ESCRT proteins and the regulation of endocytic delivery to lysosomes

J. Paul Luzio*,1, Siân C. Piper*, Katherine Bowers†, Michael D.J. Parkinson*, Paul J. Lehner* and Nicholas A. Bright*

*Cambridge Institute for Medical Research, University of Cambridge, Cambridge CB2 0XY, U.K., and †Institute of Structural and Molecular Biology, Division of Bioscience, University College London, London WC1E 6BT, U.K.

Abstract
In mammalian cells, there is evidence of cargo specificity in the requirement for particular ESCRT (endosomal sorting complex required for transport) proteins to sort cargo into the luminal vesicles of MVBs (multivesicular bodies). We have focussed on studying the ESCRT requirements for delivery of MHC class I to lysosomes following polyubiquitination by the Kaposi’s sarcoma-associated herpesvirus protein K3. Down-regulation of polyubiquitinated cell-surface MHC class I in HeLa cells stably expressing K3 is achieved via clathrin-mediated endocytosis, followed by sorting into the luminal vesicles of MVBs and eventual delivery to lysosomes. Depletion of ESCRT-I and some ESCRT-III components interferes with this sorting and allows recycling of MHC class I to the cell surface. Depletion of ESCRT-II components has no effect on K3-mediated down-regulation of MHC class I and no gross morphological effect on endocytic compartments. Thus virally polyubiquitinated MHC class I does not require all of the ESCRT proteins in order to be sorted into the luminal vesicles of MVBs. However, there may be a further requirement for ESCRT-III proteins to ensure the efficient fusion of MVBs with lysosomes.

The formation of MVBs (multivesicular bodies) on the endocytic pathway to lysosomes
In mammalian cells, many endocytosed cell-surface proteins, including receptors and bound ligands, are internalized via clathrin-coated pits and transferred successively to early endosomes and late endosomes before delivery to lysosomes for degradation by proteases. Live-cell imaging studies have shown that late endosomes are formed subsequent to large (400–800 nm) vesicles arising from a dynamic early-endosome network and undergo conversion such that they lose the small GTPase Rab5 and recruit Rab7 [1]. Late endosomes have more luminal vesicles than early endosomes and are often described as MVBs [2,3]. The luminal vesicles are enriched in either lysobisphosphatidic acid or PtdIns3P [4]. Many endocytosed membrane proteins are ubiquitinated, with ubiquitination acting as a signal for sorting into the luminal vesicles of MVBs [2,5]. Not all MVBs are functionally equivalent, and it has been shown that EGF (epidermal growth factor) bound to EGFR (EGF receptor)-traffics through a subpopulation of MVBs that are morphologically identical with other MVBs, but are distinguished because they contain annexin 1 and lack lysobisphosphatidic acid [6]. The final step of delivery of endocytosed macromolecules to lysosomal proteases requires kissing and direct fusion events between late endosomes and lysosomes. These events have been studied in compartment ablation/electron microscopy experiments, in cell-free assays and by confocal microscopy in living cells (reviewed in [7]).

In yeast, structural and functional studies have led to a model in which the formation of luminal vesicles in endosomes and the sorting of ubiquitinated proteins into these vesicles requires the sequential recruitment and assembly on to the endosome-limiting membrane of four ESCRT (endosomal sorting complex required for transport) proteins (ESCRT-0, -I, -II and -III) [2,8]. Each ESCRT is made up of soluble cystolic proteins, the class E Vps (vacuolar protein sorting) proteins. Following the deubiquitination of membrane protein cargo and its sorting into a newly formed luminal vesicle, the ESCRTs are disassembled by the recruitment and activity of the AAA (ATPase associated with various cellular activities) Vps4p. The mechanism by which the ESCRTs promote negative curvature in the limiting membrane of the endosome to form luminal vesicles is not well understood. However, evidence from a study on the overexpression of the ESCRT-III protein CHMP (charged multivesicular body protein) 4 in mammalian cells showed that it can polymerize to form curved filaments on the cytoplasmic face of the plasma membrane that can promote negative membrane curvature in the presence of dominant-negative Vps4 [9]. The existence of multiple mammalian homologues of some of the unique yeast class E Vps proteins has argued for greater complexity of the role of ESCRTs in mammalian MVB formation and cargo sorting, including the possibility of cargo-specific requirements for specific ESCRT proteins.

Key words: endosomes, endosome, lysosome, multivesicular body (MVB), MHC class I.
Abbreviations used: CHMP, charged multivesicular body protein; EGF, epidermal growth factor; EGFR, EGF receptor; ESCRT, endosomal sorting complex required for transport; Hrs, hepatocyte growth factor-regulated tyrosine kinase substrate; KSHV, Kaposi’s sarcoma-associated herpesvirus; K3, K3, MHC class 1; Vps, vacuolar protein sorting.

1To whom correspondence should be addressed (email jpl10@cam.ac.uk).
ESCRT requirements for sorting virally ubiquitinated MHC class I molecules into MVBs

In order to study the mammalian ESCRT system in greater detail, we have focused on the down-regulation of cell-surface MHC class I molecules mediated by KSHV (Kaposi’s sarcoma-associated herpesvirus). MHC class I molecules enable cytotoxic T-lymphocytes of the immune system to survey the proteome of all nucleated cells and thus detect viruses and other intracellular parasites. Many viruses down-regulate MHC class I molecules using a variety of mechanisms. One such mechanism employed by KSHV is the expression of an integral membrane protein, K3, which is a ubiquitin E3 ligase capable of promoting the polyubiquitination of MHC class I molecules. This occurs through the formation of a Lys63-linked ubiquitin chain added to a conserved lysine residue in the middle of the cytosolic tail of each MHC class I molecule [10]. Following the expression of K3 in HeLa cells, newly synthesized MHC class I molecules traffic through the secretory pathway, acquire endo H (endoglycosidase H) resistance and reach the plasma membrane from where they are rapidly endocytosed and degraded as a result of K3-mediated polyubiquitination. Evidence for degradation by lysosomal proteases was provided by the demonstration of protection from degradation by ammonium chloride, chloroquine and the vacular H+ -ATPase inhibitors bafilomycin A1 and concanamycin A. Using depletion by RNAi (RNA interference) in HeLa cells expressing K3, we have shown that endocytosis of the polyubiquitinated MHC class I molecules is dependent on clathrin and the clathrin adaptor epsin 1 [11]. Down-regulation of these MHC class I molecules also requires ESCRT proteins. This was first shown in RNAi experiments in which the ESCRT-I protein Tsg101 (tumour susceptibility gene 101) was depleted [12]. This treatment resulted in protection of MHC class I molecules from degradation and their recycling to the cell surface. No accumulation of polyubiquitinated MHC class I molecules was observed, suggesting that deubiquitination occurs rapidly after internalization from the plasma membrane. Depletion of Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate), an ESCRT-0 protein, similarly led to a rescue of MHC class I molecules on the surface of K3-expressing HeLa cells [10].

Whereas the data from Hrs- and Tsg101-depletion experiments were consistent with a role of ESCRT-0 and ESCRT-I in down-regulation of polyubiquitinated MHC class I molecules, depletion of ESCRT-II proteins had no effect [13]. This surprising result was confirmed in an independent study [14]. In addition, Langelier et al. [14] demonstrated that depletion of the ESCRT-III protein CHMP6 did not prevent the down-regulation of virally polyubiquitinated MHC class I molecules. These data are in marked contrast with data obtained for hepcidin-mediated ferroportin down-regulation, which does require ESCRT-II [15], as does degradation of the chemokine receptor CXCR4 [16]. Data on the effects of ESCRT-II depletion on the widely studied EGF-EGFR system are less clear, with Langelier et al. [14] reporting an ‘intermediate’ effect on the delivery of EGF to lysosomes, Malerød et al. [16] observing reduction of both EGF and EGFR degradation, but our own experiments demonstrating no effect on the degradation of EGF [13]. The clear differences in ESCRT-II requirements for down-regulation of different ubiquitinated cargoes imply some form of cargo specificity in the requirements for ESCRT-II. The lack of a CHMP6 requirement for down-regulation of virally polyubiquitinated MHC class I molecules is consistent with ESCRT-II linking ESCRT-I to ESCRT-III via CHMP6. However, down-regulation is blocked by depletion of other ESCRT-III proteins. Thus, for down-regulation of virally polyubiquitinated MHC class I molecules, a different and presently unknown linkage between ESCRT-I and the remainder of ESCRT-III may be required. This is shown diagrammatically in Figure 1.

The role of the ESCRT pathway in the fusion of MVBs with lysosomes

An interesting question that remains unresolved, at least in terms of molecular mechanism, is whether correct functioning of the ESCRT pathway is necessary for subsequent fusion of MVBs with lysosomes. Depletion of the human ESCRT-III protein CHMP3 inhibits EGFR degradation, but not the kinetics of signal termination, and has been shown to result in the accumulation of the EGFR in MVBs in cultured cells [17]. This was interpreted as being the result of an inhibition of fusion of MVBs with lysosomes, implying a function for mammalian CHMP3 that is distinct from its role in MVB biogenesis and sorting. Similarly, it was observed that cells

Figure 1 | The ESCRT pathway
A model for the sequential recruitment of ESCRT-0, -I, -II and -III to ubiquitinated cargo at the limiting membrane of the endosomes and the possible need for an alternative link (?) between ESCRT-I and ESCRT-III for the sorting of some cargoes, such as polyubiquitinated MHC class I molecules, into the luminal vesicles of MVBs. Also shown is the possible requirement for polymerization of the ESCRT-III protein CHMP4 for formation of luminal vesicles and sorting of cargo. ub, ubiquitin. Modified from [5] with permission. © 2007 Elsevier.
from a mouse in which the ESCRT-III-associated protein CHMP5 was knocked out form MVBS, but showed reduced degradation of receptors and fluid-phase markers [18]. Because CHMP6 is clearly required for the delivery of only some membrane proteins into the luminal vesicles of MVBS, the study of delivery of ligands from endosomes to lysosomes following its depletion may prove to be particularly informative in the future in studying events on the pathway of delivery to lysosomes that are downstream of MVBS formation.

**Conclusions**

The observations that virally ubiquitinated MHC class I molecules may be down-regulated from the cell surface and sorted into the luminal vesicles of MVBS in the absence of ESCRT-II and Vps20/CHMP6 implies that ESCRT requirements for cargo sorting into MVBS are cargo-specific and that ESCRT-II and Vps20/CHMP6 are not absolutely necessary for the formation of MVBS. In addition, there must be an alternative linkage that does not involve ESCRT-II, between ESCRT-0/ESCRT-I and ESCRT-III to enable the sorting of MHC class I molecules into MVBS. It is interesting to note that the cargo-specific requirements for ESCRT proteins to mediate sorting into MVBS may have an analogy with the budding of retroviruses from the plasma membrane. Different combinations of ESCRT proteins are used to facilitate budding of specific viruses, e.g. the budding of avian sarcoma virus requires ESCRT-II, but the budding of HIV-1 does not [14,19].

**Funding**

Our experimental work was funded by grants from the Medical Research Council [grant number GP310915 (to J.P.L.)], the Wellcome Trust [grant number 084957 (to P.J.L.)] and the British Heart Foundation [grant number FS/02/045] (to K.B.). M.D.J.P. is a Medical Research Council-funded research student. S.C.P. was a Wellcome Trust-funded research student. The Cambridge Institute for Medical Research is supported by a Strategic Award [079895] from the Wellcome Trust.

**References**


Received 15 November 2008
doi:10.1042/BST0370178