The delivery of endocytosed cargo to lysosomes

J. Paul Luzio¹, Michael D.J. Parkinson, Sally R. Gray and Nicholas A. Bright
Cambridge Institute for Medical Research and Department of Clinical Biochemistry, University of Cambridge, Cambridge CB2 0XY, U.K.

Abstract
In mammalian cells, endocytosed cargo that is internalized through clathrin-coated pits/vesicles passes through early endosomes and then to late endosomes, before delivery to lysosomes for degradation by proteases. Late endosomes are MVBs (multivesicular bodies) with ubiquitinated membrane proteins destined for lysosomal degradation being sorted into their luminal vesicles by the ESCRT (endosomal sorting complex required for transport) machinery. Cargo is delivered from late endosomes to lysosomes by kissing and direct fusion. These processes have been studied in live cell experiments and a cell-free system. Late endosome–lysosome fusion is preceded by tethering that probably requires mammalian orthologues of the yeast HOPS (homotypic fusion and vacuole protein sorting) complex. Heterotypic late endosome–lysosome membrane fusion is mediated by a trans-SNARE (soluble N-ethylmaleimide-sensitive factor-attachment protein receptor) complex comprising Syntaxin7, Vti1b, Syntaxin8 and VAMP7 (vesicle-associated membrane protein 7). This differs from the trans-SNARE complex required for homotypic late endosome fusion in which VAMP8 replaces VAMP7. VAMP7 is also required for lysosome fusion with the plasma membrane and its retrieval from the plasma membrane to lysosomes is mediated by its folded N-terminal longin domain. Co-ordinated interaction of the ESCRT, HOPS and SNARE complexes is required for cargo delivery to lysosomes.

The endocytic pathway to lysosomes
Endocytic uptake from the cell surface may occur through clathrin-mediated or clathrin-independent routes [1]. After internalization, integral membrane proteins including receptors and bound ligands are delivered to the pleomorphic membrane-bound endosomal system before delivery to lysosomes [2]. Sorting within the endosomal system is complex, involving ligands being released from their receptors into the acidic endosome lumen, recycling of receptors to the cell surface and the sorting of ubiquitinated membrane proteins into ILVs (intraluminal vesicles), mediated by the ESCRT (endosomal sorting complex required for transport) proteins. On the endocytic route to degradation by lysosomal proteases, endocytosed macromolecules pass through early and late endosomes before delivery to lysosomes. The formation of late endosomes from early endosomes requires the conversion from a Rab5-positive organelle into a Rab7-positive organelle, a process regulated by the HOPS (homotypic fusion and vacuole protein sorting) complex [3]. While ESCRT proteins are recruited to early endosomes they continue to function in the formation of ILVs as the endosome matures, such that late endosomes have the morphology of MVBs (multivesicular bodies). Indeed it has been suggested that the late endosome is a term more appropriately used for the mature MVB that has lost all recycling proteins and is competent to fuse with the lysosome [4].

The method of delivery of endocytosed macromolecules from endosomes to lysosomes was for long a matter of dispute with maturation, vesicular transport, kissing and direct fusion all being suggested [5,6]. Live cell experiments employing time-lapse confocal fluorescence microscopy demonstrated that kissing and direct fusion (Figure 1) were able to account for content mixing between endosomes and lysosomes in NRK (normal rat kidney) fibroblasts [7]. In these experiments, in which lysosomes and endosomes were respectively preloaded with rhodamine dextran and Oregon Green 488 dextran, content mixing only occurred between organelles in direct physical contact and could occur via tubular extensions from either organelle. Content mixing by kissing often preceded fusion but was not a prerequisite for it. Clearly, direct fusion can account for the mixing of ILVs, as well as soluble luminal content, with lysosomal proteases and other degradative enzymes. A more recent live cell study has shown that one receptor, the Fc receptor FcRn, is transferred into the limiting membrane during kissing and then internalizes to the lysosome lumen [8].

The molecular mechanism of delivery from endosomes to lysosomes
Direct fusion of late endosomes with lysosomes produces what we have termed a hybrid organelle, from which the lysosome has to be re-formed via a maturation process. The first evidence for the formation of a hybrid organelle came from cell-free studies of endosome–lysosome fusion in which an organelle with a density intermediate between
Figure 1 | Delivery to lysosomes by kissing and direct fusion

The pathways by which endocytosed cargo is internalized from the plasma membrane to early endosomes and then to late endosomes. Late endosomes then deliver their cargo to lysosomes either by (A) kiss and run (or kiss and linger) or (B) direct fusion. Lysosomes must be re-formed from the subsequent hybrid organelles by a maturation process that will involve condensation of content, removal of non-lysosomal membrane proteins and presumably recycling of SNAREs.

That of endosomes and lysosomes was formed [9]. Using immunoelectron microscopy the hybrid organelle fraction was shown to be enriched with organelles containing both markers of lysosomes and endosomes. The cell-free assay for endosome–lysosome fusion has allowed us to establish the core machinery of fusion [2,9–12]. This machinery includes cytosolic factors, the formation of a trans-SNARE (soluble N-ethylmaleimide-sensitive factor-attachment protein receptor) complex and a late requirement for Ca2+, released from the lumen of the fusing organelles. The cytosolic factors include NSF (N-ethylmaleimide-sensitive factor), soluble NSF-attachment proteins, a Rab protein, probably Rab7, and tethers, probably including the HOPS proteins. The identity of the trans-SNARE complex was determined largely from antibody inhibition experiments and was shown to be Syntaxin7, Vti1b, Syntaxin8 and VAMP7 (vesicle-associated membrane protein 7). This differs from the trans-SNARE complex required for homotypic late endosome fusion in which VAMP8 replaces VAMP7. VAMP7 is also required for lysosome fusion with the plasma membrane where it forms a trans-SNARE complex with Syntaxin4 and SNAP23 (23 kDa synaptosome-associated protein) [13].

The sorting of VAMP7

VAMP7 is a key SNARE for lysosome fusion with late endosomes and with the plasma membrane and its presence on the lysosome membrane may help to define the lysosome. Like most other SNAREs, it does not contain short linear motifs of the form YXXØ (where X is any amino acid and Ø is a bulky hydrophobic amino acid) or [DE]XXXLL, which are standard trafficking motifs, yet it requires sorting to the lysosome and retrieval following fusion. VAMP7 is one of three mammalian SNAREs with a 120-amino-acid N-terminal longin domain. This folded longin domain can bind to the SNARE motif in VAMP7 via an intramolecular reaction, but can also function to target VAMP7 to lysosomes. Thus, when a reporter construct consisting of the extracellular and transmembrane domains of the plasma membrane protein CD8 fused to the longin domain of VAMP7 was expressed in fibroblastic cells, it was efficiently localized to late endosomes/lysosomes [14]. In yeast, two-hybrid cDNA library screens, using the longin domain as the bait protein, two binding partners required for VAMP7 traffic have been identified. The first was the δ subunit of AP (adaptor protein)-3 [15] and the second was the clathrin and clathrin adaptor AP-2-binding protein Hrb (HIV Rev binding protein) [14]. Hrb is a 562-amino-acid protein with a folded ArfGAP domain (residues 11–134) close to its N-terminus followed by what is predicted to be an unstructured tail. X-ray crystallography showed that residues 168–176 of Hrb pack against a helix in the VAMP7 longin domain, with the interaction being mediated predominantly by hydrophobic side chains. Mutations in the longin domain that abolished binding to Hrb resulted in a VAMP7 mutant that accumulated at the cell surface. The hydrophobic groove on the longin domain that
bounds Hrb also binds the SNARE motif in VAMP7. Thus the longin domain is only available to mediate trafficking when the VAMP7 is in an open conformation, such as when it is in a SNARE complex. Hrb can bind to the Syntaxin4, SNAP23, the VAMP7 SNARE complex, and to recombinant VAMP7 longin domain, but not to the recombinant whole cytosolic domain in which the longin domain will be bound back to its SNARE motif. Combining this information with what is known about the binding of the longin domain to the δ subunit of AP-3 and the subcellular localization of AP-3, it is possible to hypothesize that, after lysosome fusion with the cell surface, Hrb is required to recover VAMP7 from the cell surface when it is in a cis-SNARE complex and that after endocytosis AP-3 functions within the endosomal system to deliver VAMP7 efficiently to late endosomes/lysosomes.

Conclusions
Considerable progress has been made in discovering the major protein machineries required for the delivery of endocytosed cargo to lysosomes. We have also gained insights into how SNARE proteins required for fusion are sorted. However, a major remaining challenge is to understand the co-ordination of the various protein machineries required for lysosome–endosome fusion. We know that lysosomes fuse preferentially with late endosomes rather than early endosomes [9] and we also know that late endosomes that fuse are relatively depleted in cation-independent mannose 6-phosphate receptors [16]. However, we do not know what identifies a late endosome as being ready for fusion. One possibility is that the ESCRT proteins that are required to form MVBs and sort ubiquitinated membrane proteins into ILVs play an additional role. In this context, it is interesting to note that two ESCRT proteins have been indirectly implicated in having a role in fusion (discussed in [17]) and that there are reports of interactions between ESCRT proteins and HOPS proteins (discussed in [18]).

Funding
Our experimental work was funded by a programme grant (to J.P.L.) and a studentship (to M.D.J.P.) from the Medical Research Council. The Cambridge Institute for Medical Research is supported by a Strategic Award [number 079895] from the Wellcome Trust.

References