Molecular mechanisms of membrane trafficking. What do we learn from Dictyostelium discoideum?

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Abstract

Thanks to increasing interest, understanding of the molecular mechanisms underlying intracellular membrane transport is truly emerging. An even more rapid advance is expected since the recognition that efforts to unravel both membrane traffic and cytoskeleton need to be concerted. The two main membrane traffic pathways are the biosynthetic/secretory route, where transport occurs from the ER via the Golgi apparatus to the plasma membrane and the endocytic route involving the flow from the plasma membrane to the degradative compartment. These two pathways intersect at many compartments, both comprise multiple transport steps and both involve efficient sorting of membrane and soluble proteins. Here, we review recent studies on the dissection of endocytic trafficking in Dictyostelium discoideum and integrate the information in the light of knowledge acquired from other systems.


PMID: 10575697
DOI: 10.1016/s1434-4610(99)70026-x
PROTIST NEWS

Molecular Mechanisms of Membrane Trafficking. What do we Learn from Dictyostelium discoideum?

Thanks to increasing interest, understanding of the molecular mechanisms underlying intracellular membrane transport is truly emerging. An even more rapid advance is expected since the recognition that efforts to unravel both membrane traffic and cytoskeleton need to be concerted. The two main membrane traffic pathways are the biosynthetic/secretory route, where transport occurs from the ER via the Golgi apparatus to the plasma membrane and the endocytic route involving the flow from the plasma membrane to the degradative compartment. These two pathways intersect at many compartments, both comprise multiple transport steps and both involve efficient sorting of membrane and soluble proteins. Here, we review recent studies on the dissection of endocytic trafficking in Dictyostelium discoideum and integrate the information in the light of knowledge acquired from other systems.

Dictyostelium discoideum as Model System

Morphological studies of animal cells and quantitative assays to measure traffic in vitro and in vivo together with genetic approaches using Drosophila melanogaster, Saccharomyces cerevisiae and D. discoideum have been applied to unravel molecular mechanisms of membrane trafficking in the endocytic pathway. Isolation and characterisation of yeast mutants with endocytosis defects has been especially productive in identifying molecules involved in the internalisation step (Geli and Riezman 1998). D. discoideum offers unique advantages for the investigation of the endomembrane system. This cellular social amoeba lives on the forest floor feeding on bacteria and yeast; laboratory strains can grow in liquid media. Vegetative cells exhibit phagocytosis and pinocytosis rates 2–10 fold higher than those of professional phagocytes such as macrophages and neutrophils (Thilo 1985). In comparison, yeast cells pinocytose about six times less fluid-phase marker per cell mass than macrophages (Riezman 1985; Swanson et al. 1985). In addition, endocytosis is essential for D. discoideum, whereas it is dispensable for yeast, which uses it mainly for uptake of mating factor (Riezman 1993). Adherent D. discoideum cells have diameters of 10–20 µm and an overall morphology closely resembling the one of leukocytes, making these cells very well suited to microscopical investigations of endosomal compartments down to the ultrastructural level. Finally, D. discoideum is easy and inexpensive to grow, allowing large scale biochemical investigations. Extensive genetic screening by random "restriction enzyme mediated insertion" (REMI) mutagenesis is used to identify genes involved in various cellular processes. It is also worth mentioning that an international genome sequencing project was recently established (Loomis 1998).

A Model for the Endocytic Pathway in D. discoideum

Figure 1 schematically integrates recent data on the endocytic pathway in D. discoideum. As not all processes presented have been demonstrated yet, the figure should serve as a framework to organise the present discussion and as working model for future studies. At first glance some discrepancies seem apparent between the endosomal pathways of D. discoideum and mammalian cells. One of our objectives is thus to emphasise that differences in the balance of transport steps between different organisms should not mask a high degree of similarity.

Uptake Mechanisms

Four pinocytic pathways have been identified in mammalian cells, differing in the size of the pinching vesicles and the composition of their respective..
Figure 1a. A working model presenting the uptake mechanisms and major steps of endosomal membrane transport in *D. discoideum*. Fluid-phase markers are mainly taken up by macropinocytosis, but other micropinocytic uptake mechanisms likely exist. Soon after shedding their actin-coat (●), pinosomes acquire proton pumps (●) and lysosomal enzymes with GlcNAc-1-P modification, such as cysteine proteases (●). The endosomes rapidly acidify to pH values between 4.5–5. Proton-pump delivery may occur via Rab4- (●) regulated trafficking of clathrin-coated (xxx) vesicles between the contractile vacuole system (CV) and the endosomes. Both organelles share the identical proton pump. Alternatively, the proton pumps may be delivered directly from the Golgi apparatus (§). Delivery of lysosomal enzymes with M-6-P modification (●) occurs after an increase in lumenal pH. Enzymes with GlcNAc-1-P and M-6-P modification only rarely co-localize. No classical early endosomal recycling compartment has been identified yet. The pH then raises and reaches nearly neutral values in vacuolin-positive (●) post-lysosomes, from where excess fluid phase is egested from the cell. Concomitantly with the concentration of fluid-phase markers, Rab7 (●) and dynaminA- (●) positive vesicles are likely involved in retrieval of lysosomal enzymes and proton pumps from this compartment. The function of the contractile vacuole (CV) is dependent on Rab11 (●).

Figure 1b. Immunofluorescence staining of antigens localised to the endosomal compartments indicated in Figure 1a. F-actin (●) is in the cortical actin cytoskeleton and on early and late vacuoles of the endosomal system (stained with phalloidin coupled to Texas-Red). Coronin (●) is an actin-binding protein localised to the cortex and in the actin coat of newly formed macropinosomes, as well as post-lysosomes before they acquire vacuolin (monoclonal antibody 176-3-6 (de Hostos et al. 1993), gift from Dr. G. Gerisch, MPI for Biochemistry, Munich). The vacuolar-H^+-ATPase (●) is associated with membranes of the contractile vacuole complex and, to a much lesser extent with endosomes (monoclonal antibody 221-35-2 against the A-subunit of the vacuolar-H^+-ATPase (Neuhaus et al. 1998)). The common Antigen-1 (●) is a M-6-P-containing epitope shared by *D. discoideum* lysosomal enzymes such as (-mannosidase and (-glucosidase (Freeze et al. 1990) (monoclonal antibody 221-342-5 (Neuhaus et al. 1998)). Dynamin A (●) is a GTPase potentially involved in vesicle scission and is localised around post-lysosomal vacuoles in a punctate fashion (GFP-dynaminA (Wienke et al. 1999)). Vacuolin (●) is localised on post-lysosomes and is involved in exocytosis from this compartment (monoclonal antibody 221-1-1 (Jenne et al. 1998), gift from Dr. M. Maniak, MRC Laboratory for Molecular Cell Biology, London).
coats. Although the clathrin-dependent receptor-mediated endocytosis pathway has received the most attention in the past, it has become clear that the alternate pathways also serve important physiological functions. Macropinocytosis leads to bulk fluid-phase uptake through actin driven membrane ruffles which extrude and fold back onto the plasma membrane, thereby entrapping large portions of extracellular fluid (Hewlett et al. 1994; Swanson 1989). At least two other uptake mechanisms exist in vertebrate cells, one using small non-coated vesicles that may be important for membrane homeostasis in a clathrin-independent fashion (Cupers et al. 1994; Hansen et al. 1991) and the caveolae, which are flask-shaped, glycolipid- and cholesterol-enriched invaginations involved in transcytosis in endothelial cells (Palade 1953) and in internalisation of some GPI-anchored proteins (Parton et al. 1994; Rothberg et al. 1992).

Suppression of clathrin heavy chain synthesis reduces total fluid-phase uptake in D. discoideum by 80% (O’Halloran and Anderson 1992). This reduction might be due directly to decreased internalisation or to a secondary defect in other intracellular membrane trafficking steps. In this respect it is remarkable that clathrin heavy chain mutant cells contain no detectable tubular contractile vacuole network (Neuhaus and Soldati, unpublished results; O’Halloran and Anderson 1992). Internalisation of macromolecules via cell surface receptors has not been observed in D. discoideum. In addition, it was calculated by comparing internalised volume and membrane surface that the average diameter of primary pinosomes is 0.6 μm (Thilo and Vogel 1980). Extensive observation of living D. discoideum cells with confocal microscopy further showed that newly formed pinosomes average 1.6 μm in diameter and that the frequency of macropinosome formation may account for nearly all fluid-phase uptake (Hacker et al. 1997).

Nevertheless, other macropinocytic uptake mechanisms likely exist in this organism, as cells overexpressing Rap1, a member of the Ras-like superfamily of GTPases, show a 60% reduction in fluid-phase uptake but completely lack visible macropinocytic structures (Seastone et al. 1999). It is also noteworthy that detergent insoluble lipid rafts, which have been implicated in caveolae-mediated endocytosis in mammalian cells, were found in D. discoideum (Xiao and Devreotes 1997).

Phagocytosis happens via two different mechanisms. The better-known one is zippering, which is a triggered process induced by particle attachment to the plasma membrane followed by directed pseudopod progression and finally complete wrapping of the particle. The second phagocytosis mechanism closely resembles macropinocytosis and employs membrane ruffles that fold back forming large vacuoles containing fluid-phase, either alone (macropinocytosis) or together with particles (un-specific phagocytosis). Both mechanisms are differently regulated in mammalian cells (Swanson and Baer 1995). Zippering and membrane ruffling have both been observed in D. discoideum (Hacker et al. 1997; Maniak et al. 1995).

### Molecular Aspects of the Internalisation Processes

Actin cross-linking proteins and small guanosine triphosphatases (GTPases) of the Rac and Rho family that regulate and organise the actin cytoskeleton are important in the process of ruffle extension in D. discoideum (Seastone et al. 1998; Seastone et al. 1999). A subset of actin-binding proteins associate transiently with the phagocytic cup of amoebae such as talin (Niewöhner et al. 1997) and coronin, which is thought to regulate the G- to F-actin equilibrium (Maniak et al. 1995). Furthermore, macropinocytosis and phagocytosis share many components of the cytoskeletal machinery involved in formation of membrane ruffles, but their trigger and regulation are probably different (Maniak 1999).

Mechanochemical enzymes are required for the dynamics of phagocytic cup formation, both for the closure of the cup (Swanson et al. 1999), and for the intracellular processing of the engulfed particle or fluid-phase. Recently, myoi, the only known member of the class VII in D. discoideum, was shown to play a specific role in the uptake of particles (Dr. M. Titus, personal communication). More generally, the biochemical characteristics of class I myosins have suggested roles in membrane dynamics (Coluccio 1997; Hasson and Mooseker 1995). It was proposed that class I myosins are important for localised actin polymerisation that cause deformation of the plasma membrane and generation of projections preceding the formation of macropinosomes/phagosomes (Ostap and Pollard 1996). Myosins of the class I have been localised to membrane ruffles and phagocytic cups in D. discoideum (Fukui et al. 1989; Morita et al. 1996), Entamoeba histolytica (Voigt et al. 1999), Acanthamoeba castellanii (Baines et al. 1992; Baines et al. 1995; Jontes et al. 1998) and macrophages (Allen and Aderem 1995; Swanson et al. 1999). D. discoideum cells lacking either myosin IB, IC or IK exhibit significant reduction of the initial rate of particle uptake [Jung et al. 1996; Schwarz EC, Neuhaus EM, Kistler C, and Soldati T:...
Dictyostelium MyoK, a novel type of myosin with crucial functions in motility and maintenance of cortical integrity [submitted], and double deletion mutants (myoA-/myoB-, myoC-/myoB-) show conditional integrity (submitted), and double deletion mutant Dictyostelium MyoK, a novel type of myosin with crucial functions in motility and maintenance of cortical integrity (Kohtz et al. 1990; Salisbury et al. 1980). Involvement of the actin cortex in the mobility of GFP-labelled clathrin-coated pits was recently shown in living mammalian (Gaidarov et al. 1999) and D. discoideum cells (Damer et al. 1998; Mol Biol Cell 9: 130a).

Myosins of the class I may power the movement of the newly formed vesicles through the actin cortex, or may act at a late stage of vesicle formation, like the detachment of the endocytic vesicles from the plasma membrane or internal vacuoles. MYO5 and MYO3, the myosins of class I in yeast, are required for a budding event in receptor-mediated endocytosis (Geli and Riezman 1996; Goodson and Spudich 1995). Homologues of these yeast class I myosins are implicated in endocytosis in many other organisms and may well execute a variety of similar tasks. E. histolytica myosin IB is localised on intracellular vesicles and on macropinosomes and phagosomes in a punctate fashion (Voigt et al. 1999). Aspergillus nidulans myosin IA displays a punctate staining in the cytoplasm and at the growing tip of hyphae (Yamashita and May 1998). Acanthamoeba castellani myosin IB is localised on intracellular phagocytic and macropinocytic structures (Baines et al. 1992; Ostap et al. 1997; Mol Biol Cell 8: 370a). In an analogous way, myosin IB from D. discoideum is enriched in an early endosomal fraction (Neuhaus and Soldati, unpublished results). Moreover, another type of myosin I, the mouse myosin Ia is associated with endosomes and lysosomes in hepatoma cells (Raposo et al. 1999).

Is there Early Recycling in Dictyostelium discoideum?

The cytoskeletal coat is released soon after closure of the macropinocytic cup (Hacker et al. 1997). Pinosomes then rapidly acidify and acquire lysosomal marker (Aubry et al. 1993; Maniak 1999; Padh et al. 1993). Acidification could take place via proton-pump (vacuolar-H+-ATPase) delivery from the Golgi apparatus or via membrane-trafficking events between the endosomes and the contractile vacuole, which share the identical proton pump (Heuser et al. 1993; Temesvari et al. 1996). The contractile vacuole system in D. discoideum is a morphologically highly complex organelle that is primarily responsible for removing excess water (Heuser et al. 1993). It is striking that no classical early endosomal recycling compartment has been unambiguously identified in D. discoideum.

In mammalian cells, recycling of endocytosed plasma membrane proteins and lipids requires passage through a series of compartments. After internalisation, clathrin-coated vesicles deliver their cargo to early sorting endosomes that exhibit tubular-vesicular morphology. Due to their acidic internal pH, early sorting endosomes facilitate the dissociation of many receptor-ligand complexes. Dissociated ligands and other fluid-phase components are then delivered to late endosomes while lipids and other membrane components are removed from the early sorting compartment and enter a pericentriolar recycling endosome (Apodaca et al. 1994; Daro et al. 1996). Receptor recycling occurs from two distinct early endosomal compartments with fast and slow kinetics (Sheff et al. 1999). In mammalian cells, Rab4 and Rab11 have been implicated in recycling to the plasma membrane. Rab4 seems to be a regulator of fast transferrin receptor recycling, it is present mainly on early sorting endosomes, but absent from pericentriolar recycling endosomes (Daro et al. 1996; van der Sluijs et al. 1992). Rab11 localises to the pericentriolar recycling endosomes and regulates transport from the early sorting endosome to this compartment (Ullrich et al. 1996).

RabD, the D. discoideum Rab4 homologue, is highly enriched on acidic lysosomes, and found to a lesser extent on pinosomes and post-lysosomes. In addition, RabD co-localises with the vacuolar-H+-ATPase in the reticulum of the contractile vacuole system (Bush et al. 1994). Cells expressing RabD dominant negative mutants are impaired in their ability to regulate water homeostasis. Efflux from lysosomes to post-lysosomes is also impaired, resulting in a prolonged transit time and decreased number of post-lysosomal vacuoles (Bush et al. 1996). Interestingly, PI3-kinase, shown to interact with Rab5 in mammalian cells (Simonsen et al. 1998), regulates traffic from lysosomes to post-lysosomes in D. discoideum (Buczynski et al. 1997b), perhaps by modulating RabD activity.

In addition, a D. discoideum Rab11 homologue was recently identified and localised to the contractile vacuole system. Cells expressing a Rab11 dominant negative mutant show a swollen contractile vacuole reticular network and are impaired in contractile vacuole function (Bush et al. 1998; Mol Biol Cell 9: 464a). Together these observations indicate that RabD may regulate transport of vesicles rich in...
proton pumps between endosomes and the contractile vacuole complex.

The hypothesis, that the distribution of proton pumps depends on membrane trafficking between the two compartments is further supported by the finding that acidosomes, proton pump-rich vesicles likely originating from the contractile vacuole, and endosomes can fuse in vitro (Padh et al. 1991). Interestingly, the inability to correctly sort the proton pumps between endosomes and contractile vacuole is one of the major phenotypes of clathrin heavy chain deficient D. discoideum cells (O'Halloran and Anderson 1992). The controversial finding that cAMP receptors are collected in a contractile vacuole-related compartment after internalisation (Padh and Tanjore 1995) may represent the only direct indication of an endosomal function of the contractile vacuole system.

Recent intriguing evidence implicates endolysosomal vesicles in the non-classical exocytosis of a "signal peptide-less" protein from mammalian cells (Andrei et al. 1999). Interleukin 1b is translocated from the cytosol into these vesicles and later secreted in response to extracellular ATP and osmotic conditions. A similar phenomenon has been described in D. discoideum where CAD-1, a Ca²⁺-dependent cell adhesion molecule, is post-translationally translocated inside the contractile vacuole. Export of CAD-1 to the cell surface is performed directly from this compartment, as it is inhibited by suppression of the water-pumping activity (Sesaki et al. 1997).

Altogether these results support the assumption that the contractile vacuole and the endosomal system are connected by membrane-trafficking events and that the contractile vacuole system could represent an ancestral precursor of the mammalian recycling endosome.

The Digestive Compartments

The lysosomes of D. discoideum are functionally and morphologically similar to mammalian lysosomes, they are acidic, hydrolase-rich and 0.3–1.0 µm in size (Cardelli 1993). Lysosomal enzymes are synthesised as precursor polypeptides, proteolytically processed and targeted to endosomes and lysosomes (Bush and Cardelli 1989; Cardelli et al. 1986; Mierendorf et al. 1985; Wood and Kaplan 1985). In mammalian cells the delivery of lysosomal enzymes bearing a mannose-6-phosphate (M-6-P) modification requires binding to M-6-P receptors and transport from the TGN to the late endosomes via clathrin-coated vesicles (Hille-Rehfeld 1995; Pfeffer 1991). Even though the mammalian M-6-P receptor recognises D. discoideum lysosomal enzymes, no such receptor has been identified in D. discoideum questioning the role of M-6-P in lysosomal enzyme targeting in this organism.

In addition, D. discoideum lysosomal enzymes also carry other sugar modifications and are sequentially delivered to the maturing endosomes, in apparent correlation with changes in lumenal pH (Souza et al. 1997). Vesicles with GlcNAc-1-P modified proteins such as cysteine proteases fuse with phagosomes in the first minutes after ingestion; fusion with vesicles containing M-6-P modified hydrolases occurs later. The two sets of proteins reside in morphologically and functionally distinct compartments. A retrieval system for cysteine proteases, using a receptor recognising GlcNAc-1-P is debated.

Retrieval of Lysosomal Enzymes

Rab 7 has been shown in vitro to be a positive regulator of homotypic fusion between early endosomes from D. discoideum (Laurent et al. 1998) as well as between late endosomes in HeLa cells (Papini et al. 1997). It has also been shown to enhance the rate of transport between early and late endosomes in a variety of cell types (Feng et al. 1995; Mukhopadhyay et al. 1997; Vitelli et al. 1997).

D. discoideum Rab7 has been localised around lysosomal and post-lysosomal compartments in a discontinuous, punctate fashion. The fluid phase remains longer in the lysosomes of cells overexpressing a dominant negative form of Rab7; these cells also contain more and larger acidic lysosomes (Buczynski et al. 1997a). Rab7 was proposed to play an important role at a late step of the endosomal pathway, maybe regulating retrieval of lysosomal enzymes and vacuolar-H⁺-ATPase from post-lysosomes to lysosomes, a process coupled to efficient egestion of fluid phase. The small punctate structures observed may represent small vesicles budding from post-lysosomes and/or fusing with lysosomes.

Very recent findings show that dynamin A, a D. discoideum homologue of the vertebrate GTPase proposed to pinch off clathrin coated vesicles (Baba et al. 1995; Damke et al. 1994), localises in punctate cytoplasmic structures often surrounding vacuolin-positive post-lysosomal vacuoles (Wienke et al. 1999). Furthermore, cells lacking dynamin A exhibit alterations in the progression of fluid phase, indicative of a functions of dynamin A in late endosomal trafficking, perhaps acting in the same step as Rab7.
Exocytosis from Post-Lysosomes

In *D. discoideum*, after progression through the endosomal pathway and digestion of nutrients, the fluid phase enters big, nearly neutral post-lysosomal compartments where it is concentrated and finally egested. In a way reminiscent of macropinosomes, these post-lysosomes are again surrounded by actin and sequentially acquire coronin and vacuolin, which targets them for exocytosis (Jenne et al. 1998). Similar large non-acidic endosomal vacuoles exist in other protists such as *E. histolytica* (Aley et al. 1984). *D. discoideum*, like other lower eukaryotes, partly secretes its lysosomal enzymes (Florin et al. 1986), which is likely due to incomplete retrieval from the post-lysosomal compartment.

In mammalian cells, lysosomes were considered as a terminal degradative compartment into which transport is mostly unidirectional. Exceptions to this classical point of view have now been reported. Backward flow from the acidic lysosomes to more neutral endosomes has been shown for at least some markers (Bright et al. 1997; Mullock et al. 1998). Elevation of the intracellular Ca\(^{2+}\) concentration in rat kidney fibroblasts induces fusion of lysosomes with the plasma membrane and exocytosis of lysosomal enzymes (Rodriguez et al. 1997). The involvement of Ca\(^{2+}\)-transients in the invasion mechanism of the parasite *Trypanosoma cruzi*, which occurs by fusion of lysosomes with the plasma membrane (Tardieux et al. 1992), suggests that lysosome exocytosis is a process active in most cell types. In this context it is also relevant that specialised Ca\(^{2+}\)-dependent secretory vesicles such as neutrophil azurophil granules, mast cell specific granules and cytotoxic lymphocyte lytic granules share characteristics with lysosomes, maybe reflecting a common biogenesis (Griffiths 1996).

Together these findings suggest that fusion of lysosomes/post-lysosomes with the plasma membrane may be a ubiquitous form of regulated exocytosis in most eucaryotic cells. The linearity of the major endosomal flux in *D. discoideum* which leads to the exocytosis of fluid-phase markers and partial secretion of lysosomal enzymes from the post-lysosomal compartment may therefore be considered as an ancestral form of the role of lysosomes in intracellular membrane traffic in higher eucaryotic cells.

Acknowledgements

We wish to thank James Cardelli, John Heuser, Markus Maniak and Margaret Titus for stimulating discussions on these topics during the last years. EMN was supported by a doctoral fellowship from the Max-Planck Gesellschaft.

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Membrane Traffic in *Dictyostelium discoideum*


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