

# Macropinosome Maturation and Fusion with Tubular Lysosomes in Macrophages

Esther L. Racoosin and Joel A. Swanson

Department of Anatomy and Cellular Biology and the Program in Cell and Developmental Biology, Harvard Medical School, Boston, Massachusetts 02115

**Abstract.** Macropinosomes formed by addition of recombinant macrophage colony-stimulating factor (rM-CSF) to mouse macrophages migrate centripetally and shrink, remaining detectable by phase microscopy for up to 15 min. This longevity allowed us to study how macropinosomes age. Macropinosomes were pulse labeled for 1 min with fixable fluorescein dextran (FDx10f), a probe for fluid phase pinocytosis, and chased for various times. To quantify changes in their antigenic profile, pulse-labeled macropinosomes of different ages were fixed and stained for immunofluorescence with a panel of antibodies specific for the transferrin receptor (TfR), the late endosome-specific, GTP-binding protein rab 7 or lysosomal glycoprotein A (Igp-A), and the percentage of antibody positive, FDx10f-labeled macropinosomes was scored. Some newly formed macropinosomes were positive for TfR, but few were rab 7 or Igp-A-positive. With intermediate chase times (2–4 min), staining for rab 7 and Igp-A increased to >60%, while TfR staining declined. After a long chase (9–12 min), rab 7 staining returned

to low levels while Igp-A staining remained at a high level. Thus, macropinosomes matured by progressive acquisition and loss of characteristic endocytic vesicle markers. However, unlike a maturation process, their merger with the tubular lysosomal compartment more nearly resembled the incorporation of a transient vesicle into a pre-existing, stable compartment. Shortly after their formation, FDx10f-labeled macropinosomes contacted and merged with Texas red dextran (TRDx10)-labeled tubular lysosomes. This occurred in two steps: macropinosomes acquired Igp-A first, and then several minutes later the cation-independent mannose-6-phosphate receptor (CI-MPR) and markers of lysosomal content (cathepsin L or pre-loaded TRDx10), all apparently derived from tubular lysosomes. Thus, macropinosome progress through macrophages showed features of both the maturation and vesicle shuttle models of endocytosis, beginning with a maturation process and ending by merger into a stable, resident lysosomal compartment.

**T**HE earliest descriptions of pinocytosis noted that pinosomes changed as they aged. Lewis (1931) observed by time-lapse microcinematography that macrophages actively formed large endocytic vesicles containing fluid from the surrounding medium. These pinosomes, later known as macropinosomes, formed at the cell margin, moved centripetally and shrank as they approached the nucleus. Addition of neutral red to the culture medium, a method now known to label acidic organelles, resulted in accumulation of the dye preferentially in older macropinosomes located near the nucleus. Later, Cohn and Benson (1965) found that pinocytosis could be stimulated in murine peritoneal macrophages by calf serum. As phase-bright macropinosomes moved towards the nucleus they became increasingly phase dense, and vesicles containing acid phosphatase “entered into” them. Taken together, Lewis’ and Cohn’s experiments suggested that macropinosomes gradually acidified and acquired lysosomal hydrolases.

Since then other endocytic organelles have been discov-

ered and characterized. Clathrin-coated vesicles internalize receptor-bound ligand by budding from the plasma membrane and fusing with, or fusing together to become, early endosomes (reviewed by Pearse and Robinson, 1990; Rodman et al., 1990; Wileman et al., 1985). In early endosomes, many ligands dissociate and are sorted away from their receptors (Dautry-Varsat et al., 1983; Mellman et al., 1986), which in some cases return to the cell surface. Some endocytosed material is transferred from early endosomes to late endosomes (or pre-lysosomes) (Schmid et al., 1988; Stoorvogel et al., 1991), which have been described as multivesicular compartments bearing receptors, ligands, and other solutes destined for degradation (Griffiths et al., 1988). Some sorting occurs in the late endosomes as well. Finally, the classic lysosome provides the terminal compartment of the endocytic pathway, where proteins and other macromolecules are degraded by acid hydrolases (reviewed by Kornfeld and Mellman, 1989). Early endosomes, late endosomes, and lysosomes can be distinguished by their con-



**Table I. Antigenic Markers for Endocytic Organellar Proteins**

Antigen	Antibody	Reference
TfR	R17 217.1.3 (hybridoma-conditioned medium)	Lesley et al., 1984 (American Type Culture Collection)
rab 7	Affinity-purified rabbit antiserum	Chavrier et al., 1990
CI-MPR	Affinity-purified rabbit antiserum	MacDonald et al., 1989
lgp-A	Rat mAb	Rabinowitz et al., 1992
lgp-B	Rabbit antiserum	Granger et al., 1990
Cathepsin L	Affinity-purified rabbit antiserum	Dong et al., 1989

kothen, Germany) equipped for phase-contrast optics and for epifluorescence, using Texas red and fluorescein filter sets. Using a 100× objective lens, 35 to 50 macropinosomes (in 20 to 40 cells) were examined for each time point. Each quantitative experiment was performed in entirety at least three times. An FDx10f-labeled vesicle was considered a macropinosome if it was also phase-bright. Next, the corresponding antibody-labeled image was examined using a Texas red filter set, and the macropinosome was scored positive if it labeled uniformly with Texas red. Macropinosomes only partially labeled with antibodies were scored as negative. Pulse-labeling experiments were repeated at least three times for each antibody. Double-immunolabeling experiments were scored similarly, except that antibody-labeled vesicles were first checked for coincidence with a phase-bright vesicle, then were checked for the other antibody. All micrographs were taken using T-MAX 3200 film (Eastman Kodak, Rochester, NY) at ASA 1600 or 3200.

## Results

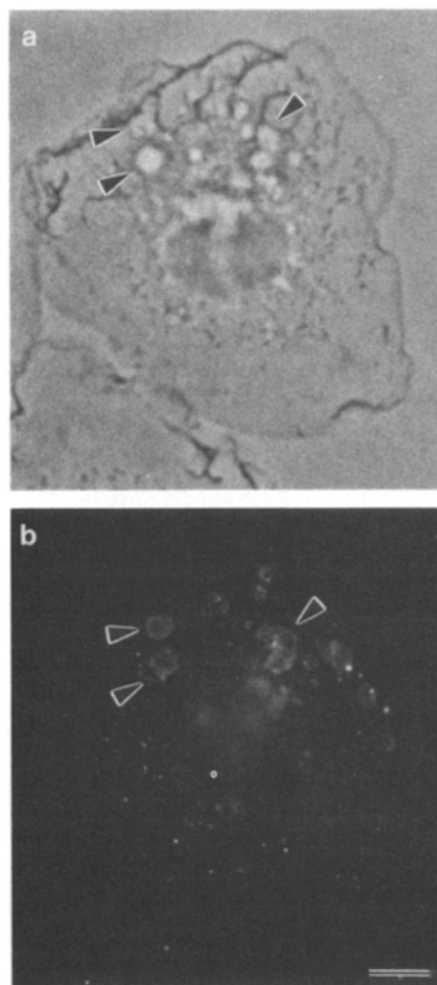
### Pulse Labeling of Macropinosomes

Addition of rM-CSF to macrophages previously starved of the growth factor stimulated the formation of phase-bright macropinosomes. They formed most often at the cell margin, and shrank as they moved centripetally (Racoosin and Swanson, 1992). To follow the changes that occurred after their formation, we labeled macropinosomes by incubating macrophages briefly in medium containing rM-CSF and fixable fluorescein dextran (FDx10f), a probe for fluid-phase pinocytosis (Fig. 1). FDx10f-labeled, phase-bright macropinosomes ( $\geq 1\text{-}\mu\text{m}$  diam) occurred at both the margins and the interior of the cell. FDx10f also labeled smaller pinosomes (Fig. 1 *b*) that, in contrast to macropinosomes, were not phase bright.

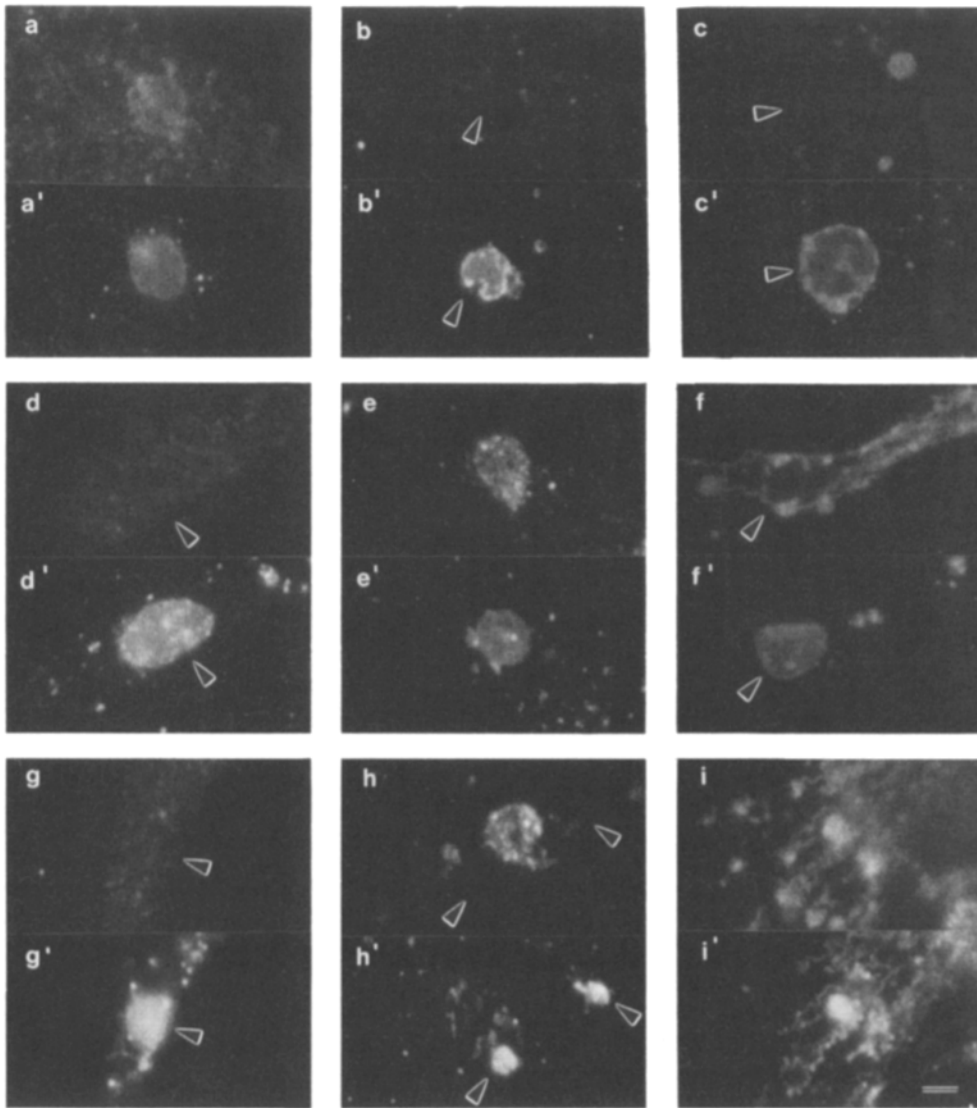
### Macropinosomes of Different Ages Exhibit a Changing Profile of Endocytic Vesicle Membrane Proteins

Macropinosomes have features of both early and late endosomes (Racoosin and Swanson, 1992). Previously endocytosed fluorescein-labeled transferrin is sorted from macropinosomes early after their formation, and previously endocytosed diI-labeled acetylated low-density lipoprotein stays within the macropinosome as it is transported to the lysosome for degradation. We next asked if, after their formation, macropinosomes acquire membrane proteins characteristic of other endocytic compartments. Macrophages were pulse labeled with medium containing FDx10f+ rM-CSF for 1 min, and then were washed rapidly and chased for increasing times in the absence of probe and in the continuing presence of rM-CSF. Cells were then fixed and prepared for immunolabeling with one of several antibodies (Table I). Three of the antibodies used were directed against the murine transferrin receptor (TfR), the ras-like GTP-binding

protein, rab 7, and the lysosomal glycoprotein lgp-A; these markers are characteristic of early endosomes (Yamashiro, et al., 1984), late endosome (Chavrier et al., 1990), and lysosomes (Chen et al., 1985), respectively. After a 1-min



**Figure 1.** Macrophages incubated in medium containing fluorescent dextran and M-CSF form fluorescent, phase-bright macropinosomes. Macrophages cultured on glass coverslips were incubated at 37°C for 1 min in medium containing 1.2 mg/ml fixable fluorescein dextran (FDx10f) and 3,000 U/ml M-CSF. Cells were then chilled rapidly on ice, washed with cold buffer lacking FDx10f, fixed, and prepared for microscopy. Phase-contrast (*a*) and fluorescence (*b*) micrographs were taken of the same cells. Phase-bright, fluorescent macropinosomes are indicated with arrowheads. Smaller FDx10f-labeled micropinosomes evident in *b* (punctate fluorescence) are not phase-bright. Bar, 5  $\mu\text{m}$ .

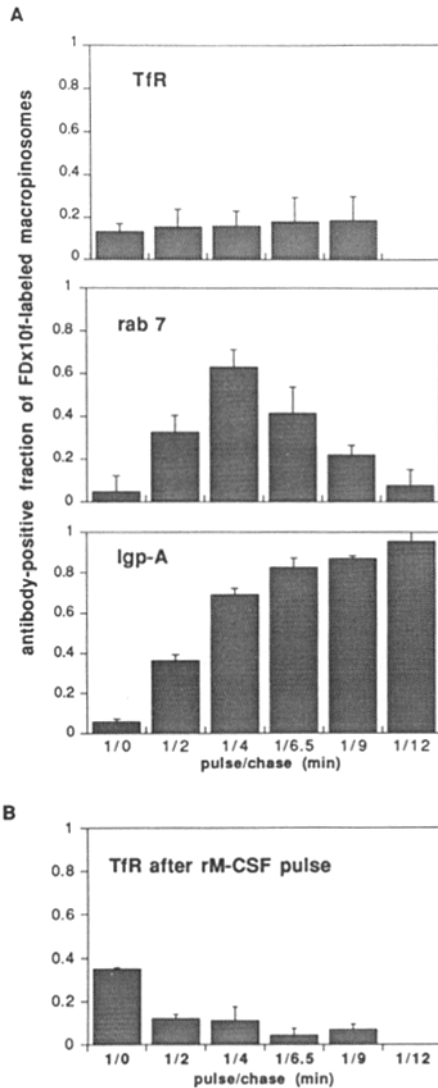


**Figure 2.** Macropinosomes of different ages label with antibodies against different endocytic vesicle membrane proteins. Macropinosomes were pulse labeled by incubating macrophages with medium containing 1.2 mg/ml FDx10f and 3,000 U/ml rM-CSF for 1 min at 37°C, washed rapidly at the same temperature, and then chased at 37°C in the absence of FDx10f, but in the continuing presence of rM-CSF for 0 (*a-c*), 4 (*d-f*), or 9 (*g-i*) min. Cells were then rapidly chilled and fixed. Next, macrophages were incubated with monoclonal rat anti-mouse TfR (*a*, *d*, and *g*), rabbit anti-recombinant rab 7 (*b*, *e*, and *h*), and monoclonal rat anti-mouse Igp-A (*c*, *f*, and *i*). Macrophages were then incubated with Texas red-labeled goat anti-rat or -rabbit secondary antibody and prepared for microscopy. For each lettered panel, the Texas red antibody-labeled image is above the FDx10f-labeled macropinosome. Note that shortly after their formation, macropinosomes are labeled with antibodies against transferrin receptor (*a* and *a'*); 4 min later, against rab 7 (*e* and *e'*) and 9 min later, Igp-A (*i* and *i'*). Bar, 1  $\mu$ m.

pulse without chase, some macropinosomes labeled with anti-TfR antibody (Fig. 2, *a* and *a'*) but not with anti-rab 7 (Fig. 2, *b* and *b'*) or anti-Igp-A (Fig. 2, *c* and *c'*). When cells were chased for 4 min, we observed many macropinosomes labeled with anti-rab 7 (Fig. 2, *e* and *e'*), but anti-TfR staining was diminished (Fig. 2 *d* and *d'*, and see below); in addition, some macropinosomes labeled with anti-Igp-A. After a 9-min chase, more macropinosomes labeled with anti-Igp-A (Fig. 2, *i* and *i'*), and fewer labeled with anti-rab 7 (Fig. 2, *h* and *h'*) or anti-TfR (Fig. 2, *g* and *g'*). These results indicated that after formation, macropinosomes acquired, and then lost specific membrane markers for organelles in the endocytic pathway.

Preparations such as those shown in Fig. 2 were then quantified. FDx10f-labeled macropinosomes that were homogeneously labeled with the test antibody were scored as positive (Fig. 3 *A*). The percentage of rab 7- and Igp-A-positive macropinosomes increased early and concurrently. The percentage of rab 7-positive macropinosomes then decreased while Igp-A staining increased. After 10 min, there were very few rab 7-positive macropinosomes.

Unexpectedly, the quantitative analysis of TfR labeling did not support the initial morphological impression. The percentage of TfR-positive macropinosomes (Fig. 3 *A*, *top*) was low and relatively constant throughout the time course, indicating that only a small number of new macropinosomes contained TfR, and that TfR did not recycle from the macropinosome. As this data was also inconsistent with our earlier observation of fluorescein-labeled transferrin movement through macropinosomes and with the double-labeling experiments (see below), we considered that TfR traffic might be variable during the first few minutes of M-CSF treatment. TfR trafficking has been shown to be altered by growth factors, and M-CSF causes TfR redistribution to the cell surface of bone marrow-derived macrophages (Lokeshwar and Lin, 1990). We therefore asked if preincubation with rM-CSF followed by a pulse-chase protocol would restore a TfR traffic more consistent with earlier observations. Macrophages were preincubated for 5 min in medium containing rM-CSF before performing a pulse-chase experiment. This resulted in a higher percentage of TfR-positive macropinosomes that then decreased with chase time (Fig. 3 *B*). The



**Figure 3.** With increasing age, macropinosomes progressively acquire and lose endocytic vesicle membrane proteins. Macrophages prepared as described in Fig. 2 were examined using phase-contrast and epifluorescence microscopy. (A) The fraction of FDx10f-labeled macropinosomes labeled with antibody against Tfr (top), rab 7 (middle), or lgp-A (bottom), were scored for each chase time. (B) Macrophages were incubated for 5 min in RB-containing rM-CSF only, and then were pulsed with FDx10f and chased as described above. Cells were then labeled with the anti-Tfr antibody and scored. Experiments were performed at least three times for each antibody; the average of these experiments  $\pm$ SD is displayed in the graph.

time course of macropinosome labeling with rab 7 was not altered by M-CSF preincubation (data not shown). Taken together, the quantitative data suggest a model in which macropinosomes are initially Tfr-positive early endosomes, and then are remodeled into late endosomes (Tfr negative, rab 7 and lgp-A positive). As rab 7 labeling decreases, the macropinosome may be further modified by the continued addition of lgp-A.

### Double Immunolabeling Reveals Distinct Distributions of Endocytic Vesicle Membrane Proteins

If FDx10f-labeled macropinosomes of different ages bear different endocytic membrane proteins, it follows that macropinosomes containing Tfr should not also have rab 7 or lgp-A. Moreover, many rab 7-positive macropinosomes should also contain lgp-A. To test this, we examined phase-bright macropinosomes of rM-CSF-treated macrophages immunolabeled with combinations of antibodies. Tfr-positive macropinosomes (Fig. 4, a and c) were positive for neither rab 7 (Fig. 4 b) nor lgp-B (used instead of anti-lgp-A; Fig. 4 d). In contrast, some rab 7-positive macropinosomes were lgp-A-positive (Fig. 4 f). It is also noteworthy that lgp-A-positive tubular structures were never labeled with anti-rab 7 antibodies (Fig. 4 e); a result consistent with the observation that macropinosomes lose rab 7 labeling before the contents of tubular lysosomes are transferred to macropinosomes (see below).

Quantitative analysis of these results (Table II) showed that nearly every Tfr-positive macropinosome lacked both rab 7 and lgp-B. Likewise, neither rab 7-positive nor lgp-B-positive macropinosomes labeled with anti-Tfr-antibody. Thus, recycling of Tfr from the macropinosome was complete before the organelle acquired rab 7 and lgp A or B.

### Macropinosome Fusion with Tubular Lysosomes

Previous studies showed that the lysosomal compartment in murine bone marrow-derived macrophages exists as an extended, interconnected network, and that it can be labeled by incubating cells in medium containing probes for fluid-phase pinocytosis, such as Lucifer yellow (Swanson et al., 1987) and HRP (Heuser, 1989). In addition, diI-labeled acetylated low-density lipoprotein, a fluorescent ligand for the macrophage scavenger receptor, labels macropinosomes (Racoosin and Swanson, 1992), and eventually reaches a tubular compartment.

To determine when macropinosomes fuse with the tubular lysosomal compartment, we pre-labeled tubular lysosomes by pinocytosis of Texas red-labeled dextran,  $M_r$  10,000 (TRDx10; 30-min pulse, 1-h chase), then pulse-labeled macropinosomes with FDx10f + rM-CSF followed by chases for increasing times. We then determined when the two fluorescent markers labeled the same macropinosome. Fig. 5 shows the corresponding distributions of FDx10f (Fig. 5, a, c, and e) and TRDx10 (Fig. 5, b, d, and f). Immediately following

**Table II. Fraction of Macropinosomes Containing More Than One Endocytic Membrane Marker**

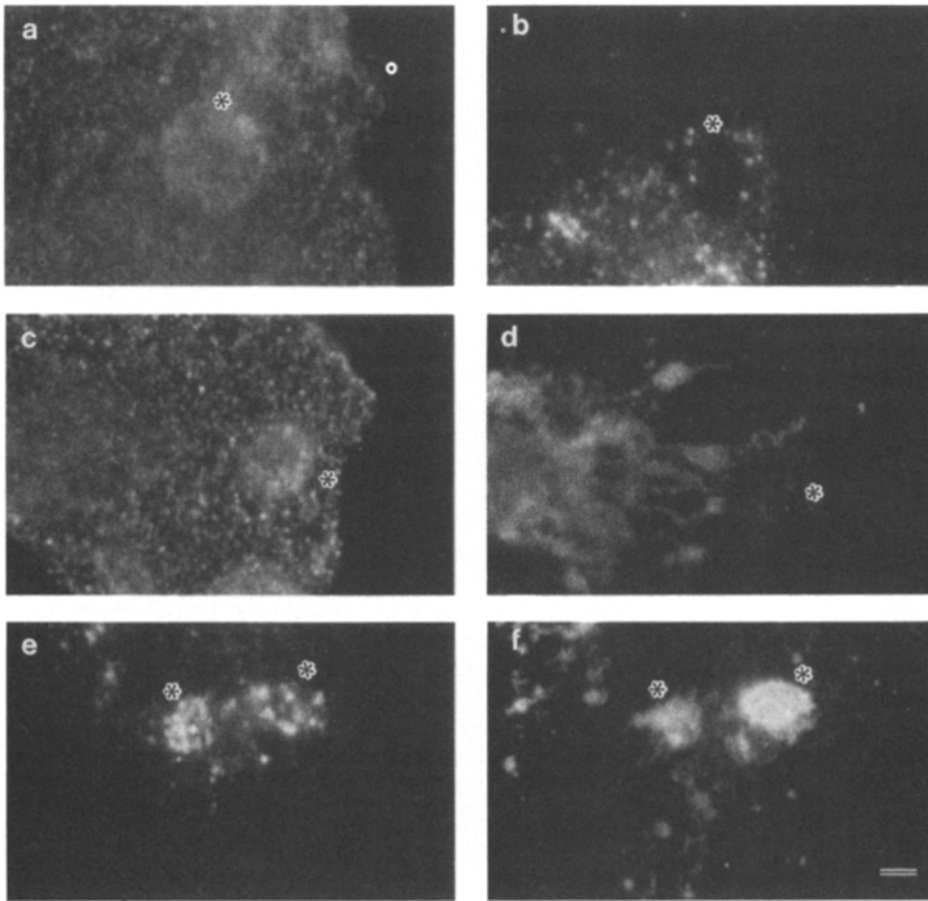
	Tfr	rab7	lgp-A/B
Tfr	—	1/159*	4/152‡
rab 7	0/152	—	78/163§
lgp-A/B	2/169‡	63/164§	—

Macrophages were treated as described in Fig. 5. Double antibody-labeled macropinosomes were scored positive if a phase-bright macropinosome labeled with one antibody was also labeled with the other antibody. The data represents the combined results of two experiments.

\* Indicates the number of Tfr-positive macropinosomes/total rab 7-positive macropinosomes counted.

‡ Stained with anti-lgp-B.

§ Stained with anti-lgp-A.



**Figure 4.** Double immunolabeling of macrophages reveals distinct distributions of endocytic vesicle membrane proteins on macropinosomes. Macrophages were incubated for 4.5 min at 37°C with medium containing rM-CSF, and then fixed and labeled with mixtures of antibodies against TFR and rab 7 (*a* and *b*); TFR and lgp-B (*c* and *d*); and rab 7 and lgp-A (*e* and *f*), and fluorescent anti-Ig secondary antibodies. Corresponding areas of cells are marked with an asterisk. Macropinosomes labeled with the anti-TFR antibody do not label with either anti-rab 7 or anti-lgp-B (*a* and *c*). In contrast, macropinosomes sometimes label with both anti-rab 7 and -lgp-A antibodies (*e* and *f*). Bar, 1  $\mu$ m.

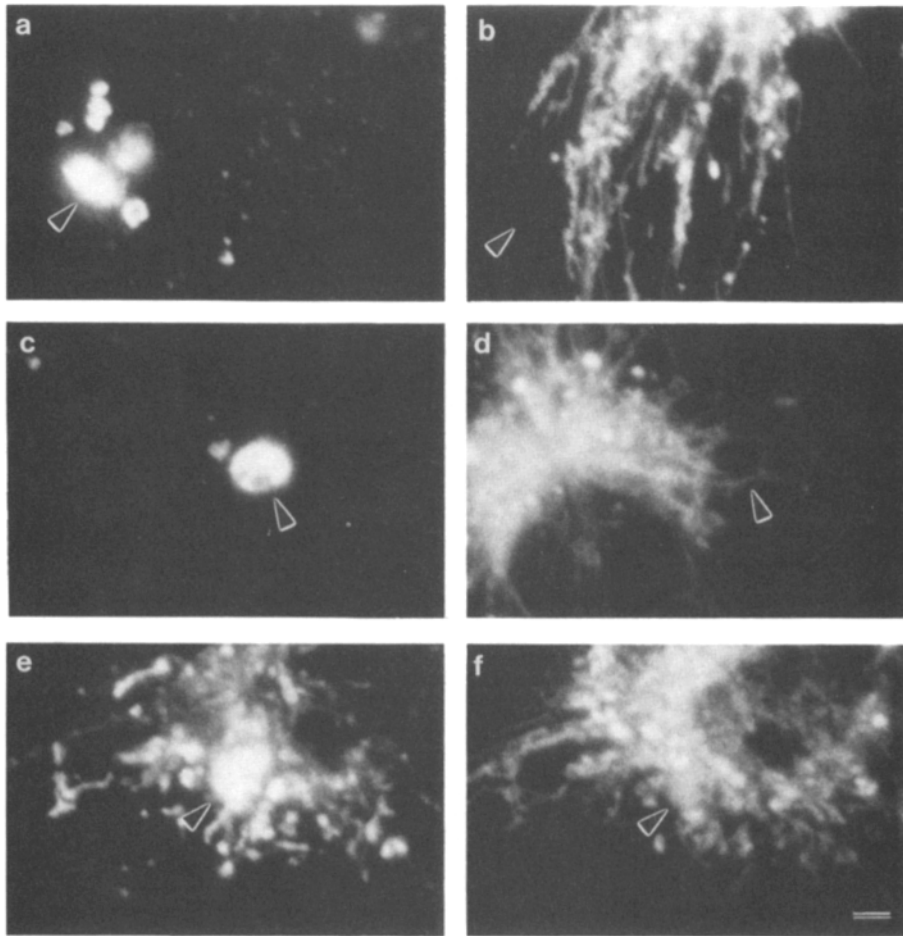
a 1-min pulse of FDx10f, macropinosomes formed near the cell periphery (Fig. 5 *a*) did not contact TRDx10-labeled tubular lysosomes (Fig. 5 *b*). 4 min after the 1-min pulse, tubular lysosomes (Fig. 5 *d*) appeared to wrap around the macropinosome (Fig. 5 *c*). After a 9-min chase (Fig. 5, *e* and *f*) the two probes coincided. Taken together with the result that lgp-A was delivered to macropinosomes as early as 3 min after formation (see Fig. 3, *lgp-A* graph), this delayed entry of TRDx10 into macropinosomes suggested that lysosomal membrane proteins were delivered to macropinosomes before lysosomal contents.

#### ***An lgp-A-labeled Macropinosome Intermediate May Precede Complete Fusion with Tubular Lysosomes***

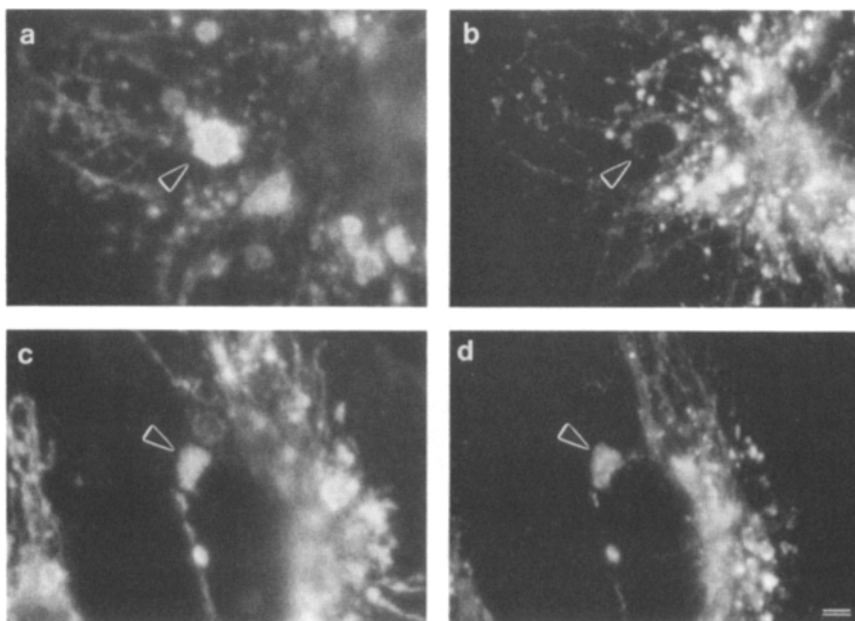
Macropinosome fusion with lysosomes appeared to occur in two steps. Tubular lysosomes prelabeled by pinocytosis by FDx10f (Fig. 6, *b* and *d*) also labeled with anti lgp-A (Fig. 6, *a* and *c*). However, some lgp-A-positive macropinosomes were surrounded by an FDx10f-labeled tubular compartment yet did not contain FDx10f (Fig. 6, *a* and *b*, *arrowheads*). Other macropinosomes were doubly labeled (Fig. 6, *c* and *d*, *arrowheads*). Although the age of macropinosomes could not be estimated in this experiment, their heterogenous labeling with FDx10f and lgp-A indicated a transient condition of the macropinosome in which lgp-A had been transferred from tubular lysosomes but lysosomal contents had

not. Alternatively, there may be an lgpA-positive intermediate compartment, lacking lysosomal content markers, that interacts with macropinosomes before the lysosomes do.

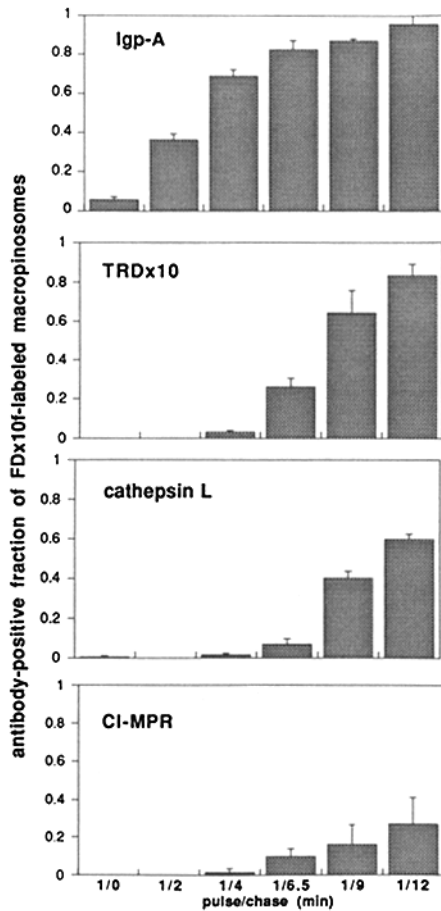
We next determined the time course of fusion between FDx10f-labeled macropinosomes and lysosomes, using various markers for late endocytic compartments. These included TRDx10 as a lysosome content marker, an antibody directed against the late endosome-specific cation-independent mannose-6-phosphate receptor (CI-MPR), and an antibody against the major excreted protein of mouse transformed fibroblasts (Dong et al., 1989), that is identical to the lysosomal enzyme cathepsin L and displays a tubular distribution in bone marrow-derived macrophages (data not shown). The kinetics indicated that lgp-A delivery from lysosomes and/or the *trans*-Golgi network into macropinosomes preceded delivery of the other markers of lysosomes and CI-MPR (Fig. 7). Notably, the kinetics of association of TRDx10, cathepsin L, and CI-MPR with macropinosomes were similar, although the percentage of antibody- or TRDx10-positive macropinosomes at the 12-min chase period varied from 32% for CI-MPR to 83% for TRDx10. We speculate that the low percentage of CI-MPR-positive macropinosomes indicated that at steady-state, CI-MPR receptors were associated mostly with other types of vesicular compartments. Indeed, throughout our experiments, anti-CI-MPR-labeled mostly small vesicular compartments and only occasionally ( $\sim$ 15–20% of cells) labeled lgp-A-positive



**Figure 5.** Macropinosomes fuse with pre-labeled tubular lysosomes. The tubular lysosomal compartment was labeled by endocytosis of TRDx10 as described in Materials and Methods. Macrophages were then incubated for 1 min in medium containing 1.2 mg/ml FDx10f and rM-CSF, and then chased for 0 (*a* and *b*), 4 (*c* and *d*), or 9 (*e* and *f*) min in warm medium lacking FDx10f but containing rM-CSF. After these incubations, cells were fixed at 37°C, and prepared for microscopy. *a*, *c*, and *e* are fluorescein images; *b*, *d*, and *f* are Texas red images. Arrowheads mark corresponding areas of cells. Fusion of a macropinosome with a tubular lysosome is seen in *e* and *f*, as indicated by TRDx10 labeling within the FDx10f-labeled macropinosome. Bar, 2  $\mu$ m.



**Figure 6.** IgG-A-labeled macropinosomes fuse incompletely with pre-labeled tubular lysosomes. The macrophage tubular lysosomal compartment was labeled as described in Fig. 5, except that FDx10f was used as the fluorescent marker. Next, cells were incubated for increasing times in medium containing rM-CSF, and then were washed and fixed at 37°C. Macrophages were then labeled with antibody against IgG-A, followed by a Texas red-labeled secondary antibody. (*a* and *c*) IgG-A-labeled images; (*b* and *d*) The corresponding FDx10f-labeled tubular lysosomal image. Note that IgG-A labels much of the FDx10f-labeled tubular compartment, but that the IgG-A-labeled macropinosomes do not always contain FDx10f (compare *a* and *b*, arrowheads, with *c* and *d*). Bar, 2  $\mu$ m.

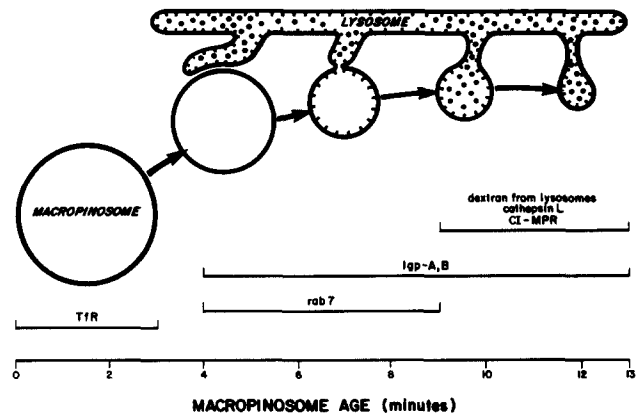


**Figure 7.** Delivery of Igp-A to macropinosomes precedes delivery of other lysosomal or late endosomal markers. The tubular lysosomal compartment was pre-labeled with TRDx10 (second panel) or was not pre-labeled with fluid-phase markers (first, third, and fourth panels). Macropinosomes were then pulse labeled and chased, as described in Fig. 2. Macrophages were then extracted and labeled with anti-CI-MPR (fourth panel), or -cathepsin L (third panel) antibodies. Antibody- and TRDx10-positive macropinosomes were then scored as described for Fig. 3. Three time course experiments were performed for each antibody or TRDx10 and are displayed as the average percentage for each time point  $\pm$ SD. The graphs for TRDx10, cathepsin L, and CI-MPR are compared with the anti-Igp-A results of Fig. 3.

tubular compartments. In any case, the more prevalent colocalization of pre-loaded TRDx10 and cathepsin L with FDx10f-labeled macropinosomes indicated that lysosomal contents transferred efficiently into macropinosomes before they merged into the tubular lysosomal network.

## Discussion

The macropinosome has the size and longevity needed to follow the progress of an individual vesicle through the endocytic pathway. A newly formed macropinosome begins as an early endosome derived from the plasma membrane, rapidly matures into a late endosome, and then merges completely into the tubular lysosomal compartment. The merger is gradual, in that lysosomal membrane arrives first, and lysosomal content is transferred later. As it moves toward the



**Figure 8.** Macropinosomes mature and merge with tubular lysosomes. The horizontal axis indicates the time-dependent acquisition and loss of endocytic membrane proteins (horizontal bars), as the macropinosome merges into the tubular lysosomal compartment. Tubular lysosomal membrane (Igp A/B, hatched outline) appears in macropinosomes early, and the contents of the tubular lysosomes (small, closed circles) arrive later. The origin of the rab 7 is not known, but could occur by vesicle-mediated delivery or by direct insertion of rab 7 into macropinosome membrane from the cytosol.

nucleus and shrinks, the macropinosome interacts with the tubular lysosomal compartment, receiving lysosomal membrane proteins Igp A and B. Approximately 9 to 12 min after formation, the contents of macropinosomes and tubular lysosomes mix (Fig. 8). Macropinosome movement through the endocytic pathway thus exhibits features of both the vesicle shuttle and maturation models of endocytosis, in that it initially acts as a maturing vesicle, but then merges with the stable lysosomal compartment.

## The Macropinosome as Early Endosome

According to the two models of endocytosis, early endosomes either are formed from the fusion of nascent pinosomes (Murphy, 1991), or preexist in the cell as stable organelles (Griffiths and Gruenberg, 1991). The de novo formation of macropinosomes containing Tfr is consistent with the first model (maturation). Approximately 35% of the newly formed macropinosomes in macrophages pre-incubated with rM-CSF contained Tfr. Subsequently, the number of Tfr-positive macropinosomes decreased, presumably due to receptor recycling. This is consistent with previous work showing that fluorescein-labeled transferrin is present in 1/3 of nascent macropinosomes, and then is recycled (Racoosin and Swanson, 1992). Both approaches show that older macropinosomes lack both transferrin receptors or fluorescein-labeled transferrin and therefore have matured from an early endosome into a later-stage endosome.

The apparent recycling of transferrin and its receptor indicates that macropinosomes interact with cytoplasmic proteins that mediate recycling. Two ras-like GTP-binding proteins, rab 4 and rab 5, have recently been associated with early endosomes (van der Sluijs et al., 1991; Chavrier et al., 1990). rab 5 was shown to be essential for vesicle fusion with early endosomes in an in vitro assay (Gorvel et al., 1991), and transfection of cultured cells with genes encoding mu-



tant rab 5 (Bucci et al., 1992) or rab 4 (van der Sluijs et al., 1992) proteins disrupted correct cycling of the transferrin receptor. We labeled macrophages with affinity-purified anti-rab 5 antibody (a gift of Dr. Marino Zerial, EMBL, Heidelberg, Germany), and observed a fine punctate labeling in cytoplasm, with no labeling of macropinosomes (data not shown). This suggests either that rab 5 does not associate with macropinosomes or that its association is too slight to detect by our methods or by that antibody. We have not yet attempted localization of rab 4.

### *The Macropinosome as Late Endosome*

The appearance of rab 7-positive, TfR-negative macropinosomes (Figs. 3 and 4) represented maturation from an early endosome to a late endosome. rab 7 has been localized in normal rat kidney cells to endocytic compartments that immunolabel with antibody against the CI-MPR (Chavrier et al., 1990), and is therefore thought to be a marker for late endosomes. In our studies, however, the kinetics of rab 7 labeling were different from CI-MPR-labeling kinetics; that is, rab 7 labeling of macropinosomes was greatly diminished before significant anti-CI-MPR labeling. Despite our inability to double-label macrophages with the two rabbit-derived antibodies, we observed that anti-rab 7 never labeled tubulovesicular organelles, whereas anti-CI-MPR-labeled tubules occasionally. Otherwise, both antibodies produced a punctate labeling of small and indistinct vesicles. Our observations of CI-MPR in some tubular structures and in the oldest macropinosomes indicates that it resides in a very late compartment of endocytosis. The labeling kinetics observed with anti-rab 7 were more consistent with the known properties of late endosomes, i.e., somewhere between early endosomes and lysosomes.

It is not known how rab 7 associates with macropinosomes. rab proteins are modified by the covalent addition of fatty acid moieties that permit membrane association (for review see Magee and Newman, 1992). Anti-rab 7 labeling of macropinosomes ranged from a lightly punctate image to a more homogenous labeling (data not shown). In addition, sometimes small rab 7-positive vesicles that lacked FDx10f appeared to be closely associated with FDx10f-labeled macropinosomes (Fig. 2), indicating that rab 7 associates with small vesicles that then fuse with maturing macropinosomes. Alternatively, rab 7 may insert directly into macropinosomes.

Increased staining for rab 7 coincided with that for Igp-A, and many macropinosomes could be double-labeled with antibodies against these proteins. Organelles with similar protein profiles have been observed by Rabinowitz et al. (1992), in murine peritoneal macrophages. These organelles were tubulovesicular, and could be labeled by endocytosis of BSA-coated colloidal gold (BSA-gold, 8-min incubation followed by a 22-min chase). The compartments that were filled with BSA-gold also labeled with antibodies against endocytic membrane proteins LAMP-1 and -2 (Igp-A and -B), CI-MPR, macrosialin, and rab 7. In addition, anti-LAMP-2 and -CI-MPR were within tubular regions of the compartment. In the present work anti-Igp-A and anti-CI-MPR (data not shown) labeled both macropinosomes and tubular lysosomes, whereas anti-rab 7 did not label tubular lysosomes. Therefore, bone marrow-derived macrophages do not ap-

pear to contain a rab 7-positive tubular compartment like that seen in the peritoneal macrophages. Instead, the compartment is more like the tubulovesicular lysosomal compartment in interferon- $\gamma$ -activated murine peritoneal macrophages, that contains both LAMP-1 and cathepsin D (Harding and Geuze, 1992).

### *Merger of the Macropinosome into the Tubular Lysosomal Compartment*

In contrast to their initial behavior, older macropinosomes merge with, rather than mature into, the tubular lysosomal compartment. FDx10f-labeled macropinosomes fused with lysosomes in a stepwise process, first becoming Igp-A positive, and then acquiring the lysosomal contents. These results are analogous to those of Harding and Geuze (1992), who showed that phagosomes containing heat-killed *Listeria* rapidly fuse with a tubulovesicular lysosomal compartment. Close association between Igp-A-positive tubular lysosomes and FDx10f-labeled macropinosomes (Figs. 5 and 6) indicates that the lysosomal glycoprotein is delivered via an incomplete fusion event that permits lateral membrane movement but not transfer of lysosomal contents. There is precedent for this in the work of Wang and Goren (1984). They showed that following phagosome-lysosome fusion in macrophages, a small fluorescent dye selectively entered phagosomes hours before a larger fluorescent dye, even though both had previously been loaded together into lysosomes. Their result supports the idea that fusion between lysosomes and phagosomes, or between lysosomes and macropinosomes occurs initially without complete transfer of lysosomal contents, perhaps by the existence of porelike structures connecting the two compartments. Later, as pores increase in size, lysosomal contents would be transferred. Transient interactions between lysosomes and macropinosomes were described by Willingham and Yamada (1978) in a process they named piranhalysis. By time-lapse video microscopy, they observed discrete lysosomes repeatedly engaging and fragmenting the macropinosome. It is not known if other organelles communicate in this way, by transient and incomplete fusion with only partial mixing of contents; but if they did, many molecular sorting problems could be avoided.

### *Macropinosome Progression Shows Features of Both Maturation and Vesicle Shuttle Models*

Macropinosome progress through the macrophage begins as a maturation process and ends as a shuttling process. Early on, a morphologically stable organelle, the macropinosome, changes its antigenic profile with age; that is, it matures. The stable, tubulovesicular lysosomal compartment interacts early and continuously with this maturing macropinosome, which merges into this compartment, rather than being molded independently into a similar one. That is, macropinosomes do not extend into tubules before joining the tubular lysosomes. Indeed, we have seen no evidence of a tubular endosomal compartment (Marsh et al., 1986; Hopkins et al., 1990; Tooze and Hollinshead, 1991). We therefore distinguish the later stages of macropinosome progress from maturation, in that we find a small vesicle shuttling into a morphologically stable, and highly plastic, preexisting compartment. We suggest that the two models of endocytosis

represent the extremes of a continuum, and that progression along the endocytic pathway is essentially a single process with variability in the relative size and stability of the interacting vesicles. A participating organelle may be considered stable or transient by the degree that it retains its defining characteristics (size, shape, content, or antigenic profile) after interacting with other organelles. Although our studies are restricted to macropinosomes, they are compatible with earlier studies of endosome progress after receptor-mediated endocytosis (Stoorvogel et al., 1991; Dunn and Maxfield, 1992). It is therefore possible that, like macropinosomes, the smaller endocytic compartments of receptor-mediated endocytosis also change first by a maturation process and later by merging into a stable terminal compartment.

The authors thank Eric Berthiaume for technical assistance. We also gratefully acknowledge the donation of antibodies from Drs. Bruce Granger, Marino Zerial, Hugh Rosen, Kevin Clairmont, and Gary Sahagian. Human recombinant macrophage colony-stimulating factor (rM-CSF) was generously supplied by Genetics Institute.

This work was supported by a grant to J. A. Swanson from the American Cancer Society. J. A. Swanson is an established investigator of the American Heart Association.

Received for publication 9 December 1992 and in revised form 4 March 1993.

#### References

- Bucci, C., R. G. Parton, I. H. Mather, H. Stunnenberg, K. Simons, B. Hoflack, and M. Zerial. 1992. The small GTPase rab 5 functions as a regulatory factor in the early endocytic pathway. *Cell*. 70:715-728.
- Chavrier, P., R. Parton, H. P. Hauri, K. Simons, and M. Zerial. 1990. Localization of low molecular weight GTP-binding proteins to exocytic and endocytic compartments. *Cell*. 62:317-329.
- Chen, J. W., T. L. Murphy, M. C. Willingham, I. Pastan, and J. T. August. 1985. Identification of two lysosomal membrane glycoproteins. *J. Cell Biol.* 101:85-95.
- Cohn, Z. A., and B. Benson. 1965. The in vitro differentiation of mononuclear phagocytes. II. The influence of serum on granule formation, hydrolase production and pinocytosis. *J. Exp. Med.* 121:835-848.
- Dautry-Varsat, A., A. Ciechanover, and H. F. Lodish. 1983. pH and the recycling of transferrin during receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA*. 80:2258-2262.
- Dong, J., E. M. Prenc, and G. G. Sahagian. 1989. Mechanism for selective secretion of a lysosomal protease by transformed mouse fibroblasts. *J. Biol. Chem.* 264:7377-7783.
- Dunn, K. W., and F. R. Maxfield. 1992. Delivery of ligands from sorting endosomes to late endosomes occurs by maturation of sorting endosomes. *J. Cell Biol.* 117:301-310.
- Gorvel, J.-P., P. Chavrier, M. Zerial, and J. Gruenberg. 1991. rab 5 controls early endosome fusion in vitro. *Cell*. 64:915-925.
- Granger, B. L., S. A. Green, C. A. Cabel, C. L. Howe, I. Mellman, and A. Helenius. 1990. Characterization and cloning of lgp 110, a lysosomal membrane glycoprotein from mouse and rat cells. *J. Biol. Chem.* 265:12036-12043.
- Griffiths, G., and J. Gruenberg. 1991. The arguments for pre-existing early and late endosomes. *Trends Cell Biol.* 1:5-9.
- Griffiths, G., B. Hoflack, K. Simons, I. Mellman, and S. Kornfeld. 1988. The mannose-6-phosphate receptor and the biogenesis of lysosomes. *Cell*. 52:329-341.
- Harding, C. V., and H. J. Geuze. 1992. Class II MHC molecules are present in macrophage lysosomes and phagolysosomes that function in the phagocytic processing of *Listeria monocytogenes* for presentation to T cells. *J. Cell Biol.* 119:531-542.
- Helenius, A., I. Mellman, D. Wall, and A. Hubbard. 1983. Endosomes. *Trends Biochem. Sci.* 8:245-250.
- Heuser, J. 1989. Changes in lysosome shape and distribution correlated with changes in cytoplasmic pH. *J. Cell Biol.* 108:855-864.
- Hopkins, C. R., A. Gibson, M. Shipman, and K. Miller. 1990. Movement of internalized ligand-receptor complexes along a continuous endosomal reticulum. *Nature (Lond.)*. 346:335-339.
- Kornfeld, S., and I. Mellman. 1989. The biogenesis of lysosomes. *Ann. Rev. Cell Biol.* 5:483-525.
- Lesley, J., R. Hyman, R. Schulte, and J. Trotter. 1984. Expression of transferrin receptor on murine hematopoietic progenitors. *Cell. Immunol.* 83:14-25.
- Lewis, W. H. 1931. Pinocytosis. *Bull. Johns Hopkins Hosp.* 49:17-27.
- Lokeshwar, B. L., and H.-S. Lin. 1990. Growth factor-dependent regulation of transferrin receptor in proliferating and quiescent macrophages. *Cell. Immunol.* 130:401-415.
- MacDonald, R. G., M. A. Tepper, K. B. Clairmont, S. B. Perregaux, and M. P. Czech. 1989. Serum form of the rat insulin-like growth factor II/mannose-6-phosphate receptor is truncated in the carboxyl-terminal domain. *J. Biol. Chem.* 264:3256-3261.
- Magee, A. I., and C. M. H. Newman. 1992. The role of lipid anchors for small G-proteins in membrane trafficking. *Trends Cell Biol.* 2:318-323.
- Marsh, M., G. Griffiths, G. E. Dean, I. Mellman, and A. Helenius. 1986. Three-dimensional structure of endosomes in BHK-21 cells. *Proc. Natl. Acad. Sci. USA*. 83:2899-2903.
- McLean, I. W., and P. K. Nakane. 1974. Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* 22:1077-1083.
- Mellman, I., R. Fuchs, and A. Helenius. 1986. Acidification of the endocytic and exocytic pathways. *Ann. Rev. Biochem.* 55:663-700.
- Murphy, R. 1991. Maturation models for endosome and lysosome biogenesis. *Trends Cell Biol.* 1:77-82.
- Pearse, B. M. F., and M. S. Robinson. 1990. Clathrin, adaptors and sorting. *Ann. Rev. Cell Biol.* 6:151-171.
- Rabinowitz, S., H. Horstmann, S. Gordon, and G. Griffiths. 1992. Immunocytochemical characterization of the endocytic and phagolysosomal compartments in peritoneal macrophages. *J. Cell Biol.* 116:95-112.
- Racoosin, E. L., and J. A. Swanson. 1989. Macrophage colony-stimulating factor (rM-CSF) stimulates pinocytosis in bone marrow-derived macrophages. *J. Exp. Med.* 170:1635-1648.
- Racoosin, E. L., and J. A. Swanson. 1992. M-CSF-induced macropinocytosis increases solute endocytosis but not receptor-mediated endocytosis in mouse macrophages. *J. Cell Sci.* 102:867-880.
- Rodman, J. S., R. W. Mercer, and P. D. Stahl. 1990. Endocytosis and transcytosis. *Curr. Opin. Cell Biol.* 2:664-672.
- Schmid, S. L., R. Fuchs, P. Male, and L. Mellman. 1988. Two distinct subpopulations of endosomes involved in membrane recycling and transport to lysosomes. *Cell*. 52:73-83.
- Stoorvogel, W., G. J. Strous, H. J. Geuze, V. Oorschot, and A. Schwartz. 1991. Late endosomes derive from early endosomes by maturation. *Cell*. 65:417-427.
- Swanson, J. A., A. Bushnell, and S. C. Silverstein. 1987. Tubular lysosome morphology and distribution within macrophages depend on the integrity of cytoplasmic microtubules. *Proc. Natl. Acad. Sci. USA*. 84:1921-1925.
- Tooze, J., and M. Hollinshead. 1991. Tubular early endosomal networks in AtT20 and other cells. *J. Cell Biol.* 115:635-653.
- van der Sluijs, P., M. Hull, A. Zahraoui, A. Tavittian, B. Goud, and I. Mellman. 1991. The small GTP-binding protein rab 4 is associated with early endosomes. *Proc. Natl. Acad. Sci. USA*. 88:6313-6317.
- van der Sluijs, P., M. Hull, P. Webster, P. Male, B. Goud, and I. Mellman. 1992. The small GTP-binding protein rab 4 controls an early sorting event on the endocytic pathway. *Cell*. 70:729-740.
- Wang, Y.-L., and M. B. Goren. 1987. Differential and sequential delivery of fluorescent lysosomal probes into phagosomes in mouse peritoneal macrophages. *J. Cell Biol.* 104:1749-1754.
- Wileman, T., C. Harding, and P. Stahl. 1985. Receptor-mediated endocytosis. *Biochem. J.* 232:1-14.
- Willingham, M. C., and S. S. Yamada. 1978. A mechanism for the destruction of pinosomes in cultures fibroblasts: piranhalysis. *J. Cell Biol.* 78:480-487.
- Yamashiro, D. J., B. Tycko, S. R. Fluss, and F. R. Maxfield. 1984. Segregation of transferrin to a mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway. *Cell*. 37:789-800.