In Vitro Fusion of Phagosomes with Different Endocytic Organelles from J774 Macrophages*

(Received for publication, May 27, 1998, and in revised form, August 3, 1998)

Andrea Jahraus‡‡, Torunn E. Tjelle‡, Trond Berg‡, Anja Habermann‡, Brian Storrie‡, Oliver Ulrich‡‡, and Gareth Griffiths‡‡

From the§Cell Biology Programme, European Molecular Biology Laboratory, Postfach 10 2209, D-69012 Heidelberg, Germany, the University of Oslo, MCB, P.O. Box 1050, Blindern, N-0316 Oslo, Norway, the Department of Biochemistry, Virginia Tech, Blacksburg, Virginia 24061-0308, and **Institut für Biochemie, Universität Mainz, Becherweg 90, D-55128 Mainz, Germany

We describe novel biochemical and electron microscopy assays to investigate in vitro fusion of latex bead phagosomes with three different endocytic organelle fractions from J774 macrophages. After formation, early phagosomes fuse avidly with early and late endosomes and for a longer period of time with lysosomes, but they subsequently become fusion-incompetent. The fusion of early, not late, phagosomes with all these endocytic fractions could be significantly stimulated by Rab5. In contrast to other cell types investigated, this Rab is uniquely enriched on both early and late endosomes in J774 macrophages. Moreover, exogenous Rab5 stimulates homotypic fusion between both sets of organelles. This was shown by a quantitative electron microscopy fusion assay that can directly assay fusion between any combination of morphologically defined organelles. By the same approach, we discovered an unexpected Rab5-stimulatable fusion between early and late endosomes in J774, but not in BHK cells. Thus, in J774 cells both Rab5 and the endocytic pathway seem to have evolved additional functions not yet seen in nonphagocytic cells.

Phagocytosis is a process crucial for immune defense, which involves the uptake of particles larger than 0.3–0.5 μm. It is used for such purposes as the clearance of pathogens by professional phagocytes (for reviews, see Refs. 1–3). These specialized cells are also able to take up particulate material such as carbon particles and asbestos and, in this way, remove potentially life-threatening material from the blood circulation. Macrophages and neutrophils, the quintessential phagocytes, have evolved a number of complementary mechanisms to kill invading pathogens, such as the oxidative burst (4), acidification of the newly formed phagosome (5, 6), and the fusion of this phagosome with endocytic organelles (7, 8). This fusion process provides access of digestive enzymes to the phagosome to digest the phagosomal content. The endocytic pathway appears to consist of up to four distinct sets of organelles through which endocytic tracers pass sequentially: the early endosome, the endocytic carrier vesicle (multivesicular body), the late endosome (prelysosomal compartment; rich in some recycling receptors such as the two mannose 6-phosphate receptors in some cells), and the lysosome (9–13). The latter two sets of organelles are structurally distinct, although they are both highly enriched in lysosomal enzymes (14). Our knowledge of the pathways and mechanistic details of the fusion process between the newly formed phagosome and these endocytic organelles is still incomplete. Up to the late 1980s, the accepted model was that phagosomes interacted exclusively with organelles classified at that time as “lysosomes” (7, 15–17). More recent in vivo studies suggested that, after internalization into macrophages, 1-μm latex bead-enclosing phagosomes preferentially fuse with late endosomes (18). Additionally, in vitro studies have also shown that bacterial phagosomes can fuse with early endosomes (19–21).

Desjardins et al. (22, 23) provided evidence that, following internalization of 1-μm latex beads into J774 macrophages, phagosomes undergo a complex series of biochemical changes (summarized by Desjardins (24); see also Ref. 25). Of relevance to the present study is the fact that phagosomes acquire the Ras-related family of Rab GTPases, in particular Rab5 and Rab7, which are now accepted as being crucial in regulating membrane organelle docking and/or fusion events in the cell (26, 27). A number of studies have shown that Rab5 and Rab7 sequentially regulate transport in the endocytic pathway of cells such as BHK and HeLa (28–33). Rab5 has been localized in a number of cells to the early endosomes, to endocytic clathrin-coated vesicles as well as on the plasma membrane (28, 34). This protein somehow regulates both the rate of docking/fusion of clathrin-coated vesicles with early endosomes as well as the homotypic fusion of early endosomes with themselves (28, 30). While latex bead phagosomes reached an apparent equilibrium of fluid content mixing with late endocytic organelles after ~60 min, Rab5 and Rab7 could be detected on isolated latex bead phagosomes for at least 15 h (22, 35). In addition to the GTP-binding proteins, the spanning membrane glycoproteins LAMP-1 and -2, abundant constituents of both late endosomes and lysosomes (11), are also acquired by phagosomes (22, 35, 36). In our system, these molecules are present on the earliest latex bead phagosome stages studied and reach their maximal concentration on phagosomes between 6–12 h, thereafter remaining relatively constant in amounts on the phagosomal membrane (22). The fact that the phagosome is a newly formed vesicle that changes its biochemical composition with time argues strongly that this organelle is a structure that “matures” (8, 24), a phenomenon defined as an irreversible physiological change, akin to an aging process (14). We have argued that early and late endosomes are stable, preexisting compartments that do not mature (14, 37), although other views, preferring maturation phenomena, have also been proposed (38,
Phagosome Fusion with J774 Macrophage Endocytic Organelles

39. Desjardins (24) has recently summarized the accumulating set of data suggesting that phagosomes dynamically fuse with and physically separate from multiple elements of the endocytic pathway, a process he has termed “kiss and run” by analogy to the ideas on the exocytotic fusion pore (40, 41).

The complexity of the phagosome maturation makes it very difficult to dissect the highly complex molecular events that phagosomes undergo in vitro. Here we introduce novel in vitro assays to investigate the fusion between isolated phagosomes and endocytic organelles of J774 mouse macrophages. We have used 1-μm latex beads as markers for phagosomes. The advantage of using these polystyrene particles is the ease and purity with which phagosomes can be isolated (22). Moreover, due to the nondegradable nature of latex, phagosomes at any stage after bead internalization can be examined. We also took advantage of a novel procedure for isolating enriched fractions of early endosomes, late endosomes, and lysosomes of J774 macrophages, with a minimum of cross-contamination between the three fractions (Refs. 42 and 43 and this study). With these tools, we could study the fusion between phagosomes and endocytic organelles in a more accessible manner. While most of this study deals with an avidin-biotin biochemical assay that is based on an earlier in vitro system (44), we have also set up a complementary EM fusion assay that is a direct estimator of the fusion between any combination of endocytic organelles with themselves or with phagosomes. To our surprise, we found that the fusion properties of these endocytic organelles and the role of Rab5 in macrophages differed significantly from that in fibroblasts. We speculate that this is related to the many specialized functions of macrophages that seemingly require a hyperactive endocytic pathway.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of Cytosol—J774A.1 mouse macrophages were maintained as described by Blocker et al. (46). For phagosome preparation, 15 10-cm dishes of macrophages were plated into 6245 × 245-mm plates (Nunc) 1.5 days before the incubation with avidin latex beads. For preparation of endocytic organelles, cells were grown to subconfluence in 10-cm dishes (Falcon) when postnuclear supernatant extracts (PNS) were used for fusion assays we used three dishes, whereas for isolated endocytic organelles six dishes were used. For cytosol preparations, macrophages were grown as spinner cultures (see “Results”). After washing twice briefly with PBS and twice for 15 min with PBS, 5 mg/ml BSA on ice, a PNS was prepared (see above). For specific labeling of early endosomes or late endosomes/lysosomes, macrophages were pulsed for 5 or 30 min, respectively, with 1.7 mg/ml bHRP. Macrophages marked for late endosomes or lysosomes were further chased for 30 or 80 min, respectively, in chase medium after washing with PBS and PBS/BSA on ice. Either a crude PNS was prepared from these cells or isolation via gradients was carried out according to Tjelle et al. (43). The rationale for these fractions was as follows. Early endosomes were isolated by homogenizing macrophages that had been pulsed for 5 min with bHRP in HB containing a protease inhibitor mixture (50 μg/ml aprotinin, 0.5 μg/ml Leupeptin, 0.5 μg/ml Leupeptin, 0.5 μg/ml Aprotinin), 1 mg/ml dithiothreitol, and 5 mg/ml DNase I to avoid contaminating DNA in the early endosomal fraction after centrifugation. Then the PNS was mixed 1:1 with 80% metrizamide in PBS, placed at the bottom of an SW40 tube, and overlayed with 8.5 ml of 17% Percoll/HB and 500 μl of HB. After centrifugation at 56,000 × g for 45 min, the early endosomes were collected from the top of the self-forming gradient. Late endosomes and lysosomes were isolated from one set of macrophages using ovalbumin-conjugated gold particles (10 nm) to achieve a density shift between late endosomes and lysosomes. The gold was internalized into J774 for 3 h at a final A260 of 6 and chased overnight to trace the bulk of gold into the lysosomal compartment. The cells were homogenized in HB in the presence of protease inhibitor mixture and dithiothreitol (see above) and centrifuged at 750 × g for 7 min. The gold-containing fraction was found in the nuclear pellet, whereas the late endosome fraction stayed in the PNS. This PNS was layered into a SW40 tube onto 8.5 ml of 17% Percoll/HB with a 2-ml 50% sucrose cushion underneath in order to eliminate gold particles left over in the gradient. After centrifugation at 56,000 × g for 1 h, the late endosomes accumulated in a band in the Percoll gradient close to the sucrose cushion. The nuclear pellet with the gold-containing lysosomes was resuspended in 2 ml of 17% Percoll/HB and layered onto 2 ml of 64% sucrose cushion in a SW50.1 tube. A centrifugation at 40,000 × g for 30 min separated the gold-filled lysosomes, which pellets with the sucrose cushion, from the nuclei remaining on top of the gradient. Fractions were previously characterized by Tjelle et al. (43). PNS and/or fractions enriched in endocytic organelles were prepared by incubating peritoneal J774 macrophages for 3–4 × 107 μg bead. The number was determined by flow cytometry or light scattering at A405. Increasing amounts of beads resulted in a linear increase in light scattering.

Phagosome Fusion—Phagosomes were fed a final concentration of 0.05% of the stock preparations of avidin beads in IM under ambient conditions for indicated times at 37 °C. After two washes with PBS, cells were chased, if necessary, for the indicated times in either chase medium (minimal essential medium, 10 mM TES, 10 mM MOPS, 15 mM HEPES, 2 mM NaH2PO4, 35 mg/ml NaHCO3, pH 7.4) under ambient conditions for indicated times extended 1 h, cells were kept in culture medium at 5% CO2. In the case of 20-min pulse/10-min chase phagosomes as well as the 1-h pulse and 1-h chase phagosomes, the fusion results seen with the three endocytic fractions were the same when either chase medium or Dubbeco’s modified Eagle’s medium were used for the isolation of phagosomes. Phagosomes were isolated as described (22, 45). A concentration of phagosomes was achieved by performing a second gradient; the phagosome band was adjusted with 62% sucrose, 3% imidazole to 35%, overlaid with homogenization buffer (HB; 250 mM sucrose, 3 mM imidazole, pH 7.4), and centrifuged in a SW 60 rotor for 20 min at 100,000 × g at 4 °C. The bead number in the 55% HB interphase from the latter gradient was determined at A405, and phagosomes were aliquoted and rapidly frozen in liquid nitrogen in the presence of 0.1 mg/ml biotin-insulin (Sigma) to quench accessible avidin sites on the beads. After thawing, about 70% of the phagosomes were enclosed by an intact membrane. The high purity of the isolated phagosomes had been shown (23).
Phagosome Fusion with J774 Macrophage Endocytic Organelles

Biochemical in Vitro Fusion of Phagosomes with Endocytic Organelles—For the biochemical fusion between phagosomes and organelles of the endocytic pathway, we modified a cell-free assay for homotypic endosome fusion (44). J774 macrophage endosomes were in a nitrogen atmosphere defrosted overnight prior to fixation. Then cells were pulsed for 1 h with avidin latex beads and chased for 1 h. One set of cells was further incubated for 5 min with 5-nm BSA-gold to label early endosomes. In these cells, a single labeling with Rab5 antibodies was done, whereas for the other set of macrophages, double-labeling was done with LAMP-1 antibodies and Rab5 antibodies to identify late endosomes as LAMP-1-positive organelles that were free of 16-nm BSA-gold. For the immunogold quantification of Rab5 on phagosomes of different ages, J774 macrophages were fed with latex beads as described above, fixed, and prepared for cryosections that were labeled with the polyclonal Rab5 antibody. The labeling was quantified using intersection points as described (50).

EM in Vitro Fusion of Phagosomes and Endocytic Organelles—Isolated phagosomes and PNS fractions containing electron-dense gold particles of different sizes and/or HRP were used. BSA-gold was prepared according to Slot and Geuze (51). Labeling of two different early endosomes was carried out by incubating J774 macrophages or BMMs for 5 min with 5- or 10-nm BSA-gold (final A_dry = 5) in IM. Late endosomes were labeled by incubating cells for 30 min with 5- or 10-nm BSA-gold (see above), respectively, and chased for 30 min in Dulbecco’s modified Eagle’s medium. During the last 5 min of this chase, the cells were fed with 10 mg/ml HRP in IM, which enabled us to identify the early endosomes. After preparation of the PNS fractions, the protein concentration was determined. Rab5-GTP was added to the fusion mixture, which contained all ingredients of the biochemical assay except biotinylated insulin. Where indicated, Rab5-Rab GDI complex was added to the tube at a final concentration of 50 nM. Late endosomes were labeled by incubating J774 macrophages or BMMs for 5 min with 5- or 10-nm BSA-gold (final A_dry = 5) in IM.

Results

Preparation of Phagosomes, Early Endosomes, Late Endosomes, and Lysosomes—To study the fusion of organelles operationally defined as early endosomes, late endosomes, and lysosomes with phagosomes from J774 mouse macrophages, we established a cell-free system related to an earlier homotypic in

endosome fusion assay (44). J774 macrophage endosomes were in a nitrogen atmosphere defrosted overnight prior to fixation. J774 macrophages were fed with latex beads as described above, fixed, and prepared for cryosections that were labeled with the polyclonal Rab5 antibody. The labeling was quantified using intersection points as described (50). The reaction was sonicated (pulsed 15 times, using duty cycle 10%)

immunogold quantification of Rab5 on phagosomes of different ages.
**Phagosome Fusion with J774 Macrophage Endocytic Organelles**

*Vito* fusion assay for early endosomes (10). The basis of this assay is the use of an avidin-biotin detection system to monitor fusion between the phagosomes enclosing avidin-conjugated beads with different endocytic organelles containing hHRP. In J774 macrophages, HRP is taken up mainly (75%) as a fluid phase marker, and the remainder binds receptors such as the mannose receptor (54).

Different maturation stages of phagosomes were obtained by internalizing avidin covalently bound to beads into macrophages using pulse-chase protocols. The earliest time point of newly formed phagosomes used in the following experiments consisted of a 20-min pulse and 10-min chase. Phagosomes at later stages of maturation were prepared by 1 h of internalization, followed by a 1-, 3-, 6-, 12-, or 24-h chase, respectively. The cells were subsequently homogenized, and the phagosomes were purified by flotation as described by Desjardins et al. (22, 23).

For the preparation of labeled endocytic counterparts, we used two different approaches. First, we used PNS from cells in which bHRP was targeted to one or all of the operationally defined endocytic organelles. Second, for most of the studies we took advantage of a recent procedure developed for J774 macrophages for preparing enriched fractions of early endosomes, late endosomes, and lysosomes (see Ref. 43 and “Experimental Procedures”). An extensive characterization of these three operationally defined endocytic compartments has been described (42, 43). To be confident that the fractions of early endosomes, late endosomes, and lysosomes prepared by this novel procedure were indeed reproducible under our present conditions, we measured the kinetics by which HRP entered or egressed from the different fractions in two different experiments (Fig. 1A and B). In all conditions, HRP was internalized into macrophages for 5 min, washed extensively, and then chased for the indicated times. At each time point, early endosomes or late endosomes and lysosomes were isolated, and the HRP content of each fraction was quantified. After a 5-min pulse, the HRP was predominantly in the early endosome fraction (Fig. 1A), and only 7.8 or 2.8% of the bulk of HRP was found in the late endosome fraction. Whereas the percentage of HRP in the late endosome pool increased for the following 45 min of chase and then decreased, the marker accumulated steadily in the lysosomal fraction with increasing chase times (Fig. 1B). With continuous chase, this marker was transported out of the early endosomes, leaving an average remaining level in that compartment of 7% after 45 min (Fig. 1A). After 15 min of chase, the HRP began to fill the late endosome fraction as well as the lysosome fraction. Whereas the percentage of HRP in the late endosome pool increased for the following 45 min of chase and then decreased, the marker accumulated steadily in the lysosomal fraction with increasing chase times (Fig. 1B). An additional experiment showed that less than 10% of biotinylated transferrin, a marker for early endosomes, was found in the late endosome fraction after 3 min of internalization (data not shown).

To further substantiate the quality of the three endocytic fractions, we assayed the early endosome markers transferrin receptor and EEA1 (55) as well as three lysosomal enzymes by Western blotting. As shown in Fig. 1C, the transferrin receptor and EEA1 were exclusively detected in the early endosome fraction, whereas the bulk of cathepsin S was found in the late endosome fraction. In contrast, cathepsin B and D proteins were in high concentrations in both late endosome and lysosome fractions. It should be noted that the bulk of all hydrolase activities is found in the lysosome fraction with two exceptions; cathepsin H was heavily enriched in the early endosome fraction by enzyme-linked immunosorbent assay, Immuno-EM, and its activity, while cathepsin S activity was predominantly found in the late endosome fractions (42). Thus, the early and late endosome compartments are reasonably well separated from each other and from lysosomes. Due to the lack of appropriate markers, we are unable to rule out the presence of a significant contamination of the lysosome fraction with late endosomes. In agreement with Tjelle et al. (43), and in contrast to all other cells examined, Rab5 is not restricted to the early endosomes in J774 cells (Fig. 1C; see below).

**Conclusion**—For the fusion assay, the fractions containing the bHRP-labeled endosomes or lysosomes and the avidin bead-containing phagosomes were mixed in duplicate on ice in the presence of an ATP regeneration or an ATP depletion system, cytosol, and biotinylated insulin in order to quench any signal that could result from broken phagosomes. After mixing, the reactions were brought to 37 °C for 80 min, a time at which kinetic studies showed the reaction was still not saturated. A
plateau was reached after 2 h (data not shown). After lysis of membranes with Triton X-100 and washing steps, using a flotation gradient, the enzymatic activity of bHRP bound to the same number of avidin beads was measured. The HRP signal from each sample was compared with known standard concentrations of bHRP in parallel with the enzymatic assay. Fusion was then expressed as ng of bHRP bound to $2 \times 10^7$ avidin beads, duplicates were averaged, and the variance was calculated. Although the absolute values of bHRP transferred to the avidin beads varied from experiment to experiment, the pattern of the results was always consistent within any one set of experiments.

As shown in Fig. 2, the fusion reaction between 1-h pulse/1-h chase phagosomes (“2-h phagosomes”) and a mixed endosome/lysosome population (40-min bHRP pulse) was dependent on the presence of cytosol from J774 cells with a maximum fusion signal at a protein concentration of 4 mg/ml. As expected, the reaction was also dependent on ATP, and no detectable fusion occurred at 4°C (data not shown). The use of increasing amounts of phagosomes or of bHRP-filled organelles led to a linear increase of signal, although at relatively high levels of bHRP input the background (−ATP) signal also increased (data not shown).

**Fusion Capacity of Phagosomes with Early Endosomes and Late Endosomes Declines Rapidly, while That with Lysosomes First Increases and Then Declines**—Phagosomes are newly formed organelles that interact with endocytic organelles and mature with time in the cell. After having established criteria for the labeling and purification of phagosomes, early and late endosomes, and lysosomes, we were now able to study the in vitro fusion capacity of phagosomes of different ages with the three different endocytic fractions. Prior to their enrichment on gradients, the endocytic organelles were loaded with bHRP in vivo, using internalization times established to get the maximum amount of tracer into the specific compartment (see Fig. 1, A and B). As shown in Fig. 3, phagosomes could specifically fuse with all three fractions. This result is in agreement with an experiment in which 2-h phagosomes were able to fuse with PNS fractions in which bHRP had accumulated in early endosomes, late endosomes, or lysosomes (data not shown). We emphasize that the magnitudes of these fusion values for the different endocytic organelles cannot be directly compared with each another, since we do not know the total surface area of membrane of the different endocytic organelles in these fractions; nor can we easily relate these parameters to their protein concentration. Nevertheless, our data suggest that, under our in vitro conditions, lysosomes are much less fusogenic than early and late endosomes.

A comparison of the extent of phagosome fusion with endocytic organelles over time showed that in all cases early phagosomes fused much more than did more mature ones. Moreover, the pattern of fusion was similar with both early and late endosomes in that the highest signal was obtained at the 30-min phagosome time point and, thereafter, the signal dropped such that after 4 h it was only slightly above background. In the case of lysosomes, a different pattern was seen. Here, the signal was initially low and rose to a peak at 2 h. Subsequently, fusion capacity dropped and was not detectable after 13 h. Although the fusion signal was (in magnitude) significantly less than that for the other two endocytic organelles, this pattern was consistently seen. This might be due to their high content of internalized gold that is used to separate them from endosomes (see Refs. 56 and 57).

Although avidin is a very protease-resistant molecule (58), we also considered the possibility that the loss of fusion signal
after a few hours was due to degradation of avidin in the hydrolytic environment of late phagosomes. To control for this, phagosomal membranes of different ages were solubilized with Triton X-100, and the naked avidin beads were exposed to the same bHRP concentration that was used in the in vitro fusion assay. Resolubilization of the beads and measurement of bound bHRP showed that up to 25 h after phagosome internalization there was no decrease in the bHRP binding capacity of the beads. This experiment also showed that under the conditions of our fusion assay the avidin binding sites on the beads were not saturated (data not shown).

Collectively, these data show that within the highly fusogenic period, phagosomes fused avidly with both early and late endosomes before they fused optimally with lysosomes. These de novo assembled organelles lost their capacity to interact with early and late endosomes after 4 h and with lysosomes at 13 h after their formation.

**Effects of Rab Proteins**—Before investigating the role of Rab GT-Pases, we first tested the poorly hydrolyzable GTP analogue GTPγS in the fusion assays. We found a dose-dependent stimulation when we preincubated membranes and cytosol with GTPγS (up to 50 μM final concentration) before and during phagosome-endosome fusion, whereas the immediate addition of ATP, which varied ±8.0% between the different concentrations of Rab GDI.

We next specifically investigated the role of a Rab5-Rab GDI complex in the fusion assay, which had earlier been shown to stimulate homotypic fusion of early endosomes in vitro in BHK cells (62, 63). We asked whether this complex could deliver measurable amounts of Rab5 onto purified phagosomes. After treatment of phagosomes for 20 min at 37 °C with 50 nM Rab5-Rab GDI followed by flotation of phagosomes, there was a 15-fold increase in the amount of exogenous Rab5 associated with phagosomes relative to endogenous Rab5 (Fig. 5). Surprisingly, phagosomes of all ages were able to recruit similar amounts of Rab5, including the fusion-deficient older ones. In contrast to phagosomes, control fish skin gelatin-coated beads (without membranes) treated in the same way with Rab5-Rab GDI did not recruit Rab5 (Fig. 5A, lane fsg). Similarly, Horiuchi et al. (64) have shown that clathrin-coated vesicles could specifically recruit Rab5 from its complex with GDI, whereas control microsomal membranes could not.

The Rab5-Rab GDI complex was next tested in the fusion assay between phagosomes of different ages and the three different endocytic organelle preparations. As shown in Fig.
6A, the fusion of both 30-min and 2-h phagosomes with early endosomes was stimulated 1.6-fold by the complex; however, as before, there was no significant fusion of 25-h phagosomes with early endosomes, nor was there any significant stimulation by the Rab5-Rab GDI complex, despite the ability of these membranes to recruit exogenous Rab5 (Fig. 5A). In a series of experiments, the range in stimulation was 1.6–4-fold over control. In addition, in some experiments a slight stimulation of the fusion of early endosomes with 25-h phagosomes could be observed (data not shown). With late endosomes, the situation was similar, a 2–4-fold stimulation was seen between this fraction and two early time points of phagosomes (Fig. 6B). In the experiment shown in Fig. 6B, we also detected a low level of fusion between late endosomes and 25-h phagosomes after the addition of the Rab5-Rab GDI complex. However, this stimulation over the background basal level of fusion was not always observed (results not shown).

A significant stimulation of the fusion of two early stages of phagosomes, but not the 25-h ones, with lysosomes was observed in the presence of Rab5-Rab GDI (Fig. 6C). Since some of the internalized gold particles that we used to density-shift the lysosomes do not leave the late endosomes, even after an overnight chase (see Fig. 7 and Ref. 18), we suspect that some of this fusion stimulation signal may be due to a contamination of the lysosomal fraction with the Rab5-enriched late endosomes (see Figs. 1C and 7). Again, due to the lack of availability of specific lysosome markers, we are unable to quantify the extent of this possible contamination.

We also investigated whether exogenous Rab5 might give rise to larger fusion profiles in vitro, as seen under in vivo conditions when either the wild type form of Rab5 or more strikingly when the GTP mutant is overexpressed (28, 33, 63). To evaluate this possibility, following the fusion of early phagosome with HRP-filled early endosomes, we quantified the average profile size of early endosomes by thin section electron microscopy and a point-counting stereological approach. If membrane organelles fuse, their average volume and, thus, their average profile size in thin sections must increase. No significant change was observed in the average area of these HRP-containing fusion profiles (an estimator of organelle volume), either in the presence or absence of either ATP or Rab5 (data not shown). Since fusion is occurring but the organelles are not increasing in volume, this finding argues that fusion must also be taking place under our in vitro conditions, consistent with the idea of a kiss and run fusion process (24).

In a series of parallel experiments with Rab7-Rab GDI complexes, we saw no functional effects on phagosome fusion with endocytic organelles, although Rab7 could also be efficiently recruited to phagosomes of all ages from a complex of Rab7 with Rab GDI (data not shown).

**Detection of Rab5 on both Early and Late Endosomes on J774 Macrophages—** Rab5 has been considered, until now, to function exclusively in the early endocytic transport loop, having been detected only on the plasma membrane, clathrin-coated vesicles, and early endosomes in fibroblasts and many other cells (28, 30, 34). The stimulation of the fusion of early phagosomes with late endosomes by Rab5 suggested that Rab5 might be localized to late endosomes as well as to early endosomes in J774 cells, although it was also possible that the fusion stimulation we measured was exclusively due to the presence of Rab5 on the phagosomal membranes. In the study by Tjelle et al. (43), Rab5 was indeed detected by immunoblotting on both early and late endosome fractions of J774 cells. We therefore investigated in more detail the localization of Rab5. As shown in Fig. 1C, endogenous Rab5 was detected on all three endocytic organelle fractions by quantitative Western blotting. As expected, the highest concentration of Rab5 was found in the early endosome fraction. Relative to this pool (100%) the late endosomes contained 57%, while the lysosomes had 18%. The latter is clearly an upper limit, since we expect that some of this signal reflects some contamination of the lysosomes by the late endosomes (see above).

To provide more direct data on the localization of Rab5, we used immunogold labeling of thawed cryosections of J774 macrophages selectively labeled with different markers in order to identify the different endocytic compartments (see “Experimental Procedures”). The data obtained by Western blotting were quantitatively confirmed by this EM experiment; Rab5 was specifically localized to early endosomes as well as late endosomes and phagosomes (Fig. 7). A small amount of label was also seen over structures operationally defined as lysosomes (heavily labeled with internalized gold particles after an overnight chase), although we again emphasize that this marker (and the absence of other suitable markers) did not enable us to unequivocally distinguish late endosomes from lysosomes in these preparations.

**In Vitro Assay by Electron Microscopy—** The finding that Rab5 was detected at significant levels on late endosomes of J774 cells and the stimulation of phagosome fusion with late endosomes by this GTPase were unexpected, since previous studies (cited above) had failed to detect this Rab protein on late endosomes in other cell types. This raised the question of whether the Rab5 pool on late endosomes could regulate late endosome homotypic fusion in J774 cells. For this and related questions, we set up an EM fusional assay that could investigate not only phagosome fusion with endosomes but also homotypic as well as heterotypic fusion between any combination of these organelles. The strength of this approach is the ease with which different electron-dense markers can be selectively targeted to the different organelles as well as the fact that fusion events are monitored directly (see Fig. 8). The rationale here was to target different sizes of gold to either early endosomes (5-min pulse) or late endosomes (30-min pulse, 30-min chase) in two sets of cells from which PNS fractions were prepared. In our experience, when a marker is destined for late endosomes it may take considerable time to chase out all of this marker from the early endosomes (see, for example, Fig. 1A). In order to avoid misidentifying these early endosomes as late endosomes in thin sections, we also labeled the early endosomes of the same set of cells with HRP for 5 min before fixation; any profile containing the HRP reaction product and gold would then be classified as an early endosome. For all fusion assays, two sets of PNSs, each with one or two different markers, were mixed with 4 mg/ml cytosol, both in the presence and absence of ATP as well as of the Rab5-Rab GDI complex. Epon thin sections were then systematically sampled for the content mixing of the different markers; this is an unbiased procedure (50, 53). Examples for some of the different fusion events are shown in Fig. 8.

As shown in Table I, phagosomes could fuse with both early and late endosomes in a Rab5-stimulatable manner, in agreement with the biochemical results. Furthermore, even homotypic fusion of late endosomes seemed to be stimulated by Rab5 in this cell line, in contrast to the situation seen in parallel incubations with BHK membranes. To our surprise, using the EM assay, we also observed a significant level of ATP-dependent fusion between early and late endosomes in the macrophages (Fig. 8D and Table I), again in contrast to what we and others have observed using BHK cells (Table 1; see Refs. 10 and 65). The addition of exogenous Rab5 also stimulated this heterotypic fusion event in J774 cells but not in BHK cells. A striking finding, evident in Table I, is the roughly similar
some time points with and without exogenous Rab5 for fusion in the absence of ATP, which varied ±19.7% from the mean.

The extent of fusion between phagosomes and the different endocytic compartments differed according to phagosome age; phagosomes older than 4 h lost their capacity to fuse in vitro with both early and late endosomes, while their ability to fuse with lysosomes was lost after 13 h. These data are in agreement with a similar lowering of phagosome fusion capacity seen at late maturation stages in vivo (67). There was an interesting difference in the pattern of in vitro fusion seen between phagosomes with early and late endosomes versus their fusion with lysosomes. Whereas the reaction with early and late endosomes was highest for the youngest phagosome stages tested, the highest rate of fusion with lysosomes was significantly delayed, peaking at 2 h of phagosome formation. This is consistent with the notion that phagosomes mature due to the sequential interaction with endosomes and lysosomes (8, 24). Interestingly, some pathogens manipulate the “normal” phagosome routes to fusion. Of particular relevance here is the recent demonstration that some pathogen-enclosing phagosomes can fuse with late endocytic structures, whereas their fusion with late endocytic organelles is

DISCUSSION

In the present study, we have set up novel biochemical and EM assays to investigate the in vitro fusion of highly purified latex bead-enclosing phagosomes of different ages with highly enriched fractions of early endosomes, late endosomes, or lysosomes, which allowed us to characterize individual stages of phagosome fusion with endocytic organelles in more detail than earlier studies (19, 20). We report five significant and novel observations. First, phagosomes can fuse with all three endocytic organelles in vitro. Second, the extent of this fusion varied drastically with phagosome age, with organelles older than a few hours losing their capacity to fuse. Third, the fusion of phagosomes up to 4 h of age (but not 25 h) with both early and late endosomes, and possibly with lysosomes, was significantly stimulated by the addition of exogenous Rab5. Fourth, in contrast to all other cells examined, Rab5 is localized to both early and late endosomes of J774 macrophages. Moreover, the addition of exogenous Rab5 enhanced not only homotypic fusion between early endosomes but also that between late endosomes from J774 cells. Finally, we describe an unexpected fusion event between early and late endosomes in J774 macrophages whose magnitude could also be elevated by the addition of Rab5 GDI.

The extent of fusion between phagosomes and the different endocytic compartments differed according to phagosome age; phagosomes older than 4 h lost their capacity to fuse in vitro with both early and late endosomes, while their ability to fuse with lysosomes was lost after 13 h. These data are in agreement with a similar lowering of phagosome fusion capacity seen at late maturation stages in vivo (67). There was an interesting difference in the pattern of in vitro fusion seen between phagosomes with early and late endosomes versus their fusion with lysosomes. Whereas the reaction with early and late endosomes was highest for the youngest phagosome stages tested, the highest rate of fusion with lysosomes was significantly delayed, peaking at 2 h of phagosome formation. This is consistent with the notion that phagosomes mature due to the sequential interaction with endosomes and lysosomes (8, 24). Interestingly, some pathogens manipulate the “normal” phagosome routes to fusion. Of particular relevance here is the recent demonstration that some pathogen-enclosing phagosomes can fuse for an extended period with the early endosomes, whereas their fusion with late endocytic structures is
inhibited (35, 68, 69). In this context, we emphasize that our results here relate specifically to the use of phagosomes enclosing nonopsonized (and nonbiodegradable) latex beads, which in their early stages seem to follow the conventional routes by which pathogens are degraded. We expect that by varying the surface properties of particles one could modulate the intracellular behavior of phagosomes, as shown for example by the recent data of Oh and Swanson (70).

The Rab proteins are now known to play an important role in the still elusive process of membrane docking/fusion (71, 72). One of the best characterized members of this family is Rab5, which is a rate-limiting component for early endosome homotypic fusion (28, 33, 63), as well as for some bacterial phagosome fusions with early endosomes (19, 68). It is the fraction of membrane-associated Rab5-GTP that seems to determine the ability of clathrin-coated vesicles and endosomes to dock and fuse, and, in this process, Rab5 recruits its downstream effector rabaptin-5 to the membrane (62); the latter protein is, like Rab5, present in similar amounts by Western blotting on phagosomes of all ages (25), but its distribution relative to the endocytic organelles of J774 cells has not been analyzed. An unexpected result in the present study was the finding that the addition of Rab5-GDI complex could stimulate phagosome fusion with late endosomes and lysosomes as well as the fact that this Rab protein could be detected in significant amounts on late endosomes and to a lower extent on lysosomes of J774.

**FIG. 7. Immunogold labeling on thawed cryosections of J774 macrophages.** A and B, the cells were fed for 2 h with 16-nm BSA-gold followed by 1-h pulse/1-h chase with latex beads before fixation. The section was double-labeled with a polyclonal rabbit Rab5 antibody (10-nm gold; arrowhead) and a polyclonal rat LAMP-1 antibody (5-nm gold; small arrow). The 16-nm BSA-gold (large arrows) is predominantly in large aggregates in vesicular structures classified as lysosomes (L). These are labeled for LAMP-1 but not for Rab5. In contrast, the profiles of late endosomes show heterogeneous structures, also LAMP-positive, that have relatively low levels of dispersed 16-nm gold (large arrow) as well as significant labeling for Rab5 (arrowhead). Bars, 100 nm. C shows a quantitation of Rab5 given as the density of gold particles per linear profile of systematically sampled inner nuclear membranes (NIM), outer nuclear membranes (NOM), plasma membranes (PM), lysosomes (Lys), phagosomes (Phag), late endosomes (LE), and early endosomes (EE). The values represent the mean, and the error bars show the S.E.
macrophages both by immuno-EM and, in similar ratios, by quantitative Western blotting. In order to investigate the significance of this latter finding in more detail, we established an EM assay to monitor fusion. The advantage of this approach, which complements the biochemical assay, is that it enabled us to analyze the in vitro fusion between any combination of phagosomes and early or late endosomes directly and at EM resolution. Overall, the results obtained by this method were in excellent agreement with those obtained using the biochemical assay. Moreover, the results of the EM assays argued further that Rab5 has evolved additional functions in J774 cells downstream of the early endosomes. First, the addition of Rab5 stimulated not only phagosome-late endosome fusion but also late endosome homotypic fusion in the organelles from these cells. The latter fusion was not detected in BHK cells, which lack detectable Rab5 on late endosomes (34). Second, exogenous Rab5 was able to stimulate a previously unobserved heterotypic fusion between early endosomes and late endosomes in J774 cells but again not in BHK cells. The latter observation is consistent with earlier data using BHK, Madin-Darby canine kidney, and neuronal cells, in which early endosomes consistently failed to fuse with late endosomes (44, 65, 73, 74).

We cannot formally rule out the possibility that what we interpret as a direct early-late endosome fusion in J774 cells was the result of an in vitro vesicular budding and fusion event, as has been shown for BHK and other cells in vivo (37, 44). Indeed, both the budding of endosome carrier vesicles and their fusion with late endosomes have been reconstituted in vitro using these cells, albeit as two separate events (75). However, since we have not seen convincing evidence for the presence of endosomal carrier vesicles in J774 cells, while a profile of early and late endosomes can often be seen in very close apposition in vivo in EM thin sections of these cells (data not shown), we currently prefer the idea that in these cells early and late-endosome fusion is a direct event.

Fig. 8. Examples of the EM fusion assay. A, a fusion reaction between a PNS from one set of cells that had 5-nm gold in late endosomes and HRP in early endosomes with 2-h phagosomes. In this image, one phagosome is shown on the left that has acquired 5-nm gold by fusion of the phagosome with a late endosome, while the two phagosomes marked by stars have acquired the electron-dense HRP activity by fusing with an early endosome. Note that both of these phagosomes seem to be enclosed by the same membrane profile (large arrow). B and C, fusion between elements of the late endosomes (5-nm gold; arrows) with other late endosomes that are marked by 10-nm gold (arrowheads). The larger aggregate of gold on the right is probably within a lysosome. D, a fusion between an early endosome and a late endosome as seen by mixing of 5-nm (arrow) and 10-nm gold (arrowhead). On the left is an early endosome profile (star) that contains HRP reaction product as well as 5-nm gold (arrowhead) that is a consequence of homotypic early endosome fusion. Bars, 100 nm.
endosomes are capable of transiently fusing with each other, perhaps by a kiss and run mechanism. If this heterotypic fusion event also occurs in vivo, this process could serve to transiently, and perhaps locally, mix the luminal contents of the early and late endosomes. Such a transient mixing process, which was possibly already seen in vivo by Berthiaume et al. (76), could have important implications for many macrophage-specific functions such as antigen presentation (77).

Since macrophages are well known for their exceptionally well developed endocytic system (78–80), we assume that the unexpected roles for Rab5 and the unusual heterotypic early endosome fusion we have found in J774 macrophages are related to the functions of these highly specialized cells. Of all the Rab proteins, Rab5 seems to be especially well suited for the role of a molecular switch that turns other processes on or off. It has an extremely high intrinsic GTPase activity compared with other members of the Rab family and appears to undergo continuous cycles of GTP binding and hydrolysis on the early endosome membrane (71). Perhaps this is the reason why the highly dynamic and specialized endocytic pathway of macrophages requires a broader cellular distribution for a “fast” molecular switch such as Rab5. When cells are induced to overexpress the GTP form of Rab5 they produce abnormally enlarged vacuoles (33, 81). Interestingly, in BHK cells some of these Rab5-enriched structures also seem to contain Rab7, usually a late endosomal marker, which is found on phagosomes, as well as on early and late endosomes in J774 cells (Refs. 22 and 43; this study, data not shown). Thus, in both cell lines some mixing of early and late endosomes by direct fusion might occur under some conditions (81).

Two general conclusions can be drawn from our data. First, with the exception of the heterologous cell fusion data and the nonfusogenic late phagosomes, all organelles enriched in Rab5 are able to fuse well with each other. This is consistent with the notion that the Rab proteins need to be present on both fusion partners for their function, in agreement with the data of Haas et al. (82). Second, the mere presence of Rab5 on both fusion partners is not sufficient to drive the docking/fusion reaction, since late phagosomes possess Rab5 and more can be recruited from a Rab5-Rab GDI complex, but these organelles are nevertheless generally fusion-incompetent. Additional factors are obviously implicated. The reasons for these differences in fusogenicity between these different stages may well be related to the significant molecular differences found to exist between the early and late phagosomes, such as their levels of annexins IV and VI (83) or their phospholipid composition (22).

The latex bead phagosome system has proven to be a versatile system for many different in vitro approaches. In addition to fusion, we have established an approach to study both microtubule-associated protein (MAP)-dependent binding to, as well as the bidirectional motility of phagosomes along microtubules (46, 84). Moreover, phagosomes bind to F-actin, in part via a myosin (85), and reagents that affect the actin cytoskeleton have significant effects on phagosome fusion with endocytic organelles. We have recently monitored actin nucleation on phagosomes, and this process also shows fascinating maturation dependent alterations. The challenge we now face is to integrate all of this information into a comprehensive model that can explain how phagosome function and dynamics are coordinated in the J774 macrophage cell line.

**Acknowledgments**—We thank Marino Zerial for constructive criticisms, many suggestions, reagents, and generous support. We also thank Janis Burkhardt and Ariel Blocker for help in the early stages of this project. Further, we are indebted to Stephen Fuller, Bernard Hoflack, and Roland Le Borgne for constructive comments and help with the manuscript. Finally, we express our gratitude to Roland Le Borgne for the gift of anti-transferrin receptor antibody, Heidrun Kirschke for providing the anti-cathepsin antibodies, Harald Stenmark for the anti-EEA1 antibody, and Peter Scheiffele for all of the help with the PhosphorImager.

**REFERENCES**

1. Brown, P. J. (1995) BioEssays 17, 109–117
2. Greenberg, S., and Silverstein, S. C. (1993) in Fundamental Immunology (Paul, W. E., ed) 3rd Ed., pp. 941–964, Raven Press, New York
3. A. Jahraus, M. Egeberg, M. Cyrklaf, A. Habermann, H. Faulstich, D. Echner, A. Pralle, H. Hörber, H. Defaqua, and G. Griffiths, manuscript in preparation.
4. H. Defaqua, M. Egeberg, A. Habermann, M. Diakonova, C. Roy, P. Mangeat, W. Voeltner, G. Marriott, J. Pfannstiel, H. Faulstich, and G. Griffiths, submitted for publication.
