

Remodeling of endosomes during lysosome biogenesis involves 'kiss and run' fusion events regulated by rab5

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Summary

The small GTPase rab5 has been shown to play key roles in the function of both endocytic and phagocytic organelles. Although these organelles share several additional common features, different processes have been proposed to explain their biogenesis. In the present study, we provide evidence that lysosome biogenesis involves mechanisms similar to those previously described for the formation of phagolysosomes. Transient interactions ('kiss and run') between endocytic organelles are shown to occur during lysosome biogenesis. These interactions are regulated initially by the GTPase activity of rab5, as demonstrated by the loss of size-selective fusion between endosomes in

cells expressing a GTPase-deficient mutant of rab5. Endocytic compartments in these cells sequentially display properties of early and late endosomes. However, the formation of lysosomes and the sorting of endocytic solute materials to small electron dense vacuoles are not affected by the rab5 mutation. Together, our results indicate that endosome maturation occurs during the early part of lysosome biogenesis. This process involves transient fusion events regulated, in part, by the small GTPase rab5.

Key words: Endosomes, Lysosomes, Membrane fusion, Kiss and run

Introduction

Endocytosis and lysosome biogenesis are coordinated processes allowing cells to fulfill a variety of functions including internalization of nutrients, the regulation of cell-surface receptor activity, and antigen processing (for reviews, see Mellman, 1996; Mukherjee et al., 1997). These functions take place in a series of endocytic compartments, including lysosomes, the so-called 'end station' where the bulk of protein degradation occurs (Kornfeld and Mellman, 1989; Storrie, 1995). In the past few years, our understanding of lysosome formation has greatly improved with the identification of several of the molecules involved in membrane interaction and fusion along the endocytic pathway, including SNARE proteins and members of the rab family of small GTP-binding proteins. These proteins are parts of the machinery required for fusion at both early and late stages of the endocytic pathway (Clague, 1999; Mullock et al., 1998). Despite our growing knowledge of the molecules involved, it is still not clear whether the materials internalized by endocytosis sequentially appear in early endosomes, late endosomes and lysosomes by the maturation of endosomes (Stoorvogel et al., 1991; Murphy, 1991) through, for example, 'kiss and run' interactions (Storrie and Desjardins, 1996), or by vesicular transport between pre-existing compartments (Griffiths and Gruenberg, 1991; Gruenberg and Maxfield, 1995). A better comprehension of this aspect of lysosome biogenesis is likely to help us define more clearly its molecular requirements.

Lysosome and phagolysosome biogenesis are considered to occur by different mechanisms. However, more and more

evidences indicate that these two pathways share common features. So far, most, if not all, of the proteins present on endosomes have also been found on phagosomes (Garin et al., 2001). Among these markers, rab5, a member of the rab family of small GTPases present on early endocytic and phagocytic structures has been particularly studied (Chavrier et al., 1990; Gorvel et al., 1991; Bucci et al., 1992; Desjardins et al., 1994). Its apparent absence from late endosomes, where rab7 is observed, was instrumental to the proposal that early and late endosomes are pre-existing organelles displaying specific sets of proteins (Griffiths and Gruenberg, 1991). However, recent evidence clearly indicates that rab5 is also associated to late endosomes in some cell types (Jahraus et al., 1998) and that several rabs can be present on the same endosomes (Sönnichsen et al., 2000). In a previous study, we used a RAW264.7 macrophage cell line expressing a GTPase-defective rab5(Q79L) mutant (Duclos et al., 2000) to demonstrate that phagolysosome biogenesis occurs through 'kiss and run' interactions (Desjardins, 1995), regulated in part by rab5. In the present study, we used the rab5 mutant cell line to determine to what extent the processes of phagolysosome and lysosome biogenesis are related.

Our results demonstrate that endosome maturation occurs during the early stages of lysosome biogenesis. Rab5 regulates parts of this process by allowing transient interactions of a 'kiss and run' nature between endosomes. Together, our results indicate that lysosome biogenesis occurs by mechanisms very similar to those involved in the maturation of phagosomes during phagolysosome biogenesis.

Materials and Methods

Cell culture and stable transfections

The murine macrophage cell line RAW264.7 (American Type Culture Collection, Rockville, MD) was cultured in DMEM, pH 7.4 supplemented with 10% heat-inactivated FBS (Life Technologies, ON, Canada), 20 mM Hepes pH 7.3–7.4, and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin), at 37°C in 5% CO₂. A rab5a-EGFP fusion was created by subcloning the rab5a(Q79L) cDNA into pEGFP-C2 (Clontech, Palo Alto, CA). Rab5a(Q79L)- and EGFP-rab5a(Q79L)-expressing macrophages were obtained by electroporation of RAW264.7 macrophages as described (Duclos et al., 2000).

Morphology of endosomes by electron microscopy

To determine the effect of rab5(Q79L) on endosome morphology, 10 mg/ml HRP (Sigma) was internalized in transfected and control cells for 30 minutes. Cells were then fixed in 1% glutaraldehyde. HRP was revealed by a DAB (Sigma, St Louis, MO) reaction as described previously (Desjardins et al., 1994). Cells were post-fixed in 1% OsO₄, dehydrated in alcohol, processed for flat embedding in Epon 812 and observed at the Zeiss CEM 902 electron microscope as described previously (Desjardins et al., 1994).

Time course of large endosomes formation

To determine the nature of the large endosomes, 1.5 mg/ml BSA-rhodamine (kindly provided by Lucian Ghitescu, Université de Montréal, Montréal) in DMEM was internalized for 2 minutes at 37°C. Cells were then washed twice in cold PBS for 3 minutes with mild agitation. BSA-rhodamine was chased for the indicated time points by incubating cells in DMEM at 37°C. Cells were then rapidly observed at the epifluorescence microscope without fixation.

To further analyze the nature of the large endosomes, 16 nm BSA-gold particles were internalized for 30 minutes without a chase to form early and late endosomes, or with a 16 hours-chase in order to empty early and late endosomes, and fill lysosomes. Cells were then fixed and processed for observation at the electron microscope as described (Desjardins et al., 1994).

Effect of bafilomycin A1 on endosome morphology

To characterize the effect of bafilomycin A1 on various endosome populations in wild-type RAW264.7 macrophages, 16 nm BSA-gold particles were internalized in macrophages for 15 minutes, followed by 0 minutes, 60 minutes or 16 hours of chase time in DMEM to fill endosomes at various stages of their maturation. Cells were then treated with bafilomycin A1 (Kamiya Biomedical Company, Tukwila, WA) in DMSO or with DMSO only for 30 minutes and prepared for electron microscopy. To characterize the effect of bafilomycin A1 on endosomes from rab5(Q79L)-expressing cells, late endosomes were formed by the internalization of 35 nm BSA-gold particles for 15 minutes, followed by a 4 hour chase in DMEM. In the same cells, early endosomes were then formed by internalization of 5 nm BSA-gold particles for 15 minutes. Cells were then treated with bafilomycin A1 or with DMSO for 30 minutes, and prepared for electron microscopy.

Antibodies and immunofluorescence microscopy

To better confirm the nature of the large endosomes, immunofluorescence analysis was performed with the following primary antibodies: the mAb 4F11 raised against a peptide from the C-terminal region of rab5 (a generous gift of Angela Wandinger-Ness, National Center for Genome Resources, Santa Fe, New Mexico); a rabbit polyclonal antibody directed against the C-terminal region of rabaptin-5 (a kind gift of Marino Zerial, Max Planck Institute for

Molecular Cell Biology and Genetics) (Stenmark et al., 1995); an affinity-purified human autoantibody to EEA1 (Mu et al., 1995); a rabbit polyclonal antibody raised against rab7 (a kind gift of Stéphane Méresse Centre d'Immunologie de Marseille-Luminy, Marseille); monoclonal rat anti-LAMP1 and anti-LAMP2 (Developmental Studies Hybridoma Bank, Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD); a rabbit polyclonal antibody raised against flotillin1 (a kind gift of Robert Parton, Centre for Microscopy and Microanalysis, Department of Physiology and Pharmacology, and Institute of Molecular Bioscience, University of Queensland, Brisbane). For all immunofluorescence experiments, cells were grown on 18 mm round coverslips. Fixation was performed either in 4% paraformaldehyde followed by 0.2% Triton X-100 permeabilization at room temperature, or in 80% methanol/20% acetone for 20 minutes at –20°C. After washes in PBS, cells were incubated in a blocking solution made of 2% BSA and 0.2% gelatin in PBS. Incubation with the primary antibodies was done for 1 hour at room temperature. After washes in PBS/1% BSA, cells were incubated with the appropriate secondary antibodies [Texas-Red-conjugated anti-mouse IgG, Texas-Red-conjugated anti-rabbit (BIO/CAN Scientific, Mississauga, ON, Canada), ALEXA-conjugated anti-human IgG or ALEXA-conjugated anti-rat IgG (Molecular Probes, Eugene, OR)] for 30 minutes in the dark, at room temperature. Coverslips were mounted on Gelvatol (Air Products & Chemicals, Allentown, PA) and observed with a Zeiss inverted epifluorescence microscope, or with a Leica confocal fluorescence microscope.

Dextran segregation

To observe segregation of fluid phase tracers, macrophages on coverslips were incubated for 30 minutes with mixtures of tetramethylrhodamine dextran, average molecular weight 70,000 (TRDx70) plus fluorescein dextran, average molecular weight 10,000 or 70,000 (FDx10 or FDx70), each at 5 mg/ml in DMEM. After extensive washes in PBS, the tracers were chased for the indicated times (0, 30, 120 or 240 minutes). Coverslips were mounted on Gelvatol and observed at the Leica confocal fluorescence microscope.

Size-selective transfer of particles at the electron microscope level

To further characterize the size-selective transfer of particles, macrophages were incubated for either 15 or 30 minutes to allow the endocytosis of a mixture of 100 nm BSA-coated latex beads and 16 nm BSA-gold particles. After extensive washes in PBS, the tracers were chased for 0, 30, or 120 minutes. Cells were then fixed and processed for observation at the electron microscope as described (Desjardins et al., 1994). Coating of latex beads with BSA was done by incubating the beads with 5 mg/ml BSA, as previously described (Duclos et al., 2000), with the difference that washes in this case were performed in distilled water at 48,300 g.

Results

Rab5(Q79L) induces the formation of giant endosomes in RAW264.7 macrophages

Previous studies have reported that the expression of an active form of rab5 (rab5(Q79L)) induces the formation of giant endosomes displaying early endocytic markers in various cell lines (Stenmark et al., 1994; D'Arrigo et al., 1997; Aballay et al., 1999). To determine whether the rab5(Q79L) mutation has a similar effect in RAW264.7 macrophages, we produced

stable cell lines (Duclos et al., 2000) and selected clones expressing low levels of the protein as measured by western blot analysis using an anti-rab5 antibody (not shown). These cells were loaded with the fluid phase marker HRP for 30 minutes in order to visualize the morphology of their endocytic organelles. Under these conditions, we observed at the electron microscope that several large vacuoles containing HRP were present in the rab5(Q79L)-expressing cells despite the low expression of the rab mutant. In contrast, HRP was present in smaller vacuoles in control cells transfected with the vector only (Fig. 1). This was observed for several transfected clones. These results indicate that transfection of the rab5a(Q79L) cDNA in macrophages has a profound effect on the morphology of endosomes.

Giant endosomes display early and late endocytic features

To determine the nature of the giant endosomes present in rab5(Q79L)-expressing macrophages, we characterized the kinetic of their formation and some of their biochemical properties. First, we followed the distribution of a fluorescent fluid tracer at various time points after its internalization. This was done by internalizing BSA-rhodamine for 2 minutes followed or not with chase incubations of increasing periods of time. The rapid screening of these cells without fixation allowed us to observe that normal macrophages display the rhodamine signal in small vesicles throughout the cytoplasm at all the time points studied (Fig. 2). In contrast, in addition to the small vesicles, large endosomes filled with BSA-rhodamine were present in rab5(Q79L)-expressing cells after the initial 2 minutes of internalization (not shown), a time expected to fill early endosomes. Large labeled vacuoles were still present after 10 minutes and even 30 minutes of chase of the BSA-rhodamine marker (Fig. 2), a period of time largely sufficient to empty early endosomes and load late endocytic organelles. However, at 60 minutes after internalization, a chase period normally used to load lysosomes, the rhodamine signal was now observed in smaller structures, possibly lysosomes (Fig. 2), while large empty vacuoles were visible in the rab5(Q79L)-expressing cells (Fig. 2, inset). These results indicate that the large endosomes formed in rab5(Q79L)-expressing cells kinetically correspond to both early and late endosomes.

Giant endosomes are not lysosomes

In order to determine whether rab5(Q79L) also affects the morphology of lysosomes, we internalized 16 nm BSA-gold particles and incubated cells for long periods of time. After 30 minutes of internalization, the gold particles were present in giant structures. BSA-gold was not observed in the numerous small dense vesicles present in these cells (Fig. 3A). In contrast, a 16 hour chase led to the accumulation of the gold particles in small dense vesicles, most likely representing lysosomes, while the giant vacuoles still present were devoid of the gold tracer (Fig. 3B). These results confirm that alteration of rab5 induces morphological changes to what can be described as an early-endosomes–late-endosomes continuum, without affecting the formation and morphology of lysosomes.

Giant endosomes gradually acquire resistance to bafilomycin A1-induced fragmentation

Previous studies indicated that early endosomes could be fragmented by treating cells with the proton pump inhibitor bafilomycin A1 (Clague et al., 1994; D'Arrigo et al., 1997). This observation prompted us to determine whether the

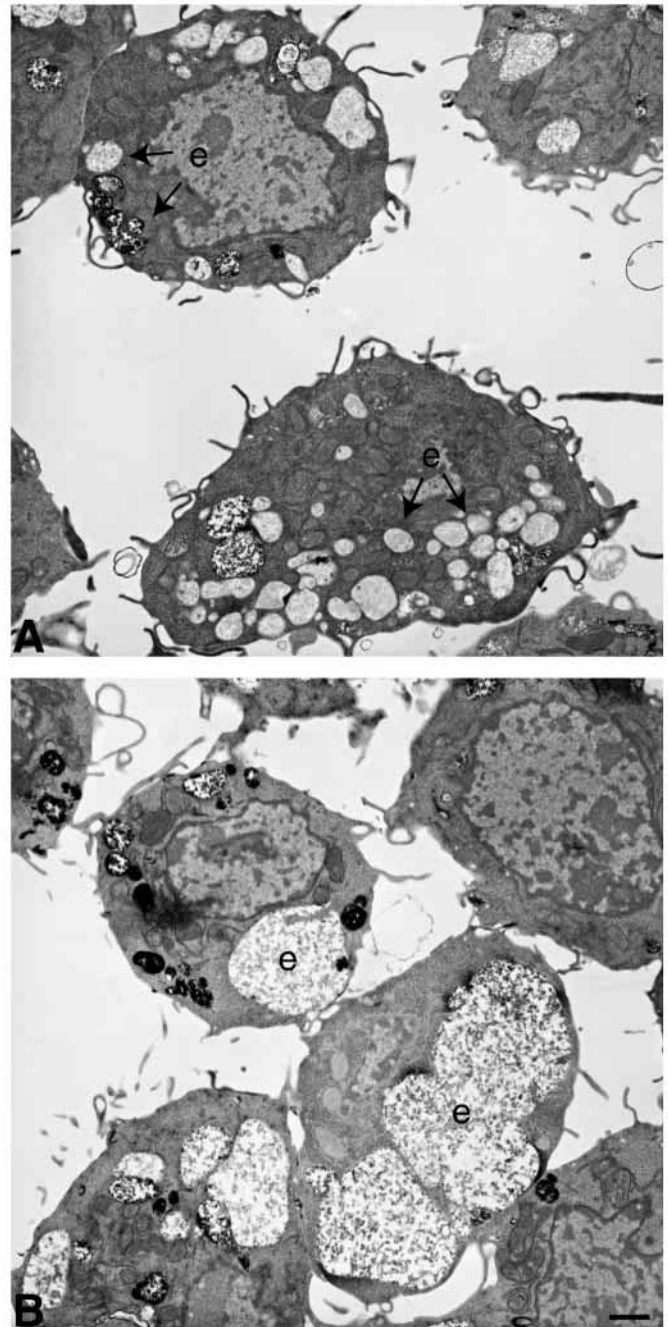


Fig. 1. Rab5(Q79L) induces the formation of giant endosomes in RAW264.7 macrophages. The effect of the rab5(Q79L) mutation on the morphology of endosomes (e) in RAW264.7 macrophages was examined at the electron microscope. Internalization of HRP (10 mg/ml) for 30 minutes in control (A) or rab5(Q79L)-expressing cells (B) led to the accumulation of this tracer in small vesicles in control cells (A), while it was found in very large endosomes (e) in mutant cells (B). Bar, 0.5 μ m.

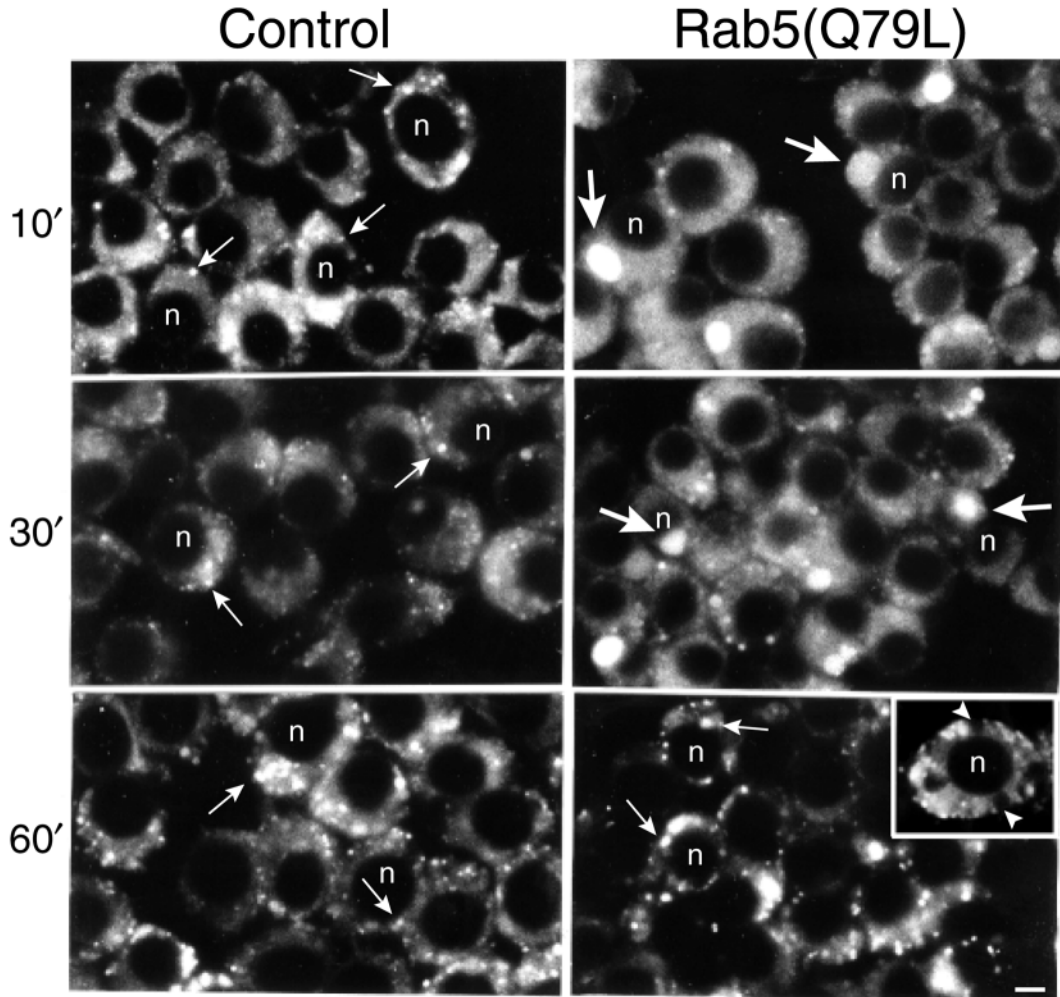


Fig. 2. Giant endosomes kinetically correspond to both early and late endosomes. To determine the nature of the large endosomes, control or rab5(Q79L)-expressing cells were allowed to internalize the fluid phase endocytic tracer BSA-rhodamine (1.5 mg/ml) for 2 minutes, followed by the indicated times of chase. While BSA-rhodamine was observed in small vesicles (small arrows) at each time point studied in control cells, in mutant cells this tracer was present in giant endosomes (large arrows) for at least 30 minutes after its internalization. However, chase of the fluorescent tracer for 60 minutes resulted in its transfer to smaller vesicles similar to those observed in control cells. The inset represents large vacuoles (arrowheads) not displaying the fluorescent tracer that could be observed at this time point in rab5(Q79L)-expressing cells. n, nucleus. Bar, 10 μ m.

large endosomes in rab5(Q79L)-expressing cells could be fragmented by this drug. By preloading cells with BSA-gold for different periods of time to fill early endosomes, late endosomes and lysosomes, we were able to show that, in control macrophages, treatment with bafilomycin A1 resulted in the fragmentation of early endosomes, but had no noticeable effect on the morphology of late endosomes and lysosomes (Fig. 4). Based on these criteria, we performed similar experiments in rab5(Q79L)-expressing cells preloaded with BSA-gold. Observation at the electron microscope clearly showed that newly formed early endosomes could be fragmented with bafilomycin A1 treatment while the late endosomes were insensitive to the drug (Fig. 5). Indeed, in cells preloaded sequentially with 16 nm and 5 nm BSA-gold to fill late endosomes with the large gold particles and early endosomes with the small gold particles, only the early structures were fragmented (Fig. 5). These results indicate that while giant early endosomes can be fragmented by bafilomycin

A1, the enlarged structures are remodeled in a way that eventually confers resistance to the fragmentation effect of this drug.

Giant endosomes display both early and late endocytic markers

The nature of the large endosomes was further studied by localizing a series of well-known endocytic markers by immunofluorescence microscopy. These included: the early endosome-associated molecules EEA1 and rabaptin-5; the late endosome marker rab7, as well as flotillin1, a protein recently shown to accumulate on subdomains of maturing phagosomes (Dermine et al., 2001); and the late endosome/lysosome markers LAMP1 and LAMP2 (Fig. 6). In control cells, where large vacuoles are not usually observed, all these markers were detected on small vesicles distributed throughout the cytoplasm (not shown). In rab5(Q79L)-expressing cells, in

addition to small vesicles, the large endosomes were also labeled for all of these markers (Fig. 6), confirming the kinetic data (Fig. 2) showing that the enlarged structures correspond to both early and late endosomes. This point was also demonstrated by the observation of EGFP-rab5(Q79L) fusion proteins on large endosomes displaying the late endocytic marker LAMP1 (Fig. 6). Of further interest, while the labeling

for LAMP1 and LAMP2 was evenly distributed on the membrane of the giant organelles, all the other markers, including flotillin1, displayed punctate patterns of labeling, which suggests that they are associated with subdomains of the endosome membrane.

Rab5 regulates the 'kiss and run' fusion between endosomes

In macrophages, the GTPase activity of rab5 regulates the transient fusion events ('kiss and run') between phagosomes and endosomes required for phagolysosome biogenesis (Duclos et al., 2000). This was demonstrated by the loss of size-selective transfer of particles between these compartments upon the expression of a GTPase-deficient form of rab5. To investigate whether rab5-regulated transient fusion events also occur along the endocytic pathway during lysosome biogenesis, we followed the fate of fluorescent dextran molecules of different sizes [average mol wt of 10,000 (green) and 70,000 (red)] after their co-internalization in macrophages. In control cells, the two dextran populations were found in the same endocytic organelles (labeled in yellow) after 30 minutes of internalization (Fig. 7). These molecules were rapidly sorted, within 30 minutes of chase, to distinct endosomes displaying red or green labeling, and then observed in segregated compartments for the entire duration of the experiment (up to 240 minutes of chase) (Fig. 7). In contrast, the small and large dextran molecules remained co-localized for as long as 150 minutes after endocytosis in rab5(Q79L)-expressing cells, their separation to distinct endocytic organelles only starting after a 240 minute chase (Fig. 7). In control cells at 240 minutes, the size segregation of the two dextran molecules did not appear as extensive as for the 30'/120' time point, which indicates that some remixing between late endocytic structures could occur, as suggested previously (Luzio et al., 2000). Together, these results indicate that the GTPase activity of rab5 regulates, to some extent, the transient nature ('kiss and run') of the fusion events occurring between endosomes in the early part of the process of lysosome biogenesis.

These results were further confirmed at the electron microscope by internalizing 16 nm BSA-gold particles and 100 nm BSA-coated latex beads. At the earliest time point observed (15 minutes of internalization), 63% and 61% of

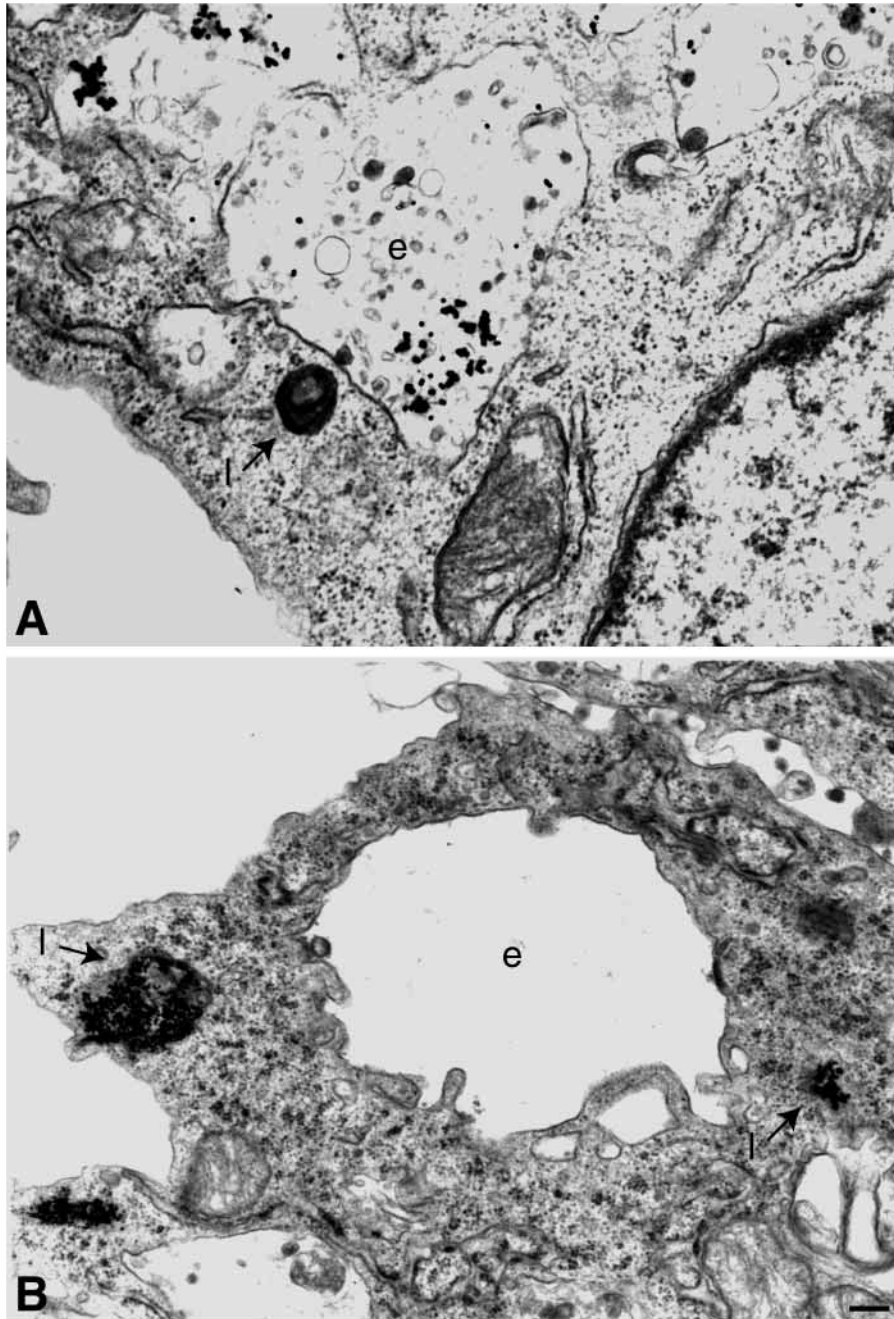


Fig. 3. Giant endosomes do not correspond to lysosomes. To further demonstrate the nature of the giant endosomes in rab5(Q79L) expressing macrophages, cells were incubated with 16 nm BSA-gold for 30 minutes, followed or not by a 16 hours chase period. After an internalization of 30 minutes (A), BSA-gold particles were found in large endocytic organelles (e). As in the kinetic experiment with the fluid phase tracer, a longer chase period (16 hours) resulted in the transfer of the BSA-gold particles to smaller electron dense vesicles (l) (B), while large vacuoles now devoid of BSA-gold particles could be observed. Bars, 100 nm.

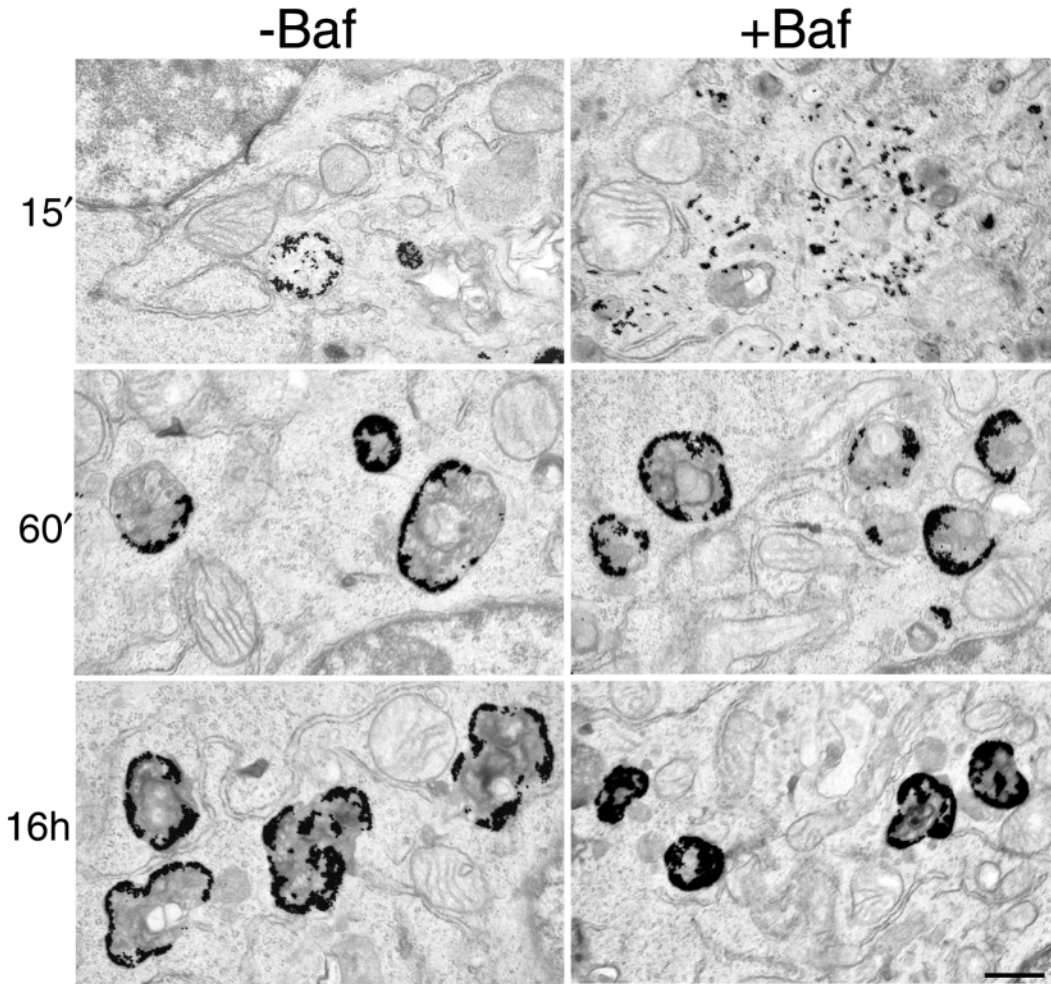


Fig. 4. Bafilomycin A1 induces fragmentation of early endosomes in control cells. To determine the effect of the proton pump inhibitor bafilomycin A1 (Baf) on endocytic organelles, macrophages were incubated in the presence of 16 nm BSA-gold particles for the indicated times in order to form endosomes of various ages. Cells were then treated for 30 minutes with 500 nM of bafilomycin A1 (right panels) or DMSO as a control (left panels), and the resulting morphology of the endocytic structures was observed at the electron microscope. As shown in the top right panel, only early endosomes have been disrupted by the bafilomycin A1 treatment. Bar, 550 nm (bottom-right panel) or 500 nm (all other panels).

endosomes contained particles of both sizes in control and rab5(Q79L)-expressing cells, respectively (Fig. 8). In control cells, the particles started to segregate into different endosomal compartments 60 minutes after their internalization (Fig. 8), and were still observed in separated endosomes 150 minutes after endocytosis (Fig. 8). In contrast, as described for the dextran molecules by fluorescence, the two sizes of particles remained co-localized for as long as 150 minutes after endocytosis in rab5(Q79L)-expressing cells (Fig. 8), which demonstrates the size-selective nature of the fusion events occurring along the endocytic pathway and is expected from 'kiss and run'-type interactions.

Discussion

Cells internalize materials from the external milieu by various processes including endocytosis and phagocytosis. Although a clearer picture of the biogenesis of phagolysosomes has emerged over the past few years (Méresse et al., 1999), the

biogenesis of endocytic organelles and lysosomes remains controversial and two main models still prevail (Gruenberg and Maxfield, 1995). According to the model of pre-existing organelles (Griffiths and Gruenberg, 1991), early and late endosomes are biochemically distinct stations along the endocytic pathway linked via shuttle vesicles. These shuttles, referred to as endocytic carrier vesicles, originate from parts of early endosomes that bud and carry the cargo molecules between stations. In its simplest form, the second model, the maturation model (Murphy, 1991), argues that early endosomes gradually transform into late endosomes and lysosomes along with their cargo. Biochemical modifications of the organelles are generated by recycling processes allowing the retrieval of early markers, and by the acquisition of newly synthesized late markers from the Golgi network. The sum of these two opposing processes generates biochemically distinct organelles with time. Understanding the overall process of lysosome biogenesis will enable a better comprehension and integration of the

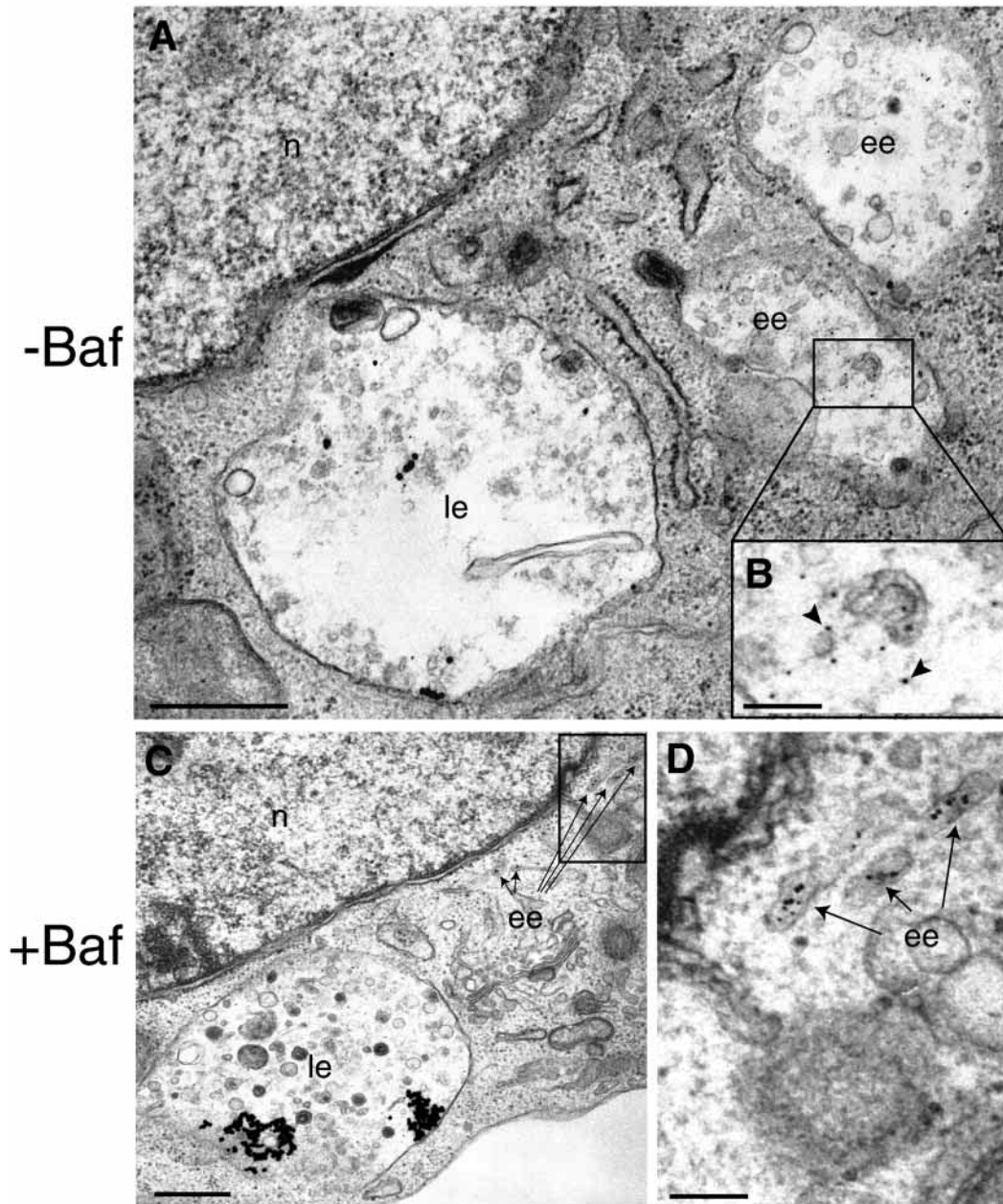


Fig. 5. Bafilomycin A1 does not induce fragmentation of giant late endosomes in rab5(Q79L)-expressing cells. To investigate the effect of bafilomycin A1 (Baf) on the giant endosomes in rab5(Q79L)-expressing cells, late endosomes (le) were formed by a 1 hour/4 hour pulse-chase of 35 nm BSA-gold particles, and the same cells were then allowed to internalize 5 nm BSA-gold particles for 30 minutes in order to load mostly early endosomes (ee) (A). The two sizes of particles are found in different endosome populations, both representing quite large endosomes. Panel B corresponds to an inset of panel A, allowing a better visualization of early endosomes containing only 5 nm BSA-gold particles. The same experiment was repeated, with cells incubated in the presence of 500 nM bafilomycin A1 for 30 minutes after the pulse-chase of gold particles. In C, we show a large late endosome filled with 35 nm BSA-gold particles, while the 5 nm BSA-gold particles are now found in very small vesiculo-tubular structures, better seen at a higher magnification (D). Bars, 250 nm (A,C); 50 nm (B,D).

molecular data generated on membrane trafficking in the endocytic pathway.

Do endosomes mature?

Rab5 was originally described as an early endosome marker (Chavrier et al., 1990). Thus, it was not surprising that mutations affecting that molecule would alter the properties of early endosomes. For example, the expression of a GTPase-defective mutant of rab5 in epithelial cells was shown to induce the formation of giant endocytic structures identified as early endosomes (Stenmark et al., 1994). Late endosomes and lysosomes were not altered in these cells, supporting the concept of pre-existing organelles (mutation to a protein residing in early endosomes would affect mainly, if not only, this compartment). However, in our study, expression of a

GTPase-deficient mutant of rab5 in RAW264.7 macrophages brought an interesting observation. Our results clearly indicate that the giant endocytic structures present in these cells display features of both early and late endosomes. This was demonstrated by kinetic studies showing that a tracer internalized by fluid phase endocytosis remained in giant endosomes for long periods of time, largely sufficient to load late endocytic organelles. In addition, the giant endosomes were shown to display archetypal markers of early endosomes such as rab5 and its effectors rabaptin5 and EEA1 (Chavrier et al., 1990; Stenmark et al., 1995; Mu et al., 1995), as well as several of the markers known to associate with late endosomes. These include the well-studied rab7 (Chavrier et al., 1990; Zerial and Stenmark, 1993; Méresse et al., 1995), LAMP1 and LAMP2 (Griffiths et al., 1988; Kornfeld and Mellman, 1989; Rabinowitz et al., 1992), and flotillin1, a membrane-associated

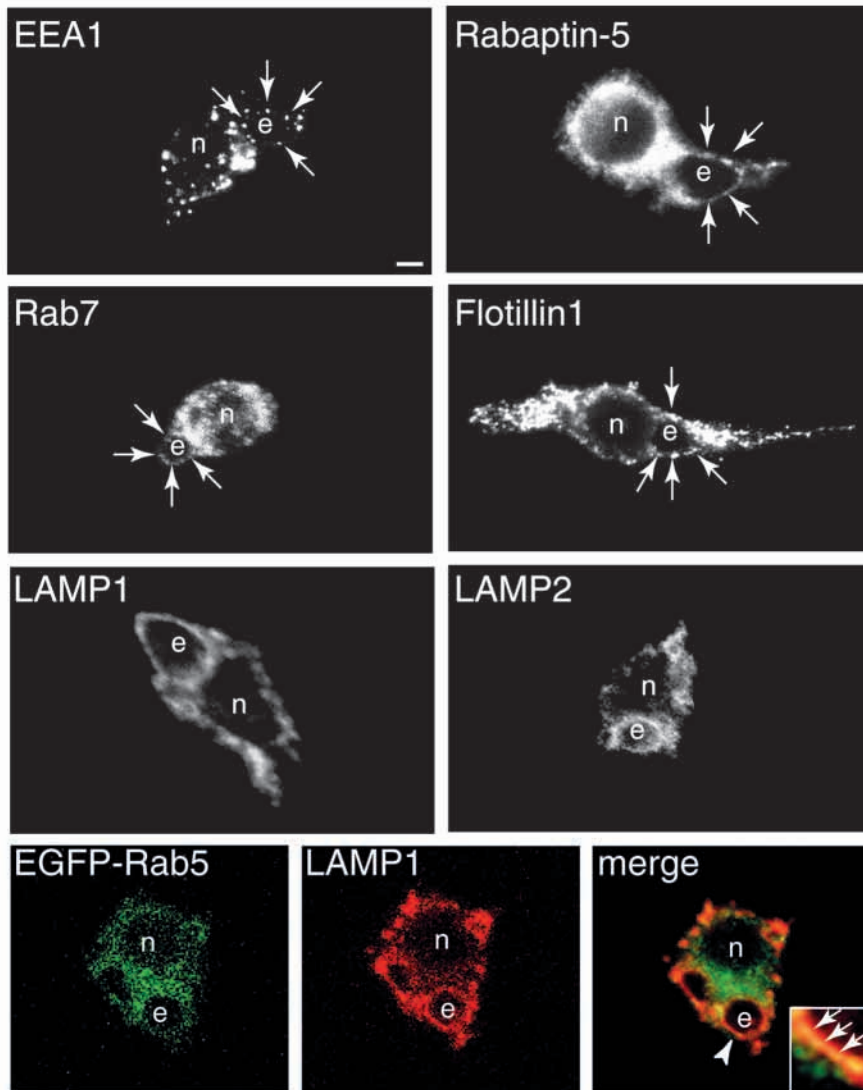


Fig. 6. Giant endosomes display early and late endosome/lysosome markers in RAW264.7 macrophages. To better confirm the nature of the large endocytic structures, rab5(Q79L)-expressing macrophages were grown on coverslips, fixed and probed with antibodies against different endosomal markers. Cells were then observed at the epifluorescence microscope. The early endosomal markers EEA1 and rabaptin-5 decorated large vesicles in a punctate pattern (arrows). The same phenomenon was observed with rab7, as well as with the recently described late endosome marker flotillin1. The large vacuoles were also positive for the late endosomal/lysosomal markers LAMP1 and LAMP2. However, these markers defined a continuous pattern around the large endosomes. Co-localization of rab5(Q79L) (designated as EGFP-rab5) with LAMP1 was observed using confocal fluorescence microscopy in cells expressing EGFP-rab5(Q79L). Arrowhead in the merge panel points at the section magnified in the inset, where arrows point at the yellow dots representing endosomal membrane domains on which EGFP-rab5(Q79L) and LAMP1 co-localize. n, nucleus; e, endosome. Bar, 10 μ m.

molecule recently shown to accumulate on maturing phagosomes (Dermine et al., 2001). Moreover, we showed that several of the large endosomes display early (rab5) and late (LAMP1) endocytic markers simultaneously, in agreement with a recent report (Rosenfeld et al., 2001). Partial co-localization of these markers was also observed in control cells. However, because of the small size of the endocytic structures in these cells, it was difficult to determine whether both markers were effectively present on the same endosomes. Thus, our results suggest that rather than being composed of distinct early and late endosomes, macrophages possess a heterogeneous population of endocytic organelles displaying mixed features. This supports previous results showing that, in J774 macrophages, in addition to its association to early endosomes, rab5 is present on late endocytic organelles, a feature proposed to be unique to this cell type (Jahraus et al., 1998).

Further observation indicated that the enlarged endocytic structures present in macrophages expressing the Q79L mutant of rab5 sequentially displayed characteristics of both early and late endosomes. Indeed, we showed that treatment of cells with

bafilomycin A1, a vacuolar proton pump inhibitor, induced the fragmentation of early endosomes in normal macrophages, as previously described in other cell lines (Clague et al., 1994; D'Arrigo et al., 1997), with no noticeable effect on the size or overall morphology of late endosomes and lysosomes. By internalizing BSA-gold of two different sizes for various periods of time, we were able to fill the early and late endosomes of a given cell with different tracers. In Q79L mutant cells treated with bafilomycin, the enlarged endosomes containing the early endocytic tracer were fragmented, whereas the morphology of enlarged endosomes containing the late endocytic tracer was not altered. These results confirmed that the enlarged endocytic structures induced by the Q79L mutation are dynamically modified and acquire with time the property of late endocytic structures to resist the fragmenting effect of bafilomycin. Together, these results indicate that endosomes are gradually modified during a process allowing their acquisition of late endocytic markers and features (resistance to bafilomycin fragmentation). These series of events are reminiscent of the maturation process taking place during phagolysosome biogenesis, where a sequential

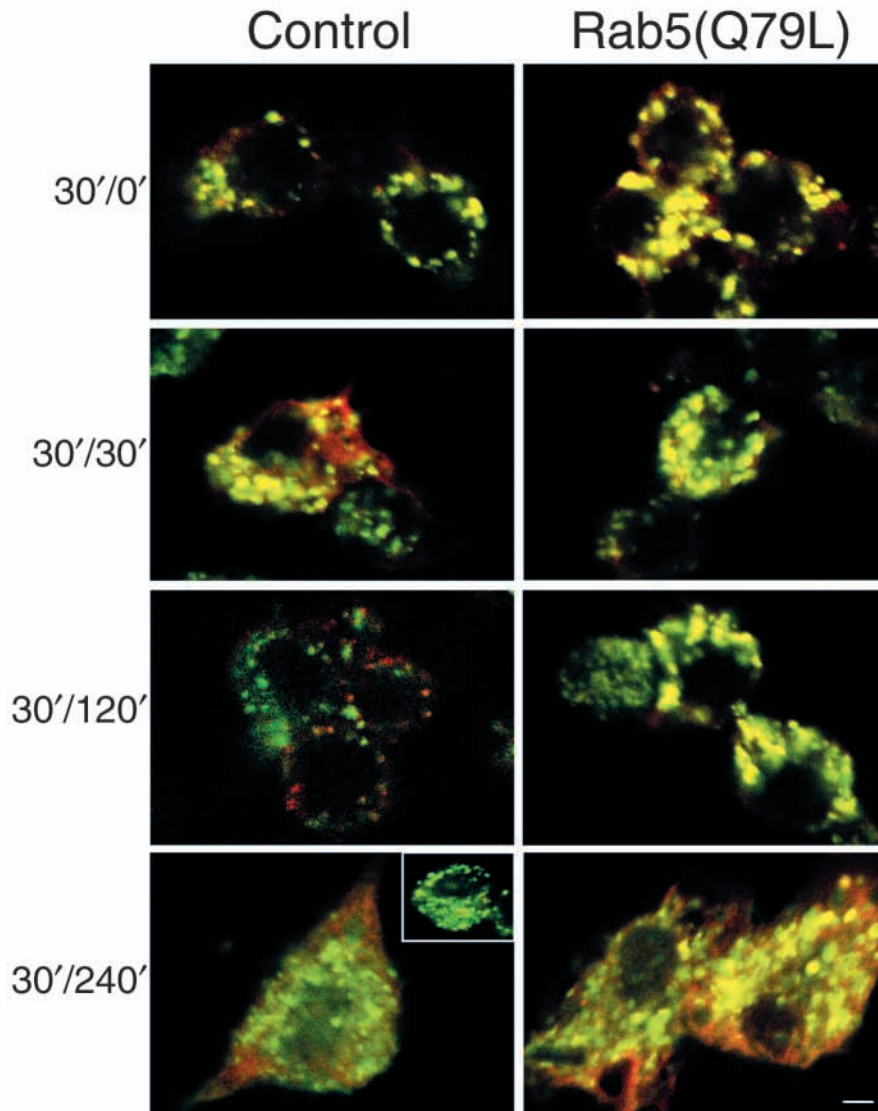


Fig. 7. Rab5 regulates the 'kiss and run' fusion between early/late endosomes. To determine whether rab5 modulates 'kiss and run' interactions between endosomes, cells were incubated with mixtures of dextrans (FDx10 and TRDx70 or FDx70 and TRDx70) for 30 minutes, then washed and further incubated for the indicated times. The distribution of dextrans was observed by confocal fluorescence microscopy. Immediately after internalization, the different-sized dextrans (FDx10, green signal and TRDx70, red signal) were co-localizing in the same organelles, labeled in yellow, in control and rab5(Q79L)-expressing cells. After a 30 minute chase, distinctly labeled endosomes were observed in control cells allowed to internalize the mixture of FDx10 and TRDx70. At the same time point in mutant cells, the different-sized dextrans were still co-localizing in the endocytic compartments. After a 120 minute chase, the segregation of different-sized dextrans was almost complete in control cells, in contrast with the mutants, where yellow endosomes showing the co-localization of the FDx10 and TRDx70 could still be observed. After a longer chase period (240 minutes), the size-dependent segregation was still observed in control cells, while the separation of the different-sized dextrans started to become apparent in mutant cells. The inset represents a control cell after a pulse-chase of 30/240 minutes of FDx70 and TRDx70, which clearly demonstrates that the segregation of dextrans depends on the size of the molecules, and not on the properties of the different fluorophores, since similar-sized dextrans co-localize in the same vesicles. Bar, 10 μ m.

acquisition of rab GTPases has been observed (Desjardins et al., 1994), and where 'kiss and run' fusion events regulated by rab5 have been shown to occur (Duclos et al., 2000).

As shown both at the light and electron microscope, albeit that the expression of rab5Q79L had a profound effect on the morphology of early-late endosomes, the mutation did not affect the transfer of the endocytic tracers to small dense lysosomes. These results suggest that although rab5 is an important regulator of fusion along the early part of lysosome biogenesis, it is unlikely to be involved in the late endosomes to lysosomes traffic. At this point, it is still unclear how the terminal formation of the lysosomal compartment occurs. It could happen by fragmentation of the late endosomes or, as proposed by Luzio et al. (Luzio et al., 2000), by the formation of a hybrid organelle between late endosomes and lysosomes, followed by condensation of the content of this organelle as well as selective recycling of membrane constituents.

The membrane of endosomes displays microdomains
The enlarged endosomes formed in rab5(Q79L)-expressing

cells increased our ability to observe the pattern of distribution of endocytic markers at the surface of endosomes. We found that several of these markers, including flotillin1, displayed a punctate pattern on endosomes. Interestingly, flotillin 1, a marker also displaying a punctate distribution on phagosomes, has recently been shown to accumulate in lipid rafts domains on the membrane of these organelles (Dermine et al., 2001). There is now increasing evidence suggesting the existence of specialized microdomains on the membrane of endocytic organelles (for reviews, see Gruenberg, 2001; Miaczynska and Zerial, 2002). For example, early endosomes and recycling endosomes have been shown to be enriched in lipid rafts (Mukherjee et al., 1998; Gagescu et al., 2000). Several reports have indicated the presence of members of the docking and fusion machinery, such as SNAREs, on specialized lipid microdomains (Schnitzer et al., 1995; Galli et al., 1996; Lafont et al., 1999). Others have reported the co-localization of rab5 and one of its effectors (EEA1) on microdomains at the surface of endosomes, although not formally identified as lipid rafts (McBride et al., 1999). The punctate distribution of EEA1 has also been observed on the membrane of phagosomes (S.D. and

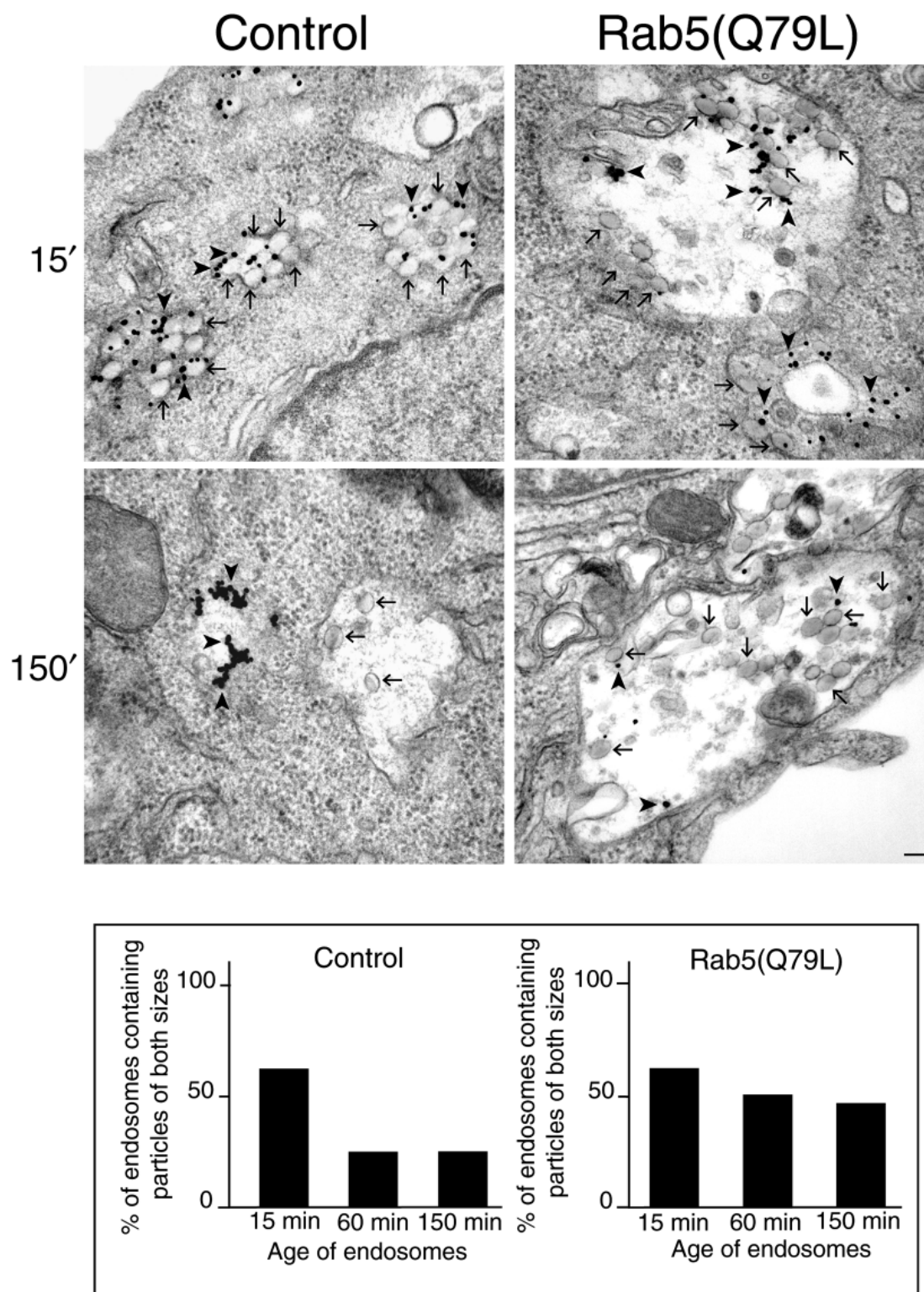


Fig. 8. 'Kiss and run' fusion between early/late endosomes. To further characterize and quantitate the transient 'kiss and run' interactions between endosomes, cells were pulse-chased with a mixture of 100 nm BSA-coated latex beads and 16 nm BSA-gold particles for the indicated times. Endosomes containing either a mixture of 100 nm (arrows) and 16 nm (arrowheads) particles, or only one of the tracers were quantified at the electron microscope. The results obtained indicate that, at the earliest time point observed (15 minutes of internalization), the majority of endosomes contained the mixture of particles in control and rab5(Q79L)-expressing cells. At 150 minutes after endocytosis, the two tracers were segregated in different endosomes in control cells, while a large proportion of endosomes still contained both of them in rab5(Q79L)-expressing cells. Quantitative analysis of these results is presented in the bottom panel. Bar, 100 nm.

M.D., unpublished). In living cells, rab5 was shown to accumulate on microdomains representing 'hot spots' for fusion on endosomes (Roberts et al., 1999). Furthermore, rab4, rab5 and rab11 have recently been shown to define distinct membrane domains on endosomes in the recycling pathway (Sönnichsen et al., 2000). Unraveling the exact function of these microdomains on endocytic organelles will provide a better understanding of lysosome biogenesis.

'Kiss and run' and lysosome biogenesis

We proposed that because most of the molecules found in endosomes are also present in phagosomes, the two pathways share common mechanistic features (Storrie and Desjardins, 1996). For example, all the small GTPases of the rab family identified on endocytic structures have been found on phagosomes (Garin et al., 2001). One of these molecules, rab5, was shown to promote 'kiss and run' interactions between endosomes and phagosomes during phagolysosome biogenesis, through its GTPase activity (Duclos et al., 2000). We show here that rab5 plays a similar role by allowing transient fusion events between endocytic organelles during the early parts of lysosome biogenesis. As a consequence, endocytic tracers of various sizes, co-internalized in macrophages, are rapidly segregated in distinct populations of endosomes. This size-selective transfer most probably reflects the occurrence of fusion events between endosomes containing the small and large tracers with endosomes devoid of tracer, as proposed previously (Berthiaume et al., 1995). Indeed, in rab5 Q79L-expressing cells, the ability to promote 'kiss and run' fusion is lost and endosomes fuse in ways allowing the constant mixing of tracers. With time, endosomes are remodeled to a point where rab5 is either no longer present or functional, allowing 'kiss and run' fusion events to resume and the efficient sorting of tracers to lysosomes to occur.

Based on our results, and by analogy with phagolysosome biogenesis, we propose a model in which endocytic organelles are part of a continuum of vesicles dynamically remodeled. This remodeling, regulated by protein complexes such as rab5 and its effectors, as well as members of the SNARE family of proteins, involves series of transient interactions of a 'kiss and run' nature. These interactions could take place at specialized membrane domains, as suggested by the punctate distribution of a number of proteins involved in membrane fusion at the surface of endocytic organelles. In this model, the GTPase activity of rab5 would act as a timer (Rybin et al., 1996), regulating the fusion/fission process between endosomes. Rab5-driven transient interactions would allow the maintenance of the size and integrity of endocytic organelles in the early parts of the pathway. Small GTPases other than rab5 could be involved in the subsequent steps of lysosome biogenesis (Mullock et al., 1998). A potential candidate being rab7, a protein present on late endosomes (Feng et al., 1995; Méresse et al., 1995; Papini et al., 1997; Vitelli et al., 1997) recently shown to be essential for the maintenance of the perinuclear lysosome compartment (Bucci et al., 2000). In the near future, proteomics and lipidomics studies of isolated endosomes and phagosomes will probably contribute to uncover all the molecules and their interactions involved in the pathways of lysosome and phagolysosome biogenesis.

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