

Biogenesis of Phagolysosomes Proceeds through a Sequential Series of Interactions with the Endocytic Apparatus

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Abstract. We have examined the modifications occurring during the transformation of phagosomes into phagolysosomes in J-774 macrophages. The use of low density latex beads as markers of phagosomes (latex bead compartments, LBC) allowed the isolation of these organelles by flotation on a simple sucrose gradient. Two-dimensional gel electrophoresis, immunocytochemistry, and biochemical assays have been used to characterize the composition of LBC at different time points after their formation, as well as their interactions with the organelles of the endocytic pathway. Our results show that LBC acquire and lose various markers during their transformation into

phagolysosomes. Among these are members of the rab family of small GTPases as well as proteins of the lamp family. The transfer to the LBC of lamp 2, a membrane protein associated with late endocytic structures, was shown to be microtubule dependent. Videomicroscopy showed that newly formed phagosomes were involved in rapid multiple contacts with late components of the endocytic pathway. Collectively, these observations suggest that phagolysosome formation is a highly dynamic process that involves the gradual and regulated acquisition of markers from endocytic organelles.

PHAGOCYTOSIS is the process by which cells internalize large particulate material destined to be degraded by lysosomal enzymes. It is predominantly carried out by specialized cells of the polymorphonuclear family and monocytic lineage such as neutrophils and macrophages. This process can be divided into three distinct steps: (a) attachment of the particle to the cell surface, mediated by receptors at the surface of the cell; (b) engulfment, characterized by a flow redistribution of the plasma membrane to surround the particle; and (c) formation of a phagolysosome (Siverstein et al., 1989). The internalization process is complete when a single fusion event at the tip of the surrounded particle yields a membrane-bound organelle of plasma membrane origin, the phagosome, which transiently displays a pH above neutrality. Phagosomes are then transformed into functional phagolysosomes as they receive hydrolases and their internal pH decreases to five or below (Geisow et al., 1981). While it is still not yet clear if phagosomes acquire hydrolases by fusing with terminal lysosomes, recent studies suggest that phagosomes can fuse with late endosomes (Rabinowitz et al., 1992), and possibly with earlier endocytic organelles (Hart and Young, 1991; Mayorga et al., 1991; Pitt et al., 1992a).

We observed in a previous study that latex beads accumulate in compartments that displayed characteristics of late endosomes after long periods of internalization (Rabinowitz

et al., 1992). In the present study, we have characterized the process by which latex bead-containing compartments (LBC)¹ acquire late endocytic characteristics. The results obtained show that LBC are gradually transformed with time and transiently display a protein composition characteristic of late endosomes. Among these proteins are members of the rab family of small GTPases known to have regulatory roles in fusion processes (Pfeffer, 1992; Zerial and Stenmark, 1993), and integral membrane proteins of the lamp family that are localized mainly to late endocytic structures (Kornfeld and Mellman, 1989). Videomicroscopy observations suggest that these markers are transferred to the LBC through a highly dynamic process involving multiple contacts with endocytic organelles.

Materials and Methods

Cell Culture and Phagosomes Formation

J-774 mouse macrophages were grown in 10-cm dishes in DME supplemented with 10% fetal calf serum, 1% glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a 5% CO₂ atmosphere. The cells were used between passage 5 and 25.

The phagosomes (LBC) were formed by the internalization of latex beads (0.8 µm in diameter, 10% suspension, blue dyed; Sigma, St. Louis, MO), diluted 1:200 in culture medium (4 ml/dish) at 37°C. The internalization of the latex beads was done at 37°C and was followed by washes in PBS 0.01 M, pH 7.2, (3 × 10 min) at 4°C on a rocker. The chases were performed

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1. *Abbreviation used in this paper:* LBC, latex bead-containing compartments.

at 37°C in culture medium. Internalization and chase times varied depending on the experiments as mentioned in the figure legends. All pulses and chases were performed at 5% CO₂ unless otherwise indicated. The internalization times varied from 5 min to 1 h, while the chase times varied from 0 to 20 h.

Cell Fractionation

Isolation of LBC was performed using a modification of a method described by Wetzel and Korn (1969). After the final pulse and chase of latex beads, the cells were washed in cold PBS (3 × 5 min) and scraped with a rubber policeman in PBS at 4°C. The cells were pelleted and washed for 5 min in homogenization buffer (250 mM sucrose, 3 mM imidazole, pH 7.4) at 4°C (Gorvel et al., 1991). They were then pelleted again, resuspended in 1 ml of homogenization buffer, and homogenized on ice in a Dounce homogenizer with a tight-fitting pestle. The homogenization was carried out until about 90% of cells were broken without major breakage of the nucleus, as monitored by light microscopy. By this procedure we have estimated that over 90% of the content of fluids in the phagosomes is latent (Jahraus et al., unpublished data). Unbroken cells were pelleted in a 15-ml Falcon tube at 1,200 rpm for 5 min at 4°C and the supernatant, containing the LBC, was recovered. The LBC were then isolated by flotation on a sucrose step gradient (all sucrose solutions are wt/wt in 3 mM imidazole, pH 7.4) as follows. The LBC supernatant from five subconfluent 10-cm dishes (about 1 ml) was brought to 40% sucrose by adding the same volume of a 62% sucrose solution. This 40% sucrose supernatant was loaded on top of a 1-ml cushion of 62% sucrose. We then added 2 ml of 35% sucrose, 2 ml of 25% sucrose, and 2 ml of 10% sucrose solutions. Centrifugation was done in swinging bucket rotor (SW40; Beckman Instruments, Palo Alto, CA) for 1 h at 100,000 g at 4°C. The LBC band was collected from the interface of the 10 and 25% sucrose solutions and resuspended in 12 ml of cold PBS. The LBC were finally pelleted by a 15 min centrifugation at 40,000 g in an SW 40 rotor at 4°C.

Metabolic Labeling of LBC

Cells at ~50% confluence were first incubated 3 × 10 min at 37°C in low methionine culture medium (10% of the normal methionine content) to deplete the cells of methionine. The cells were then incubated for 15 h in low methionine culture medium supplemented with 400 μCi/dish of [³⁵S]methionine in 4 ml of medium (Amersham International, Amersham, UK). Internalization and chase of the latex beads were then performed for various time points in the presence of [³⁵S]methionine at 37°C. The cells were then washed with cold PBS and processed for cell fractionation as described above.

In some cases, after the 15-h metabolic labeling, the cells were washed 3 × 5 min in PBS and 3 × 5 min with normal culture medium at 37°C in the incubator. A chase of the [³⁵S]methionine was then performed by incubating the cells for 3 h in normal medium to empty ER and the Golgi of the labeled lamp proteins. LGPs, a group of proteins homologous to the lamps, leave the Golgi and reach their final destination after biosynthesis with a *t*_{1/2} of 30–45 min (Green et al., 1987; Arterburn et al., 1991). LIMP III, a rat protein related to lamp 1 has a *t*_{1/2} of about 60 min, and 80% of that protein is found in lysosomes after 2 h of synthesis (Barriocanal et al., 1986). Thus, a chase of 3 h should be sufficient to ensure that the bulk of radioactive lamps have left the Golgi. Phagosomes were then formed in these cells in normal medium as usual.

Two-dimensional Gel Electrophoresis

Separation of proteins according to their isoelectric point and molecular weight was done using high resolution two-dimensional (2-D) gel electrophoresis based on the method of Bravo (1984), as described previously (Wandinger-Ness et al., 1990; Huber et al., 1993). For every sample of a given experiment, the same amount of radioactivity was applied to the isoelectric focusing. The second dimension was performed on 15% SDS-acrylamide gels for 12 h as described previously (Huber et al., 1993). The gels were then fixed in 45% methanol and 7% acetic acid, and fluorographed using an autoradiography enhancer (Dupont, New England Nuclear Research Products, Boston, MA). Films to be compared were exposed for the same length of time.

Transfer to Nitrocellulose Blots and [³²P]GTP-overlay

GTP-binding proteins were visualized by overlaying with [³²P]GTP after separation by 2-D gel electrophoresis as described above, and transfer under renaturing conditions to nitrocellulose as described previously (Huber et

al., 1993). Briefly, the transferred blots were rinsed for 30 min in GTP-binding buffer (50 mM NaH₂PO₄, pH 7.5, 10 μM MgCl₂, 2 mM DTT, 0.2% Tween 20, and 4 μM ATP as competing substrate), and incubated in [³²P]GTP (1 μCi/ml, specific activity 2,903 Ci/mmol, 1 Ci = 37 GBq) for 2 h at room temperature. The blots were then washed for 60 min with several changes of GTP-binding buffer and air dried. The proteins which bound [³²P]GTP were visualized by autoradiography using Kodak Xomat Ar film with an intensifying screen, at -80°C for 12 to 36 h.

Immunoblots and Immunoprecipitation

The kinetic analysis of rab 5 proteins, rab 7 proteins, and lamp 2 proteins association with LBC fractions was performed using Western blot analysis. For each time point, the proteins from the same number of LBC (as determined by forward light angle scattering analysis in a flow cytometer) were lysed in Laemmli buffer and separated by SDS-PAGE. Proteins were then transferred onto nitrocellulose filters using standard procedures. Rab 5 protein was revealed using a monoclonal antibody (2A4 D12 No. 15) directed against a peptide from the COOH-terminal region (kind gift of Dr. A. Wandinger-Ness, Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL), while rab 7 protein was revealed using a rabbit polyclonal antibody (whole serum) against a COOH-terminal peptide (kind gift of Dr. M. Zerial, EMBL, Heidelberg, Germany) (Chavier et al., 1990). Lamp 2 was revealed using a rat monoclonal antibody (ABL-93, IgG2a ascites supernatant) (Chen et al., 1985). Lamp 2 from a metabolically labeled LBC fraction was also identified on 2-D gels after immunoprecipitation using standard procedures and the antibody described above.

Epon Embedding and Immunocytochemistry on Cryosections

For the study of the transfer of a fluid phase tracer from the late endocytic structures to the LBC, HRP (10 mg/ml in culture medium) was internalized in cells for 10 min at 37°C. The cells were then washed with cold PBS, and HRP was chased in culture medium for 90 min at 37°C. After washing in cold PBS, latex beads were continuously internalized for times ranging from 5 to 60 min in culture medium at 37°C. Cells were then washed in cold PBS and fixed in the dishes with 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 30 min. Samples were then processed to reveal HRP with the DAB reaction and embedded in Epon as described previously (Marsh et al., 1986).

The kinetic analysis of the appearance of lamp 2 on LBC was quantitated using immunocytochemistry on cryosections. For this, BSA-gold (bovine serum albumin tagged to 16-nm gold particles) prepared according to Slot and Geuze (1985) was internalized for 30 min at 37°C. The cells were then washed with cold PBS (3 × 5 min), and BSA-gold was chased overnight at 37°C. After washing with PBS, cells were allowed to internalize latex beads for 20 min followed by chases for various periods of time as described above. Cells were then washed with cold PBS, and detached from the dishes by adding 1 ml of EGTA (50 mM in PBS, pH 7.4) for 45 min at 4°C on a shaker. The cells were then collected in an Eppendorf tube and washed with cold PBS. The pellet of cells was then fixed overnight at 4°C with 8% paraformaldehyde in 250 mM Hepes, pH 7.4, and processed for frozen sectioning (Griffiths et al., 1984). Lamp 2 was localized on thawed cryosections using specific rat monoclonal antibodies (Chen et al., 1985), as described previously (Griffiths et al., 1984). Quantitative analysis of the labelings was performed by counting the number of gold particles per length of LBC membrane. This membrane was identified as being the one directly surrounding the latex beads.

To study the role of microtubules in the transfer of lamp 2 from late endocytic organelles to LBC, cells were treated the following way. Cells were allowed to internalize latex beads for 20 min in normal condition. The cells were then washed at 4°C for 30 min in PBS with or without 10 μM nocodazole, and the beads chased for 2 h at 37°C with or without nocodazole. As a control, some of the cells treated with nocodazole were allowed to recover by washing away the drug, and latex beads were then chased for 2 h in normal conditions. Cells were then processed for cryosections, and lamp 2 revealed as described above.

Biochemical Analysis of HRP Transfer to LBC and Enzyme Markers

The kinetic experiments described for EM were essentially repeated (using HRP at 2 mg/ml) and the transfer of HRP to LBC analyzed biochemically after cell fractionation and LBC isolation. In addition, in some experiments

latex beads were internalized first for 1 h and chased for 1 h, and cells were then allowed to internalize HRP for 5 min followed by various chase times. The HRP enzymatic activity was measured by spectrophotometry as described previously (Gruenberg et al., 1989), on each part of the gradient (bands and sucrose cushions). The amount of HRP activity on the first band (phagosomes fraction) was then divided by the sum of the values over the rest of the gradient. The total recovery of HRP activity recovered on the gradient was over 90% of the amount loaded.

β -hexosaminidase and galactosyl transferase activity were measured on each part of the gradient as follows. For β -hexosaminidase detection, 100 μ l of each sample was added to 400 μ l of 100 mM Na acetate, pH 4.8, 0.2% Triton X-100, 1 mM 4 methyl umbelliferyl β -N-acetyl glucosamine. The samples were incubated overnight at 37°C and the reaction was stopped by adding 750 μ l of 1 M Na carbonate. The product of the enzyme activity was then read with the fluorimeter (excitation 365 nm, emission 440 nm). Galactosyl transferase activity was measured as described by Bergeron et al. (1982).

Video Microscopy

For the video microscopy studies, the cells were used at low confluency to ensure maximum spreading. Cells were plated on glass coverslips 12–15 h prior to the experiments. Late endocytic structures were first labeled by the internalization of 16 nm BSA-gold (used at OD 4.0 at 255 nm) prepared according to Slot and Geuze (1985), for 30 min at 37°C. Cells were then washed in cold PBS (3 \times 5 min), and the BSA-gold was chased for 90 min at 37°C. Latex beads diluted 1:500 to 1:1,000 in "air culture medium" (Eagle's minimum essential medium, 4 mM NaHCO₃, 10 mM HEPES, 11 mg pyruvate/100 ml, 0.64% glucose, 2 mM L-glutamine) were added on ice for 30 min (to allow binding to the cell surface without internalization). The unbound beads were removed by gentle washes in PBS, and coverslips were mounted in a closed chamber for observation on the video microscope by bright-field as described previously (Parton et al., 1992).

Results

Our goal in the present work was to characterize the transformation of newly formed latex bead phagosomes into phagolysosomes both by biochemical approaches and by electron microscopy at the individual organelle level. A prerequisite for the biochemical analysis was the availability of a method to isolated pure LBC.

Isolation of LBC

To isolate LBC, J774 macrophages were incubated with latex beads for 30–60 min at 37°C to allow phagocytosis to occur. After bead internalization, the cells were washed and warmed for various periods of time. Cells were then homogenized, and the bead-containing compartments isolated at the interface of 10–25% sucrose by flotation on a discontinuous sucrose gradient using modifications of methods described earlier (Wetzel and Korn, 1969; Muller et al., 1980a). Thin section EM of the LBC fractions showed that the vast majority of the isolated beads in these LBC fractions were surrounded by a continuous and closely apposed membrane. No contamination by other organelles was observed in these EM preparations (see Fig. 3).

To analyze the protein composition of the LBC fractions, cells were metabolically labeled with [³⁵S]methionine for 15 h, and allowed to internalize beads for 60 min, followed by a 60-min chase in the absence of beads. High resolution 2-D gel electrophoresis showed that the polypeptide profile of the LBC (Fig. 1c) was significantly simplified when compared to the total membrane (Fig. 1b) and the postnuclear supernatant (Fig. 1a) fractions. There was a significant enrichment of a number of polypeptides in the LBC relative to the total membrane fraction. For example, an abundant pro-

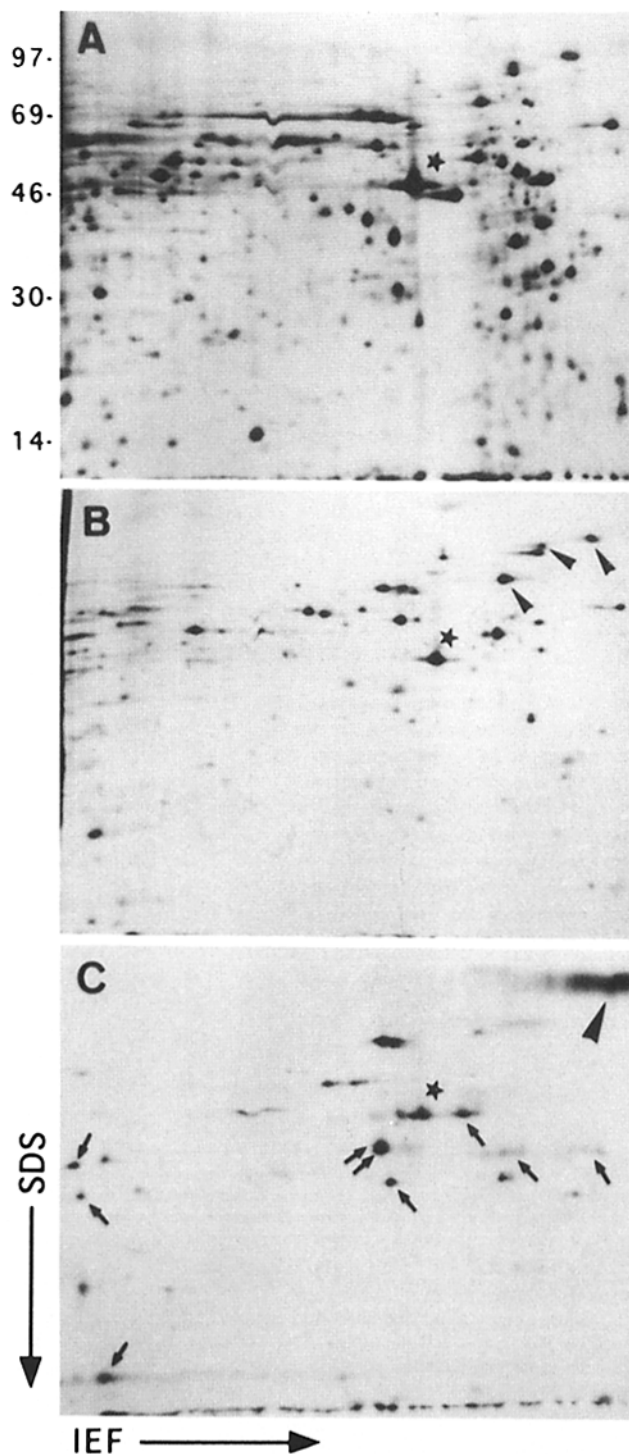
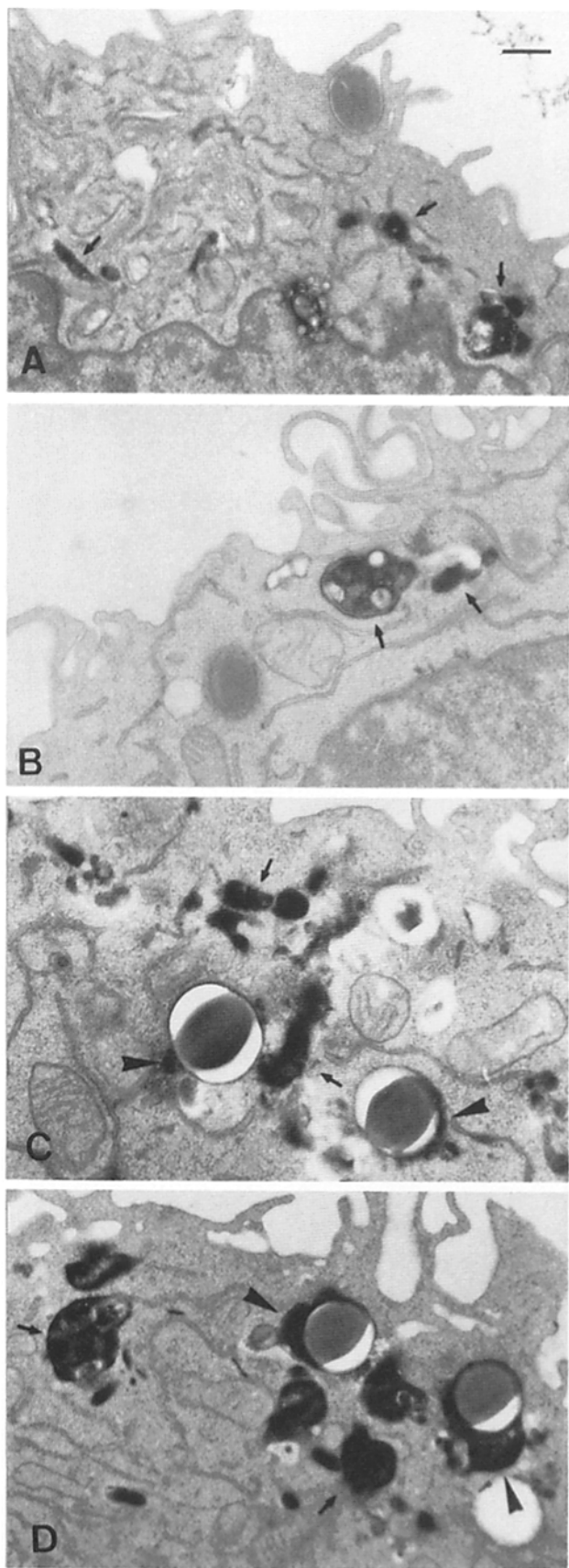


Figure 1. High resolution 2-D gel polypeptide pattern of post-nuclear supernatant (A), total cellular membrane fraction (B), and LBC (C) from cells that were allowed to internalize latex beads for 60 min followed by a 60-min chase. The amounts of radioactivity loaded on each gel were equivalent. The star indicates actin. (B) Arrowheads point at known ER proteins (from left to right, BiP, endoplasmic reticulum chaperone, and calnexin). (C) The arrowhead indicates the position of lamp 2, the major enriched protein in LBC fractions. Arrows show as yet unidentified proteins that are enriched in LBC fractions. The molecular weight markers are indicated on the left of panel.



tein in the LBC fractions was lamp 2 (Fig. 1 c), identified by immunoprecipitation, which was at the limit of detection in the total membrane fraction or PNS. To further assess the purity of these fractions, the protein profile of LBC was analyzed on 2-D gels for the presence of known markers of the endoplasmic reticulum, such as endoplasmic, BiP, and calnexin (Celis et al., 1992), which are common contaminants of other cellular fractions. Although these markers were present in the total membrane fraction (Fig. 1 b), they were undetectable in the LBC fractions (Fig. 1 c). The LBC fractions were also free of Golgi contamination as measured by galactosyltransferase activity (results not shown).

An important advantage of this flotation method is that the latex beads float the membranes enclosing them to a density on the gradient where no protein was detectable in the absence of beads. In addition, when cells were allowed to internalize the fluid phase marker HRP for various periods of time at 37°C in the absence of latex beads, no HRP activity could be detected at the interface between 10 and 25% sucrose. This shows that no endocytic structures are present in this fraction in the absence of beads.

Transfer of a Fluid Tracer from Late Endocytic Organelles to LBC

In order to investigate the interactions between late endocytic structures and newly formed phagosomes, we first analyzed the rate at which the fluid phase tracer HRP, preloaded into late endocytic structures, floats up with the LBC. Entry of HRP into LBC was assessed both morphologically and biochemically. For EM analysis, cells were allowed to internalize HRP for 10 min at 37°C, followed by a chase of 90 min in HRP-free medium to ensure that all the tracer has left the early parts of the endocytic pathway. Subsequently, latex beads were added and allowed to enter the cells for various periods of time ranging from 5 to 60 min before fixation, DAB cytochemistry, and embedding in Epon. EM clearly showed the dense product of the DAB reaction in pleiomorphic and vesicular structures close to the nucleus (Fig. 2). The latex beads internalized in these cells first appeared in LBC devoid of HRP (bead internalization times of 5 to 10 min) (Fig. 2 a and b). In contrast, after 30 min, the vast majority of the bead-containing compartments displayed HRP reaction product (Fig. 2 c). However, the apparent density of the HRP reaction product increased until a maximum was reached after 60 min of bead internalization (Fig. 2 d). EM of isolated LBC fractions after similar kinetic experiments confirmed these findings (Fig. 3). The HRP product

Figure 2. Electron micrographs showing a kinetic analysis of HRP transfer from late endocytic organelles to LBC. HRP was first internalized and chased to late endocytic organelles for 90 min. Latex beads were then internalized for various periods of time without a chase period. (A) 5-min pulse. A latex bead surrounded by pseudopodia is present at the cell surface. The electron dense reaction product of HRP is present in vesicular and tubular structures in the perinuclear region. (B) 10-min pulse. The latex bead (arrowheads) is inside the cell but not in the structures that react for HRP. (C) 30-min pulse. Most LBC contain HRP reaction product. (D) 60-min pulse. LBC are uniformly filled with HRP reaction product. In all figures arrows point at late endocytic structures filled with HRP, while the arrowheads indicate LBC-containing HRP. Bar, 0.5 μ m.

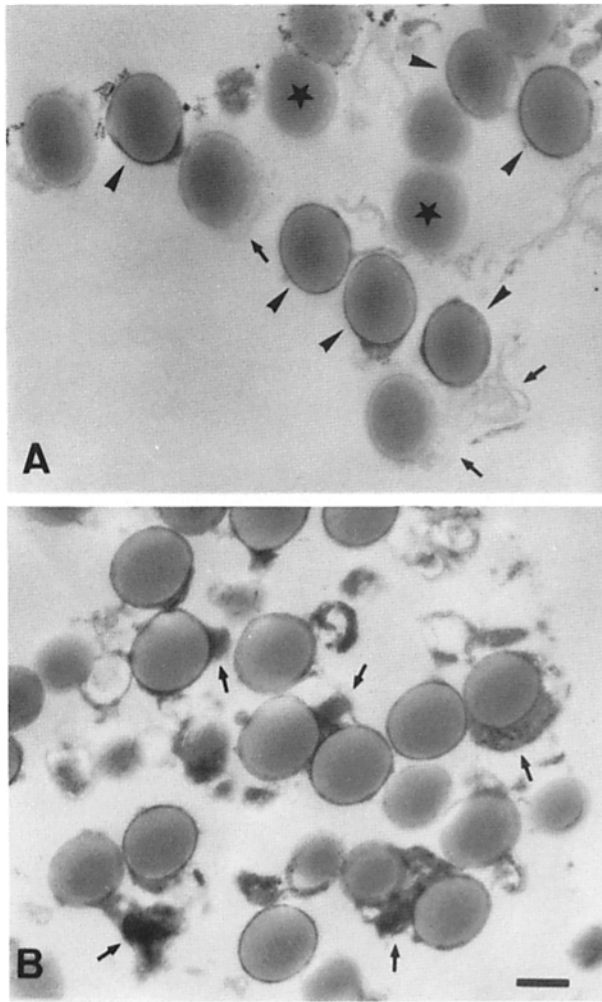


Figure 3. Electron micrograph of isolated phagosomes after HRP transfer from late endocytic organelles. HRP was first internalized and chased to late endocytic structures for 90 min. Latex beads were then internalized for 15 min (A), or for 15- plus 120-min chase (B). The LBC fraction of these cells was then prepared and processed for EM. (A) arrowheads indicate phagosomes displaying a ring-like HRP reaction product. The arrows indicate phagosome membrane outpockets unlabeled with HRP. The stars indicate latex beads not surrounded by reaction product. (B) arrows point at outpockets filled with HRP product. Bar, 0.5 μm .

was already associated with LBC after 15 min of bead internalization (Fig. 3 a). At this early time point, HRP was apparent in most of the LBC as a thin ring surrounding the latex bead while the outpockets of membrane associated with the LBC were not labeled (Fig. 3 a). In contrast, after 2 h of bead chase the reaction product of HRP completely filled the membrane-enclosed space around the beads (Fig. 3 b).

Quantification of the transfer of HRP from endosomes to the LBC population was then performed on isolated fractions. In one set of experiments HRP was first internalized for 10 min and chased for 90 min to the late endocytic compartments, all at 37°C. Latex beads were then internalized for 15–30 min and chased for various periods of time. The cells were then homogenized and the HRP activity associated with the bead compartments of each time point was determined after isolation on sucrose gradients. For each time

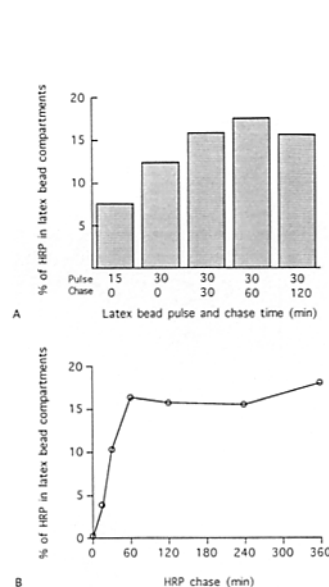


Figure 4. Biochemical analysis of the transfer of HRP from endocytic organelles to phagosomes. (A) HRP was internalized and chased to late endocytic compartments for 90 min. Latex beads were then internalized and chased as indicated. The amount of HRP in LBC fractions as a percentage of the total cell associated HRP was then determined. (B) LBC were formed by internalization of latex beads for 60 min followed by a 60-min chase. HRP was then internalized for 5 min by endocytosis and chased in culture medium as indicated. The amount of HRP in the LBC fractions was then determined as in A.

point, the compartments associated with the same number of beads, measured by FACS analysis (see Materials and Methods) were analyzed. The analysis showed that an increasing amount of HRP was transferred to the LBC fractions with time. A maximum of $\sim 20\%$ of the total internalized HRP was present in the bead-containing compartments after 60 to 90 min of bead internalization (Fig. 4 a). Alternatively, beads were internalized first for 60 min followed by a 60-min chase. Cells were then fed HRP for 5 min which was then chased for increasing times (Fig. 4 b). A maximum of $\sim 18\%$ of the total HRP loaded in cells was transferred to LBC fractions within 60 min after which the proportion of HRP associated with the LBC fraction remained constant up to 6 h. Thus, an apparent equilibrium between the fluid content of LBC and late endocytic organelles was reached after ~ 60 min. These results showed that LBC interact, presumably by fusion, with endocytic compartments. Other evidence that the luminal content of late endocytic organelles was transferred into LBC was also provided by the finding that $\sim 15\%$ of the total cell-associated β -hexosaminidase activity was detected in LBC fractions prepared from cells that had internalized latex beads for 60 min followed by a 60-min chase.

Kinetic Analysis of the LBC Protein Composition

To further investigate the modification of latex bead phagosomes during their transformation into phagolysosomes, kinetic experiments were performed to study their total protein composition using high resolution 2-D gel electrophoresis. Cells were metabolically labeled for 15 h, and bead uptake was done in the continuous presence of label. The results showed complex patterns of polypeptides at the various time points, in gels loaded with the same amount of radioactivity and exposed for the same periods of time. The exposures presented in Fig. 5 show only the most abundant proteins associated with the LBC fractions; at each time point studied, longer exposure revealed a number of additional spots (not shown). Most of the proteins observed in the earliest LBC fractions (30-min pulse of beads without chase) (Fig. 5 a)

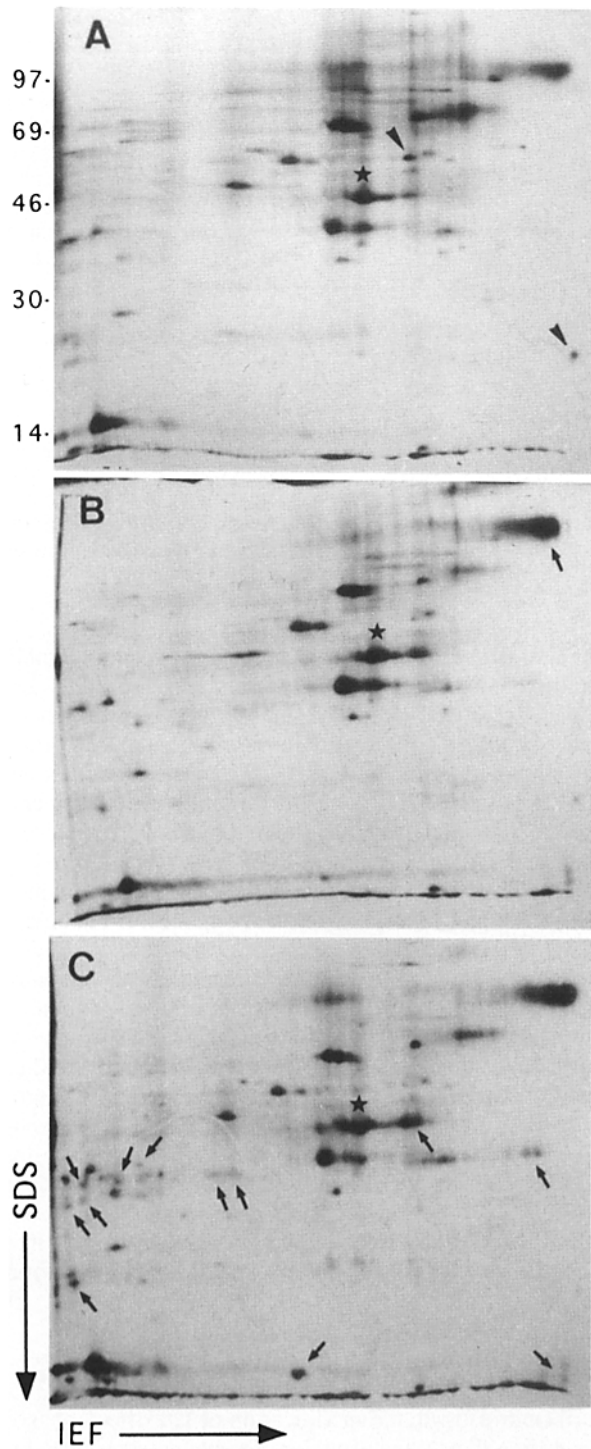


Figure 5. 2-D gel showing the polypeptide composition of LBC formed after 30 min of latex beads pulse (A), 60 min pulse and 60 min chase (B), and 60 min pulse and 6 h chase (C). The stars indicate the position of actin. (A) Arrowheads show proteins present at the earliest time but absent at the later time points. (B) The arrow shows lamp 2. In C the arrows indicate proteins appearing with time that are not detected at the earlier time points.

were also present at later times (60-min pulse, 60-min chase) (Fig. 5 b) (60-min pulse, 6-h chase) (Fig. 5 c), in agreement with previous reports (Muller et al., 1980a,b; Pitt et al., 1992b). However, LBC fractions at later time points dis-

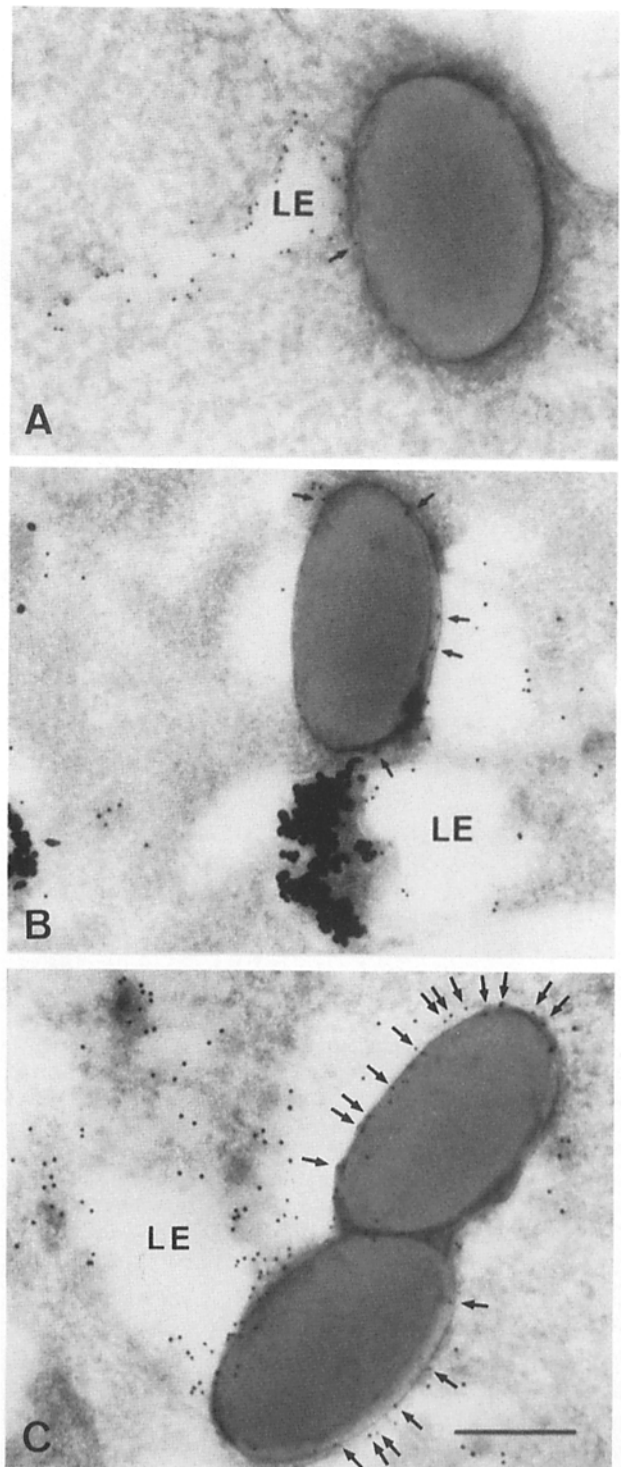


Figure 6. Thawed cryosections showing the localization of lamp 2 in J774 macrophages. BSA-gold 16 nm was internalized and chased overnight to label late endocytic structures. Thawed frozen sections were labeled with anti lamp 2 and 9 nm protein A-gold (arrows indicate some of the gold particles). Phagosomes were formed by a 20-min incubation with latex beads. (A) Cells fixed without a further chase. Note the absence of lamp labeling around the membrane surrounding the latex bead, while a profile of a late endosome that label strongly for lamp is in close contiguity with the latex structure. (B) After the beads were chased for 30 min, a significant amount of lamp labeling is detectable on the membrane around the beads. (C) After a 120-min chase, a strong labeling for lamp 2 is seen around the latex beads. LE, late endocytic organelles. Bar, 0.5 μ m.

Table I. Labeling Density of Lamp 2 over Latex Bead Compartments in Normal Cells and Cells Treated with Nocodazole

Latex bead pulse and chase	Density of labeling (gold particles/ μm length of membrane)		
	Normal cells	Nocodazole-treated cells*	Recovery after nocodazole†
20-min pulse, 0-min chase	0.9 \pm 0.2	ND	ND
20-min pulse, 30-min chase	1.9 \pm 0.4	ND	ND
20-min pulse, 60-min chase	2.7 \pm 0.4	ND	ND
20-min pulse, 120-min chase	6.0 \pm 0.6	1.4 \pm 0.1	3.8 \pm 0.3

* Latex beads were internalized in normal medium for 20 min and chased for 120 min in medium with nocodazole.

† Latex beads were internalized as in *, washed, and further chased for an additional 120 min in normal medium.

ND, not determined.

played an enrichment of certain proteins, including lamp 2 (Fig. 5, *b* and *c*) identified by immunoprecipitation (results not shown), and numerous new polypeptides, especially in the range of 35 to 55 kD (Fig. 5 *c*). These proteins remain to be identified.

Kinetics of Lamp Protein Acquisition by LBC

Changes in the content of lamp 2 during phagolysosome biogenesis were then examined in more detail. The lamp proteins are a family of highly glycosylated membrane proteins of ~100–115 kD of unknown function, which are abundant in late endocytic structures and absent, or in very low concentration, on the plasma membrane and early endocytic organelles (Kornfeld and Mellman, 1989). We could therefore assay lamp 2 in the LBC to follow the modification of phagosomes from the early stage, after their formation from the plasma membrane, to the stage where they acquire late endocytic characteristics.

The amount of lamp 2 associated with the LBC fractions increased with time, as shown by quantitative analysis of immunogold labeling on thawed cryosections of macrophages (Fig. 6 and Table I), and by Western blot analysis on isolated phagosomes formed after 0, 2, and 15 h of latex beads chase (Fig. 7 *a*). For the EM experiments, BSA-gold was preinternalized and chased for several hours prior to the latex beads internalization to facilitate the identification of the late endocytic structures. Under these conditions, this tracer distributes between the pleomorphic late endosomes and the spherical terminal lysosomes (Rabinowitz et al., 1992). At the earliest time point of latex beads internalization (20-min pulse without chase) (Fig. 6 *a*), labeling for lamp 2 molecules was associated with the membranes of pleomorphic late endosomes and lysosomes identified by the accumulated BSA-gold. Although, in many cases the newly formed LBC

appeared to be in close contact with lamp 2-labeled structures, at this early time point the membrane closely surrounding the latex beads showed very low labeling for this protein. With increasing chase times of latex beads, the density of labeling for lamp 2 increased on the LBC membrane (Fig. 6 *b* and *c*). The quantitative analysis of the labeling of the LBC membrane for lamp 2 showed a sixfold increase within 2 h of latex bead chase (Table I). Moreover, analysis of the distribution of labeling density for lamp 2 on individual profiles of latex bead-enclosing membranes also revealed that LBC acquired this protein in a gradual manner with time (results not shown).

We then assessed if the lamp proteins acquired by LBC came from late endocytic structures, as opposed to a direct transfer of newly synthesized proteins from the TGN. For this, cells were labeled with [³⁵S]methionine for 15 h, followed by a chase of 3 h, a period sufficient to empty the ER and Golgi complex of labeled lamp proteins (Barriocanal et al., 1986; Green et al., 1987; Arterburn et al., 1991). Latex beads were then internalized for 1 h and chased for 1 h in normal medium. The LBC were then isolated as described above, and their proteins analyzed by 2-D gel electrophoresis. Labeled lamp 1 and lamp 2, identified by their migration pattern on the gels (Chen et al., 1985), were present in the LBC fractions (results not shown). The identity of lamp 2 was further confirmed by immunoprecipitation (not shown). No noticeable decrease was observed in the levels of these proteins relative to cells in which the golgi pool was not depleted. These results suggest that a large amount of the lamp proteins are effectively transferred to the LBC from post-Golgi stations, the late endosomes and lysosomes.

GTP-binding Proteins Associated with LBC

Western blot analysis with an anti-rab 5 monoclonal antibody and a polyclonal anti-rab 7 antibody was performed on LBC fractions isolated at various time points. The results showed that rab 5 decreased continuously over 0, 2, and 15 h of latex bead chase (Fig. 7 *b*), while rab 7 increased between 0 and 2 h of chase and decreased between 2 and 15 h of chase (Fig. 7 *b*).

We next asked whether other GTP-binding proteins were associated with LBC fractions at various times after their formation using [³²P]GTP-overlay combined with high resolution, 2-D gel electrophoresis. The autoradiograms obtained showed a complex pattern of GTP-binding proteins in the various LBC fractions (Fig. 8). From these proteins, four could be identified by the coordinates of their position on gels, using as a reference the same proteins overexpressed in BHK cells (L. Huber, unpublished results). In addition to the

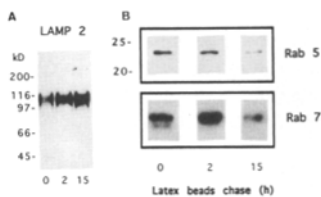


Figure 7. Western blotting of LBC fractions with (A) anti-lamp 2 antibody and (B) anti-rab 5 and rab 7 antibodies. Phagosomes were formed by internalization of latex beads for 20 min followed by further incubation without beads for

the indicated time. After fractionation, phagosomes were isolated and their proteins were separated by SDS-PAGE and transferred to nitrocellulose filters which were incubated with anti-lamp 2, anti-rab 5 or anti-rab 7 antibodies. The reaction was developed using enhanced chemiluminescence. Markers indicate molecular mass (kD).

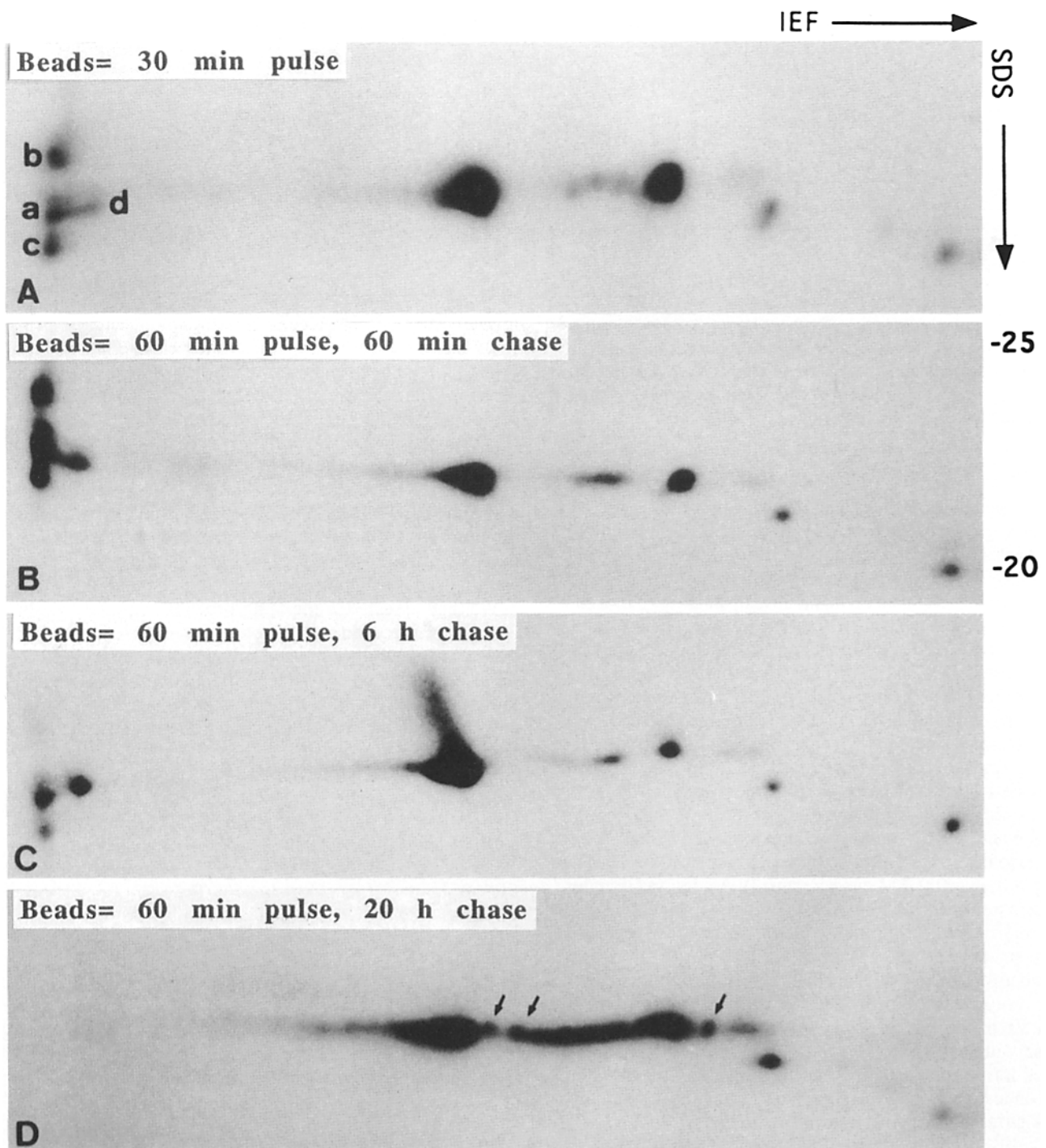


Figure 8. Analysis of GTP-binding proteins from phagosome preparations at various time points. Proteins were separated by 2-D IEF/SDS-PAGE, transferred onto nitrocellulose filters, incubated with [α - 32 P]GTP, and visualized by autoradiography. (A) Phagosomes formed after 30 min of latex bead internalization. (B) Phagosomes formed after 1 h of bead internalization followed by a chase of 1 h. In C the chase time was increased to 6 h and in D for 20 h. a, rab 5a; b, rab 5b; c, rab 5c; d, rab 7. Arrows indicate potentially new GTP-binding proteins associated with very late LBC fractions. The other prominent GTP-binding proteins that are seen at all times remain to be identified.

late endosomal marker rab 7, we could also identify different isoforms of rab 5. Rab 5 was shown to be localized to the plasma membrane, including clathrin-coated pits and vesicles, and the early endosomes (Chavrier et al., 1990; Bucci et al., 1992). Molecular cloning has revealed the existence of three different isoforms of rab 5, termed 5a, 5b, and 5c

(Chavrier et al., 1992) which appear to share the same localization and functional characteristics (Bucci et al., 1992; Bucci and Zerial, personal communication). All three isoforms of rab 5, as well as a relatively low amount of rab 7, were detectable on the LBC at the earliest time point (30 min of bead internalization without chase) (Fig. 8 a). After 30

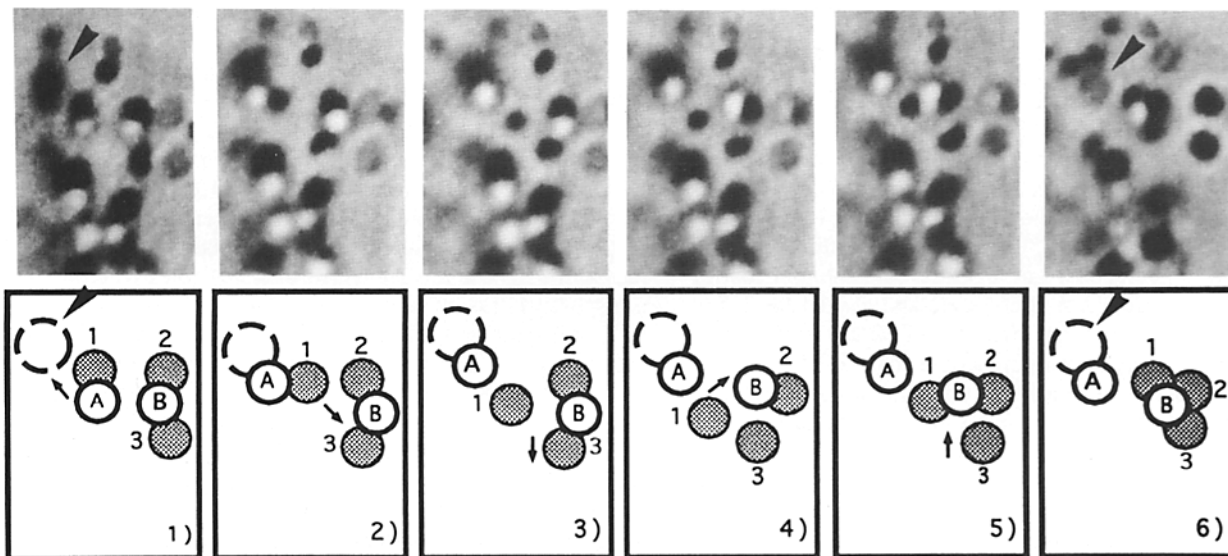


Figure 9. An example of a sequence of interactions between phagosomes and late endocytic organelles as seen by video microscopy. Upper panels show the perinuclear area of a cell incubated first with BSA-gold for 30 min followed by a chase of 90 min, and subsequently with latex beads for 30 min at 4°C to promote binding to the cell surface. The observations were then started at 37°C. The frames presented in the upper panel were recorded 30 min after the beginning of the observation and cover a period of 15 s. The lower panels show schematic representation of interacting organelles in part of these images. *A* and *B* are phase-lucent LBC. *1*, *2*, and *3* are gold-filled organelles. Arrows indicate the movement of structures between frames. Note the apparent attachment and separation of labeled structures, as well as the rotation of late endocytic organelles around LBC as seen in frames 5 and 6. Large arrowheads indicate a reference structure in both panels.

min of bead internalization followed by a chase of 1 h, rab 7 protein appeared to increase with respect to the rab 5 proteins (Fig. 8 *b*). When the chase time was increased up to 6 h, the signal for the rab 5 proteins (mainly rab 5 *b*) was significantly lower, while a relatively large proportion of rab 7 protein remained (Fig. 8 *c*). Finally, very late LBC fractions (20 h of chase) appeared devoid of rab 5 and rab 7 proteins by this approach (Fig. 8 *d*). At all time points, we failed to detect a polypeptide at the position expected for rab 4, another rab protein associated with endocytic organelles (see van der Sluijs et al., 1992). In addition to the known rab proteins two major GTP-binding spots as well as additional minor spots were visible at all times of bead internalization. The identity of these polypeptides remains to be elucidated. Moreover, at the longest time of bead chase (20 h), a time when neither rab 5 nor rab 7 could be detected on LBC, additional unidentified GTP-binding polypeptides appeared (Fig. 8 *d*).

Video Microscopy

Video microscopy was used to assess the nature of the interactions between LBC and endocytic organelles in living cells. It was technically not feasible in this approach to look at possible interactions of the LBC with early endocytic organelles since endocytic markers are only transiently occupying these compartments. However, we could easily label late endocytic organelles and look at their interactions with latex phagosomes. For this, BSA-gold was fed to cells for 30 min and chased for 90 min to load late endocytic structures (late endosomes and lysosomes). When viewed by bright-field microscopy, the structures containing the BSA-gold appeared as black, near-spherical organelles of relatively uniform size. Internalization of latex beads in gold-loaded cells

led to the formation of LBC that appeared as white refractile structures (Fig. 9). As is evident in Fig. 9, the two sets of markers could be easily distinguished from each other in all frames.

Time-lapse video microscopy over long periods of time revealed that both gold-filled and bead-filled structures made frequent contacts. Two aspects of this process are presented on Fig. 9. First, multiple contacts occurred between LBC and late endocytic structures. In some cases, the gold-labeled endocytic structures moved peripherally and made contact with the bead structures near the plasma membrane. Although on some occasions late endocytic structures appeared to be stably attached to LBC, in most of the cases these attachments were transient. The dense structures appeared to separate from the bead-containing structures and subsequently make close contact with other nearby LBC in a repetitive manner throughout the time course of our experiments. The second type of movement we observed was an apparent rotation of the gold-labeled structures around the surface of the LBC. Although the structures appeared to be in close contact, the resolution was not sufficient to determine whether fusion and transfer of content from late endocytic organelles to LBC occurred. Indeed, both structures appeared to remain distinct despite these contacts, and the late endocytic organelles were often able to detach from the LBC after the rotation. Collectively, these results argue that the interaction between LBC and late endosomes/lysosomes is highly dynamic.

Role of Microtubules in the Interactions between LBC and Late Endocytic Structures

We have shown in a previous study that the interactions between late endocytic organelles and LBC are dependent on the presence of a well-organized microtubule network in nor-

mal rat kidney (NRK) cells (Jahraus et al., 1994). Further, the movement of late endocytic organelles and LBC in macrophages was greatly inhibited when microtubules were depolymerized by nocodazole treatment (J. Burkhardt, unpublished results). Thus, we asked if the maintenance of a well-organized microtubule network was important for the transformation of LBC with time. To address this question we used immunocytochemistry to analyze the transfer of lamp 2 from late endocytic organelles to LBC, under normal conditions and in nocodazole-treated cells. Latex beads were internalized for 20 min in the absence of nocodazole and chased for 2 h in either the absence or in the presence of 10 μ M nocodazole. The quantitative electron-microscopic analysis of lamp 2 labeling showed that the amount of this protein was about five-fold lower on LBC formed after a chase in the presence of nocodazole, compared with control cells (Table I). The effects of nocodazole were reversible; the lamp 2 labeling increased significantly when nocodazole was washed away and the latex beads were chased for an additional 2 h in normal medium. These experiments demonstrate a role for microtubules in the transformation of phagosomes into phagolysosomes.

Discussion

Newly formed phagosomes have been shown to have a polypeptide composition indistinguishable from that of the plasma membrane (Muller et al., 1980a,b). However, within minutes, the composition of phagosomes is modified, both as a consequence of their interactions with organelles of the endocytic pathway (Hubbard and Cohn, 1975; Kielian and Cohn, 1980; Muller et al., 1980a; Pitt et al., 1992a,b) and of recycling processes (Muller et al., 1980b; Mayorga et al., 1991; Pitt et al., 1992a). Indeed, previous studies have shown that phagosomes can fuse with endocytic organelles, both in vivo and in vitro (Mayorga et al., 1991; Hart et al., 1991; Pitt et al., 1992a; Rabinowitz et al., 1992). However, the mechanisms of these interactions and the events leading to the formation of phagolysosomes are still poorly understood. In the present work we studied the changes which occur in the protein composition of latex bead phagosomes and the nature of their interactions with late endocytic organelles. The strength of our approach was to use complementary biochemical and morphological techniques to assess, at the population and the individual organelle levels, the nature of phagosome transformation with time.

Using a flotation method to isolate LBC we could show that a significant amount of tracers internalized into endosomes by fluid phase endocytosis was transferred to the LBC fraction. The kinetic analysis of this transfer was consistent with the notion that the fluid content of late endosomes and LBC completely intermixed within 60–90 min. That such a transfer occurred was also demonstrated by the acquisition of a significant amount of β -hexosaminidase by LBC. In contrast with the relatively rapid transfer of the luminal contents, the process of transfer of a membrane marker from late endocytic organelles to LBC required a considerably longer period of time (\sim 6 h). Previous studies showed that phagosomes start to acquire lamp proteins early after their formation (Joiner et al., 1990; Pitt et al., 1992 b). Our study extends these findings by showing that the transfer of these molecules takes place via an incremental process, over a

long period of time, as shown by quantitative analysis of individual latex bead organelles.

The complexity of the modifications of LBC with time and of their interactions with endocytic organelles was particularly evident in our analysis of the 2-D gel patterns and, in particular, in the GTP-binding proteins associated with LBC. Most of the proteins acquired by LBC with time are unknown and their role remains to be identified. One class of proteins we could identify was the small GTPases of the rab family. These are known to play key roles in vesicular transport and fusion processes, both in the biosynthetic and endocytic pathways (Hall, 1990; Gruenberg and Clague, 1992; Pfeffer, 1992; Schekman, 1992; Zerial and Stenmark, 1993). Until now, they have not been implicated in phagocytosis or in phagosome-lysosome fusion events. Our data indicate that, throughout their transformation, the LBC composition of GTP-binding proteins varies significantly. Rab 5 was present on LBC fractions from the earliest time point assayed. The presence of the three recently described isomers of this rab protein on the LBC membranes could most easily be explained by their acquisition from the plasma membrane during the internalization process. However, other markers that appear to be cointernalized from the plasma membrane with latex beads, such as the mannose, Fc, and transferrin receptors disappear within 30 min from phagosomal fractions (Pitt et al., 1992b), while rab 5 associated with LBC in our study for up to 15 h. This suggests that, rather than being a passive marker cointernalized with the latex beads, rab 5 may play a more important role in the biogenesis of phagosomes and phagolysosomes. Recent studies have shown that rab 5 regulates homotypic fusion events between early endosomes (Gorvel et al., 1991; Bucci et al., 1992). It is therefore conceivable that phagosomes could also fuse with early endosomes, as proposed by Stahl and colleagues (Mayorga et al., 1991; Pitt et al., 1992a,b). However, this process would have to take place without transfer to the LBC of rab 4, which has also been localized to early endosomes (van der Sluijs et al., 1991). This protein was not detected by GTP-overlay in our preparations, in spite of the high affinity of this protein for [³²P]GTP in the detection system that we use (van der Sluijs et al., 1992; Huber and Peter, submitted for publication). Clearly, further studies are required to assess the potential role of rab 5 during phagocytosis as well as the significance of the sequential loss of the rab 5 isomers from phagosomes.

The finding that a significant amount of rab 7, an established late endosome marker (Chavrier et al., 1990), was associated with the LBC confirmed our earlier immunocytochemical observations on peritoneal macrophages (Rabinowitz et al., 1992). In the present study, the kinetic analysis of rab 5 and rab 7 proteins strongly suggests that both are present on the same latex-enclosed membrane at certain times during phagosomes transformation. However, our attempts to show this point by immunolabeling were not successful, probably due to the relatively low endogenous amounts of these rab proteins in the macrophage cell line that we used.

At very late time points LBC lack detectable rab 5 and rab 7 proteins, but still display a high amount of lamp 2, indicating that LBC might somehow transform into terminal lysosomes, as proposed previously (Lang et al., 1988; Tassin et al., 1990; Montgomery et al., 1991). Indeed, lysosomes were shown to be devoid of rab 5 and rab 7 proteins, while

being enriched in lamp proteins (Kornfeld and Mellman, 1989; Chavrier et al., 1990; Rabinowitz et al., 1992). Nevertheless, at late time points, a number of other GTP-binding proteins were associated with the LBC. Although no GTP-binding proteins were detected on dense lysosome fractions by van der Sluijs et al. (1991), several were associated with the lysosomal fractions analyzed by Sai and Okhuma, (1992). While the significance of these differences is presently unclear, we speculate that the sequential appearance and disappearance of GTP-binding proteins on LBC reflects their role in regulating the timing and specificity of fusion events along the phagocytic pathway. We are currently testing the hypothesis that the loss of rab proteins at the late time points may correlate with a significant reduction in the rate of fusion between LBC and endocytic organelles.

An important clue about the cellular events occurring during the transformation of phagosomes into phagolysosomes came from our video observations on living cells. Although the resolution of this approach did not enable us to detect fusion events, the data showed clearly that multiple transient contacts occurred between LBC and late endocytic organelles throughout the time course of our observations. Despite resolution limitation, the video analysis was a crucial factor in convincing us that the biogenesis of phagolysosomes is extremely dynamic. Several observations indicate that the intracellular movement of these organelles and their interactions require microtubules. Chemicals and mycobacteria that inhibit phagosome-lysosome fusion also inhibit the saltatory movement of late endocytic organelles (Hart et al., 1983, 1987). In *Toxoplasma gondii*-infected cells, such inhibition is accompanied by a block in the transfer of lysosomal membrane glycoproteins to the phagosomes (Joiner et al., 1990). In a previous study we found that the interactions between lysosomes and late bead-containing organelles in NRK cells were dependent on microtubules (Jahraus et al., 1993). In the present study, preliminary video-microscopic observations suggest that the LBC movement and contact with late endocytic organelles are significantly decreased in nocodazole-treated cells. Consistent with this, the acquisition of lamp 2 by LBC was significantly inhibited by nocodazole, an effect that could be overcome, at least in part, when this drug was washed out.

Collectively, these results convinced us that the fusion events between phagosomes and late endosomes were more complex than we had previously imagined (Rabinowitz et al., 1992). In that study we envisaged that latex phagosomes fuse with late endosomes in a simple one-step fashion. In the latter model we would have expected, for example, both membrane and luminal markers to equilibrate rapidly after the fusion event. However, the data in the present study lead us to favor the alternative working hypothesis. Shortly after their formation, latex bead-containing phagosomes become competent to fuse, in a microtubule-dependent way, with late endosomes, as proposed recently for macropinosomes (Racoosin and Swanson, 1993). However, rather than proposing single one-hit fusion events, we envisage a more dynamic situation where these organelles are involved in transient fusion followed by rapid fission events. Such a mechanism has been proposed to explain the dynamic homotypic interactions observed between elements of both early and late endosomes (Gruenberg et al., 1989; Gruenberg and Clague, 1992). Moreover, incremental processes carried on

by multiple fusion-fission events could allow rapid mixing of luminal contents while limiting the diffusion of membrane proteins, and might provide a way to regulate the rate of intermixing of membranes that initially have distinct biochemical composition. A similar idea has been proposed previously to explain the differences in the rate of transfer of different soluble tracers from lysosomes to phagosomes (Wang and Goren, 1987).

In conclusion, we have shown that the use of latex beads as phagocytic markers can facilitate a molecular description of the interactions between the phagocytic and endocytic pathways. In addition to being more dynamic than previously appreciated, these interactions are especially interesting in that they display membrane traffic features expected of both vectorial and lateral fusion events.

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