ESCRT proteins, endosome organization and mitogenic receptor down-regulation

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Abstract
Mitogenic tyrosine kinase receptors such as the EGFR (epidermal growth factor receptor) are endocytosed once they are activated at the cell surface. After reaching the early endosome, they are ubiquitinated within their cytosolic domain and are consequently sorted away from recycling receptors. They are then incorporated into intraluminal vesicles within the MVB (multivesicular body) en route to the lysosome, where they are degraded. MVB formation requires the stabilization of the vacuolar domain of the early endosome, the segregation of degradative cargo within this domain (with subsequent incorporation of receptors such as EGFR into intraluminal vesicles) and the physical separation and movement of this domain away from the tubular regions of the early endosome. How these different aspects of MVB biogenesis are coupled is unknown, but ESCRTs (endosomal sorting complexes required for transport) have been identified as key molecular players in driving mitogenic receptor sequestration and formation of intraluminal vesicles.

The present review summarizes recent findings within the field and from our laboratory regarding the detailed function of ESCRTs and associated proteins in driving the ubiquitin-dependent sorting of EGFR and in maintaining the domain organization of the early endosome.

Introduction
Down-regulation of mitogenic tyrosine kinase receptors is a process of fundamental importance, since defective receptor down-regulation results in a failure to turn off mitogenic signalling pathways and is associated directly with disease [1]. Mitogenic receptors such as the EGFR (epidermal growth factor receptor) are endocytosed once they are activated at the cell surface. After reaching the early endosome, they are ubiquitinated within their cytosolic domain and consequently sorted away from recycling receptors. They are then incorporated into intraluminal vesicles within the MVB (multivesicular body) en route to the lysosome, where they are degraded (Figure 1A). Although phosphotyrosine phosphatases are important for down-regulating mitogenic signalling, the relocation of the receptor kinase domain into intraluminal vesicles and away from the cytosolic space is central to switching off receptor signalling, and mutations in components of the pathway are linked to loss of cell-cycle control [1–4]. Although EGFR and other signalling receptors are transported through the MVB pathway, other classes of receptors may be recycled to the plasma membrane. These receptors, such as TFR (transferrin receptor), are sorted away from EGFR into tubular regions of the early endosome.

The importance of the MVB pathway is underscored by it being used to regulate the activity of other signalling receptors such as Notch [2] and GPCRs (G-protein-coupled receptors) [5]. In specialized secretory cells, the intraluminal vesicles of the MVB are secreted as ‘exosomes’, which play a major role in antigen presentation and other intercellular signalling pathways [6]. The MVB pathway is also closely linked to autophagosome generation, and mutations in MVB components may be central to the development of neurodegenerative disorders [7,8]. In addition, many molecular components involved in forming MVB also function in the budding from cells of retroviruses such as HIV, in a process that is topologically similar to MVB formation [9].

ESCRT (endosomal sorting complex required for transport) protein sorting to MVBs involves several steps. These include selection of MVB cargo away from recycling receptors such as TFR, and the recruitment of cytosolic factors that drive the inward invagination of the MVB-limiting membrane. MVB sorting must also involve the physical separation of EGFR-rich domains away from the tubular elements of the early endosome, a process supported by microtubule motors such as cytoplasmic dynein and the kinesin KIF16B [10,11]. Detailed mechanistic studies have shown that MVB formation involves the sequential recruitment to the endosome of cytosolic factors (Figure 1B). Most are within ESCRTs [12,13]. In mammalian cells, these include the stable complexes ESCRT-0 (including Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) and STAM (signal-transducing adaptor molecule)), ESCRT-I (comprising TSG101 (tumour susceptibility gene 101), VPS (vacuolar protein sorting), CHMP-2 (apoptosis-linked protein X), ALG-2 (apoptosis-linked gene 2)-interacting protein X, OHRP, KIF16B) and ESCRT-II (including CHMP-3, Alix, and Alix-interacting protein X). These proteins, working with ESCRT-0, drive the inward invagination of the MVB limiting membrane.

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Figure 1 | The multivesicular body pathway

(A) Ubiquitinated receptors such as EGFR are sorted away from recycling receptors such as TFR to intraluminal vesicles within a domain of the early endosome that develops into the MVB and matures into the late endosome. (B) Sorting of cargo to the MVB involves the binding of ESCRTs to ubiquitin (Ub) on cargo cytoplasmic domains, to sequester them away from recycling receptors. ESCRT-III binding probably drives intraluminal vesicle formation. Bro1p acts in combination with Doa4p.

Although many ESCRT interactions have been mapped using yeast two-hybrid interaction studies and in vitro biochemical experiments [12,13], the precise functions of each complex are still not known in detail, and the underlying mechanism of MVB formation remains uncertain. Functional studies in yeast point to an ordered recruitment to the endosome [14], with ESCRTs 0–II probably involved in ubiquitin-dependent sequestration of receptors. Each of these complexes contains proteins that possess ubiquitin-binding motifs. For example, Hrs and STAM contain UIMs (ubiquitin interaction motifs) and bind ubiquitin directly via these [15]. TSG101 contains an N-terminal UEV (ubiquitin enzyme variant) domain that drives the recognition of ubiquitin by ESCRT-I [16]. VPS36 contains a GLUE [GRAM-like ubiquitin binding in EAP45 (ELL-associated protein 45)] domain, also capable of binding ubiquitin [17,18]. ESCRT-III is believed to drive membrane invagination, although the mechanism by which this is achieved remains uncertain [19]. ESCRTs are finally recycled off the membrane by the AAA (ATPase associated with various cellular activities) VPS4, and it is possible that this ATPase cycle is linked directly to altering membrane curvature. As well as ESCRTs and VPS4, other factors are involved in ubiquitin-dependent cargo selection and MVB formation. In particular, deubiquitinating enzymes play a major role in MVB formation. AMSH [associated molecule with the SH3 (Src homology 3) domain of STAM] binds both to STAM and to ESCRT-III components [20], whereas UBPY (ubiquitin-specific protease Y) also binds to ESCRT-III [21].

ESCRTs and endosome structure

On the basis mainly of studies in yeast, the primary process catalysed by ESCRTs and associated factors is thought to be the formation of intraluminal vesicles within the MVB and the sorting of cargo into these vesicles [14]. However, in addition to inward invagination, microscopical studies show that other elaborate changes also occur in the form and distributions of endosome tubules and vacuoles when ligand binding induces EGFR to internalize. In particular, the size and volume density of MVBs increases, in addition to the number of intraluminal vesicles per MVB [22]. These changes underline the contribution that the morphology of the endosome plays in selectively routing cargo. Despite its importance, little is known of how the structural organization of the endosome is achieved. In particular, the relationship between the series of protein–protein interactions that selectively...
sort trafficking membrane proteins and the membrane shape changes that involve tubulation and vacuolation remain to be determined. In this respect, it is perhaps significant that at least some endogenous ESCRT components have been localized predominantly to endosomal tubules, with relatively low concentrations found on early and late endosomal vacuoles [23]. Hence, ESCRTs may contribute to the overall organization of the endosome into vacuolar and tubular domains.

For this reason, we have examined the ultrastructural changes to the endosomal system that occur as a consequence of depleting ESCRT activity. Abrogation of ESCRT-I activity by microinjecting function-blocking antibody [24] or by RNAi (RNA interference)-based depletion of ESCRT-I [25] causes a block in forward transport of EGFR to the lysosome and the accumulation of EGFR in endosomal structures positive for the early endosomal marker EEA1 (early endosome antigen 1), as assessed by fluorescence microscopy. These early endosomes appear highly clustered compared with early endosomes in untreated cells. They also are highly stained with antibodies recognizing ubiquitinated proteins, suggesting that turnover of ubiquitinated cargo by deubiquitinating enzymes is prevented under these conditions [24].

Further analysis using electron microscopy showed that depletion of ESCRT-I results in the impairment of a range of endosomal activities [25]. First, forward transport of EGFR to late endosomal compartments is prevented. In addition, forward transport of a fluid-phase marker, HRP (horseradish peroxidase), is also blocked. Instead, markers accumulate in structures that could be identified as early endosomes on the basis of their density of labelling with antibodies against the early endosomal marker, SNX1 (sorting nexin 1), and the lack of labelling with antibodies against the late endosomal/lysosomal marker, LAMP1 (lysosome-associated membrane protein 1). These defects in transport to the lysosome are accompanied by profound structural reorganization of early endosomes. Although early endosomes normally exhibit a pattern of tubular and vacuolar domains, these are replaced by multiciesternal structures resembling the ‘Class E’ compartments that define several ESCRT mutants in Saccharomyces cerevisiae [14]. Both EGFR and HRP accumulate in these structures. Although the multiciesternal structures contain some internal membranes, EGFR sorting to internal vesicles is inhibited.

Defects in endosomal function upon depletion of ESCRT-I are not confined to the MVB pathway. In addition, recycling of TIR from the endosome to the cell surface and mannose 6-phosphate receptor from the endosome to the Golgi complex are also inhibited [25]. Dual-labelling experiments using gold-conjugated anti-EGFR and anti-TIR demonstrated that ESCRT-I depletion also impairs the selection of EGFR away from TIR within the limiting membrane of the early endosome, indicating that the normal domain organization of the endosome has been lost. The cisternae formed upon ESCRT-I depletion are interleaved by a fine matrix. This matrix may be composed of structural components that are normally organized in order to generate MVB, but which bind to the endosome in an unregulated fashion upon loss of ESCRT-I. In conclusion, since both cargo recognition and endosome morphogenesis are disrupted upon depletion of ESCRT-I, this protein complex not only might simply drive the selection of cargo into intraluminal vesicles, but also might provide an essential link between cargo recognition and recruitment of structural proteins that drive membrane deformation en route to generating MVBs.

**Bro1-related proteins**

As well as ESCRTs, other components are vital for MVB formation. One important example is the yeast protein Bro1p, which acts quite late in the MVB pathway. Although its activity is not fully defined, one of its roles appears to be the recruitment and activation of the deubiquitinating enzyme Dos4p, an enzyme that removes ubiquitin from cargo immediately before the formation of intraluminal vesicles [26]. We have recently been engaged in the search for the mammalian orthologue of Bro1p. The closest structural homologue to Bro1p is the protein Alix [ALG-2 (apoptosis-linked gene 2)-interacting protein X], which has two N-terminal domains, the Bro1 domain and the V domain, in common with Bro1p [27]. A significant amount of circumstantial evidence suggests that Alix is required for MVB formation. First, *in vitro* interaction studies show that Alix can bind several of the ESCRTs [28,29]. Secondly, Alix is localized to endosomal membranes [23] and, like ESCRTs, is relocalized to aberrant endosomes induced by expression of dominant-negative VPS4 [30]. Thirdly, Alix is enriched in preparations of exosomes from immune cells [31]. Fourthly, retroviruses such as HIV utilize ESCRT-III subunits to bud from cells, and Alix is essential for the budding of a subset of these viruses. During this process, Alix binds to a YPXL motif within a domain of the viral Gag protein that is essential for budding [28,29,32].

Irrespective of this circumstantial evidence, direct functional evidence for Alix supporting MVB formation is lacking, with several studies finding that EGFR degradation is unaffected by Alix depletion (e.g. [33,34]). We also examined the activity of Alix during MVB sorting. We found that attachment of the HIV viral domain carrying a YPXL peptide onto the cytoplasmic domain of TIR diverts the receptor from the tubular regions of the early endosome to the lumen of MVB, providing correlative evidence supporting a role for Alix in MVB sorting. However, we found that sorting of this chimaera and degradation of the EGFR are only moderately affected in cells almost entirely lacking Alix [35]. We concluded that, although Alix may have some role in MVB sorting, a related protein is likely to have a more dominant role.

We therefore examined the effect of depleting a structurally related protein, HD-PTP (His domain phosphotyrosine phosphatase). HD-PTP is a putative tumour-suppressor gene [36] which has recently been shown to bind some ESCRTs *in vitro* [37], but is otherwise largely uncharacterized. Indeed, RNAi depletion of HD-PTP reduces transfer of fluid-phase markers and EGFR to lysosomes and causes the accumulation of ubiquitinated proteins on endosomal
components, very similar to the phenotype seen when ESCRT-I is depleted. In addition, lysosomes are dramatically enlarged in HD-PTP-depleted cells, and recycling of TR to the cell surface from the early endosome is impaired [35]. Hence, multiple pathways arising from the endosome are defective when HD-PTP is lost.

These RNAi experiments formed the basis for examining the structural requirements of HD-PTP for supporting endosomal sorting, since RNAi-depleted cells could be transfected with an RNAi-resistant form of HD-PTP and its mutants. We established that EGFR trafficking and turnover of ubiquitinated protein cargo on endosomes requires the Bro1 domain of HD-PTP, which contains a binding site for the ESCRT-III subunit, CHMP4B, that is conserved between HD-PTP and Alix. Surprisingly, however, mutational analysis demonstrated that binding of CHMP4B is not strictly essential for HD-PTP function within the context of the full-length protein. Presumably, residues within C-terminal regions of HD-PTP allow the full-length protein to bypass an absolute requirement for CHMP4B binding.

As with disruption of ESCRT-I, depletion of HD-PTP causes profound changes to the structural organization of the early endosome. Early endosomes appear highly clustered by fluorescence microscopy, and this has been confirmed by electron microscopy [35]. The normal balance between vacuolar and tubular regions of the early endosome are replaced by an arrangement of tubules and small vesicular elements that are held in large clusters. These clusters are reminiscent of the structures seen upon depletion of ESCRT-I, but the multicisternal elements characteristic of ESCRT-I depletion were not observed. EGFR fails to be incorporated into MVBS, but instead remains associated with tubular elements within the endosomal clusters.

In summary, disruption of some ESCRTs and associated factors in mammalian cells leads to impairment in the sorting of ubiquitinated cargo proteins into intraluminal vesicles within MVBS. However, detailed examination by electron microscopy indicates that these proteins are important for other aspects of endosome morphogenesis, including the maintenance of structural boundaries within the early endosome and the maturation of endosomes into intermediates that are capable of delivering their content to lysosomes.

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References


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