Formation of multivesicular endosomes in Dictyostelium

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Summary

Multivesicular endosomes are present in virtually every eucaryotic cell, where they arise by intra-endosomal budding of the limiting endosomal membrane. Some genetic diseases such as Chediak-Higashi syndrome are characterized by enlarged membrane-filled endosomes. The same altered endosomal morphology can be observed in cells exposed to certain drugs, for example U18666A. The mechanisms involved are still poorly characterized, partially because this atypical budding event is particularly difficult to observe in mammalian cells. Taking advantage of the simplicity of the endosomal structure in Dictyostelium discoideum, we could visualize intra-endosomal budding at the ultrastructural level. In this model organism, the drug U18666A was shown to stimulate intra-endosomal budding, while an inhibitor of PI 3-kinase activity was found to have no effect on this process. Inactivation of a Dictyostelium gene with similarity to the gene responsible for Chediak-Higashi syndrome did not alter the intra-endosomal budding or the accumulation of intra-endosomal membranes. Thus, although treatment with U18666A and inactivation of the Chediak-Higashi gene cause similar morphological defects in mammalian cells, observations in a different model reveal that their respective modes of action are different.

Key words: Multivesicular endosome, Budding, Chediak-Higashi syndrome, Dictyostelium discoideum

Introduction

In mammalian cells, endosomes are membrane compartments into which the extracellular material internalized by the cells is delivered. At the ultrastructural level they are characterized by the presence of internal membranes in their lumen, presenting either a vesicular or a lamellar appearance. Intra-endosomal membranes contain some of the integral membrane proteins present in the limiting membrane, and therefore are thought to arise by invagination and vesiculation of the limiting endosomal membrane (intra-endosomal budding) (Murk et al., 2003; Piper and Luzio, 2001). Indeed invaginations of the limiting endosomal membrane that might correspond to intra-endosomal budding have been occasionally observed by electron microscopy (Hirsch et al., 1968; Murk et al., 2003; Van Deurs et al., 1993). Inclusion in intra-endosomal membranes is a key event in the endocytic pathway and it is in particular a mechanism to selectively target membrane proteins for degradation within lysosomes (Katzmann et al., 2002). Studies in yeast cells have identified intra-endosomal targeting signals such as ubiquitin (Reggiori and Pelham, 2001) and endosomal sorting complexes that ensure the selective inclusion of proteins in intra-endosomal membranes (Katzmann et al., 2002).

The observation of multivesicular endosomes is difficult, because of their small size and the tight packing of membranes in these compartments. Only electron microscopy provides the resolution necessary to observe intra-endosomal membranes accurately. However, this precludes observation in living cells, so to date the actual formation of an intra-endosomal vesicle has never been clearly observed. Indeed when observing a potential intra-endosomal budding profile in a fixed cell, it is impossible to decide with certainty if it represents an intra-endosomal budding, the fusion of a lumenal vesicle with the limiting membrane, or even a simple fold in the limiting membrane. These limitations severely handicap the study of diseases in which endosomal morphology is altered. In humans, at least three genetic pathologies are linked to alterations in endosomal morphology: Chediak-Higashi (CH) syndrome (Shiflett et al., 2002), Niemann-Pick C disease (Vanier and Millat, 2003) and Tangier disease (Oram, 2002). In these three conditions, abnormally large membrane-filled endosomes are present and contain an unusually high amount of cholesterol. These giant cholesterol-rich endosomal structures might be caused by a stimulation of intra-endosomal budding, by a stabilization of intra-endosomal structures, by a decrease in the fusion of intra-endosomal vesicles with the limiting membrane, or by an entirely different mechanism. A large number of drugs have been discovered and characterized on the basis of their ability to perturb intracellular cholesterol transport and metabolism in mammalian cells. Some of them, e.g. imipramine or U18666A (U18), also cause the intracellular accumulation of enlarged endosomes filled with membranous material (Liscum and Munn, 1999). The precise mechanism by which U18 alters endosomal morphology is not known. Treatment with U18 mimics genetic pathologies such as CH at the morphological level, however it is not clear whether these
two phenotypes are caused by similar alterations in endosomal dynamics.

The slime mould Dictyostelium discoideum is widely used to study the endocytic and phagocytic pathways (Rupper and Cardelli, 2001). We took advantage of the simplicity of Dictyostelium endosomes to study the formation of multivesicular endosomes. This system allows a direct observation of intra-endosomal budding and revealed that this process is highly stimulated by the action of U18. Using this system we also demonstrated that phosphoinositide 3-kinase (PI 3-kinase) and LVS, the Dictyostelium homolog of the gene involved in CH syndrome, are not essential for the intra-endosomal budding process in Dictyostelium.

Materials and Methods

Cells and media

The subclone DH1-10 (referred to as wild-type (Cornillon et al., 2000)) of the Dictyostelium wild-type strain DH1 (Caterina et al., 1994) and the Dictyostelium bwB null strain (Cornillon et al., 2002) were used in this study. Cells were grown in HL5 medium (Cornillon et al., 2000) at 20°C. For bacterial feeding, Dictyostelium cells were grown in the presence of Escherichia coli DH5α bacteria.

For pulse-chase analysis, cells were labeled for 3 hours in HL5 containing 0.4 mCi/ml of [35S]methionine (trans [35S]-label; ICN Radiochemicals). Labeled cells were washed and chased for 0, 1, 2 or 4 hours by incubating in HL5 containing unlabeled methionine and 20 μg/ml U18666A (Biomol, Zürich, Switzerland), as indicated. At each time point, cells were collected, pelleted by centrifugation and frozen at –70°C before detergent lysis and immunoprecipitation of p80 with H161, as described previously (Ravanel et al., 2001). Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions.

Electron microscopy

To analyze the morphology of endosomal membranes, cells were incubated in the indicated conditions. They were then fixed for 1 hour in HL5 medium containing 2% glutaraldehyde and 0.3% osmium tetroxide. Then the cells were embedded in Epon resin and processed for conventional electron microscopy as described previously (Orci et al., 1973).

To analyze the composition of endosomal membranes, cells were fixed for 1 hour at room temperature in HL5 containing 2% paraformaldehyde and 0.2% glutaraldehyde. Fixed cells were rinsed three times with phosphate buffer (0.1 M, pH 7.4) and the cells processed for cryosectioning essentially as described previously (Liou et al., 1996). Briefly, the cell pellet was infiltrated with sucrose and frozen in liquid nitrogen. Frozen sections were cut with a Leica FCS cryotome, transferred to grids, and incubated with H161 monoclonal antibody specific for the p80 endosomal protein, then with a gold-coupled antibody to mouse immunoglobulins. As described previously (Ravanel et al., 2001), the H161 antibody stains endosomal compartments intensely and specifically.

For freeze-fracture, cells were fixed for 1 hour at room temperature in HL5 containing 2.5% glutaraldehyde, and the samples were infiltrated for 1 hour in 30% phosphate-buffered glycerol and frozen in Freon 22 that had been cooled with liquid nitrogen. Cells were fractured and shadowed in a Balzers BAF 301 apparatus (High Vacuum Corp., Balzers, Lichtenstein). The replicas were washed in a sodium hypochlorite solution, rinsed in distilled water and mounted on Formvar- and carbon-coated copper grids. Epon sections, cryosection and cryofracture were examined and photographed in a Philips CM-10 transmission electron microscope (Philips, Eindhoven, The Netherlands) at calibrated magnifications.

Fluorescence microscopy

Cells attached to glass coverslips in HL5 medium were treated for 2 hours with 20 μg/ml U18666A, then fixed in HL5 containing 4% paraformaldehyde, rinsed with PBS and permeabilized in PBS containing 0.1% saponin. Cells were processed for immunofluorescence by successive incubations with H161 monoclonal antibody and with a mixture of an Alexa 488-coupled donkey anti-mouse Ig and 250 μg/ml filipin III (Sigma). Pictures of cells were taken with an AxioCam camera (Carl Zeiss).

Acidic endocytic compartments were observed in living cells using LysoSensor (Molecular Probes). For this, DH1-10 and bwB null cells were grown on a glass coverslip and then incubated in the presence of 1 μg/ml of LysoSensor for 15 minutes before observation with a LSM 510 Zeiss confocal microscope.

Internalization of the fluid phase was measured as described previously (Cornillon et al., 2000). Briefly 10⁵ cells were resuspended in 1 ml of HL5 medium containing 0.5 mg/ml of FITC-dextran (Molecular Probes, Eugene, Oregon, USA). The cell suspension was incubated on a rotating shaker for 30 minutes, then washed twice with ice-cold HL5. The internalized material was quantified using a fluorescence activated cell sorter (FACSCalibur, Beckton-Dickinson, San Jose, CA, USA).

Results

Dictyostelium amoeba can be fed either with bacteria or by growing them in nutrient rich medium (HL5 medium). Cells fed with bacteria contain endosomes filled with membranous structures (Hohl, 1965) (Fig. 1A), reminiscent of mammalian multivesicular and multilamellar endosomes. The origin of these intra-endosomal membranes is not fully understood, they may derive from the limiting endosomal membrane and/or incorporate incompletely digested bacterial material. However, as described previously, when cells are grown in HL5 medium, Dictyostelium endosomes appear as large vacuoles mostly devoid of internal membranous material (Ravanel et al., 2001; Ryter and de Chastellier, 1977) (Fig. 1B). These results indicate that the occurrence of internal membranes in Dictyostelium endosomes is a physiological event modulated by growth conditions.

Exploiting the simplicity of Dictyostelium endosomes when cells are grown in liquid medium, we studied the effects of the drug U18 on their structure. In liquid medium, treatment with U18 for 2 hours led to the accumulation of large multivesicular structures, reminiscent of those observed in mammalian cells (Fig. 1C). This process is very fast in Dictyostelium cells compared to mammalian cells: only very minor effects are seen in mammalian cells treated for 2 hours with U18, and enlarged endosomes only become apparent after 6 to 24 hours of U18 treatment (data not shown).

Since untreated Dictyostelium endosomes are essentially devoid of internal membranes, and massive membrane accumulation is evident after 2 hours of U18 treatment, analysis at shorter treatment times would be expected to produce evidence of intra-endosomal vesicle formation and clues about the mode of action of U18. When cells were treated for very short periods of time with U18 (5-15 minutes), the initial steps of multivesicular endosome formation could indeed be observed. Inward budding profiles were clearly seen and these buds invariably appeared to contain cytosolic material (Fig. 2). These invaginations of the limiting endosomal membrane were easily distinguished from the majority of intra-endosomal vesicular profiles that appeared
empty and were never seen in continuity with the limiting membrane. Empty intra-endosomal vesicles were readily detected after 5 minutes of U18 treatment. They accumulated with time, making putative buds more and more difficult to visualize, owing to the increased complexity of the endosomal structure (Fig. 1 and data not shown). In every case, the membrane delimiting putative buds and intra-endosomal vesicles had a typical bilayer structure (Fig. 2). One simple interpretation of these findings is that inward buds give rise to intra-endosomal vesicles from which the cytosolic content is rapidly lost. Our observations provide no evidence about how the cytosolic material is lost (i.e. degraded or recycled back to the cytosol). However vesicles containing partially condensed cytosolic material were often seen after short U18 treatments (Fig. 2B), and might represent intermediate stages between the budding profiles and the empty intra-endosomal vesicles. Intra-endosomal buds occasionally appeared elongated (Fig. 2C), and may give rise to more lamellar intra-endosomal structures, as seen for example in Fig. 1. In addition to allowing a clear visualization of the inward budding process, these results demonstrated that U18 modifies the endosomal structure by dramatically increasing the rate of intra-endosomal budding.

In order to determine the nature of the compartment where membrane accumulation was taking place, we performed immunolabeling of cryosectioned samples with an antibody directed against p80, a specific marker of Dictyostelium endosomes (Ravanel et al., 2001). This revealed that the inward budding seen after a short U18 treatment occurs in a compartment positive for p80, i.e. an endosomal compartment (Fig. 3B). The more complex structures resulting from accumulation of internal membranes after 2 hours of treatment were also localized in p80-positive endosomal compartments (Fig. 3C). In addition to establishing the identity of the affected compartments, these results also show the sorting of the p80 protein. Prior to U18 treatment, p80 is present in the limiting endosomal membrane, and no intra-endosomal membranes are present (Fig. 3A). After a short U18 treatment (15 minutes), p80 was incorporated into the intra-endosomal membranes (Fig. 3B). After longer periods of treatment (2 hours, Fig. 3C), a large fraction of the endosomal p80 (approximately two thirds in Fig. 3C) appeared associated...
with the limiting endosomal membrane, suggesting that p80 was gradually depleted from intra-endosomal membranes. Pulse-chase analysis indicated that the p80 protein was very stable both in untreated and in U18-treated cells (Fig. 3D), suggesting that this gradual exclusion was not caused by a selective degradation of p80 in intra-endosomal membranes, but rather by a specific retrieval to the limiting endosomal membrane.

The tight packing of intra-endosomal structures and the intrinsic limitations of immunolocalization made it difficult to determine the exact proportion of the p80 protein associated with the limiting membrane, and thus to bring quantitative evidence for an exclusion of p80 from intra-endosomal membranes after a prolonged exposure to U18. In order to more clearly demonstrate differences between the composition of intra-endosomal membranes and of the limiting endosomal membrane, we performed freeze-fracture analysis of cells treated with U18. In freeze-fracture replicas of endosomal membranes the fracture plane splits the membrane along their hydrophobic interior and exposes the inner aspect of either the cytosolic leaflet (protoplasmic, P-face) or the lumenal leaflet (exoplasmic, E-face). Intramembrane particles correspond to integral membrane proteins, and the particle density of fracture faces affords a relative measure of the protein content of a membrane (Branton, 1966; Yu and Branton, 1976). Multivesicular endosomes resulting from a 2 hours treatment with U18 could easily be identified based on their specific morphology. While the limiting endosomal membrane presented a high density of particles, both the P and E faces of intra-endosomal membranes appeared very poor in particles, indicating that these membranes are depleted in integral membrane proteins (Fig. 4). This indicates that a large number of integral membrane proteins are excluded from the intra-endosomal membranes after a 2 hours treatment with U18. As in mammalian cells, the production of intra-endosomal structures in Dictyostelium cells is accompanied by a sorting of membrane components.

In order to analyze the lipid composition of membranes accumulated in multivesicular endosomes, we performed a double staining of U18-treated cells with p80 and filipin, a sterol-specific marker (Fig. 5). In untreated Dictyostelium cells filipin stained numerous membranes, including p80-positive endosomes. In cells treated with U18 for 2 hours, filipin intensely stained a few intracellular structures, which were also positive for p80. These results revealed that membrane accumulation in Dictyostelium endosomes was accompanied by an accumulation of sterols. This feature is similar to observations in mammalian cells, where large multivesicular endosomes, induced by pathological situations or by drug treatments, contain high amounts of cholesterol (Liscum and Munn, 1999).

Together these data suggest that multivesicular endosomes formed in Dictyostelium cells are similar to those observed in mammalian cells in several respects: morphology,
accumulation of sterols and active sorting of membrane proteins. As in mammalian cells, U18 promotes the formation of huge membrane-filled endosomes in Dictyostelium, and our results indicate that this is caused by an increase in intra-endosomal budding. Using this system, we analyzed the role of two cellular components potentially implicated in the biogenesis of endosomes, the enzyme phosphatidylinositide 3-kinase (PI 3-kinase) and the LvsB/CH protein, in the formation of intra-endosomal vesicles.

Recent studies have highlighted the role of PI 3-kinases in the control of the endocytic pathway (Fernandez-Borja et al., 1999; Futter et al., 2001; Odorizzi et al., 2000; Petiot et al., 2003), although their exact role in transport, membrane sorting, or endosome biogenesis is still being debated. To evaluate the role of PI 3-kinase activity in intra-endosomal budding in our system, we exposed cells to LY-294002 (LY), a well-characterized inhibitor of PI 3-kinase activity in Dictyostelium (Funamoto et al., 2001; Rupper and Cardelli, 2001). Exposure to 30 µM LY inhibited fluid phase uptake (Fig. 6A), a macropinocytic process controlled by PI 3-kinase in Dictyostelium (Rupper and Cardelli, 2001). Exposure to 30 µM LY inhibited fluid phase uptake (Fig. 6A), a macropinocytic process controlled by PI 3-kinase in Dictyostelium (Rupper and Cardelli, 2001), demonstrating that PI 3-kinase activity was effectively inhibited under the conditions of our assay. This effect was virtually immediate and reversible, while treatment with higher concentrations of LY were not fully reversible (data not shown). Treatment of cells with LY alone did not affect endosome structure (Fig. 6B), nor did it prevent the formation of intra-endosomal structures in U18-treated cells (Fig. 6). Intra-endosomal budding profiles seen after short U18 treatment were identical in the presence or absence of LY (Fig. 6C), and the accumulation of intraendosomal membranes after longer U18 treatment was also not affected by the addition of LY (Fig. 6D). Thus the effect of U18 on inward budding is independent of the PI 3-kinase activity. These results also indicate that the activity of PI 3-kinase is not essential for the intra-endosomal budding process.

Alterations in the endosomal structure are also seen in genetic diseases such as the CH syndrome. Cells from CH patients contain unusually large endosomes filled with membranous material and have abnormal secretion of lysosomal enzymes (Shiflett et al., 2002). It is not clear if the accumulation of intra-endosomal membranes is the primary cause of the CH phenotype, or a secondary consequence. In Dictyostelium six gene products (called LvsA to F) exhibit a BEACH (Beige and Chediak-Higashi) homology domain, and the most homologous to the CH protein is LvsB (Cornillon et al., 2002; Harris et al., 2002). In lvsB mutant cells the structure and function of endosomal compartments are altered in a similar manner to that observed in mammalian CH cells: endosomes appear bigger (Fig. 7A) (Harris et al., 2002) and secretion of lysosomal enzymes is altered (Cornillon et al., 2002; Harris et al., 2002). This phenotype is not observed in lvsA, C, D, E or F mutants, suggesting that LvsB plays a unique role in endosomal physiology in Dictyostelium. Despite this
This does not rule out the possibility that a subset of integral endosomal vesicles and the limiting endosomal membrane. and that a very active exchange takes place between the intracellular membranes of membrane proteins from intracellular membranes. Our results also suggest that LvsB does not play an essential role in the formation of intra-endosomal budding. A detailed ultrastructural analysis revealed that endosomes in lvsB cells did not exhibit any unusual accumulation of internal membranes. Moreover, upon U18 treatment, intra-endosomal budding in lvsB cells appeared identical to budding in wild-type cells and led to a similar accumulation of intra-endosomal membranous structures. Taken together, these results suggest that LvsB does not play an essential role in intra-endosomal budding.

Discussion
In this study we have examined the formation of multivesicular endosomes in Dictyostelium cells. The intricate nature of endosomes makes structural analysis complex in mammalian cells. On the contrary in Dictyostelium cells, the endosomal structure is relatively simple. It is also flexible, and can be modulated in particular by culture conditions or by the use of drugs like U18666A. These unusual features allowed us to study, at the ultrastructural level, the formation of multivesicular endosomes. The formation of intra-endosomal vesicles was clearly visualized, in particular the appearance of intra-endosomal budding profiles. Our results also suggest that membrane sorting allows the retrieval of a large number of integral membrane proteins from intra-endosomal membranes and that a very active exchange takes place between the intra-endosomal vesicles and the limiting endosomal membrane. This does not rule out the possibility that a subset of integral membrane proteins might remain or even be concentrated in intra-endosomal membranes, as reported in mammalian cells (Piper and Luzio, 2001).

In yeast cells, a detailed ultrastructural analysis led to the observation of intra-endosomal budding profiles and integral membrane proteins were depleted from these budding membranes (Muller et al., 2000). This intra-endosomal budding is morphologically equivalent to our observations, but there are a number of differences in the experimental conditions and in the results obtained. In particular, in our system, budding is induced by exposure to U18, a drug known to affect endosomal structure in mammalian cells, but which to our knowledge has not been used in yeast cells. Another major difference is the observation that in yeast, exclusion of integral membrane proteins was shown to accompany the invagination of autophagic tubules, while in our model, membrane sorting appears to occur at a later stage. More detailed studies will, however, be necessary to determine the site and mechanisms of sorting of membrane proteins in multivesicular endosomes and to establish a comparison with sorting mechanisms observed in other systems (Katzmann et al., 2002; Reggiori and Pelham, 2001).

Another aspect of the process of intra-endosomal budding is the sequestration of a portion of cytosol. This has been shown previously to lead to the eventual degradation of cytosolic components and even of entire organelles (Wang and Klionsky, 2003). This process is termed microautophagy, as opposed to macroautophagy, a process where nascent autophagosomes enclose a portion of cytosol in a double membrane and later fuse with lysosomes. While a lot of attention has been devoted to the characterization of macroautophagy, both at the morphological and molecular level, molecular details and functional importance of the microautophagic pathway are largely unknown (Wang and Klionsky, 2003). Intra-endosomal budding as observed in Dictyostelium also leads to the sequestration of cytosolic material. Our observations agree with the notion that the cytosolic material enclosed in intra-endosomal vesicles is condensed and degraded shortly after their budding, although further experiments will be necessary to confirm this point. The relative ease with which intra-endosomal budding can be induced and visualized in Dictyostelium might help to clarify its potential importance, its relationship with the macroautophagic pathway, and its regulation in response to nutrients or to various classes of drugs.

One of the major advantages of using Dictyostelium as a model system is to allow the simple evaluation of the effect of various compounds or mutations on the biogenesis of multivesicular endosomes. In particular we tested whether the activity of the PI 3-kinase is essential for the formation of intra-endosomal membranes by exposing cells to an inhibitor of this class of enzymes. Intra-endosomal budding as well as accumulation of intra-endosomal membranes were clearly unaffected by the inhibition of PI 3-kinase activity. While these results demonstrate unambiguously that PI 3-kinase does not play an essential role in the formation of intra-endosomal membranes, they are perfectly compatible with the notion that PI 3-kinase can regulate this process, assuming that this regulation is bypassed by U18 treatment in our experiments.

Similarly, our results suggest that LvsB does not play an essential role in intra-endosomal budding or in the
accumulation of intra-endosomal membranes. It is striking that in this system several features of the CH phenotype are reproduced in the lvsB mutant (enlarged endosomes, abnormal secretion of lysosomal enzymes), while no abnormal accumulation of intra-endosomal membranes is observed. Thus accumulation of intra-endosomal membranes does not necessarily accompany the CH phenotype and it is presumably a secondary consequence of some other defect. The increase in endosome-endosome fusion, reported previously in lvsB mutant cells (Harris et al., 2002), might be the primary cause of their phenotype. According to this interpretation, the fact that giant CH endosomes are filled with membranous structures in mammalian cells might simply reflect the fact that constitutive intra-endosomal budding is more active in mammalian cells than in Dictyostelium.

Although mammalian cells treated with U18 display a phenotype very similar to CH cells, our analysis suggests that these phenotypes might be caused by very different mechanisms: U18 directly promotes intra-endosomal budding, while LvsB/CH primarily controls the size of endosomes, possibly by affecting their rate of fusion, and does not affect the biogenesis of intra-endosomal membranes.

In addition to CH protein, many gene products have been implicated in the formation of multivesicular endosomes in mammals and in yeast (Katzmann et al., 2002; Pelham, 2002). Many of these genes are also found in the Dictyostelium genome. The relative ease with which mutants can be generated in Dictyostelium should aid the assessment of the role of these gene products in the intra-endosomal budding process and contribute to a better understanding of the establishment and maintenance of the endosomal structure.

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