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The Hrs/STAM Complex in the Downregulation of Receptor Tyrosine Kinases

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Cell surface receptor proteins that have undergone endocytosis are transported to the endosome. From the endosome, ligand-activated receptor tyrosine kinases are further transported to the lysosome for degradation, a process called "receptor downregulation." By contrast, nutrient receptors, such as those for low-density lipoprotein and transferrin, are recycled back to the plasma membrane. Sorting of these two types of receptors occurs at the endosome, where ubiquitination of receptor proteins serves as the sorting signal. Namely, ubiquitinated receptors are incorporated into the lysosomal degradation pathway, whereas those that are not ubiquitinated are returned to the cell surface. Hrs and STAM are proteins that form a complex on the endosomal membrane. Recent studies have shown that the Hrs/STAM complex binds ubiquitin moieties and acts as sorting machinery that recognizes ubiquitinated receptors and transfers them to further sequential lysosomal sorting/trafficking processes.

Key words: endosome, multivesicular body, protein sorting, receptor downregulation; ubiquitination.

Abbreviations: CPS, carboxypeptidase S; EGF, epidermal growth factor; LDL, low-density lipoprotein; MVB, multivesicular body; RNAi, RNA interference; RTK, receptor tyrosine kinase; Ub, ubiquitin; UIM, ubiquitin-interacting motif; vps, vacuolar protein sorting.

Upon stimulation with growth factors, their cell surface receptors with intrinsic tyrosine kinase activity are rapidly internalized as a complex with bound ligands *via* clathrin-coated vesicle-mediated endocytosis. The growth factor/receptor tyrosine kinase (RTK) complex is eventually transported to the lysosome and degraded by acid hydrolases (Fig. 1). This process, called "receptor downregulation," is a cellular strategy to attenuate the cell proliferation signal triggered by growth factors and to prevent the overgrowth of the cell. Conjugation of ubiquitin (Ub) to RTKs has been shown to play an essential role in their downregulation. Moreover, this ubiquitination does not serve as a signal for proteasomal degradation but as a sorting signal for transport to the lysosome. Here, we review recent findings on the mechanisms of sorting ubiquitinated RTKs at the endosome and the role of the Hrs/STAM complex in this process.

The recycling versus multivesicular body pathways

Not only activated RTKs but also nutrient receptors, such as those for low-density lipoprotein (LDL) and Fe^{3+}-bound transferrin, undergo endocytosis mediated by clathrin-coated vesicles. All the receptors are first transported to the early endosome where they are sorted to different pathways. The LDL and transferrin receptors release LDL and Fe^{3+}, respectively, at the early endosome and are recycled back to the plasma membrane *via* the recycling endosome (the recycling pathway; Fig. 1) (*1*). By contrast, ligand-activated RTKs are incorporated into luminal vesicles of the endosome that bud inward from its limiting membrane (Fig. 1) (*1*, *2*). As endosomes that contain such luminal vesicles are called the multivesicular body (MVB), this trafficking route is referred to as the MVB pathway. The MVBs either maturate to or fuse with the late endosome, and subsequent fusion of the late endosome with the lysosome results in release of the luminal vesicles containing activated RTKs into the lumen of the lysosome (*1*, *2*).

The MVB pathway is conserved from yeasts to higher eukaryotes. In the budding yeast *Saccharomyces cerevisiae*, some newly-synthesized vacuolar hydrolases are also sorted for the MVB pathway in their biosynthetic trafficking to the vacuole, a counterpart of the mammalian lysosome. Carboxypeptidase S (CPS), one of such hydrolases, is synthesized and transported to the vacuole as a transmembrane precursor, proCPS (*2*, *3*). During the trafficking pathway, its short N-terminal region faces the cytoplasm, whereas its catalytic domain faces the lumen of passing organelles until it arrives at the endosome (Fig. 1). There, the membrane-spanning proCPS is incorporated into the MVB luminal vesicles and eventually transported to the vacuolar lumen, where it is converted to a mature soluble hydrolase by proteolytic cleavage at a specific luminal juxtamembrane site (Fig. 1).

Monoubiquitination as the MVB sorting signal

How are endocytosed membrane proteins that are incorporated into the MVB luminal vesicles and destined for the lysosome/vacuole (*i.e.*, activated RTKs in mamma-

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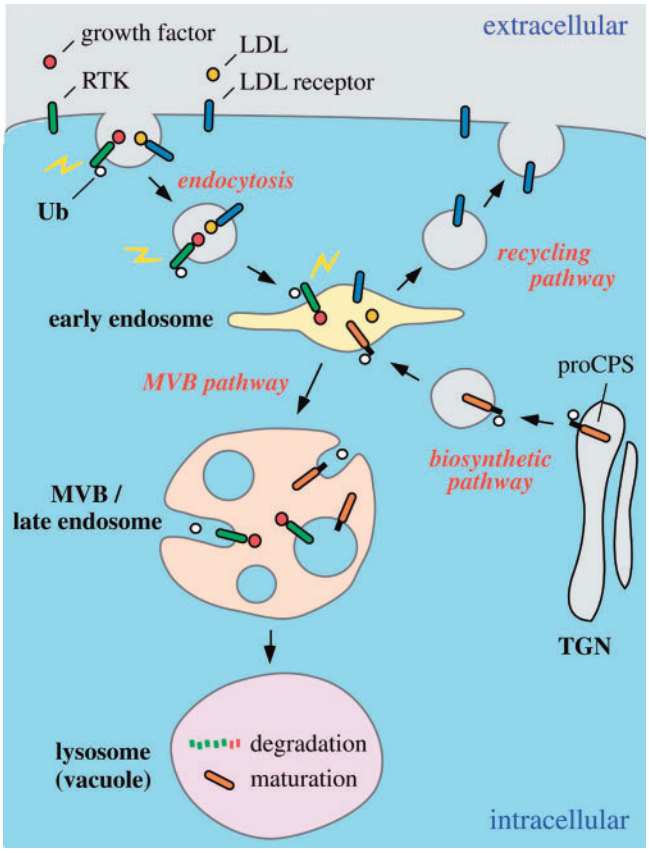

Fig. 1. **Membrane traffic *via* the endosome.** Traffic of growth factor-activated RTK and LDL receptor from the cell surface to the endosome (endocytosis), further traffic of RTK to the lysosome (MVB pathway), and recycling of LDL receptor to the cell surface from the endosome (recycling pathway) in mammalian cells are illustrated. Also, traffic of proCPS from the *trans*-Golgi network (TGN) to the vacuole *via* the endosome (biosynthetic and MVB pathways) in yeast, is shown. See the text for details.

lian cells and biosynthetic hydrolase precursors in yeast) and those that are returned to the cell surface (*i.e.*, LDL and transferrin receptors) sorted at the early endosome? The mechanism was initially elucidated in yeast. proCPS is monoubiquitinated (conjugated with a single Ub moiety) in its short cytoplasmic region, and this ubiquitination serves as a signal for the protein to be incorporated into the MVB luminal vesicles (Fig. 1) (*3*, *4*). proCPS mutants which do not undergo ubiquitination stay on the limiting membrane of the MVB and are eventually mislocalized on the vacuolar limiting membrane (*3*). On the contrary, if a single Ub sequence or a peptide containing the ubiquitination site of proCPS is artificially fused to cytoplasmic regions of membrane proteins that are normally transported to the vacuolar limiting membrane, the fusion proteins are mislocalized to the lumen of the organelle (*3*, *5*). In addition to CPS, two other vacuolar enzymes, a polyphosphate endophosphatase Phm5 and a hypothetical haem oxygenase Hmx1, are shown to be sorted to the vacuolar lumen by ubiquitination (*4*). Unlike the 26S proteasome system that requires polyubiquitination (conjugation of four or more Ub moieties in tandem) of target proteins to be recognized as a degradation signal, monoubiquitination is sufficient as the MVB sorting signal (*3*, *5*). Before ubiquitinated cargoes are incorporated into the MVB luminal vesicles, the conjugated Ub moieties are cleaved by a deubiquitinating enzyme Doa4 and recycled to the cytoplasm for reuse (Fig. 1) (*2*).

Stimulation of mammalian cells with growth factors induces ubiquitination of their receptors. Based on the smeary electrophoretic pattern of ubiquitinated RTKs on SDS–polyacrylamide gels, they have been believed to be polyubiquitinated and undergo proteasomal degradation. However, recent studies have shown that RTKs are monoubiquitinated on several different lysine residues (multiply-monoubiquitinated or multiubiquitinated), and that this type of ubiquitination is sufficient for the degradation of activated RTKs (*6*, *7*). These results suggest that their ubiquitination does not serve as the proteasomal degradation signal but as the MVB sorting signal at the endosome (Fig. 1). In addition, RTKs are transmembrane proteins that are embedded in transport vesicles within cells. Therefore, it is unlikely that they enter the narrow portal of the cylindrical 20S proteasome core for degradation unless their cytoplasmic regions are shedded from the vesicles by proteolytic cleavage. Taken together, it is more likely that degradation of RTKs in the lysosome, but not in the proteasome, plays a major role in their downregulation.

c-Cbl is an E3 Ub ligase for RTKs (*8*). v-Cbl, an oncogenic counterpart of c-Cbl, consists only of its N-terminal tyrosine kinase-binding domain that mediates binding to RTKs. When overexpressed, v-Cbl dominant-negatively blocks ubiquitination of activated RTKs, leading to constitutive activation of RTKs and cellular transformation (*8*). A mutant of the hepatocyte growth factor receptor, c-Met, that lacks c-Cbl–binding ability has also been shown to act as a transforming protein due to a defect in undergoing downregulation (*9*). These results suggest an essential role for the ubiquitination of RTKs in attenuating growth factor signaling, of which deregulation leads to tumor development.

Structure and localization of the Hrs/STAM complex

Hrs (hepatocyte growth factor-regulated substrate) and STAM (signal-transducing adaptor molecule) were both identified as tyrosine phosphorylated proteins in cells stimulated with growth factors and cytokines (*10*–*15*). Therefore, they were initially implicated in the signaling of cell growth and differentiation. Detailed characterization of these molecules, however, has demonstrated that they are key players in the downregulation of RTKs. As shown in Fig. 2, Hrs and STAM contain several domains and peptide motifs that play important roles in their function. The roles of these domains and motifs will be described later.

Whereas only one *HRS* gene is found in any single organism, mammals have two *STAM* genes encoding STAM1 and STAM2 (also known as Hrs-binding protein, Hbp) (*12*–*15*). EAST (EGF receptor-associated protein with SH3 and TAM domains) is a STAM ortholog in chicken with higher homology to STAM2 than to STAM1 (*16*). STAM1 and STAM2 have a 53% overall amino acid sequence identity to each other, comprise the same

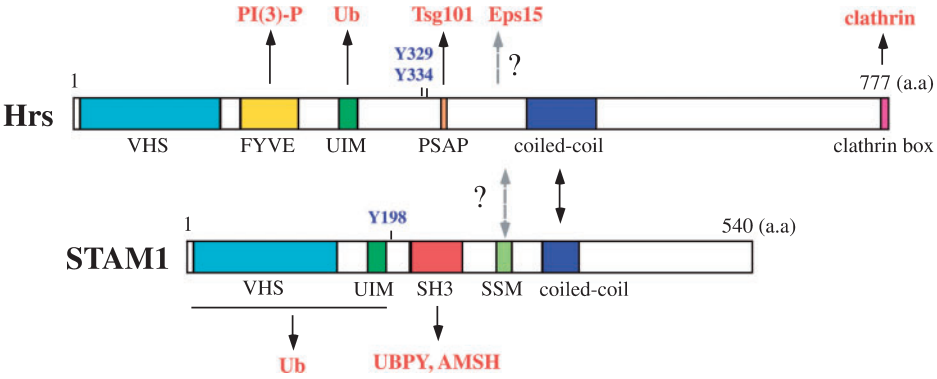

Fig. 2. **Schematic structure of the Hrs/STAM complex.** Domain structures and binding molecules of human Hrs and STAM1 are shown. Tyrosine phosphorylation sites (Y334 and Y329 for Hrs; Y198 for STAM1) are also indicated. Hrs and STAM bind to each other through their coiled-coil regions. How the STAM-specific motif (SSM) of STAM stabilizes its Hrs binding is unknown (*17*). The Eps15-binding site of Hrs has not been precisely mapped (*32*). PI(3)-P, phosphatidylinositol(3)-phosphate; SH3, Src homology 3.

domain organization, and are currently believed to have a redundant function. Hrs and STAM bind to each other *via* their coiled-coil regions (Fig. 2) (*11*, *15*). The STAM-specific motif (SSM), a short sequence consisting of approximately 25 amino acids and conserved in STAM proteins in higher eukaryotes, is also required for stable binding to Hrs (Fig. 2) (*17*). Depletion of Hrs by RNA interference (RNAi) causes a significant decrease in the amount of STAM proteins (*17*). Similarly, simultaneous depletion of both STAM1 and STAM2 causes a moderate (~50%) decrease in the Hrs protein level (our unpublished results), suggesting that Hrs and STAM are stabilized by forming a complex with each other. Therefore, Hrs and STAM seem to function solely as a protein complex.

The Hrs/STAM complex is localized on the cytoplasmic face of the early endosome (*17–19*). Hrs has a FYVE (Fab1/YOTB/Vac1/EEA1) domain, a double zinc finger domain that is structurally conserved among eukaryotic proteins (Fig. 2). The FYVE domain specifically binds phosphatidylinositol(3)-phosphate which is enriched in the endosomal membrane (*20–22*), thereby functioning to anchor Hrs on the endosome (*23–26*). STAM does not have such a lipid-binding domain and is shown to be localized on the early endosome by binding to Hrs (*17*). On the early endosomal membrane, there are specific areas to which a flat clathrin coat is attached. Localization of Hrs is restricted to this microdomain of the endosome, although the function of the clathrin coat is unclear (*27*, *28*). Hrs binds the clathrin heavy chain *via* a clathrin box, a short peptide motif located at the C-terminus of Hrs (Fig. 2) (*27*). Therefore, the Hrs/STAM complex might recruit the clathrin coat to the microdomain of the endosome, or *vice versa*, through direct interaction.

The budding yeast *S. cerevisiae* has single Hrs and STAM orthologs, Vps27 (vacuolar protein sorting 27) and Hse1 (Hbp/STAM/EAST 1), respectively. Vps27 was identified through genetic screening of vacuolar protein sorting (*vps*) mutants, which are defective in the biosynthetic trafficking of an acid hydrolase, carboxypeptidase Y, to the vacuole (*29*). Among the *vps* mutations, *vps27* is grouped into the class E category, which exhibits defects in protein transport *via* the endosome (*30*). Although not identified in the initial *vps* screening, recent studies have demonstrated that disruption of the *HSE1* gene also leads to the class E *vps* phenotype (*31*). In addition, it is shown that Vps27 and Hse1 proteins form a complex on the endosomal membrane (*31*).

The Hrs/STAM complex as the Ub receptor in MVB sorting

Hrs was shown to interact with Eps15 (EGF pathway substrate 15) and sorting nexin 1, both of which are involved in the downregulation of the epidermal growth factor (EGF) receptor (*32*, *33*). In addition, overexpression of Hrs leads to the inhibition of EGF receptor degradation (*27*, *33*), although it could be a secondary effect due to general defects in endosomal function because Hrs overexpression drastically changes the morphology of the endosome (*18*). On the other hand, loss of function of Hrs by gene targeting causes the enlargement of the early endosome in mouse cells (*24*), which is reminiscent of the enlarged endosome called "class E compartment" in class E *vps* mutant yeast cells (*30*). These initial findings suggested the involvement of the Hrs/STAM complex in the downregulation of RTKs *via* the MVB pathway. A short peptide motif of approximately 20 amino acids has been identified as a Ub-binding site in the S5a subunit of the 19S regulatory subunit of the 26S proteasome (*34*). A breakthrough in understanding the molecular function of the Hrs/STAM complex was brought in 2001 by Hofmann and Falquet, who found through a bioinformatic database search that this Ub-binding sequence, which they termed the Ub-interacting motif (UIM), is present in several endocytic proteins including Hrs and STAM, as well as their yeast orthologs Vps27 and Hse1 (Fig. 2) (*35*). Subsequently, several groups have demonstrated that both Hrs and STAM bind Ub directly (*19*, *28*, *31*, *36–41*). In Hrs, the UIM serves as the major Ub-binding site (*28*, *31*, *37–40*). In STAM, in contrast, the N-terminally-located VHS (Vps27/Hrs/STAM) domain plays an essential role in Ub binding cooperatively with the following UIM (Fig. 2) (*41*).

In *vps27* or *hse1* mutant yeast cells, ubiquitinated cargoes, such as proCPS and endocytosed Ste3, a receptor for the **a**-factor mating pheromone, are not transported to the lumen of the vacuole but are mislocalized to its limiting membrane (*31*, *37*). This phenotype cannot be suppressed by expressing their mutants lacking functional UIMs, suggesting that the binding of the Vps27/Hse1 complex to ubiquitinated cargoes is an essential step in MVB sorting (*31*, *37*). Neither can it be suppressed by a Vps27 mutant lacking the Hse1-binding site, suggesting that the complex formation of these proteins is also required for the sorting function (*42*). In mammalian cells, overexpression of Hrs (*27*, *28*, *38*) or STAM (*41*) induces the accumulation of ubiquitinated proteins on

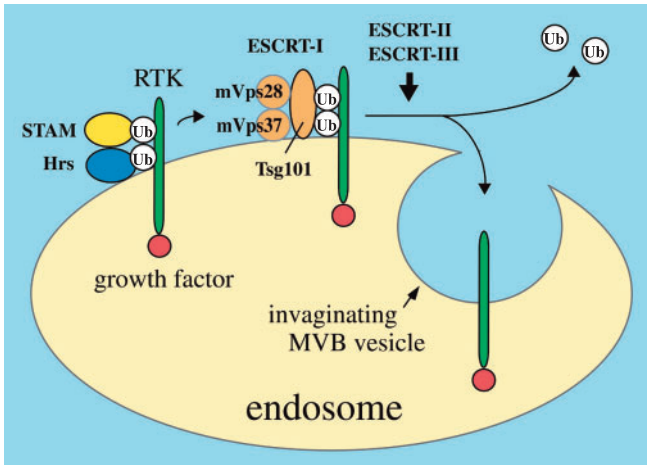

Fig. 3. **A model for the function of the Hrs/STAM complex.** A current model for the function of the Hrs/STAM complex in MVB sorting of multiubiquitinated RTKs on the early endosomal membrane is illustrated. mVps28 and mVps37 are mammalian orthologs of yeast Vps28 and Vps37, respectively. See the text for details. By analogy to yeast cells, RTKs are believed to be deubiquitinated before being incorporated into the MVB luminal vesicles.

the endosome. Neither Hrs mutants lacking UIM (*43*) nor STAM mutants lacking the VHS domain or UIM (*41*) can cause these effects, suggesting essential roles of these domains in recognizing ubiquitinated cargoes. Complex formation of Hrs and STAM is also essential for the endosomal accumulation of ubiquitinated proteins, as the effect of STAM2 is lost by deleting the Hrs-binding coiled-coil region (*17*). In addition, knockdown of endogenous Hrs by RNAi (*19*, *44*, *45*) as well as knockout of both STAM1 and STAM2 by gene disruption (*46*) inhibits the degradation of ligand-activated RTKs. Also in the fruit fly *Drosophila*, disruption of the *HRS* gene has been shown to cause defects in the downregulation of RTKs as well as that of other signaling receptors, including Thickveins (a receptor serine/threonine kinase for a transforming growth factor-β family ligand, Decapentaplegic), Patched and Smoothened (a receptor and a signal-transducing membrane protein for Hedgehog, respectively), and Notch (*36*, *47*). Impaired downregulation of these receptors in *hrs* mutant flies leads to enhanced activation of downstream signaling molecules (MAP kinase and Smad for RTKs and Thickveins, respectively) and to elevated expression of their target genes (*36*, *47*). Taken together, it is proposed that the Hrs/STAM complex and its yeast ortholog Vps27/Hse1 sort ubiquitinated cargo proteins including ligand-activated RTKs by recognizing their Ub moieties on the early endosome (Fig. 3).

Eps15 is a protein that binds the adaptor protein 2 (AP2) complex of the clathrin-coated pit on the cytoplasmic face of the plasma membrane and plays an essential role in the endocytosis of cell surface receptors (*48*). Hrs binds Eps15 (*32*). EAST, a chicken ortholog of STAM, also binds this protein either directly or *via* Hrs (*16*). Similarly to Hrs and STAM, Eps15 is tyrosine phosphorylated and monoubiquitinated in response to EGF stimulation (*48*). More interestingly, it contains two tandem UIMs at the C-terminus and binds ubiquitinated proteins through these motifs (*39*). Eps15 is partially colocalized with clathrin and ubiquitinated proteins on the Hrs/STAM-positive microdomains of the early endosomal membrane (*19*). Therefore, in addition to a role in clathrin-mediated endocytosis, it is possible that Eps15 acts as the third component of the MVB sorting complex that recognizes ubiquitinated cargoes on the early endosome.

The Hrs/STAM complex recruits ESCRT-I

Currently, 18 genes belong to the class E *VPS* category in yeast. In addition to the complex of Vps27 and Hse1, other class E Vps proteins form complexes that serve as the sorting machinery for ubiquitinated cargoes on the endosomal membrane. These include ESCRT (endosomal sorting complex required for transport) -I (composed of Vps23, Vps28, Vps37) (*3*), ESCRT-II (composed of Vps22, Vps25, Vps36) (*49*), and ESCRT-III (composed of Vps2, Vps20, Vps24, Snf7/Vps32) (*50*). Among these complexes, ESCRT-I and ESCRT-II have been shown to directly interact with ubiquitinated cargoes (*3*, *51*). Proteins that are orthologous to the components of the yeast ESCRT complexes are present in mammals, and they form similar complexes in mammalian cells (Fig. 3) (*2*). Vps23 and its mammalian ortholog Tsg101 (tumor susceptibility gene 101) are a component of ESCRT-I and contain a Ub E2 variant (UEV) domain. This domain has a sequence homology to the catalytic domain of the E2 Ub-conjugating enzymes but lacks an enzymatic activity due to the absence of the critical cysteine residue. However, it binds the Ub moiety of ubiquitinated proteins (Fig. 3) (*3*). The components of ESCRT-I shuttle between the endosomal membrane and the cytoplasm. It has been shown that Vps23 and Tsg101 also bind Vps27 and Hrs, respectively, partially *via* the tetrapeptide PSAP sequence in Hrs or similar motifs in Vps27 (Fig. 2) (*42*, *45*, *52–54*). The recruitment of Vps23 and Tsg101 to the endosomal membrane is impaired in the absence of Vps27 or Hrs (*52*, *53*). In addition, mutants of Vps27 and Hrs with impaired binding to Vps23 or Tsg101 are defective in MVB sorting (*42*, *52*) and downregulation of RTKs (*45*). Therefore, Vps27 and Hrs are proposed to facilitate the complex formation of ESCRT-I on the endosome by recruiting Vps23 or Tsg101, and to transfer ubiquitinated cargoes, which are initially recognized by the Hrs/STAM complex, to ESCRT-I for further processes of vacuolar/lysosomal trafficking (Fig. 3).

The Hrs/STAM complex is also required for MVB formation *(i.e.*, invagination and budding of the endosomal limiting membrane into its lumen), as there are no or reduced MVBs in *vps27* mutant yeast cells (*31*), in *hrs* mutant *Drosophila* cells (*36*), and in mammalian cells in which Hrs is depleted by RNAi (*53*). However, the ESCRT-I recruitment by the Hrs/STAM complex may not be prerequisite for MVB formation, because the phenotype in *vps27* mutant yeast cells can be suppressed by expressing a Vps27 mutant lacking the Vps23-binding site (*42*). The phenotype can also be suppressed by Vps27 mutants lacking functional UIMs but not by a mutant lacking the Hse1-binding site, suggesting that MVB formation depends on the formation of the Vps27/Hse1 complex but is independent of its interaction with ubiquitinated cargoes (*31*, *42*).

Gag proteins of several enveloped viruses including human immunodeficiency virus type 1 (HIV-1) contain a tetrapeptide P(S/T)AP motif in their late domains (*55*). The P(S/T)AP motifs have been known to be essential for the budding of viruses from host cells (*55*). They have recently been shown to compete with the PSAP motif of cellular Hrs in Tsg101 binding and to recruit Tsg101 to the site of viral budding (*i.e.*, plasma membrane) in order to utilize the MVB formation machinery for viral budding (*54*).

Regulation of the activity of the Hrs/STAM complex

As mentioned above, both Hrs and STAM undergo tyrosine phosphorylation upon stimulation with growth factors. Therefore, their function is likely to be regulated by tyrosine phosphorylation. Using mass spectrometry, a phosphorylation site in EGF-stimulated cells has been assigned to tyrosine 334 of human Hrs (Fig. 2) (*43*, *56*). Tyrosine 329 is also assigned as an additional potential phosphorylation site (Fig. 2) (*43*, *56*). However, the biological significance of phosphorylation of the tyrosine residues 334 and 329 is unclear, as mutating both residues to phenylalanine does not affect (1) the binding of Hrs to ubiquitinated proteins, (2) the ubiquitination of Hrs itself (see the next paragraph), (3) the activity of Hrs to change the morphology of the early endosome, or (4) the activity of Hrs to inhibit ligand-induced RTK degradation when overexpressed (*43*). In addition, tyrosine phosphorylation of this Hrs mutant is not completely abolished after EGF stimulation, suggesting the presence of an additional phosphorylation site(s) (*43*). For STAM proteins, tyrosine 198 of human STAM1 and the corresponding residue in STAM2 are identified as potential phosphorylation sites in EGF-stimulated cells (Fig. 2) (*56*). No further study, however, has been performed.

Hrs and STAM proteins are also ubiquitinated. The mode of the ubiquitination, however, is unclear. Whereas Polo *et al.* (*39*) reported that Hrs undergoes ubiquitination in response to EGF stimulation, other groups showed that the ubiquitination is constitutive and not enhanced by growth factor stimulation (*40*, *43*). Whereas Katz *et al.* reported that Hrs is poly- or multi-ubiquitinated (*40*), others showed that it is monoubiquitinated (*39*, *43*). STAM1 is also shown to be constitutively monoubiquitinated (*40*). There are several possibilities on the role of ubiquitination of the Hrs/STAM complex. The Ub moieties of the complex might compete with those of ubiquitinated cargoes in binding to the sorting machinery, such as the Hrs/STAM complex itself and ESCRT-I, thereby the ubiquitination regulates the cargo recognition in some way. They might also recruit other Ub-binding proteins to the site of MVB sorting. For example, Eps15 and Tsg101 bind Ub. As Eps15 and Tsg101 also bind Hrs directly, the ubiquitination of the Hrs/STAM complex may enhance or stabilize its interaction with Eps15 and/or Tsg101. Eps15 and Tsg101 also undergo ubiquitination (*48*, *57*). Therefore, the Hrs/STAM complex may in turn bind to their Ub moieties. For Tsg101, it is proposed that ubiquitination inactivates its function by inducing its translocation from the site of MVB sorting (*57*). The UIM of Hrs is essential for its ubiquitination, as Hrs is not ubiquitinated when the UIM is mutated (*39*, *40*). However, it is difficult to investigate the role of ubiquitination of the Hrs/STAM complex by using UIM mutants because this motif also serves as a binding site for other ubiquitinated proteins. It is, therefore, necessary to identify the ubiquitination sites of these proteins to study the role of their ubiquitination.

The Hrs/STAM complex binds deubiquitinating enzymes

The Src homology 3 (SH3) domain of STAM proteins binds two structurally unrelated proteins, UBPY (Ub isopeptidase Y) and AMSH (associated molecule with the SH3 domain of STAM), *via* a unique motif PX(V/I)(D/N)RXXKP in these proteins (Fig. 2) (*58*, *59*). UBPY, also termed as USP8, is a deubiquitinating enzyme of the Ub-specific protease family. It is homologous to a yeast protein Doa4. As free Ub molecules are depleted in the *doa4* mutant yeast cells, Doa4 is implicated in deubiquitinating endosomal cargoes before they are incorporated into the MVB luminal vesicles and preventing Ub molecules from degradation in the vacuole (Fig. 1) (*60*). Therefore, UBPY might have a similar function in mammalian cells. Another possibility is that UBPY regulates the downregulation of RTKs negatively or positively by deubiquitinating RTKs or endocytic proteins, such as Hrs and STAM.

Whether AMSH is involved in MVB sorting has been even less clear. However, AMSH has the JAMM (Jab1/MPN domain-associated metalloisopeptidase) motif. Through this motif, Rpn11, a component of the lid complex of the 19S regulatory subunit of the 26S proteasome in yeast, cleaves Ub moieties from polyubiquitinated proteins before they are digested in the 20S proteasomal core complex (*61*, *62*). Very recently, AMSH has also been demonstrated to possess a JAMM motif–associated Ub isopeptidase activity (*63*). Furthermore, depletion of AMSH by RNAi leads to the accelerated degradation of ligand-activated EGF receptor, suggesting that AMSH regulates the RTK downregulation negatively (*63*). Whether UBPY and AMSH have a redundant role or have specific roles on distinct substrates is a subject for future study.

Role of the Hrs/STAM complex in development

Drosophila mutant embryos lacking zygotic as well as maternal Hrs proteins exhibit numerous defects during early gastrulation, which is consistent with the ubiquitous expression pattern of Hrs in fly embryos (*36*). Although knockout mice lacking Hrs also die during embryogenesis (around embryonic day 11), they exhibit a defect in a specific developmental process called "ventral folding morphogenesis" that occurs at embryonic days 8 to 9 (*24*). Due to the defect in this process, *hrs* mutant embryos display two bilaterally-located beating heart tubes (*cardia bifida*) and exposure of their ventral region to the outside of the yolk sac (*24*). In *hrs* mutant embryos, apoptosis is observed specifically in cells of the definitive endoderm prior to the onset of ventral folding, suggesting that dysfunction of the endoderm cells leads to the phenotype (*24*). Knockout of either *STAM* gene exhibits an adult (*STAM1*) or no (*STAM2*) phenotype (*64*, *65*). The *stam1* mutants display several defects, such as decrease

in body weight and loss of hippocampal neurons, and die within 6 months of age (*64*). However, double knockout mice that lack both STAM1 and STAM2 are reported to exhibit the same phenotype as the *hrs* mutant. They display a defect in ventral folding morphogenesis and die by embryonic day 11.5 (*65*). These results suggest that STAM1 and STAM2 play redundant roles in mammalian development, and that Hrs and STAM proteins are involved in the same cellular process.

Hrs, and probably also STAM proteins, are ubiquitously expressed in mouse embryos. Therefore, it is even surprising that the phenotype of the *hrs* mutation as well as the *stam1/stam2* double mutation in mice is restricted to a specific cell type (definitive endoderm cells) and a specific developmental process (ventral folding). In *Drosophila*, downregulation of several signaling receptors is affected in the absence of Hrs (*36*, *47*). Therefore, the mouse phenotype may be due to deregulation of an as yet identified specific signaling from or toward the endoderm cells.

Perspectives

Both Hrs and STAM bind Ub directly. Another Ub-binding protein, Eps15, could also be involved in MVB sorting by associating with the Hrs/STAM complex. This raises the question of how this sorting complex with multiple Ub-binding sites recognizes ubiquitinated cargoes on the endosomal membrane. As activated RTKs are multiubiquitinated, a possibility is that individual components of the complex bind different Ub moieties conjugated to different lysine residues of a single RTK to enhance the cargo-binding affinity (Fig. 3). Anyway, to understand the precise sorting mechanism, it is important to elucidate the entire subunit composition as well as the tertiary structure of the MVB sorting complex. Besides, it is important to elucidate the role of tyrosine phosphorylation and ubiquitination of Hrs and STAM to understand how the activity of the sorting complex is regulated in response to RTK activation.

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