mg/ml; 30 min at room temperature). The particles were then washed three times with PBS supplemented with 1% BSA and stored in PBS, pH 7.4, containing 1% BSA, 0.1% Na3Cit, and 5% glycerol. 105 particles bound approximately 25 ng of avidin-HRP.

Phagosome-Lysosome Fusion Assay in SL-O Permeabilized Cells—
Prior to ligand uptake, J774-E cells (1 × 106/well) were cultured overnight, washed, and resuspended in cold HBSS ( Hank’s balanced solution buffered to pH 7.4 with 10 mM Hepes, 10 mM TES, and BSA (10 mg/ml). Avidin-HRP (125 μg/ml) was internalized for 30 min at 37 °C following prebinding for 60 min at 4 °C. The cells were washed twice with cold HBSS. Internalized avidin-HRP was chased for 70 min at 37 °C for transport to lysosomes (11). Mannan (1 mg/ml) was added to avoid possible reinternalization of avidin-HRP recycled to the extracellular media. After washing, the cells were allowed to bind biotinylated-β-glucuronidase-conjugated particles (20 particles/cell, at 4 °C for 60–90 min). After phagocytosis (5 min; 37 °C), cells were cooled and washed with cold HBSS (2 ×) and cold SL-O binding buffer (115 mM KOAc, 25 mM Hepes, 0.5 mM MgCl2, 0.9 mM CaCl2, pH 7.4). Activated SL-O (1.27 μg/ml) was added for 30 min at 4 °C. SL-O (1 mg/ml in Hepes (25 mM); pH 7.2, BSA (1 mg/ml), and dithiothreitol (2 mM)) was activated for 30 min at 37 °C and diluted with binding buffer. After SL-O binding, cells were washed twice with binding buffer and permeabilized carried out for 5 min at 37 °C in fusion buffer (115 mM KOAc, 25 mM Hepes, 2.5 mM MgCl2, 2.5 mM CaCl2, 5 mM EDTA, pH 7.4, 1 mM dithiothreitol containing an ATP-depleting system (2.5 mM glucose and 12.5 units hexokinase/m), and avidin (100 μg/ml) to minimize fusion during SL-O treatment and to scavenge biotinylated particles on the cell surface, respectively. SL-O (1.27 μg/ml) was sufficient to permeabilize 70–80% of J774-E cells, as assessed by the release of lactate dehydrogenase (M, 135,000), a cytosolic marker. Under these conditions, release of HRP loaded in early endosomal or lysosomal compartments and β-hexosaminidase (an endogenous lysosomal marker) was < 5% of cell content. After permeabilization, cells were incubated for 30 min at 4 °C to deplete cytosol, gently washed twice with fusion buffer and incubated in 200 μl of fusion buffer or gel-filtered cytosol (4 mg/ml) supplemented with an ATP-regenerating system (4, 6). After 30 min at 4 °C, cells were incubated for 45 min at 37 °C to allow fusion to occur. The preparations were cooled to 4 °C and lysed in solubilization buffer (1% Triton X-100, 0.2% methylbenzethonium chloride, 1 mM EDTA, 0.1% BSA, 0.15 mM NaCl, 10 mM Tris-HCl, pH 7.4) in the presence of avidin (200 μg/ml) as scavenger. Avidin-HRP-biotinylated-particle complexes were washed 4 times with solubilization buffer by using a magnet, and the HRP enzymatic activity associated with the particles was measured at 490 nm using 0.75 mg/ml O-phenylenediamine and 0.006% H2O2 as substrates in sodium acetate (50 mM), pH 5.6. Values are expressed as percentage of control cytosol-dependent fusion. Total HRP activity recoverable on biotin-β-glucuronidase magnetic beads was determined by lysing cells in the absence of scavenger. In fusion assays following lysis in the presence of scavenger (200 μg/ml avidin), 30–50% of the total precipitable HRP activity was routinely recovered on magnetic beads.

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FIG. 1. Requirements for reconstitution of phagosome-lysosome fusion in SL-O permeabilized cells. A, avidin-HRP was internalized for 30 min at 37 °C and chased for 70 min at 37 °C. After washing of cells, biotinylated-β-glucuronidase-conjugated particles were bound to cells and internalized for 5 min at 37 °C. Subsequently, cells were permeabilized by SL-O and incubated for 45 min at 37 °C in fusion buffer supplemented with the indicated concentrations of gel-filtered cytosol with ATP-regenerating (closed circles) or depleting (open circles) systems. The reaction was stopped by cooling on ice and cells were lysed with solubilization buffer in the presence of scavenger. The particles were washed with solubilization buffer, and HRP enzymatic activity (in avidin-biotin complexes) associated with the particles was quantitated. B, fusion was performed as described under the following conditions: CON, 4 mg/ml cytosol, ATP-regenerating system; ATP regimen, 4 mg/ml cytosol, ATP-depleting system; ATP-S, 4 mg/ml cytosol, 1 mM ATP-S instead of ATP and ATP-regenerating system; NOC, 50 μM nocodazole plus complete conditions as in CON. C, fusion was performed with the indicated concentrations of GTP-S under complete conditions. Values were expressed as percentage of cytosol-dependent fusion obtained with 4 mg/ml cytosol and ATP-regenerating system.
Preparation of Cytosol—Cytosol from J774-E macrophages was prepared as described previously (9) and stored at −80 °C. Cytosol preparations included a protease inhibitor mixture, 2 μg/ml aprotinin, 2 μg/ml antipain, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride. NSF-depleted cytosol was prepared as described previously (12). Cytosol and NSF-depleting cytosol were gel filtered through Sephadex G-25 spin columns equilibrated in fusion buffer immediately before use, and protein concentrations were measured using the Bio-Rad protein reagent and BSA as a standard.

Treatment of Permeabilized Cells with Rab-GDI—Cytosol depleted permeabilized cells were incubated in fusion buffer for 30 min at 25 °C in the presence or absence of Rab-GDI (10 μM). The cells were washed twice with fusion buffer and then incubated for 45 min at 37 °C in fusion buffer supplemented with gel-filtered cytosol and an ATP-regenerating system in the presence or absence of Rab-GDI (10 μM). To follow removal of Rab proteins by Rab-GDI, treated cells were washed once with fusion buffer, lysed with SDS-polyacrylamide gel electrophoresis sample buffer, and boiled for 5 min. Following SDS-polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose, and the membranes were blocked overnight at 4 °C using standard procedures. Rabβ was detected using 4F11, an anti-Rabβ mouse IgG2a monoclonal antibody (6), and developed with HRP-conjugated goat antimouse IgG by the ECL detection system (Amersham Corp).

RESULTS

Reconstitution of Phagosome-Lysosome Fusion in SL-O-permeabilized Cells—Fig. 1A shows fusion of phagosomes with lysosomes. Both exogenous cytosol and an ATP regenerating system are required to attain maximal fusion activity. Cytosol-dependent fusion was proportional to the concentration of cytosol. Background activity, fusion in the absence of added cytosol, was approximately 30% of fusion in the presence of cytosol (4 mg/ml) and was subtracted from the data shown in Fig. 1A. When an ATP depleting system was added in the presence of exogenous cytosol, about 50% inhibition of fusion activity was observed (Fig. 1, A and B). Alternatively, substituting ATPγS, a nonhydrolyzable analogue of ATP, for ATP completely prevented cytosol-dependent fusion (Fig. 1B), indicating that ATP hydrolysis is required for fusion. It is possible that the depletion of ATP was incomplete and/or that during the depletion process, some fusion events that were already underway were allowed to go to completion. In addition, nocodazole inhibited cytosol-dependent fusion. In some experiments, nocodazole had an additional inhibitory effect on cytosol-independent fusion, expressed as negative values in Fig. 1B.

Monomeric and heterotrimeric GTP-binding proteins play key roles in regulating many intracellular transport processes (13–14). Addition of GTPγS (50 μM) to permeabilized cells inhibited cytosol-dependent fusion by about 50% (Fig. 1C). Maximal inhibition occurred at GTPγS (1 mM) (Fig. 1C). These results suggest that GTP hydrolysis regulates fusion of phagosomes with lysosomes.

We also examined the reconstitution of phagosome-lysosome fusion in the permeabilized cells by electron microscopy. As shown in Fig. 2, the permeabilization of cells by SL-O allowed us to better visualize the membrane structures of intracellular organelles. Both phagosomes and lysosomal compartments containing 20-nm colloidal gold particles remained intact. We first determined whether fusion of phagosomes with lysosomes in permeabilized cells required cytosol. In the absence of exogenous cytosol, fusion was low (Fig. 2A). Quantitation of gold distribution in 20 permeabilized cells indicated that 16% of phagosomes were gold-positive. Incubation of the permeabilized cells with cytosol (4 mg/ml) and an ATP regenerating system resulted in the colocalization of 20 nm of gold with phagocytic particles in larger vesicles (Fig. 2B). Approximately 64% of phagosomes were gold-positive when the experiment was carried out under complete conditions. When permeabilized cells were treated with GTPγS (1 mM) in the presence of cytosol (4 mg/ml) and an ATP regenerating system, 19% of the

**Fig. 2. Morphological characterization of phagosome-lysosome fusion in SL-O-permeabilized cells.** For morphological experiments, mannose-BSA-coated colloidal gold (20 nm) was used instead of avidin-HRP as in Fig. 1. The pulse-chase experiment was exactly as described in Fig. 1. After 5 min of phagocytosis, cells were permeabilized by SL-O and incubated for 45 min at 37 °C in fusion buffer, including an ATP generating system. A, without cytosol; B, supplemented with gel-filtered cytosol (4 mg/ml); and C, same as panel B but with added GTPγS (1 mM). Cells were fixed with glutaraldehyde and processed for electron microscopy. L, lysosomal compartments; PL, phagolysosomes. Bar, 0.5 μm.
20-nm colloidal gold particles were colocalized in the compartments containing phagocytic particles (Fig. 2C). These results are consistent with those obtained with the biochemical fusion assay (Fig. 1 A–C) and indicates that GTPγS inhibits fusion.

Rab Proteins Regulate Fusion between Phagosomes and Lysosomes—Since the removal of Rab proteins from membranes has been shown to inhibit Rab function (6, 13, 15), we tested the effect of Rab-GDI in the in vitro phagosome-lysosome fusion assay. Permeabilized cells were incubated with 10 μM Rab-GDI in the presence of GDP (100 μM) for 30 min at 25 °C. At this temperature, very little fusion was observed (data not shown). After treatment, cells were washed to remove Rab-Rab-GDI complexes and incubated for 45 min at 37 °C in the presence of Rab-GDI (10 μM) to allow fusion to occur. As shown in Fig. 3A, cytosol-dependent fusion was inhibited by about 60%. When different amounts of Rab-GDI were added to the assay, we found that as little as 5 μM Rab-GDI was effective (~10%) in blocking cytosol-dependent fusion (data not shown). Pretreatment with GDP alone did not inhibit cytosol-dependent fusion (Fig. 3A). Under the same conditions, we also measured the removal of Rab proteins from the permeabilized cells by Rab-GDI using Rab5 as an indicator. As shown in Fig. 3B, Rab5 was substantially released from membranes by Rab-GDI treatment in the presence of GDP but not in the absence of GDP.

NSF Is Required for Phagosome-Lysosome Fusion—To examine the functional involvement of NSF (N-ethylmaleimide (NEM)-sensitive cytosolic factor) in phagosome-lysosome fusion, we tested the effect of a monoclonal IgM antibody (4A6) that recognizes native NSF. When permeabilized cells were pre-treated with anti-NSF IgM antibody (100 μg/ml) in the presence of cytosol and ATP, cytosol-dependent fusion was almost completely inhibited (Fig. 4). The inhibition by anti-NSF IgM antibody was dose-dependent. Fusion was inhibited by about 40% by addition of 10 μg/ml anti-NSF IgM (data not shown). Addition of recombinant NSF wild type restored 50% of total fusogenic activity in anti-NSF IgM antibody-treated permeabilized cells. We have also observed that NSF-depleted cytosol did not substantially support fusion activity (39.2 ± 4.3% of total fusion activity), whereas control IgM-treated cytosol supported 80.7 ± 7.7% of total fusion activity. These results indicate that NSF is required for phagosome-lysosome fusion, as in other homotypic and/or heterotypic fusion processes (16–18).

**DISCUSSION**

Fusion between phagosomes and lysosomes is of interest particularly with regard to the mechanism by which pathogenic microorganisms survive inside phagosomes by interfering with membrane fusion (19). Generally, newly formed phagosomes fuse with endocytic organelles and lysosomes in vivo. In previous studies, we have shown that fusion of early phagosomes with early endosomes can be reconstituted in vitro using a cell-free system (2, 4, 6). Fusion of late endosomes with lysosomes (20) has been studied in vitro; however, neither the fusion of phagosomes with lysosomes nor its regulation has been investigated by in vitro reconstitution. Here we developed an in vitro reconstitution assay for phagosome-lysosome fusion using SL-O-permeabilized J774-E macrophages. The assay is based on the high affinity binding of avidin-HRP, previously loaded into lysosomes, to the surface of internalized particles bearing biotinylated-β-glucuronidase, a mannosylated glycoprotein internalized via the macrophage mannose receptor. We cannot completely rule out possible fusion of phagosomes with late endosomes; however, previous studies have shown that a 30-min pulse and a 70-min chase prior to permeabilization is more than sufficient to place the bulk of our probe in the dense lysosomal compartment (11). Morphological studies with colloidal gold confirmed the biochemical observations reported. A recent paper by Tjelle et al. showed that colloidal gold is transported to a lysosomal compartment in J774 macrophages (21). Their published images of gold-laden lysosomes are quite similar to ours.

Our results show that phagosome-lysosome fusion is dependent on energy and exogenously supplied cytosol, in agreement with requirements observed for other membrane fusion events (4, 9, 20, 22). Desjardins et al. and Blocker et al. have shown that transfer of lamp 2 or HRP (preloaded into lysosomal compartments) into phagosomes containing latex beads is inhibited when microtubules are depolymerized by nocodazole (5, 23). Our data show that nocodazole inhibits phagosome-lysosome fusion in vitro. In fact, nocodazole was a more potent inhibitor than ATPγS in our assay. This may be due, in part, to an effect of the drug on phagosome-lysosome fusion in the remaining intact cells. Since intracellular movement of some organelles occurs along microtubules (24), these results suggest that phagosomes also move along microtubules and that this movement is required for subsequent fusion with lysosomes.

When permeabilized cells were pre-incubated with GTPγS, phagosome-lysosome fusion was substantially inhibited, suggesting that one or more GTPases are involved in the process. Moreover, our data show that Rab-GDI inhibits fusion between phagosomes and lysosomes. Since Rab-GDI is known to inhibit several vesicular transport pathways by removal of Rab proteins from intracellular membranes (13, 15), this finding suggests the involvement of at least one Rab GTPase in phagosome-lysosome fusion. It is well known that Rab5 is localized
on early endosomes and functions in fusion among early endosomes (25–28). Rab7 is found on late endocytic compartments (29) and regulates transport steps between early endosomes and late endosomes/lysosomes (30, 31) and in yeast, between late endosomes and vacuoles (equivalent to lysosomes in mammalian cells) (32) or possibly fusion between vacuoles (17). Similarly, in the phagocytic pathway, Rab5 is present on early phagosomes and Rab7 is present on late phagosomes (5). In addition, it was recently demonstrated that Rab5 regulates early phagosome-early endosome fusion in an in vitro reconstituted cell-free system (6). All these observations indicate similar functional roles for each of the Rab proteins in both the phagocytic and endocytic pathways. However, further study is needed to clarify which Rab proteins are in fact involved in the fusion of phagosomes with lysosomes. NSF, an ATPase required for transport events at multiple stages of the secretory pathway (33–34), is also required for transport along the endocytic and phagocytic pathways. For example, fusion among early endosomes (9, 16, 36), between late endosomes and lysosomes (20), and between early phagosomes and early endosomes (4, 6) is inhibited by NEM or by antibodies against NSF, suggesting that these processes are regulated by NSF. In addition, recent studies using NSF mutants revealed that two distinct mutations in the first ATP-binding domain that affect either ATP binding or hydrolysis inhibit intra-Golgi transport (37) and fusion among early endosomes (18). In the present study, we provide evidence that hydrolysis of ATP is required for phagosome-lysosome fusion, suggesting the requirement for an ATPase. We also found that both NEM (data not shown) and antibodies against NSF inhibit fusion and that addition of recombinant NSF wt partially restores the fusogenic activity in the presence of antibodies. These results indicate that NSF is in fact required for phagosome-lysosome fusion. Since several lines of evidence suggest that NSF functions as a general component of membrane docking/fusion machinery together with SNAPs and SNAREs (38–39), it is possible that NSF-SNAP-SNARE complexes are involved in phagosome-lysosome fusion. In this regard, it has been shown that the SNAP25 proteins, syntaxin 2, 3, and 4, are present on the phagosomal membranes (35). Further studies, perhaps using in vitro fusion assays, may delineate the role played by the NSF-SNAP-SNARE machinery in phagosome-lysosome fusion.

In conclusion, we have developed a biochemical assay to reconstitute fusion between phagosomes and lysosomes in SL-O-permeabilized cells. Using this system, we found that fusion is dependent on energy and exogenously supplied cytosol and is inhibited by nocodazole, suggesting that fusion occurs via microtubule-dependent transport. In addition, rab proteins and NSF are required for fusion between phagosomes and lysosomes. The results indicate that phagosome-lysosome fusion requires components similar to that of other intracellular vesicular transport.

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