Membrane dynamics and the biogenesis of lysosomes (Review)

J. Paul Luzio††, Viviane Poupon‡, Margaret R. Lindsay†, Barbara M. Mullock†, Robert C. Piper‡ and Paul R. Pryor†

† Cambridge Institute for Medical Research and Department of Clinical Biochemistry, University of Cambridge, Addenbrooke’s Hospital, Hills Road, Cambridge CB2 2XY, UK
‡ Department of Physiology and Biophysics, University of Iowa, Iowa City, IA 52242, USA

Summary
Lysosomes are dynamic organelles receiving membrane traffic input from the biosynthetic, endocytic and autophagic pathways. They may be regarded as storage organelles for acid hydrolases and are capable of fusing with late endosomes to form hybrid organelles where digestion of endocytosed macromolecules occurs. Reformation of lysosomes from the hybrid organelles involves content condensation and probably removal of some membrane proteins by vesicular traffic. Lysosomes can also fuse with the plasma membrane in response to cell surface damage and a rise in cytosolic Ca2+ concentration. This process is important in plasma membrane repair. The molecular basis of membrane traffic pathways involving lysosomes is increasingly understood, in large part because of the identification of many proteins required for protein traffic to vacuoles in the yeast Saccharomyces cerevisiae. Mammalian orthologues of these proteins have been identified and studied in the processes of vesicular delivery of newly synthesized lysosomal proteins from the trans-Golgi network, fusion of lysosomes with late endosomes and sorting of membrane proteins into lysosomal vesicles. Several multi-protein oligomeric complexes required for these processes have been identified. The present review focuses on current understanding of the molecular mechanisms of fusion of lysosomes with both endosomes and the plasma membrane and on the sorting events required for delivery of newly synthesized membrane proteins, endocytosed membrane proteins and other endocytosed macromolecules to lysosomes.

Keywords: Endocytosis; multivesicular body; endosome–lyso- some fusion; membrane traffic; organelle biogenesis.

Introduction
Lysosomes have long been regarded as the terminal degradative compartment of the endocytic pathway in animal cells. They are defined as membrane bound organelles of ~0.5 μm diameter containing a variety of acid hydrolases. Their limiting membrane is enriched in integral membrane glycoproteins, variously described as lysosome associated membrane proteins (lamps), lysosome integral membrane proteins (limps) or lysosome glycoproteins (lgps). Lysosomes can be distinguished from endosomes by the lack of mannanose 6-phosphate receptors (MPRs) and recycling plasma membrane receptors (Kornfeld and Mellman 1989, Luzio et al. 2000, Mullins and Bonifacino 2001). They have an heterogenous morphology when observed by EM, often appear electron dense by comparison with cytosol and contain internal, i.e. lumenal, membrane vesicles and/or membrane whirls (Holtzmann 1989). Lysosomes typically constitute 0.5–5% of the cell volume and are concentrated near microtubule organizing centres (Matteoni and Kreis 1987). In both plant cells and yeast, the vacuole may be regarded as the functional equivalent of the lysosome (Klionsky et al. 1990, Klionsky 1997). In recent years, genetic studies of the yeast vacuole and of the biogenesis and function of lysosome-related organelles in animal cells have led to the identification of many proteins involved in the biogenesis and function of both these organelles and of lysosomes. Lysosome-related organelles in animal cells that have been particularly important in this respect include the pigment granules in the Drosophila eye, melanosomes in skin melanocytes and secretory lysosomes in cells of haematopoietic origin. All of these organelles are like lysosomes in that they contain acid hydrolases and lamps but no MPRs and are accessible to endocytosed markers, at least during their formation. They also contain specific proteins not found in conventional lysosomes.

The discovery of lysosomes
Lysosomes were originally discovered by de Duve in 1949, as a result of the latency of acid phosphatase activity in sub-cellular fractions and their role in the proteolysis of endocy- tosed macromolecules, in phagocytosis and in autophagy, was rapidly established (reviewed in Bainton 1981, de Duve 1983). In addition, mainly from experiments in protozoa, it was recognized that lysosomes could eliminate undigested residues by fusion with the plasma membrane. Although by the time of her review, Bainton (1981) had drawn the conclusion that ‘the lysosomal system is not just a garbage dump’, a rather static view of the lysosome as a terminal degradation compartment or garbage disposal unit was widely held until recent years. Two developments in particular have changed this perception. First, the realization that lysosomes interact with late endosomes by undergoing many ‘kiss and run’ events and/or direct fusion and, secondly, that both lysosome-related organelles called secretory lysosomes and conventional lysosomes can fuse with the plasma membrane in response to a rise in cytosolic calcium concentration.

Lysosomes as dynamic organelles
The overall route map of the endocytic pathway (Figure 1) is well understood with delivery of endocytosed macromole-
cules first to early endosomes, then late endosomes and finally lysosomes (reviewed in Mellman 1996, Mukherjee et al. 1997, Marsh 2001). Delivery from late endosomes to lysosomes has been the subject of considerable experimental investigation, testing different hypotheses (Figure 2). Whilst some maturation events undoubtedly do occur in the late endocytic pathway (see below), maturation of endosomes to lysosomes is no longer regarded as the main, or indeed likely, means of delivery by most commentators. Similarly, whilst vesicular transport is clearly of major importance in transferring content between donor and acceptor organelles elsewhere in secretory and endocytic pathways (Mellman and Warren 2000), there is little evidence for vesicular traffic between late endosomes and lysosomes. To explain the size dependent, differential transfer of newly endocytosed molecules into lysosomes and observations from living cells in which labelled late endocytic organelles continuously bump into each other, Storrie and Desjardins (1996) proposed the ‘kiss and run’ hypothesis of lysosome biogenesis. The occurrence of complete fusion, possibly developing from initial kisses, was demonstrated following the development of cell-free assays to study content mixing between endosomes and lysosomes derived from rat hepatocytes (Mullock et al. 1998). The product of content mixing was found to reside in a hybrid organelle, which was intermediate in density between late endosomes and lysosomes and shown by EM to contain markers derived from each starting organelle. The discovery of this hybrid organelle is consistent with a view of lysosome function in the endocytic pathway in which lysosomes act as storage organelles for acid hydrolases and fuse with late endosomes to create a digestive organelle or ‘cell stomach’ (Griffiths 1996). Thus, not withstanding the presence of some active hydrolases in late endosomes (Casciola-Rosen and Hubbard 1991, Pillay et al. 2002), the hybrid organelle may act as the major site for hydrolysis of endocytosed macromolecules. Lysosomes are reformed from the hybrid organelles by a maturation process which includes condensation of content and the removal of some membrane proteins and soluble content by vesicular carriers (Pryor et al. 2000a). An important point to note is that, according to the definitions of lysosomes and endosomes based on the absence or presence of MPRs, a newly formed hybrid organelle, where digestion of endocytosed macromolecules by lysosomal hydrolases will commence, is by definition an endosome, whereas at some point in the maturation process for the reformation of a lysosome it will have lost its MPRs and be defined as a lysosome.

In some cell types, there is evidence that the lysosomal compartment is modified to contain cell type-specific proteins that are subject to regulated secretion. The resulting Ca^{2+}-regulated secretory granules include the lytic granules in cytotoxic T cells and natural killer cells, dense granules in platelets and histamine-containing granules in mast cells. They are collectively referred to as secretory lysosomes which serve as both degradative and secretory compartments and are most usually found in cells of the haematopoietic lineage. A more extensive list of these organelles and their properties is found in the excellent review by Blott and Griffiths (2002). Often included amongst secretory lysosomes are the storage compartments in antigen presenting cells, i.e. macrophages, B cells and dendritic cells. These are known as MIICs, which store major histocompatibility complex Class II molecules. They also contain endocytosed antigen (Harding et al. 1991). Activation of dendritic cells results in processing and conversion of the antigens to peptide-MHC II complexes and causes tubules and vesicles

Figure 1. A model of endocytic biosynthetic and autophagic routes of delivery to lysosomes. Arrows indicate traffic routes between compartments. In this model direct fusion between late endosomes and lysosomes is shown, but see Figure 2 for alternative methods of delivering endosomal content to lysosomes.

Figure 2. Diagrammatic representation of hypotheses to explain delivery of endocytosed macromolecules from late endosomes to lysosomes.
Membrane traffic into lysosomes

There are three well described routes for delivery of macromolecules to lysosomes: endocytosis, the biosynthetic route (s) and autophagy (Figure 1).

Endocytosis

Whilst receptor mediated endocytosis via clathrin coated pits is probably the best understood uptake route for extracellular macromolecules to be delivered to lysosomes, it is certainly not the only route. A variety of non-clathrin mediated endocytic uptake pathways have been identified including phagocytosis, caveolae-mediated uptake, macropinocytosis and a constitutive cholesterol-sensitive non-clathrin-mediated uptake pathway carrying lipid rafts to the Golgi complex (all reviewed in Nichols and Lippincott-Schwartz 2001). Of these non-clathrin mediated uptake pathways, the most widely studied has been phagocytosis. Recent exciting findings include the observation of fusion between the endoplasmic reticulum and plasma membrane to generate the phagosome membrane in macrophages (Gagnon et al. 2002) and molecular details of phagosome-lysosome fusion from in vitro assays (Jahraus et al. 1998, 2001, Peyron et al. 2001).

In the conventional endocytic pathway, there remain disputes about whether membrane traffic from early-to-late endosomes occurs via maturation (Murphy 1991, Stoovogel et al. 1991) or an endocytic carrier vesicle (ECV) formed as a result of the recruitment to early endosomes of an isoform of the COP-I coat responsible for retrograde traffic from the Golgi complex to the ER (reviewed in Gu and Gruenberg 1999). Nevertheless, both the maturation and ECV models provide for intermediates between early and late endosomes, ensuring the morphological integrity of the latter compartment (Gruenberg and Maxfield 1995). As described above, ‘kiss and run’ events and direct fusion between late endosomes and lysosomes likely provide the major means of delivery to lysosomes. The overall morphology of endosomal compartments is, in major part, a consequence of the many fusion events occurring in the endocytic pathway, in particular homotypic fusions of early endosomes, late endosomes (Antonin et al. 2000) and lysosomes (Ward et al. 1997), as well as heterotypic fusions between endocytic vesicles and early endosomes, ECVs and late endosomes and late endosomes and lysosomes. Very little or no fusion has been observed between early endosomes and either late endosomes or lysosomes (Aniento et al. 1993, Ward et al. 2000).

Biosynthetic routes

Most newly synthesized acid hydrolases are delivered to lysosomes after being tagged with mannose 6-phosphate in the cis-Golgi and binding to MPRs in the trans-Golgi network (TGN). The bound hydrolases are first delivered to endosomes, where they dissociate from the receptors as a result of the acidic lumenal pH, allowing the receptors to recycle to the TGN. Fusion events between endosomes and pre-existing lysosomes may be the major means of delivery from endosomes to lysosomes (Luzio et al. 2000). Until recently, it was thought that the MPRs were sorted into clathrin coated pits at the TGN by means of the adaptor protein AP-1. This adaptor is one of four: AP-1, AP-2 (used in clathrin coated pits at the plasma membrane), AP-3 and AP-4 (see below). All these adaptors are heterotetrameric proteins utilised ubiquitously in post-Golgi secretory and endocytic pathways. Electron microscopy, protein:protein interaction studies and, most recently, structural biology (Collins et al. 2002) have strongly suggested that adaptor complexes have similar structures, resembling Mickey Mouse, with a core or ‘head’ consisting of medium (μ) and small sub-units and the amino-terminal domains of two large sub-units (α/γ and β), flanked by flexibly-hinged ‘ears’ consisting of the carboxyterminal domains of the two large sub-units. The results of experiments with μ1-deficient mouse fibroblasts suggest that the major requirement for AP-1 is in returning empty MPRs from endosomes to the
TGN (Meyer et al. 2001). It now seems more likely that the major clathrin adaptors required for traffic of MPRs and bound hydrolases from the TGN to endosomes are the GGAs (Golgi-localized, γ-ear-containing, ADP ribosylation factor-binding proteins) which are monomeric and only distantly related to the heterotetrameric APs (Dell’Angelica and Payne 2001, Puertollano et al. 2001, Robinson and Bonifacino, 2001, Zhu et al. 2001). However, there are also data showing the biochemical interaction of GGAs and AP-1 and demonstrating their colocalization in clathrin-coated buds at the TGN. These data suggest that the role of GGAs may be to package MPRs into AP-1-containing clathrin coated vesicles for transport from the TGN to endosomes (Doray et al. 2002).

Newly synthesized integral membrane proteins destined for the limiting membrane of lysosomes are delivered either by direct or indirect (via the plasma membrane) traffic routes from the TGN to lysosomes. The evidence for individual lamps taking mainly one or other of these routes has come from kinetic studies of delivery and from the endocytic uptake of anti-lamp antibodies or surface labelled lamps from the plasma membrane. Thus, the type I integral membrane proteins lamp-1 and lamp-2, which between them account for over 50% of the lysosomal content of lamps (Andrejewski et al. 1999), are delivered from the TGN with half times of 30–90 min, mainly via an intracellular route (Barriocanal et al. 1986, D’Souza and August 1986, Green et al. 1987, Carlsson and Fukuda 1992, Akasaki et al. 1995, 1996). In contrast, lysosomal acid phosphatase, also a type I membrane protein, is delivered with a half time of 5–7 h, mainly via the cell surface (Braun et al. 1989). Lamp-1, lamp-2 and lysosomal acid phosphatase all have short, 10–20 amino acid, cytoplasmic tails containing tyrosine-based motifs that determine their lysosomal targeting (Hunziker and Geuze 1996). The tyrosine-based signals are of the form YXXØ, where X is any amino acid and Ø is a bulky hydrophobic amino acid, and are clearly related to internalization signals of the same type, which interact with the µ2 sub-unit of the AP-2 adaptor found in clathrin-coated pits at the cell surface (Owen and Evans 1998, Bonifacino and Dell’Angelica 1999). The specific residues used in the XØ positions can affect the amount of direct vs indirect targeting to lysosomes (Obermuller et al. 2002, Rous et al. 2002). In lamps, the YXXØ motif is often, but not always (Irke et al. 2000), preceded by a G, which if mutated to A results in increased trafficking via the cell surface. Not all lamps use tyrosine-based targeting signals for delivery to lysosomes. Cytosplasmic di-leucine-based motifs are also used, but their site of interaction with adaptor sub-units is poorly understood.

There is also evidence that the luminal and transmembrane domains of some lamps also contain targeting information (discussed in Reaves et al. 1998). Further complexity arises in the case of polytopic lamps with several transmembrane domains. In the case of the cystine transporter cystinosin, which is predicted to contain seven transmembrane domains and a 10 amino acid carboxyterminal cytoplasmic tail containing the sequence GYDQL, lysosomal targeting is dependent both on this tail and what appears to be a novel targeting motif, containing the sequence YFPQA at its core, within the third cytoplasmic loop (Cherqui et al. 2001).

The nature of the vesicular carriers used by lamps on the direct route from the TGN to late endocytic compartments has been difficult to establish. Whilst some early evidence suggested that there may be some lamp-1 in AP-1 associated clathrin coated vesicles budding from the TGN (Honing et al. 1996), the observation of neither a change in steady state distribution nor mis-sorting to the cell surface of lamp-1 in µ1A-deficient cells suggested that AP-1 was unlikely to play a major role in lysosomal targeting (Meyer et al. 2000). GGAs also appear to play no role in lamp-1 delivery to lysosomes (Puertollano et al. 2001). Although a function for AP-4 has been suggested in targeting lamps, this adaptor is present in cells in low concentration and, to date, there is no direct evidence of an involvement in the traffic of any endogenous lamp (Aguilar et al. 2001). In contrast, there is now compelling evidence that the adaptor protein most important for the traffic of lamps from the TGN to lysosomes by the direct route is AP-3. Mutations in AP-3 occur naturally in animals including fruit flies and humans, leading to alterations of eye colour in the former and a rare genetic disease in the latter, as a result of defects in delivery of proteins to lysosome-related organelles, i.e. Drosophila eye pigment granules and platelet dense core granules, respectively (reviewed in Hirst and Robinson 1998, Huizing et al. 2001a, Mullins and Bonifacino 2001). Indeed, in Drosophila, eye colour mutants due to mutations in the genes encoding each of the AP-3 sub-units have now been identified (β, garnet; β/3, ruby; µ3, carmine; s3, orange). In mice, the coat colour mutants pearl and mocha are, respectively, caused by mutations in the genes encoding β/3B and s, resulting in abnormal membrane traffic to melanosomes.

Evidence that AP-3 is also responsible for targeting lamps to conventional lysosomes originally came from experiments where endogenous lamps were observed to traffic to lysosomes via the cell surface in cells in which functional AP-3 concentrations were reduced by transfecting with anti-sense oligonucleotides to µ3A (Le Borgne et al. 1998) or in AP-3 deficient human cells (Dell’Angelica et al. 1999). More recently, this result has been repeated in mouse pearl cells with, in addition, the demonstration of ‘rescue’ after transfection and expression of β/3A or B (Peden et al. 2002). A problem with these experiments has been that, whilst wild type lamps are observed to traffic more via the cell surface in AP-3 deficient cells, they can still reach lysosomes. Indeed, it is difficult, if not impossible, to observe any deficiency of lamp content in lysosomes of AP-3 deficient fibroblastic cells. This is, presumably, because when the lamps reach the plasma membrane they are efficiently internalized by clathrin-mediated endocytosis as a result of the interaction of YXXØ motifs with AP-2. Further evidence for the function of AP-3 has come from the authors’ own experiments on lamp-3 (CD63), which has four transmembrane domains and a classical lysosomal targeting motif, GYEVM, at the end of a short carboxyterminal cytoplasmic tail. Mutation of the targeting motif to GYEVI produced a mutant lamp-3 tail that, by yeast two hybrid analysis, was as good as the wild type tail in binding to µ3, but no longer had any capability of binding to µ2. When expressed in pearl or mocha mouse cells, the lamp-3 mutant was trapped at the plasma membrane and not delivered to lysosomes. In ‘rescued'
mocha cells expressing the δ sub-unit, transfected expressed GYEVI mutant of lamp-3 was delivered to lysosomes and not observed at the cell surface (Rous et al. 2002). Although it now seems clear that AP-3 is involved in the traffic of membrane proteins to conventional lysosomes, the intracellular site of action of AP-3 remains poorly defined. It has been localized by microscopy at, or close to, the TGN (Simpson et al. 1996) and on endosomes (Dell’Angelica et al. 1998). Study of the trafficking of the AP-3 dependent GYEVI mutant of lamp-3 suggests that it does not traffic via the early endosomal subcompartment in which, during its traffic itinerary, the TGN integral membrane protein TGN38 is sorted for delivery to the TGN (Rous et al. 2002). Wherever in the TGN/endosomal system AP-3 functions to deliver lamps, fusion events between late endosomes and lysosomes will ensure delivery to the latter. An additional issue with regard to the function of AP-3 is whether it requires clathrin for vesicle formation. There have been reports of partial colocalization of AP-3 and clathrin in cells, but the clathrin binding site on β/3 is not required for its function (Peden et al. 2002). The evidence that AP-3 is required for the direct route to deliver lamps to lysosomes from the TGN is consistent with the existence in yeast of an AP-3 trafficking route from the late Golgi to the lysosome (Cowles et al. 1997, Piper et al. 1997, Stepp et al. 1997).

Autophagy

In a well-fed mammalian cell, endocytosis and biosynthetic delivery are likely to be the major routes of membrane delivery to lysosomes. However, upon nutrient starvation, the process of autophagy (strictly, macroautophagy) is stimulated, resulting in the envelopment of a portion of cytoplasm by membrane to form an autophagosome which is able to fuse with lysosomes (Lawrence and Brown 1992, Yamamoto et al. 1998). The origin of the autophagosome membrane remains controversial, but recent genetic screens in yeast have identified many genes which encode proteins essential to the autophagic pathway (reviewed in Seaman and Luzzio 2001, Noda et al. 2002). The study of these proteins and their mammalian homologues is enabling rapid progress in understanding the molecular mechanisms underlying autophagosome formation and consumption (Mizushima et al. 2001, Nara et al. 2002, Noda et al. 2002). In yeast, fusion of autophagosomes with the vacuole requires many of the same gene products as those required for delivering newly synthesized vacuolar proteins (Darsow et al. 1997, Rieder and Emr, 1997). By analogy, it is therefore, likely that, in mammalian cells, the molecular mechanism of lysosome–endosome fusion will be similar to that of lysosome–autophagosome fusion.

Lysosome–endosome fusion and lysosome biogenesis

When proposing the 'kiss and run' hypothesis, Storrie and Desjardins (1996) wrote that 'when considering lysosome biogenesis ... the devil is in the details', which they paraphrased as 'the answer is in the mechanism'. The availability of a cell-free system to study late endosome–lysosome fusion and the discovery of the hybrid organelle has opened up the investigation of fusion events between late endosomes and lysosomes. Several clues have come from studying the function of mammalian homologues of proteins involved in delivery of macromolecules to vacuoles of the yeast Saccharomyces cerevisiae, specifically those proteins encoded by VPS (vacuolar protein sorting) and VAM (vacuolar morphology) genes (Rothman and Stevens 1986, Robinson et al. 1988, Bonangelino et al. 2002, Seeley et al. 2002). These genes have been discovered in a variety of screens designed to isolate mutants with abnormal sorting of newly synthesized proteins, in particular carboxypeptidase Y, to the vacuole and/or abnormal vacuolar morphology. Newly synthesized carboxypeptidase Y is targeted from the Golgi to the vacuole as the result of the binding of a tetrapeptide sequence within its pro-domain to a transmembrane receptor Vps10p. For full activity, carboxypeptidase Y requires proteolytic cleavage within the vacuole to produce the mature form of the enzyme. Several of the VPS and VAM genes were identified in more than one screen and have both VPS and VAM designations, as well as PEP designations based on reduced appearance of active carboxypeptidase Y in the vacuole (Jones 1977). The vps mutants have been divided into six classes (A–F) with respect to vacuolar morphology, function and extent of protein sorting defects. Often, members of a class act on the same segment of the vacuolar protein sorting pathway (reviewed in Seaman and Luzzio 2001). Several late acting Vps/Vam proteins have been shown to be required for homotypic fusion of yeast vacuoles, a process that has also been reconstituted in a cell-free system and is now well understood, with much molecular detail available on priming, tethering, docking and fusion steps (reviewed in Wickner 2002).

All of the membrane fusion events occurring throughout the endocytic pathway are consistent with the action of the common cytosolic fusion machinery, requiring NSF (N-ethylmaleimide sensitive factor) and SNAPs (soluble NSF attachment proteins), that functions according to the tenets of the SNARE (SNAP receptor) hypothesis. This hypothesis, proposed pairing between specific v- (vesicle) and t- (target organelle) SNAREs, forms a functional trans-SNARE complex, which is at the centre of the docking and fusion of vesicles with acceptor compartments (Rothman 1994). Heterotypic fusion between late endosomes and lysosomes to form hybrid organelles in the rat liver cell-free system is ATP, cytosol and temperature dependent and requires the presence of NSF, SNAPs and a small GTPase of the Rab family (Mullock et al. 1998). The Rab requirement was identified because fusion was inhibited by Rab-GDI (GDP dissociation inhibitor). It has not been formally shown in the mammalian cell-free system which Rab is required for late endosome–lysosome fusion. The best candidate is Rab7, the mammalian homologue of Ypt7p (Vam4p), which is known to be required for yeast vacuole fusion (Wickner 2002), although Rab 14 has also been suggested (Harris and Cardelli 2002). Fusion of late endosomes with lysosomes can also be inhibited by the fast acting calcium chelator, BAPTA (1,2-bis[2-aminophosphorylethyl]N,N,N′-tetraacetate acid) and is calmodulin dependent (Pyor et al. 2000a). The availability of two inhibitors, Rab-GDI and BAPTA, has
allowed the experimental ordering of the processes they inhibit, with Rab-GDI acting very early in the fusion process and BAPTA very late (Pryor et al. 2000a). By analogy with what is known about homotypic yeast vacuole fusion where Ypt7p is involved in recruiting putative tethering proteins and there is a late calcium/calmodulin requirement for membrane fusion, it has been possible to model for the mechanistic steps involved in late endosome–lysosome fusion (Figure 3).

**Tethering**

There is a growing body of evidence that in many, if not all, membrane fusion events on membrane traffic pathways, specific tethering processes occur prior to trans-SNARE complex formation, where tethering is defined as involving links that extend over distances > 25 nm from a given membrane surface, in contrast to docking which holds membranes within a bilayer’s distance, <5–10 nm of one another (Pfeffer 1999). The physical existence of tethers late in the endocytic pathway is not in dispute, since several groups have observed fine striations, consistent with the existence of tethers, between adjacent late endosomes and lysosomes in morphological studies on cultured cells (van Deurs et al. 1995, Futter et al. 1996, Bright et al. 1997). Consistent with the model (Figure 3) in which the Rab acts early and is involved in the recruitment of tethers are data from experiments in which Rab7 was over-expressed in mammalian cells and caused lysosome aggregation (Bucci et al. 2000). Over-expression of dominant negative mutants causes dispersion of lysosomes and they become inaccessible to endocytosed molecules. The presence of a Rab may not be the only requirement early in the fusion process. The phosphatidylinositol 3-kinase (Ptd-Ins 3-kinase) inhibitor wortmannin also reduces fusion (Mullock et al. 1998).

Although a function for 3-phosphorylated phosphatidylinositides, including phosphatidylinositol 3-phosphate (Ptd-Ins 3-phosphate), the product of Ptd-Ins 3-kinase activity, have not been demonstrated in the fusion of late endosomes with lysosomes, they are known to be involved in fusion events elsewhere in the endocytic pathway. In particular, Ptd-Ins 3-phosphate patches act synergistically with Rab5 in recruiting tether proteins necessary for the homotypic fusion of early endosomes (Simonsen et al. 1998). The best candidates for proteins involved in the tethering complexes functioning late in the endocytic pathway of mammalian cells are the mammalian homologues of the yeast proteins Vps11p, Vps16p, Vps18p, Vps33p, Vps39p and Vps41p (Seals et al. 2000, Wurmser et al. 2000). In yeast, these proteins act together as a complex (the HOPS, homotypic fusion and vacuole protein sorting, or Class C Vps, complex) required both for homotypic vacuole fusion and the docking of vesicles delivering cargo on the biosynthetic pathway to the vacuole (Sato et al. 2000). The complex binds to Ypt7 to initiate docking and to a t-SNARE, Vam3p, enabling it to regulate the formation of the functional trans-SNARE complex required for membrane fusion. There is some evidence that a core Vps18p/Vps11p complex may function as a tethering complex at multiple steps in membrane traffic routes to the yeast vacuole, not simply at the final step of delivery to the vacuole (Srivastava et al. 2000). Homologues of the proteins making up the yeast HOPS complex exist in both *Drosophila* and mammals. In both cases, they have been implicated in the functioning of the late endocytic pathway. Both VPS18 and VPS33 have *Drosophila* homologues, respectively deep orange and carnation, mutations which affect the lysosome-related pigment granules in fly eyes and, hence, eye colour (Sevrioukov et al. 1999). As in yeast, they are associated in a large oligomeric complex. Mammalian homologues have been cloned, shown by

![Figure 3](image-url)

**Figure 3.** The mechanism of late endosome–lysosome fusion. The diagram shows the possible stages of fusion of late endosomes with lysosomes and reformation of lysosomes from the subsequent hybrid organelles. For simplicity, a single t-SNARE (Q-SNARE) is shown. Tethering is shown preceding trans-SNARE assembly and fusion. Ca$^{2+}$ release from the organelle lumens (for simplicity, shown here only from the endosome) is necessary for fusion. Reformation of lysosomes from the hybrid organelles should require a process to recycle the v-SNARE (R-SNARE) for subsequent rounds of fusion and the condensation of lumenal content to produce dense core lysosomes.
immunofluorescence microscopy to be associated with endocytic organelles and make up a large oligomeric complex that interacts with the mammalian homologue of Vam3p, syntaxin 7 (Huizing et al. 2001b; Kim et al. 2001; Figure 4). Sequence analysis of the animal homologues has revealed remarkable similarity in the domain structure amongst Vps11p, Vps8p, Vam39p and Vps41p and thrown light on the possible mechanism of oligomerization. All of these proteins contain a clathrin heavy chain repeat (CLH) domain that was originally analysed in the clathrin heavy chain and is thought to promote the oligomerization of CLH-containing proteins (Ybe et al. 1999). Mammalian Vps11p, Vps8p and Vps41p also contain a complete or partial region that encodes a RING-H2 domain. In both yeast and Drosophila, point mutations in key cysteine residues of the RING-H2 domain of Vps11p show that it is required for function (Preston et al. 1991, Sevrioukov et al. 1999). Recent evidence from over-expression of mammalian Vps39p shows that it causes clustering of late endosomes and lysosomes (Capian et al. 2001). This effect was observed in the presence of dominant negative Rab7, implying that it functions downstream of, or in parallel to, Rab7.

**Trans-SNARE complex formation**

Syntaxin 7 has been firmly implicated as a SNARE required for both heterotypic fusion of late endosomes with lysosomes and homotypic fusion of late endosomes. Using cell-free systems, antibodies to this SNARE have been shown to inhibit both of these fusion events (Antonin et al. 2000, Mullock et al. 2000, Ward et al. 2000). In addition, over-expression of the cytoplasmic domain inhibits delivery of endocytosed markers to late endocytic compartments (Nakamura et al. 2000). Whilst it is reassuring that there are now data linking this SNARE to a possible tethering complex, described above, an unresolved question is how syntaxin 7 can apparently mediate both homotypic and heterotypic fusion processes involving late endosomes. One possibility is that it has different, combinatorial partners to form trans-SNARE complexes separately mediating the two events.

Although the SNARE hypothesis proposed specific pairing between v-(vesicle) and t-(target organelle) SNAREs, an alternative classification based on sequence alignments of the coiled coil domains and structural features observed in the crystal structure of the heterotrimeric synaptic fusion complex (Sutton et al. 1998) has been proposed. This separates SNAREs into Q-SNAREs and R-SNAREs, with four-helix bundles composed of 3 Q-SNARE and 1 R-SNARE helices being formed after SNARE complex assembly (Fasshauer et al. 1998). A refinement of this classification separates the Q-SNAREs into Qa, Qb and Qc, requiring one of each and an R-SNARE to form the functional SNARE complex (Bock et al. 2001). An alternative nomenclature for SNAREs has been proposed whereby the t-SNARE consists of a heavy chain and two light chains and the v-SNARE a single polyepitope chain (Fukuda et al. 2000). In practice, for a given SNARE complex, the v-SNARE is synonymous with the R-SNARE and the t-SNARE with the three Q-SNAREs. A refinement occurs, sometimes, when a vesicle fuses with the plasma membrane, needing only 3 SNARES, one of which contains two Q helices or, in the alternative nomenclature, a combined light chain with two helices.

**For homotypic fusion of late endosomes, antibody inhibition data has shown that the partners of syntaxin 7 in the trans-SNARE complex are syntaxin 8, Vti1b, and Vamp 8 (Antonin et al. 2000). These four SNAREs provide, respectively, the necessary Qa, Qb, Qc and R SNAREs for a functional complex. The core of this complex has been crystallized and its structure solved (Antonin et al. 2002a). Less is known about the trans-SNARE complex for heterotypic fusion of late endosomes and lysosomes. In experiments using membrane fractions of melanoma cells, which express much more syntaxin 7 than other cell types investigated, it was found that immunoprecipitation of syntaxin 7 co-precipitated Vamp 7 and syntaxin 6 along with Vti1b and Vamp 8 (Wade et al. 2001). There is no functional evidence indicating a role for syntaxin 6 late in the endocytic pathway, nor evidence of a function for Vamp 8 in heterotypic late endosome–lysosome fusion (Mullock et al. 2000). In contrast, there are data showing inhibition of late endosome–lysosome fusion by a bacterially expressed Vamp 7 lacking its transmembrane domain (Ward et al. 2000), raising the intriguing possibility that this R-SNARE can form an alternative trans-SNARE complex with syntaxin 7 for late endosome–lysosome fusion. Consistent with this suggestion, a SNARE complex consisting of orthologues of mammalian syntaxin 7, syntaxin 8, Vti1 and Vamp 7 has been isolated from Dictyostelium discoideum (Bogdanovic et al. 2002). Permeabilized mammalian cell assays, in which antibodies to specific SNAREs have been found to inhibit degradation of endocytosed epidermal growth factor, have also implicated Vamp 7 in the pathway from early endosomes to lysosomes (Advani et al. 1999). Vamp 7 (also known as toxin insensitive- or Tl-Vamp or VampSYBL1) is an unusual R-SNARE in having a relatively long (~110 amino acid) regulatory domain at its aminoterminus (Martinez-Arca et al. 2000, Filippini et al. 2001), which is predicted to have a

![Figure 4](image-url)
structural similarity to the ε sub-unit of AP-2 (Collins et al. 2002) and may well have a role in regulating fusion events. In addition, a helical amino-terminal domain in syntaxin 7 has been implicated in the regulation of SNARE complex assembly (Antonin et al. 2002b). Amongst the tethering complex proteins functioning late in the endocytic pathway, Vps33p, which is a Sec1p homologue, is also predicted to act as a negative regulator of SNARE complex assembly. Thus, despite the lack of certainty with regard to the composition of the trans-SNARE complex required for heterotypic late endosome–lysosome fusion, there are already several clues as to how assembly of this complex may be regulated.

Membrane fusion: the role of calcium

In homotypic yeast vacuole fusion, calcium is released from the lumen of the docked organelles after trans-SNARE complex formation and mediates fusion via calmodulin dependent event(s) (Peters and Mayer 1998). The calcium/calmodulin dependence has been proposed to be mediated via a protein complex which includes a protein phosphatase (Peters et al. 1999) and/or a direct interaction with V0, the membrane-integral sector of the vacuolar H+-ATPase (Peters et al. 2001). Peters et al. (2001) have proposed the formation of V0 trans-complexes, acting as proteolipid-lined channels at the fusion site. Additional protein factors, the Vtc or vacuolar transporter chaperones, are required for this process (Müller et al. 2002). Key experiments carried out by Peters and Mayer (1998) showed that the fast acting Ca2+-chelator BAPTA, the Ca2+-ionophore ionomycin and Ca2+-ATPase inhibitors all blocked homotypic vacuole fusion. They were also able to deplete lumenal Ca2+ stores by treatment with BAPTA in the presence of ionomycin and showed that such Ca2+ depleted vacuoles were unable to fuse. When lumenal Ca2+ was restored, the vacuoles again became fusion competent.

Using cell-free systems, established with sub-cellular fractions from mammalian cells, complementary data have been obtained for homotypic fusion of early endosomes (Holroyd et al. 1999) and heterotypic fusion of late endosomes with lysosomes (Pryor et al. 2000a). In both systems, fusion was inhibited by BAPTA and was also prevented by pre-incubation with a membrane permeable methyl ester of EGTA, EGTA-AM, which is cleaved within the organelle lumen resulting in chelation of lumenal Ca2+. The inhibitory effects of BAPTA and EGTA-AM were reversed by addition of CaCl2. These data strongly support a role for the release of Ca2+ from the endosome lumen in fusion events in the endocytic pathway. Experiments in which increasing concentrations of CaCl2 were added to fusion assays in the presence of BAPTA suggested that 0.3 μM Ca2+ is optimal for early endosome fusion (Holroyd et al. 1999) and 0.5 μM Ca2+ for fusion of late endosomes with lysosomes (Pryor et al. 2000a). As shown in Figure 2, there is also evidence in the latter system that the BAPTA effect occurs later in the docking/fusion process than inhibition by Rab-GDI, consistent with the observation in yeast vacuole fusion that Ca2+ release occurs after trans-SNARE complex formation. Release of lumenal Ca2+ is likely to be important in fusion events other than those occurring in the endocytic pathway (Pryor et al. 2000b). Lumenal Ca2+ has been implicated in the fusion of nuclear membranes (Sullivan et al. 1993), of regulated secretory granules required to sustain optimal exocytosis (Scheenen et al. 1998) and for vesicular traffic through the Golgi complex (Porat and Elazar, 2000).

Consistent with a requirement for release of lumenal Ca2+ to mediate lysosome-late endosome fusion, there are recent data reporting the Ca2+ concentration in lysosomes as being in the 400–600 μM range, i.e. very much higher than cytosolic concentrations (Christensen et al. 2001). This high lysosomal concentration of Ca2+ seems at odds with a study showing that endocytosed Ca2+ is rapidly lost from endosomal compartments of fibroblasts with time, such that the endosomal concentration of Ca2+ was only 3 μM, 20 min after uptake from extracellular medium containing 2 mM Ca2+. However, there is also evidence that both endosomal (Hilden and Madias 1989) and lysosomal (Lemons and Thoebe 1991) membranes contain Ca2+ transport systems, suggesting that the high intra-lysosomal Ca2+ concentration may be the result of transport across the lysosomal membrane rather than endocytosis from the extracellular medium.

The mechanism by which Ca2+ released from the lumen of mammalian endocytic compartments mediates membrane fusion is much less well understood than in yeast. The effect of calmodulin antagonists to inhibit cell-free late endosome–lysosome fusion is consistent with a calmodulin-mediated mechanism as in yeast vacuole fusion. However, other possibilities include interaction of Ca2+ with the SNARE machinery or with annexins (reviewed in Pryor et al. 2000b) or an effect on a synaptotagmin isoform. The latter is particularly intriguing, because of data showing that synaptotagmin VII is localized to lysosomes and plays a key role in Ca2+ stimulated fusion of lysosomes with the plasma membrane (see above). However, in several cell types, fusion of lysosomes with the plasma membrane is triggered when the cytosolic Ca2+ concentration rises to ~1–5 μM (Rodríguez et al. 1997, Martinez et al. 2000), ~3–15-fold higher than that found to be optimal for fusion of late endosomes with lysosomes (Pryor et al. 2000a). Indeed, Ca2+ concentrations above 1 μM inhibit heterotypic fusion of late endosomes with lysosomes (Pryor et al. 2000a). Although a wide variety of calmodulin activated enzymes, transporters, cytoskeletal proteins and other proteins are known (reviewed in Pryor et al. 2000b), no downstream calmodulin effector for Ca2+-dependent fusion events in the mammalian endocytic pathway has been described. The requirement for a downstream effector of calmodulin for cell-free late endosome–lysosome fusion is consistent with the observed displacement of the time course of BAPTA inhibition of this process from the time course when stopping the reaction by placing the assay tubes on ice (Pryor et al. 2000a).

If lumenal Ca2+ is essential for efficient membrane traffic in the endocytic pathway, one prediction is that diseases which result in an alteration of lumenal Ca2+ concentration may show defects in the endocytic pathway. In this context, it is interesting to note the recent cloning of the gene causing mucolipidoses type IV (Bargal et al. 2000, Bassi et al. 2000, Sun et al. 2000). This disease is characterized by abnormal
late endosomal/lysosomal structures which are filled with multilamellar membranous whorls and do not show the characteristic morphology of dense core lysosomes by electron microscopy. There are also defects in sorting and/or transport in the late endocytic pathway (Chen et al. 1998). The mucolipidosis IV gene encodes mucolipin-1 which is predicted to be a lysosomal integral membrane protein with several trans-membrane domains. Mucolipin-1 has sequence similarities to members of the TRP (transient receptor potential) superfamily, in particular the polycystins, and has been shown to be a novel Ca$^{2+}$-permeable channel modulated by changes in Ca$^{2+}$ concentration (LaPlante et al. 2002). There is evidence that CUP-5, the Caenorhabditis elegans homologue of mucolipin-1, functions in the biogenesis of lysosomes, possibly in the process of reformation from hybrid organelles (Fares and Grant 2002).

Membrane traffic out of lysosomes

After fusion of lysosomes with late endosomes, lysosomes are reformed from the resultant hybrid organelles. This process requires condensation of content, since lysosomes are denser than hybrid organelles. It also requires the removal of membrane proteins that need to be recycled and/or are found in late endosomes but not lysosomes. Condensation of lumenal content appears to occur by a process similar to that occurring during the formation of mature regulated secretory granules in neuroendocrine cells (Bauerfeind and Hutner 1993, Tooze 1998, Arvan et al. 2002). Just as for regulated secretory granules, there is evidence that lumenal Ca$^{2+}$ and low pH are required for formation of dense core lysosomes, since treatment of hybrid organelles with either EGTA-AM or bafilomycin A1, an inhibitor of the vacuolar proton pumping ATPase, can prevent this happening in a cell-free system (Pryor et al. 2000a).

Of membrane proteins that need to be removed from the organelle or recycled during the reformation of lysosomes, the most obvious are the MPRs, since by definition these are not found in lysosomes. The process whereby they are recycled to the TGN has been studied in several laboratories and appears mostly to occur from endosomes, such that late endosomes which fuse with lysosomes are already relatively depleted in MPRs (Hirst et al. 1998). Two proteins required for recycling of MPRs have been identified as Rab 9 and TIP47 (tall interacting protein of 47kDa). TIP47 binds to separate determinants in the cytoplasmic tails of the two MPRs and is likely to be involved in cargo selection into the recycling vesicles (Diaz and Pfeffer 1998). TIP47 also binds directly to Rab9 (Hanna et al. 2002). The nature of the coat required for the recycling vesicles is not known. As described above, AP-1 has been suggested as being involved in MPR recycling. However, it now seems clear that there is more than one recycling pathway from endocytic compartments to the TGN, which may differ both in adaptor molecules and coats (Pfeffer 2001). In yeast, the protein components of the vesicle coat required for recycling of Vps10p, the carboxypeptidase Y receptor, have been identified. These proteins are Vps5p, Vps17p, Vps26p, Vps29p and Vps35p, which together form the retromer coat complex (Seaman et al. 1998, Reddy and Seaman 2001). Homologues of each of these retromer components have been identified in mammalian cells, suggesting that this coat is universally important in retrograde traffic from endocytic compartments to the TGN (Haft et al. 2000, Pfeffer 2001). To date, there is no published evidence as to what cargo retromer coated vesicles may contain in mammalian cells.

The MPR recycling vesicles leaving late endosomes and hybrid organelles may not remove all other membrane proteins necessary for reformation of lysosomes and/or lysosome biogenesis. The Niemann-Pick C protein (NPC1), which is an integral membrane protein with 13 predicted transmembrane domains, is thought to be required for vesicular shuffling of cholesterol, derived from hydrolysed low density lipoprotein, from late endocytic organelles to the trans-Golgi network (Liscum 2000). There is evidence of colocalization of some of the R-SNARE, Vamp 7, in the shuffling vesicles, raising the possibility that these vesicles may play a role in recycling SNARE components required for fusion events late in the endocytic pathway as well as removing cholesterol, a key product of lysosomal hydrolysis (Ko et al. 2001).

Although clathrin is able to form coated vesicles on late endocytic organelles (Traub et al. 1996), it is unlikely to be involved in lysosome reformation from hybrid organelles, since cells engineered to have no clathrin have normal dense core lysosomes (Wetten et al. 2002).

Lumenal membranes in lysosomes

A widely recognized morphological feature of late endocytic organelles, including lysosomes, is the presence of lumenal membrane. Indeed, the term multivesicular body is often used instead of late endosome (Piper and Luzio 2001). Whilst some of the lumenal membrane may derive as a by-product of fusion events, resulting from the release of apposed membrane into the lumen of a fused organelle (Wang et al. 2002), this is not the source of the majority. The lumenal membrane, when compared with the limiting membrane, is enriched in lysobisphosphatidic acid (LBPA) or PtdIns 3-phosphate, the latter being in distinct regions low in LBPA (Gilfooly et al. 2000). In some cells, there is good evidence for a dynamic relationship between the lumenal and limiting membrane, such that the former can fuse with the latter, greatly increasing the surface area of the organelle (Kleijmeer et al. 2001). The lumenal membrane may be secreted as a result of fusion of late endocytic organelles with the cell surface, being released as exosomes which are known to function in intercellular communication during the immune response (Stoorvogel et al. 2002, Thery et al. 2002).

It is now recognized that the sorting of membrane proteins into lumenal membrane in the endocytic pathway is a key event in targeting these proteins for lysosomal degradation. In many cases, both in yeast and mammalian cells, an ubiquitin tag is the trigger for lumenal sorting, but some proteins enter the lumenal vesicles in an ubiquitin-indepen-
dent manner (Reggiori and Pelham 2001). Studies in yeast have identified much of the cargo selection machinery for ubiquitin-dependent sorting of proteins to be degraded into the lumenal membranes of endosomes (Conibear 2002). Three separate complexes of class E Vps proteins, known as ESCRT (endosomal sorting complexes required for transport) complexes I, II and III, acting sequentially, appear to play a major role (Katzmann et al. 2001, Babst et al. 2002a,b). ESCRT-I binds directly to ubiquitinated cargo, and mutation of the ubiquitin binding domain of the ESCRT-I component Vps23p prevents sorting of cargo into lumenal vesicles and also formation of the vesicles, indicating a coupling of the machinery for sorting and lumenal vesicle formation. ESCRT-III recruits a deubiquitinating enzyme Doa4p and, in a final step, the AAA-ATPase Vps4p catalyses the release of the machinery from the membrane. A further Class E Vps protein, Vps27p, in complex with Hse1p, also binds to ubiquitinated membrane proteins and acts as a sorting receptor for degradation (Bilodeau et al. 2002). It may act upstream of ESCRT-I or independently.

In several cases, orthologues of individual components of this yeast sorting machinery have been shown to play an equivalent role in mammalian cells. For example, mutations in Tsg101, the human orthologue of Vps23p, affect traffic of the epidermal growth factor receptor, which is normally delivered to endosomal lumenal vesicles as a necessary pre-requisite to its efficient degradation late in the endocytic pathway (Babst et al. 2000). Similarly, the mammalian Vps27p orthologue Hrs also binds ubiquitinated proteins and is able to target these for lysosomal degradation (Raiborg et al. 2002). Intriguingly, Hrs sorts ubiquitinated membrane proteins into clathrin-coated microdomains on the early endosome. EM studies have shown that these clathrin coats are devoid of the adaptors AP-1, AP-2 and AP-3 and do not cause budding off of coated vesicles (Sachse et al. 2002). They do appear to concentrate membrane proteins destined for delivery to lumenal vesicles and degradation, together with proteins such as syntaxin 7, which remains on the limiting membrane and is required for fusion events later in the endocytic pathway.

In mammalian cells, it is clear that the formation of lumenal membrane vesicles in the endocytic pathway commences at the early endosome. This internal membrane is delivered to late endosomes and, eventually, after fusion with lysosomes to the proteolytic environment of the hybrid organelle. The lumenal membrane observed in dense core lysosomes may, at least in part, constitute remnants of the endosomal lumenal vesicles delivered to the hybrid.

Conclusions

Recent advances in understanding of the molecular biology of membrane traffic pathways have contributed greatly to understanding of lysosome dynamics. The challenge now is to understand better the oligomeric protein complexes functioning in lysosome biogenesis, sorting and fusion events. This will provide insights to specificity in lysosome function, e.g. what are the differences in machinery required for fusion with late endosomes vs the plasma membrane. In turn, this should lead to an understanding of the regulation of lysosome function and, eventually, a better knowledge of abnormalities associated with disease processes involving lysosomes.

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