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REVIEW

Hrs and Endocytic Sorting of Ubiquitinated Membrane Proteins

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ABSTRACT. **Endocytosed receptors are either recycled to the plasma membrane or trapped within intralumenal vesicles of multi-vesicular bodies for subsequent degradation in lysosomes. How the cell is able to sort receptors in endosomes has so far been largely unknown. The hepatocyte growth factor regulated tyrosine kinase substrate, Hrs, is an essential protein that has been implicated in cell signalling and intracellular membrane trafficking. Very recently, several reports have demonstrated a role for Hrs in endocytic sorting of ubiquitinated membrane proteins. Here, we review current knowledge about how Hrs recognises ubiquitinated cargo that is destined for lysosomal degradation, and how Hrs may act as a key regulator of the molecular machinery involved in receptor sorting and multivesicular body formation.**

Key words: endocytosis/Hrs/multivesicular body/ubiquitin/vacuolar protein sorting

Endocytosis is an essential mechanism for receptor-mediated nutrient uptake and receptor downregulation. While nutrient receptors are typically recycled from endosomes to the plasma membrane upon endocytosis, endocytosed hormone and growth factor receptors are mostly transported to late endosomes and lysosomes for degradation. Likewise, hydrolytic enzymes are transported from the trans-Golgi network (TGN) to lysosomes via endosomes. Proteins that are destined for lysosomal degradation are sorted into intralumenal invaginations of multivesicular bodies (MVBs), which fuse with lysosomes. Certain proteins recycle to the TGN from MVBs rather than from early endosomes, indicating that sorting is still ongoing as the MVB is forming (Cavalli *et al.*, 2001; Lemmon and Traub, 2000).

For many years, scientists have been working to define the pathways that cargo molecules follow, as well as to identify the coat proteins, membrane components and regulatory factors required for sorting and trafficking in the endosomal system. Recent studies in animal cells and model organisms such as yeast have led to enormous advances in our understanding of these sorting processes. A great achievement over the past years has been the discovery of ubiquitin as a conserved signal for receptor internalisation and downregulation as well as for the targeting of newly synthesised proteins to the yeast vacuole. This review focuses on the hepatocyte growth factor regulated tyrosine kinase substrate, Hrs, which plays a key role in the molecular machinery involved in endosomal sorting of ubiquitinated proteins.

Ubiquitin as a signal for degradation

Protein ubiquitination, first found to be involved in the proteasomal destruction of soluble proteins, has recently also been implicated at multiple steps of the endocytic pathway (Hicke, 2001). In yeast, ubiquitination is a key mechanism for targeting surface proteins for internalisation from the plasma membrane, and more recently ubiquitination has also been shown to function as an endocytosis signal in mammalian cells. There is also strong evidence for a role for ubiquitin at a later step of the endocytic pathway both in yeast and mammals (Dupre *et al.*, 2001; Hicke, 2001). Monoubiquitination of the yeast a-factor receptor Ste3p and the α-factor receptor Ste2p is sufficient for internalisation from the plasma membrane and functions as a cargo sorting signal in the MVB pathway. Sorting of the yeast hydrolase

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Abbreviations: CPS, carboxypeptidase S; CPY, carboxypeptidase Y; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ESCRT, endosomal sorting complex reqired for transport; MVB, multivesicular body; TGN, trans-Golgi network; UIM, ubiquitin-interacting motif.

carboxypeptidase S (CPS) into the MVB pathway requires monoubiquitination in the short cytoplasmic tail of the protein (Terrell *et al.*, 1998; Katzmann *et al.*, 2001; Reggiori and Pelham, 2001; Urbanowski and Piper, 2001). In mammalian cells overexpression of the ubiquitin ligase c-Cbl increases ligand-induced ubiquitination and down-regulation of the epidermal growth factor receptor (EGFR) and a number of other receptor tyrosine kinases (Levkowitz *et al.*, 1998; Miyake *et al.*, 1998). Ubiquitination of the EGFR at the endosome facilitates sorting into MVBs, thereby attenuating kinase signalling (Longva *et al.*, 2002). This is in correspondence with recent findings that several endosomal proteins thought to be involved in internalisation and sorting of receptors are able to bind to ubiquitin (see below) and that ubiquitinated proteins accumulate in endosomes in EGF stimulated cells (Bishop *et al.*, 2002). While the function of ubiquitin as an endosomal sorting signal is well established, the cellular machinery that sorts ubiquitinated membrane proteins has been largely unknown until recently. This is where Hrs comes into the picture.

Hrs localises to clathrin containing microdomains on early endosomes and is essential for MVB formation

Hrs is a 115 kDa multi-domain protein that is specifically localised to the limiting membrane of early endosomes by virtue of its FYVE and coil-coil domains (Raiborg *et al.*, 2001b; Komada *et al.*, 1997). Detailed electron microscopy studies and confocal immunofluorescence analysis show that Hrs is located to microdomains on the endosomal membrane which can morphologically be distinguished as flat bilayered coats (Raiborg *et al.*, 2001a; Raiborg *et al.*, 2002; Sachse *et al.*, 2002). These bilayered endosomal coats were first described in melanoma cells, which express high levels of Hrs (Raposo *et al.*, 2001). Hrs interacts with multiple proteins including clathrin, and it can recruit clathrin to early endosomes in a PtdIns(3)P dependent manner (Sachse *et al.*, 2002; Raiborg *et al.*, 2001a). Accordingly, clathrin is detected within the Hrs microdomains along with both mono- and poly-ubiquitinated proteins, whereas EEA1 (known to be involved in early endosomal fusion) is found in different microdomains than Hrs within the same endosomal membrane. Two binding partners of Hrs, Eps15 and the signal-transducing adaptor molecule STAM, are also found within the Hrs/clathrin microdomains (H. Stenmark, unpublished results). The characteristic organisation of these proteins in coated microdomains argues for a specialised function on the endosome.

The yeast homologue of Hrs, Vps27p, belongs to the class E vacuolar protein sorting (Vps) proteins, a group of 17 proteins required for proper endosomal function including the formation of MVBs (Odorizzi *et al.*, 1998) and trafficking of certain hydrolases to the vacuole (the yeast equivalent of lysosomes) (Piper *et al.*, 1995). The knock-out of Hrs in mice is embryonic lethal and cells derived from Hrs –/– mouse embryos accumulate large endosomes (Miura *et al.*, 2000; Komada and Soriano, 1999). Moreover, Hrs was recently shown to be required for endosomal invagination and MVB formation in *Drosophila* (Lloyd *et al.*, 2002). In *Drosophila* mutants lacking Hrs, increased levels of activated epidermal growth factor receptors and Torso tyrosine kinase receptors cause enhanced tyrosine kinase signalling (Lloyd *et al.*, 2002). Consistent with this, mammalian cells devoid of MVBs due to inhibition of PtdIns(3)-kinase show increased EGF-stimulated tyrosine phosphorylation (Futter *et al.*, 2001), and overexpression of Hrs inhibits degradation of EGF receptors (Bishop *et al.*, 2002; Chin *et al.*, 2001a; Raiborg *et al.*, 2001a). Because Hrs becomes tyrosine phosphorylated upon stimulation of cells with various growth factors and cytokines (Komada and Kitamura, 1995; Asao *et al.*, 1997) phosphorylation of Hrs by activated receptors could provide a mean for modulating Hrs function and hence their own downregulation. Recent results indicate that agonist-induced phosphorylation of Hrs occurs on multiple tyrosines via non-receptor tyrosine kinases such as Src (Bache *et al.*, 2002). Hopefully, studies in Hrs-depleted mammalian cells may give more information about the relationship between Hrs function and receptor downregulation.

MVB formation is necessary to separate membrane-associated proteins destined for degradation in lysosomes (like EGF receptors) from those destined for recycling to the cell membrane (like transferrin receptors). Significantly, internalised EGF receptors are enriched in the Hrs/clathrin microdomains, whereas transferrin receptors are not (Raiborg *et al.*, 2002; Sachse *et al.*, 2002). Taken together, this suggests a role for the Hrs/clathrin microdomains in sorting of ubiquitinated membrane proteins into the MVB pathway. The coated microdomains always appear flat, but internal budding profiles are seen at the edge of coated regions (Sachse *et al.*, 2002). The role of clathrin might be to concentrate the different proteins in microdomains, although this has not yet been investigated.

Hrs binds to ubiquitin and is itself ubiquitinated

An important clue to a more detailed function of Hrs in endosomal sorting was provided from a bioinformatical screen for proteins containing putative ubiquitin interacting motifs (UIMs) (Hofmann and Falquet, 2001). This analysis identified several proteins that operate in the endocytic pathway, including Hrs and its yeast homologue Vps27p. It was soon established that mammalian Hrs (Bishop *et al.*, 2002; Raiborg *et al.*, 2002; Polo *et al.*, 2002) as well as *Drosophila* Hrs (Lloyd *et al.*, 2002) and yeast Vps27p (Shih *et al.*, 2002) are indeed able to bind to monoubiquitin *in vitro* in a UIM dependent manner, with a K_D in the high micromolar range (Raiborg *et al.*, 2002). Although the UIMs can bind to monoubiquitin, they show a preference for polyubiquitin (Polo *et al.*, 2002). Two Hrs interacting

proteins, Eps15 and STAM, also contain UIMs and are able to bind ubiquitin (Hofmann and Falquet, 2001; Polo *et al.*, 2002; H. Stenmark, unpublished results) and so is another class E protein, Vps23p/TSG101 (Bishop *et al.*, 2002; Katzmann *et al.*, 2001). This indicates that Hrs and its associated proteins as well as other class E proteins are able to recognise ubiquitinated proteins and partly explains the accumulation of ubiquitinated proteins in the Hrs/clathrin microdomains.

In addition to these findings, it was recently reported that the UIMs of several endocytic proteins such as Hrs and Eps15 are indispensable for their EGF-stimulated monoubiquitination (Polo *et al.*, 2002). The ability to both recognise ubiquitinated proteins and be ubiquitinated could provide a mean for amplification and assembly of protein complexes by the recruitment of further ubiquitin-binding proteins. Overexpression of Hrs leads to a clustering of early endosomes (Komada *et al.*, 1997), accompanied by an intracellular accumulation of both endocytosed receptors and fluid-phase markers due to an inhibition of transport from early to late endosomes and lysosomes (Bishop *et al.*, 2002; Chin *et al.*, 2001; Raiborg *et al.*, 2001a). The clustered endosomes have also been shown to accumulate ubiquitinated proteins (Bishop *et al.*, 2002). Thus, the ability of Hrs to bind to ubiquitinated proteins and simultaneously serve as a substrate for ubiquitination could be important both for cargo recognition and complex formation, thereby organising proteins that cooperate in endocytic sorting and MVB formation.

The ability to bind to ubiquitin is essential for the function of Hrs in endocytic sorting of ubiquitinated proteins, but not for MVB formation

At present it cannot be ruled out that the role of Hrs is to recruit and coordinate other components of the sorting machinery, rather than to interact directly with cargo. A direct interaction between Hrs and ubiquitinated cargo has not yet been demonstrated, and the study of such endogenous receptor candidates is hampered by the difficulties to stabilise receptor-dependent ubiquitination events in cell extracts. However, a recombinantly modified transferrin receptor (permanently fused to ubiquitin) can be co-immunoprecipitated with Hrs from a cell lysate, strongly supporting the idea that Hrs can interact with ubiquitinated cargo *in vivo*. Furthermore, overexpression of Hrs leads to the intracellular accumulation of the ubiquitinated transferrin receptor, whereas a UIM point mutant that is unable to bind ubiquitin fails to do so, further arguing for a role for Hrs in recognition of ubiquitinated receptors in mammalian cells (Raiborg *et al.*, 2002). Yeast Vps27p has two UIMs, which are required to efficiently sort ubiquitinated cargo from both the biosynthetic and endocytic pathways into MVBs. Cells carrying mutations in a single Vps27p UIM are defective in sorting of the monoubiquitinated CPS as well as the monoubiquitinated α-factor receptor Ste2p to the vacuolar lumen (Shih *et al.*, 2002), consistent with a role of Vps27p in recognition of ubiquitinated cargo.

Yeast cells lacking VPS27 exhibit a typical class E phenotype. They fail to efficiently recycle Golgi proteins such as Vps10p from the endosome, resulting in secretion of CPY, and they are unable to generate MVBs (Piper *et al.*, 1995). Ablating or mutating the UIMs of Vps27p blocks sorting of ubiquitinated cargo proteins to the vacuole lumen, whereas it only leads to a relatively weak class E phenotype (Shih *et al.*, 2002; Bilodeau *et al.*, 2002).

Altogether, these results indicate that the ability to bind to ubiquitin is essential for the function of Hrs/Vps27p in endocytic sorting and that Hrs/Vps27p can play a direct role in the recognition and sorting of ubiquitinated cargo via the UIM. In addition, other parts of Hrs/Vps27p are involved in MVB formation and CPY sorting, possibly by recruiting other class E proteins. Recent data demonstrate a role for TSG101 (the mammalian homologue of the yeast class E protein Vps23p) in virus budding, an event that is topologically similar to invagination of endosomal membranes (Garrus *et al.*, 2001). It is interesting to note that Hrs contains a motif similar to those found in Tsg101-recruiting viral proteins. This argues that the role of Hrs/Vps27p in MVB formation might be upstream of TSG101/Vps23p.

Hrs is part of a complex, ubiquitin-recognising molecular sorting machinery

Vps23p is found in a 350 kDa protein complex together with two other class E proteins, Vps28 and Vps37. This complex is named ESCRT-I, for endosomal sorting complex required for transport. ESCRT-I binds to ubiquitinated proteins and thereby directs sorting into the MVB pathway (Katzmann *et al.*, 2001). ESCRT-I seems to represent a conserved component of the endocytic sorting machinery, since TSG101 is part of a 350 kDa protein complex that contains human Vps28 and binds ubiquitin (Bishop *et al.*, 2002; Bishop and Woodman, 2001; Babst *et al.*, 2000). Recently, two complexes consisting of other class E proteins were identified and named ESCRT-II and ESCRT-III. Genetic studies indicate that these complexes act downstream of ESCRT-I in the sorting process (Babst *et al.*, 2002a; Babst *et al.*, 2002b).

There is evidence that Hrs/Vps27p also acts in cooperation with other proteins in a conserved complex. In mammalian cells, Hrs binds tightly to STAM and Eps15 (Bean *et al.*, 2000; Asao *et al.*, 1997), and is found within a 500–550 kDa complex (Bishop *et al.*, 2002; Chin *et al.*, 2001). All three proteins can bind to ubiquitin, and colocalise in characteristic microdomains on endosomal membranes (Polo *et al.*, 2002; H. Stenmark, unpublished results). In yeast, Vps27p binds tightly to the STAM homologue Hse1p. Both proteins contain UIMs and can bind to ubiquitin. Deletion

of the UIM in one of these proteins gives only a slight effect on the sorting of the ubiquitinated a-factor receptor Ste3p, while deletion of all the UIMs in both proteins leads to a profound block in sorting of Ste3p and other ubiquitinated markers (Bilodeau *et al.*, 2002). These results suggest that Vps27p and Hse1p act together to sort ubiquitinated proteins. In addition, a Hrs/STAM complex could also play a role in signal transduction, since STAM has been proposed to act downstream of Jak2 and Jak3 in cytokine signalling (Endo *et al.*, 2000; Takeshita *et al.*, 1997).

In order to be able to escort cargo into MVBs, it is conceivable that the different complexes and the clathrin coat that probably stabilises them on the membrane would need to be removed before invagination. The AAA ATPase Vps4p is thought to control the membrane association of the ESCRT complexes in yeast and mammals (Bishop and Woodman, 2001; Babst *et al.*, 1998; Babst *et al.*, 2002b) and the localisation of Hrs, STAM, EPS15 and clathrin to endosome membranes also seems to be controlled by this ATPase (H. Stenmark, unpublished results). Moreover, EGF-stimulated phosphorylation of Hrs causes a more cytosolic distribution, suggesting that phosphorylation may play a role in coat disassembly (Urbé *et al.*, 2000).

Although deubiquitination does not seem to be a prerequisite for sorting of cargo into MVBs (Katzmann *et al.*, 2001; Dupre and Haguenauer-Tsapis, 2001), it would seem important to remove ubiquitin before internalisation in order to maintain the cellular level of free ubiquitin. In yeast, the deubiquitinating enzyme Doa4 is involved in the removal of ubiquitin from endocytic cargo (Amerik *et al.*, 2000). In mammalian cells, Hrs might play an indirect role in releasing ubiquitin, since the Hrs associated protein, STAM2, interacts with the deubiquitinating enzyme UBPY (Kato *et al.*, 2000).

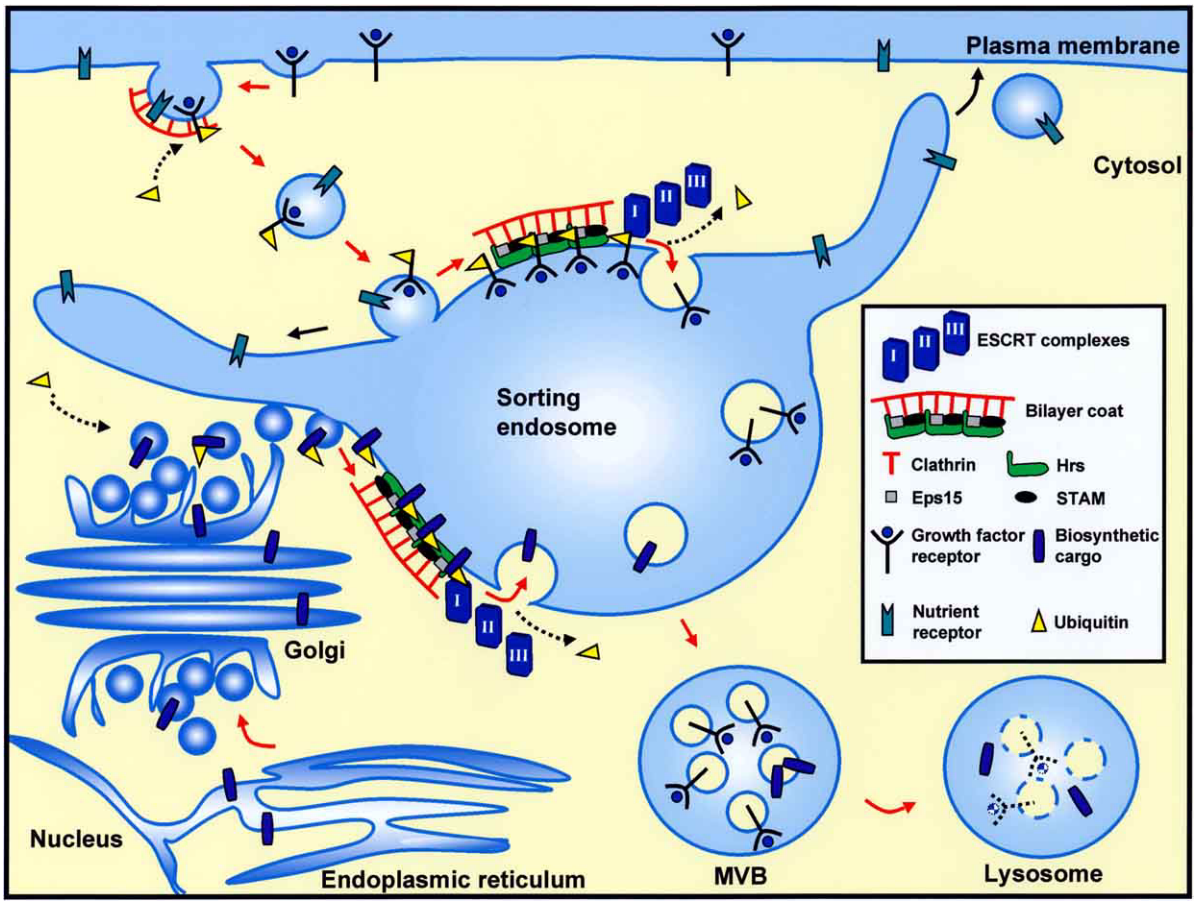

Fig. 1. Model for the role of Hrs in endocytic sorting of ubiquitinated membrane proteins into multivesicular bodies. See "*Conclusion*" for details. For simplicity, the size of the sorting endosome has been exaggerated relative to other organelles. Growth factor receptors become ubiquitinated upon ligand binding and are thus routed to MVBs and lysosomes via the Hrs pathway. In contrast, nutrient receptors are not ubiquinated and therefore do not accumulate within the Hrs-containing endosomal microdomains, enabling them to recycle to the plasma membrane. Note that the indicated endosomal routing of biosynthetic cargo via ubiquitin tagging has been shown to involve the Hrs homologue Vps27p in yeast, whereas this trafficking route has not yet been demonstrated in mammalian cells.

How do the Hrs/STAM and ESCRT complexes act in concert to facilitate endocytic sorting? One possibility is that they recognise ubiquitinated cargo in a conveyor-belt like series of sorting events which eventually leads to internalisation of cargo into MVBs.

Conclusion

Via its multiple domains, Hrs appears to act as an important endosomal scaffolding protein that is capable of both organising the sorting machinery and recruiting cargo destined for lysosomal degradation. The following findings argue in favour of this idea: 1) Hrs is specifically located to endosomal membranes via its FYVE and coil-coil domains. 2) Hrs has a UIM that is essential for the proper sorting of ubiquitinated membrane proteins, and it is found in a complex with Eps15 and STAM, which both recognise ubiquitin. 3) Hrs has a VHS domain that, in the case of GGA proteins, has been shown to interact directly with receptor tails (Nielsen *et al.*, 2001). The ligands for the Hrs VHS domain remain to be identified. 4) Hrs can bind sorting nexin-1, a protein that is found on endosomes and is implicated in EGF-receptor sorting (Cozier *et al.*, 2002; Chin *et al.*, 2001). 5) Hrs contains a motif that is predicted to interact with one of the components of the ESCRT-I complex, suggesting that Hrs can recruit the rest of the sorting machinery. It will be interesting to study if Hrs can indeed interact with ESCRT proteins. 6) Hrs can recruit clathrin to early endosomes via its C-terminal clathrin box motif (Raiborg *et al.*, 2001a). This could be important for concentrating the cargo in microdomains on the endosomal membrane before invagination and MVB formation.

Based on the available data, we present the following model for endocytic sorting of ubiquitinated membrane proteins (Fig. 1). Receptors and other membrane proteins are transported to the sorting endosome, either after internalisation from the plasma membrane by endocytosis or via transport vesicles from the trans-Golgi network. At the sorting endosome, ubiquitinated membrane proteins are recognised by the Hrs/STAM/Eps15 complex and retained in specialised microdomains on the endosomal membrane. These microdomains might be stabilised by the recruitment of clathrin by Hrs. Membrane proteins that are not ubiquitinated are free to diffuse laterally in the endosomal membrane and can be recycled to the plasma membrane or to the TGN. The membrane proteins that are retained in the microdomains can be passed on to downstream components of the sorting machinery, such as the ESCRT complexes. During a so far unknown molecular interplay, the endosomal membrane starts to invaginate at the rim of the microdomains, a process that requires Hrs and ESCRT proteins. To allow reuse of ubiquitin, ubiquitin is removed from the membrane proteins and released into the cytosol prior to enclosure of the invaginated membrane. The receptors are now trapped within MVBs. MVBs can then fuse with lysosomes and release their internal vesicles within the lysosomal lumen, where lysosomal hydrolases can be matured and activated receptors are degraded. By recognising cargo, recruiting a clathrin coat and engaging ESCRT complexes, Hrs is likely to act as a master regulator of endosomal protein sorting.

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