JCB Article

Hrs regulates multivesicular body formation via ESCRT recruitment to endosomes

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Hrs and the endosomal sorting complexes required for transport, ESCRT-I, -II, and -III, are involved in the endosomal sorting of membrane proteins into multivesicular bodies and lysosomes or vacuoles. The ESCRT complexes are also required for formation of intraluminal endosomal vesicles and for budding of certain enveloped RNA viruses such as HIV. Here, we show that Hrs binds to the ESCRT-I subunit Tsg101 via a PSAP motif that is conserved in Tsg101-binding viral proteins. Depletion of Hrs causes a reduction in membrane-associated ESCRT-I subunits, a decreased number of multivesicular bodies and an increased size of late endosomes. Even though Hrs mainly localizes to early endosomes and Tsg101 to late endosomes, the two proteins colocalize on a subpopulation of endosomes that contain lyso-bisphosphatidic acid. Overexpression of Hrs causes accumulation of Tsg101 on early endosomes and prevents its localization to late endosomes. We conclude that Hrs mediates the initial recruitment of ESCRT-I to endosomes and, thereby, indirectly regulates multivesicular body formation.

Introduction

The surface expression of many receptors is attenuated by ligand-induced endocytosis and subsequent degradation in lysosomes (Sorkin and Von Zastrow, 2002). In early endosomes, receptors destined for lysosomal degradation are sorted from those that are to be recycled to the plasma membrane. The sorting machinery recognizes special determinants on lysosome-targeted proteins, such as conjugated ubiquitin, and causes their inclusion into intraluminal vesicles of multivesicular bodies (MVBs). When MVBs fuse with lysosomes, intraluminal vesicles are degraded by lipases, and their associated proteins are degraded by proteases (Mullins and Bonifacino, 2001; Katzmann et al., 2002).

The core constituents of the machinery responsible for endosomal sorting and MVB formation have been identified by yeast genetics through the isolation of vacuolar protein sorting (*vps*) mutants (Rothman and Stevens, 1986; Odorizzi et al., 1998). Recent work has revealed that 10 out of the 17 known Vps class E proteins participate in three endosomal sorting complexes required for transport (ESCRT), ESCRT-I, -II, and -III, and genetic evidence suggests that these complexes function sequentially in protein sorting and MVB formation (Katzmann et al., 2001; Babst et al., 2002a,b). ESCRT-I consists of three proteins and is involved in the sorting of ubiquitinated proteins into MVBs. One of the subunits of ESCRT-I is Vps23p, which recognizes ubiquitin via its NH_2-terminal ubiquitin E2 variant domain (Katzmann et al., 2001). The ESCRT complexes are conserved through evolution, and the mammalian homologue of Vps23p, Tsg101, has been shown to mediate the sorting of ubiquitinated proteins into the degradative pathway, much like its yeast counterpart (Babst et al., 2000; Bishop et al., 2002). Remarkably, Tsg101 is also required for the budding of several enveloped RNA viruses such as HIV and Ebola from the plasma membrane (Garrus et al., 2001; Martin-Serrano et al., 2001). The ubiquitin E2 variant domain of Tsg101 binds to a P(S/T)AP sequence in viral proteins required for budding, and the interaction may be strengthened by the simultaneous binding to a nearby ubiquitin moiety (Pornillos et al., 2002a,b). It is thought that the binding of Tsg101 to viral proteins recruits the Vps class E machinery to the plasma membrane and engages it in a budding process topologically similar to that of MVB formation (Carter, 2002; Pornillos et al., 2002c).

One of the Vps class E proteins that has not been assigned to any of the ESCRT complexes is Vps27p and its mammalian homologue Hrs. Like Vps23p and Tsg101, Vps27p and Hrs bind ubiquitinated proteins and mediate their sorting into MVBs (Bilodeau et al., 2002; Raiborg et al., 2002; Shih et al., 2002). This raises the question of how Vps23p/Tsg101

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Key words: endocytosis; lysosome; membrane traffic; Tsg101; protein sorting

Abbreviations used in this paper: ESCRT, endosomal sorting complex required for transport; LBPA, lyso-bisphosphatidic acid; MVB, multivesicular body; siRNA, small interfering RNA; UIM, ubiquitin-interacting motif; Vps, vacuolar protein sorting.

and Vps27p/Hrs recruitment is coordinated in time and space. Here, we have tried to address this question.

Results

Tsg101 interacts with Hrs

The fact that Hrs contains a PSAP sequence (residues 348–351, Fig. 1 A), similar to that found in Tsg101-binding viral proteins (Carter, 2002; Pornillos et al., 2002c), prompted us to ask whether Tsg101 may interact with Hrs. First, we investigated this possibility using the yeast two-hybrid system. Full-length or truncated Hrs constructs were prepared as baits and Tsg101 as prey, and β-galactosidase reporter activity was measured in reporter yeast cells. The results indicated that Hrs does interact with Tsg101 (Fig. 1 B). Although the NH_2-terminal part of Hrs, containing the VHS and FYVE domains and ubiquitin-interacting motif (UIM), showed no interaction with Tsg101, the remaining part, containing the proline-rich, coiled-coil, and clathrin binding domains, bound strongly. To study if the PSAP sequence is involved in the interaction, we mutated the final proline residue in this sequence to alanine. Interestingly, this P351A mutation markedly reduced the interaction, but did not abolish it completely. Consistent with this, we also detected a weak interaction between the coiled-coil region of Hrs and Tsg101, and a Hrs construct containing only the proline-rich and coiled-coil domains of ($Hrs_{287–573}$) showed a strong interaction with Tsg101 (Fig. 1 B). In the companion paper, Pornillos et al. (2003, in this issue) show that Hrs truncated just after the coiled-coiled domain (at residue 559) binds poorly to Tsg101. Because $Hrs_{287–573}$, which binds Tsg101 strongly, contains a few additional residues after the coiled-coiled domain, it appears that these residues are important for Tsg101 binding, possibly because they allow correct folding of the coiled-coil region. Together, these results suggest that Tsg101 interacts with several interfaces of Hrs, including the PSAP sequence and the coiled-coil region. We also cannot rule out the possibility that the latter regions join to form a single Tsg101-interacting interface.

To verify these results biochemically, we next prepared a GST fusion protein of residues 287–573 of Hrs, which comprise the proline-rich domain and the coiled-coil region. The fusion protein was immobilized onto glutathione-Sepharose beads and incubated in the presence of in vitro translated ^{35}S-labeled Tsg101. SDS-PAGE analysis of the beads followed by fluorography showed that a portion of Tsg101 was indeed associated with immobilized GST-$Hrs_{287–573}$ (Fig. 1 C). Furthermore, the P351A mutation reduced the interaction to background level (the binding observed to GST alone). The interaction between GST-$Hrs_{287–573}$ and Tsg101 was sensitive to a high salt concentration but not to the nonionic detergent NP-40 (Fig. 1 C). These results are in agreement with those obtained with the two-hybrid system and show that Tsg101 can interact with Hrs, and that the PSAP sequence of Hrs is important for this interaction.

Hrs is required for the localization of ESCRT-I to membranes

Because Hrs binds to the endosome-specific lipid phosphatidylinositol 3-phosphate (Gaullier et al., 1998; Gillooly et al., 2000), this protein is likely to interact directly with the endosome membrane. The finding that Hrs interacts with Tsg101, therefore, raised the possibility that Hrs may function to recruit ESCRT-I to endosome membranes. Should this be the case, then a reduction in the cellular level of Hrs might lead to a decrease in membrane-associated ESCRT-I subunits. To investigate this possibility, we used a specific small interfering RNA (siRNA) molecule for the knockdown of Hrs expression (Elbashir et al., 2002; Bache et al., 2003). As shown in Fig. 2 (A and B), HeLa cells incubated with the siRNA duplex showed a strong decrease in Hrs expression (as assessed with Western blotting with antibodies specific for Hrs) when compared with cells that had been in-

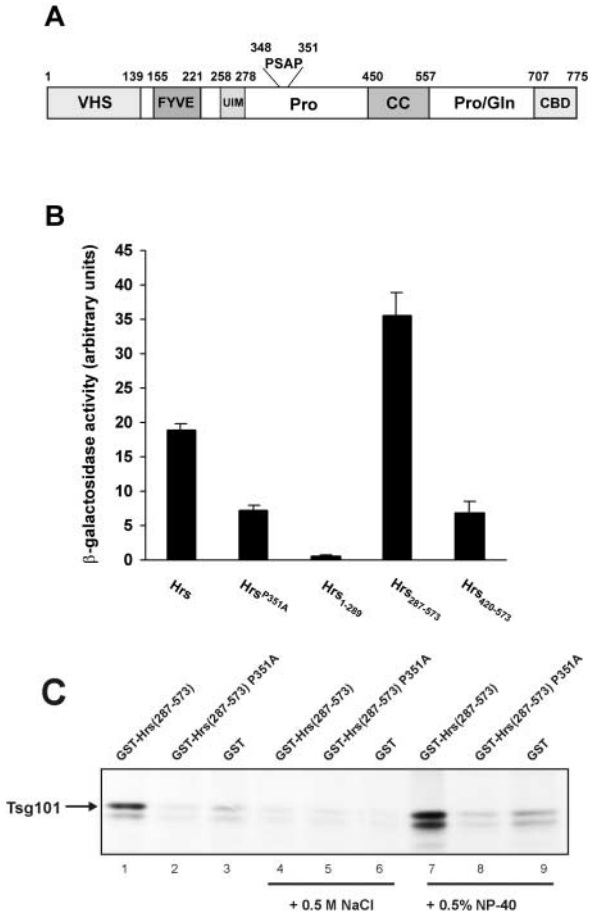

Figure 1. **Hrs interacts with Tsg101.** (A) A schematic representation of Hrs. The VHS, FYVE, coiled-coil (CC), and clathrin-binding domain (CBD) are indicated. The ubiquitin interacting motif (UIM), PSAP sequence, and proline- and proline/glutamine-rich regions are also indicated. (B) Interaction between Hrs and Tsg101 in the yeast two-hybrid system. The indicated Hrs constructs were used as bait and Tsg101 as prey. The values indicate β-galactosidase activities in arbitrary units. Neither of the bait constructs showed any significant reporter activation (<1 U) in the absence of a prey construct. All determinations were done in triplicate. Error bars denote SEM. (C) Interaction between Hrs and Tsg101 in vitro. GST alone or fused to $Hrs_{287–573}$ or $Hrs_{287–573}^{P351A}$ were immobilized on glutathione-Sepharose beads and incubated with in vitro–translated ^{35}S-labeled Tsg101 for 1 h at 4°C. The beads were washed and analyzed by SDS-PAGE and fluorography. The amounts bound in lanes 1 and 7 correspond to 2–3% of the input amount. The doublet band is presumably due to translational initiation downstream of the initiator ATG.

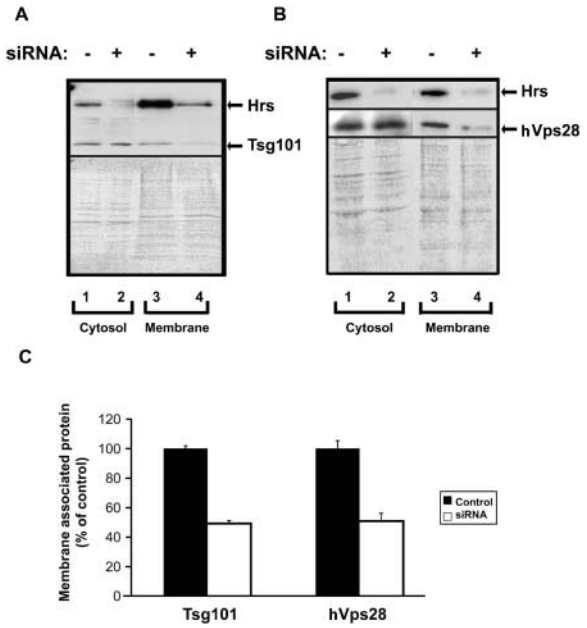

Figure 2. **Hrs is required for the efficient association of Tsg101 with membranes.** HeLa cells treated with control RNA (−) or with siRNA against Hrs (+) were fractionated into membrane and cytosolic fractions as described in Materials and methods. Hrs left in the cytosol and on membranes after siRNA treatment, as described in Materials and methods, was analyzed by SDS-PAGE (A and B, top lanes), and the corresponding levels of Tsg101 and hVps28 were shown by sequential blotting of the same membrane with anti-Tsg101 (A) or anti-hVps28 (B). The loaded amount of membrane fraction was sixfold higher than that of the cytosol fraction in A, and twofold higher in B. To visualize transferred proteins, the blots were stained with Ponceau S (bottom panels) before detection of Hrs and Tsg101 (A), and Hrs and hVps28 (B). The relative intensities of the bands from membrane fractions of control and siRNA-treated cells were quantified using ImageQuant 5.0 (C), and are presented as the average of three experiments. Error bars denote SEM.

cubated in the presence of a control RNA. Importantly, Western blotting with anti-Tsg101 antibodies showed that there was a significant reduction of membrane-associated Tsg101 levels in cells treated with siRNA to Hrs. We also investigated whether Hrs is required for membrane localization of the other known human ESCRT-I subunit, hVps28, which does not bind directly to Hrs. Like Tsg101, the amount of hVps28 in the membrane fraction was reduced by ~50% in cells treated with siRNA against Hrs (Fig. 2, B and C). In the case of both Tsg101 and hVps28, the decrease in membrane-associated proteins was less than that of Hrs in Hrs-depleted cells. We do not know the reason for this, but one explanation could be that ESCRT-I is also associated with endosomes via other molecules than Hrs (see Discussion). In any case, these results indicate that Hrs is required for efficient membrane targeting of ESCRT-I.

Hrs is required for the formation of MVBs in mammalian cells

Yeast cells devoid of the Hrs homologue Vps27p have vacuoles that lack intraluminal vesicles (even when vacuolar hydrolase activity is inhibited), and they contain a multilamellar late endosome, the class E compartment (Odorizzi et al., 1998). Similarly, Garland cells and synaptic boutons from *hrs* mutant *Drosophila* larvae have reduced numbers of MVBs (Lloyd et al., 2002). Because the endocytic pathway in mammalian cells is better characterized at the morphological level than that of yeast and flies, we found it important to study the ultrastructure of endosomes in HeLa cells depleted of Hrs with siRNA. To label endocytic compartments, the cells were allowed to endocytose HRP for 15 min before fixation and electron microscopy. Whereas early endosomes (Fig. 3 A) and MVBs (Fig. 3 B) could be readily detected in untreated cells, siRNA-treated cells (Fig. 3 C) had ~50% less MVBs (see quantification in Fig. 3 D). These results indicate that mammalian Hrs, similar to its yeast and fly counterparts, is required for MVB formation.

Hrs depletion affects the morphology of lysosomes

Because MVBs fuse with late endosomes and lysosomes (Gruenberg, 2001; Katzmann et al., 2002), we next asked whether Hrs depletion would affect the morphology of these organelles. First, we studied this by confocal microscopy using an antibody against the late endosome/lysosome marker LAMP-1. In cells treated with a control RNA molecule, numerous LAMP-1–positive structures were found in the perinuclear region, as expected (Fig. 4 E). However, in cells treated with siRNA against Hrs, the LAMP-1–positive structures were found concentrated close to the nucleus (Fig.

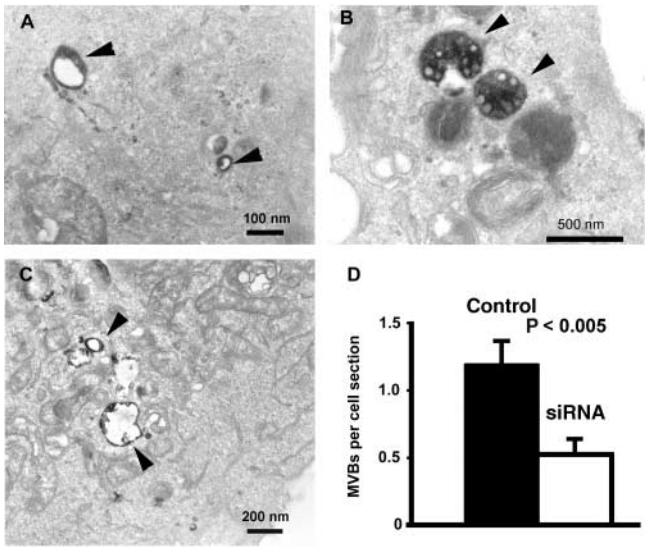

Figure 3. **Hrs is required for MVB formation.** HeLa cells treated with control RNA or siRNA against Hrs were incubated with 5 mg/ml HRP for 15 min and processed for electron microscopy. In control cells, we observed early endosomes of varying sizes (A) and MVBs (B). (C) In siRNA-treated cells we also observed early endosomes, but significantly less MVBs. (A–C) Arrowheads indicate HRP-positive structures. (D) To quantify the effect of siRNA on MVB formation, we estimated the number of MVBs per cell section. We included only MVBs with an appearance as seen in B in the estimation and omitted early endosomal structures as seen in A and C. MVBs were counted and expressed as the mean number of MVBs per cell section. Three separate experiments were performed and 20 cells were counted for each condition. Statistical significance was estimated with the *t* test. Error bars denote SEM.

4 F). Next, to study this morphological change by electron microscopy, we labeled lysosomes by performing a 3-h pulse/overnight chase incubation with BSA gold. In control cells, we observed LAMP-2 staining on gold-containing electron dense lysosomes with a membranous content (Fig. 4 A), and more occasionally on electron lucent vesicles largely devoid of endocytic tracers and with no intraluminal membranes (Fig. 4 B). In siRNA-treated cells, we observed similar LAMP-2–positive structures, but they often appeared clustered and were greatly enlarged (up to threefold increase in diameter; Fig. 4, C and D). These results indicate that Hrs is required both for MVB formation (Fig. 3) and for normal morphology of late endosomes/lysosomes.

Hrs and Tsg101 have differential intracellular localizations

Both Hrs and Tsg101 are known to localize to endosomes (Komada et al., 1997; Bishop and Woodman, 2001), but

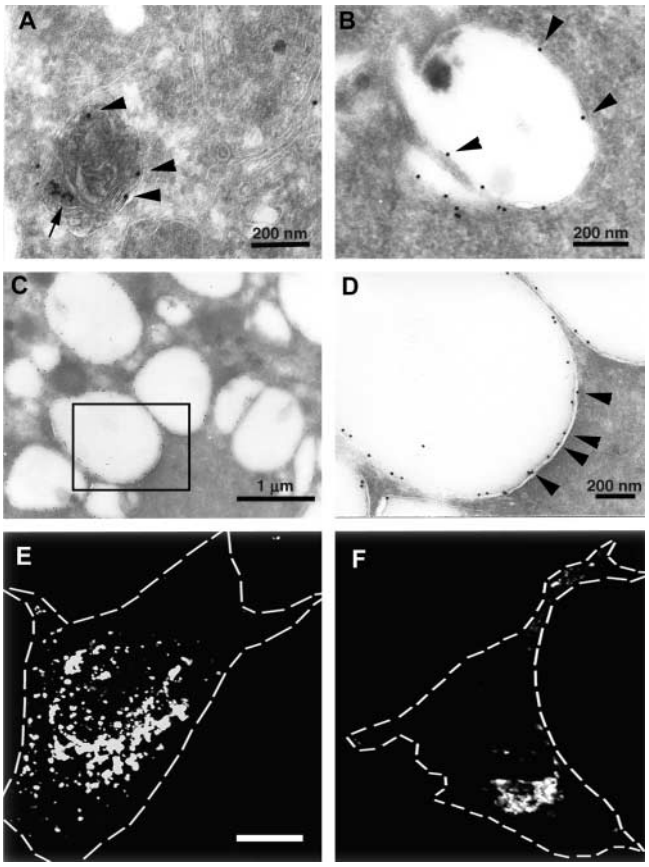

Figure 4. **Hrs depletion affects the morphology of late endosomes and lysosomes.** HeLa cells treated with control double-stranded RNA (A and B) or siRNA against Hrs (C and D) were prepared for electron microscopy or immunofluorescence microscopy as described in Materials and methods. Late endosomes and lysosomes were visualized by 7 nm internalized BSA gold (A, arrow) or staining with antibodies against LAMP-2 followed by 15 nm protein A–gold (A–D, arrowheads). The boxed area in C is shown magnified in D. Note the different sizes of bars. Immunofluorescence images were obtained by labeling with antibodies against LAMP-1 (E and F). Bar, (E and F) 5 μm. E shows a control cell, whereas F shows an siRNA-treated cell.

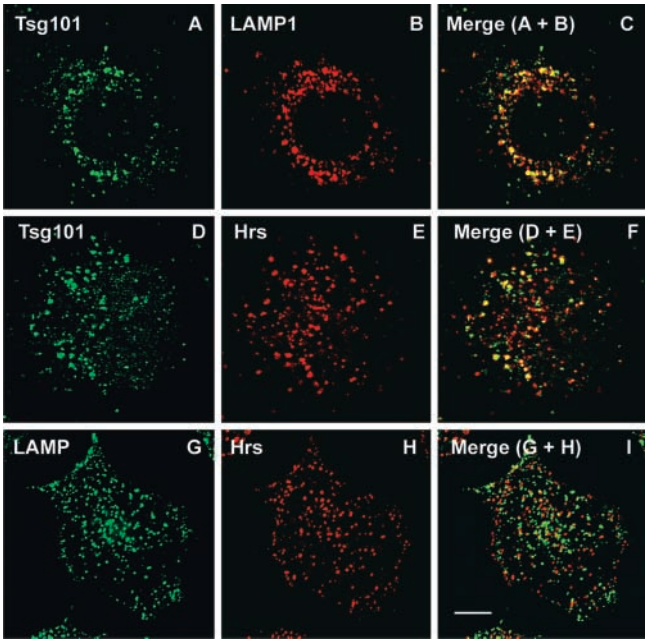

Figure 5. **Tsg101 has a broader endosomal distribution than Hrs.** HeLa cells grown on coverslips were permeabilized before fixation and double labeled with anti-Tsg101 and anti–LAMP-1 (A–C), anti-Tsg101 and Hrs (D–F), or anti-Hrs and anti–LAMP-1 (G–I). Yellow color in the merged images (C, F, and I) indicates colocalization. Bar, 5 μm.

their intracellular localizations have not been compared directly. Because Hrs is required for the endosomal recruitment of Tsg101, and because both these proteins are involved in endosomal protein sorting and MVB formation, we expected Hrs and Tsg101 to colocalize. Electron microscopy showed that overexpression of Tsg101, even at moderate levels, causes formation of aggresomes (unpublished data). To study the relative localization of Tsg101 and Hrs, we, therefore, stained HeLa cells with antibodies that detect the two endogenous proteins by immunofluorescence microscopy (Fig. 5). As expected, there was a significant overlap in the distribution of Hrs and Tsg101 (Fig. 5, D–F). However, whereas Hrs partially localized to membranes positive for the early endosome marker EEA1 (Urbé et al., 2000; Raiborg et al., 2001b) and was largely absent from LAMP-1–positive late endocytic structures (Fig. 5, G–I), Tsg101 was also found on LAMP-1–positive structures (Fig. 5, A–C). These results suggest that even though Tsg101 requires Hrs for its initial recruitment to endosomes, it stays associated with endosomes much longer than Hrs during endosomal maturation or trafficking.

Tsg101 and Hrs colocalize on endosomes that contain lyso-bisphosphatidic acid (LBPA)

Assuming that Tsg101 is recruited to endosomes by Hrs but is also found on late, LAMP-1–positive structures, we would expect a significant pool of Tsg101 to be located on intermediates between early and late endosomes. Therefore, we triple labeled HeLa cells for confocal microscopy with antibodies against Tsg101, Hrs, and LBPA, a well-characterized marker for multivesicular late endosomes (Kobayashi et al., 1998).

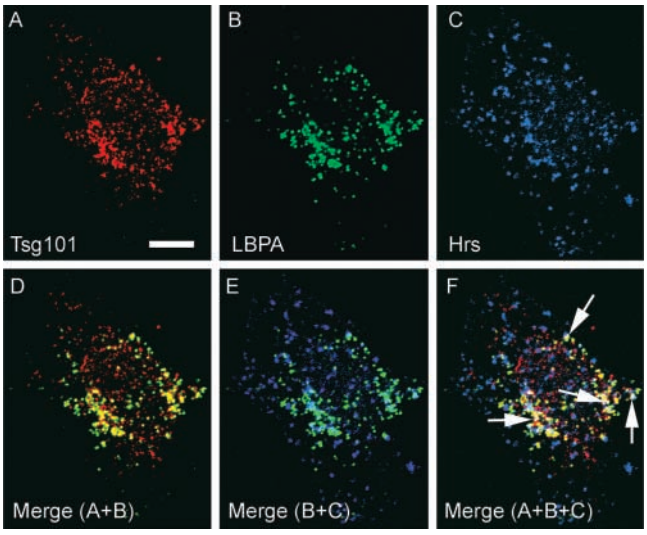

Figure 6. **Tsg101 and Hrs colocalize on LBPA-containing endosomes.** HeLa cells were permeabilized before fixation and labeled with anti-Hrs (C) and anti-LBPA (B) primary and secondary antibodies before staining with Zenon®-labeled anti-Tsg101 (A) as described in Materials and methods. Colocalization between LBPA and Tsg101 is shown in yellow (D), between LBPA and Hrs in turquoise (E), and between all three molecules in white (F). Examples of profiles positive for all three molecules are indicated by arrows. Bar, 5 μm.

We found extensive colocalization between Tsg101 and LBPA (Fig. 6, A, B, and D). In agreement with Fig. 5, some of the endosomes were positive for both Hrs and Tsg101, and most of these structures also contained LBPA (Fig. 6 A, C, and F). This suggests that Hrs and Tsg101 colocalize on a subset of LBPA-positive structures. Because Hrs colocalizes poorly with the late endosome/lysosome marker LAMP-1 (Fig. 5), we assume that these Hrs-containing LBPA-positive structures represent intermediates between early and late endosomes that are positive for LBPA but negative for LAMP1. In agreement with this idea, previous electron microscopy studies have shown that Hrs is present on the limiting membrane of MVBs (Tsujimoto et al., 1999; Sachse et al., 2002), and we observed LPBA associated with intraluminal vesicles of MVBs (unpublished data).

Overexpression of Hrs causes accumulation of Tsg101 on early endosomes and prevents its localization to late endosomes

The finding that Hrs is required for recruitment of Tsg101 to endosomes prompted us to ask whether an increased expression of Hrs would cause an increased amount of Tsg101 on early endosomes. Therefore, we compared the intracellular localization of endogenous Tsg101 in untransfected cells and cells that overexpressed Hrs. Confocal immunofluorescence microscopy showed that there was little colocalization between Tsg101 and EEA1 in untransfected cells (Fig. 7, A–C), in agreement with the finding that Tsg101 colocalizes extensively with LAMP-1 (Fig. 5, A–C). In contrast, Hrs overexpression led to increased colocalization between Hrs and Tsg101 (Fig. 7, D–F), and caused a strong redistribution of Tsg101 to early endosomes, which were now found

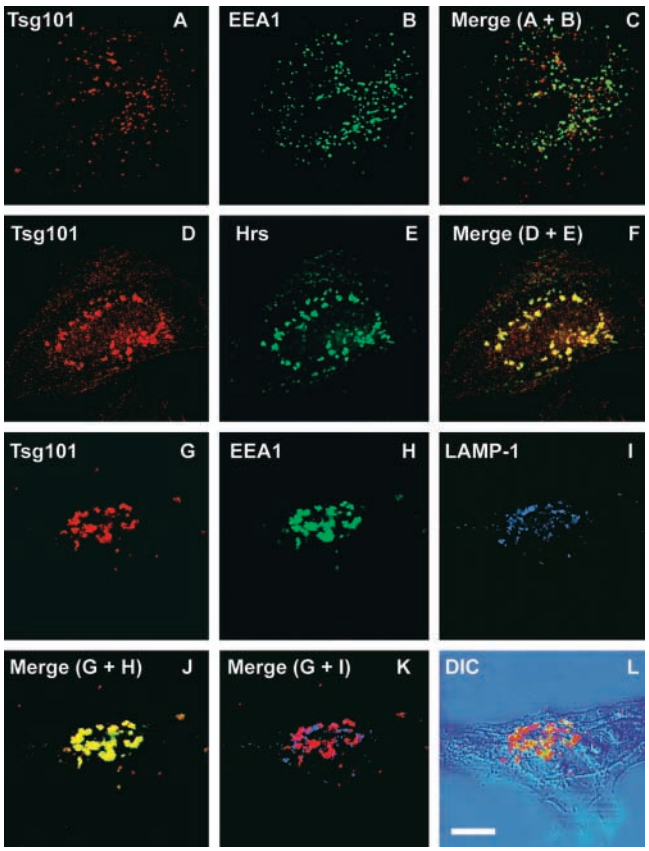

Figure 7. **Overexpression of Hrs prevents the localization of Tsg101 to late endosomes.** HeLa cells were transfected (D–L) or not (A–C) with Hrs for 48 h and permeabilized before fixation. The cells were labeled with anti-Tsg101 (A, D, and G), anti-EEA1 (B and H), or anti-LAMP-1 (I). Yellow (C, F, and J) or purple (K) color in merged images indicates colocalization. An interference contrast image of the Hrs-transfected cell (G–K) is shown in L (colors are inverted in this panel). Bar, 5 μm.

clustered in the perinuclear region (Fig. 7, G and H). Interestingly, although Tsg101 colocalized extensively with LAMP-1 in control cells (Fig. 5, A–C), Tsg101 was almost absent from LAMP-1–positive late endocytic structures in the Hrs-transfected cells (Fig. 7, G, I, and K), even though these were frequently located close to early endosomes. Instead, it colocalized strongly with EEA1 (Fig. 7, G, H, and J), which was the opposite of what was seen in untransfected cells (compare with Fig. 7, A–C). This suggests that Hrs overexpression retains Tsg101 on early endosomes and prevents its association with late endosomes and lysosomes, which is consistent with the idea that Hrs mediates recruitment of Tsg101 to early endosomes.

Discussion

In this paper, we have addressed an important question concerning the function of ESCRT complexes in MVB formation, namely, how the recruitment of these complexes to endosomal membranes is initiated. Previous work has suggested that the evolutionarily conserved ESCRT-I, -II, and -III complexes act consecutively in receptor sorting and MVB formation (Katzmann et al., 2001, 2002; Babst et al.,

2002a,b; Conibear, 2002). Here, we show that Hrs binds to the ESCRT-I subunit Tsg101 and mediates the targeting of ESCRT-I to early endosomes. Similarly, in a companion paper, Katzmann et al. (2003, in this issue) show that membrane recruitment of yeast Vps23p requires Vps27p. This indicates that Hrs/Vps27p functions upstream of the ESCRT complexes. We also show evidence that, once recruited, Tsg101 remains associated with endosomes much longer than Hrs during endosomal maturation or trafficking. Finally, we show by electron microscopy that Hrs is required both for MVB formation and for the normal morphology of endosomes and lysosomes.

What is the exact role of Hrs in MVB formation? Previous work has shown that the UIMs of Hrs and Vps27p are essential for sorting of ubiquitinated membrane proteins into the degradative pathway (Bilodeau et al., 2002; Raiborg et al., 2002; Shih et al., 2002). Interestingly, mutations in the UIMs of Vps27p specifically inhibit sorting of ubiquitinated proteins without affecting MVB formation (Bilodeau et al., 2002), suggesting that ubiquitin recognition may be uncoupled from the formation of intraluminal vesicles. Our finding that Hrs binds to Tsg101 and is required for membrane recruitment of both Tsg101 and hVps28 indicates that Hrs acts in the recruitment of ESCRT-I to endosomes. This provides the following plausible explanation for the lack of MVB formation in the absence of Hrs: because the ESCRT complexes function coordinately in the formation of inward endosomal invaginations (by mechanisms that are still not understood), a failure to recruit ESCRT-I will effectively inhibit this process.

Our finding that siRNA-mediated depletion of Hrs inhibits MVB formation agrees with previous studies of Hrs homologues in yeast and *Drosophila* (Odorizzi et al., 1998; Lloyd et al., 2002). Even though enlarged early endosomes have been observed in Hrs −/− mouse embryos (Komada and Soriano, 1999) and in Hrs-depleted HeLa cells (Bache et al., 2003), it was more surprising to observe that lysosomes increased greatly in size in HeLa cells treated with siRNA against Hrs. This indicates that Hrs not only controls MVB formation but also controls the morphology of endosomes and lysosomes.

The UIM has been shown to function as a signal for mono-ubiquitination of Hrs (Polo et al., 2002). Because Tsg101 binds with increased avidity to HIV Gag when this protein is ubiquitinated (Pornillos et al., 2002b), it is possible that ubiquitination of Hrs may strengthen its interaction with Tsg101. Ubiquitination of Hrs cannot be absolutely required, though, because $Hrs_{287–573}$, which interacted strongly with Tsg101 in vitro and in the two-hybrid system, does not contain the UIM. Even though Hrs^{P351A} had a reduced ability to bind Tsg101, overexpression of this mutant caused a similar accumulation of Tsg101 on early endosomes as wild-type Hrs (unpublished data). This can probably be explained by its residual ability to bind Tsg101 (Fig. 1 B), and the fact that we are unable to saturate Hrs binding sites on endosomes (Raiborg et al., 2001b). However, it is interesting to note that membrane recruitment of yeast Vps23p by Vps27p requires a region that contains the PSAP-like sequence PTVP, and which is also required for sorting of a ubiquitinated protein into the vacuole lumen (see Katzmann et al., 2003, in this issue). This indicates that membrane recruitment of Vps23p (Tsg101) by Vps27p (Hrs) is required for normal MVB sorting.

Previous work has shown that Hrs partially colocalizes with another FYVE domain containing protein, EEA1 (Urbé et al., 2000; Raiborg et al., 2001b). However, whereas EEA1 is strictly confined to sorting early endosomes (Wilson et al., 2000), Hrs is also present on the limiting membrane of MVBs (Tsujimoto et al., 1999; Sachse et al., 2002). Given that Hrs partially colocalizes with EEA1, we were initially surprised to find so little colocalization between Tsg101 and EEA1 (Fig. 7, A–C). However, this lack of colocalization can be explained if EEA1 localizes to sorting endosomes and Tsg101 to MVBs/late endosomes. The finding that Tsg101, but not Hrs, is present on late (LAMP-1 positive) endosomes indicates that these proteins are not permanently associated. We speculate that Hrs is involved in the initial recruitment of Tsg101 to form MVBs, and that Tsg101 remains membrane associated through interactions with other molecules. Accordingly, when Hrs is overexpressed, it accumulates on early endosomes and causes a strong redistribution of Tsg101 to these structures and an altered appearance of late endosomes. The observation that Tsg101 is associated with LBPA-positive MVBs and late endosomes, which by definition contain intraluminal vesicles (Gruenberg, 2001; Katzmann et al., 2002), raises the possibility that ESCRT proteins may remain associated with the limiting membrane of the MVB even after formation of intraluminal vesicles has taken place.

Hrs and the ESCRT complexes are essential for endosomal protein sorting and MVB formation. Here, we have identified a functional interaction between Hrs and the ESCRT-I subunit Tsg101. Viral late domains target Tsg101 and ESCRT-I to the plasma membrane via a P(S/T)AP sequence in order to facilitate viral budding (Carter, 2002; Pornillos et al., 2002c). Hrs appears to act in a similar manner at the early endosome and is critical for the formation of endosomal invaginations and vesicles. Indeed, Pornillos et al. (2003, in this issue) show in a companion paper that the PSAP-containing part of Hrs can functionally replace the PTAP-containing part of the HIV Gag protein in budding of viruslike particles from the plasma membrane, indicating that HIV Gag mimics the Tsg101-recruiting effect of Hrs. Together, with previously published results (Katzmann et al., 2001; Bilodeau et al., 2002; Bishop et al., 2002; Lloyd et al., 2002; Raiborg et al., 2002; Shih et al., 2002), these results argue that Hrs plays a dual role in endosomal protein sorting: First, it recruits ubiquitinated proteins into clathrin-coated microdomains on early endosomes. Second, it recruits the ESCRT-I complex that is essential for further sorting and MVB formation.

Materials and methods

Plasmid constructs

The DNAs encoding human Tsg101 (Li and Cohen, 1996) and hVps28 (Bishop and Woodman, 2001) were amplified by PCR from a marathon-ready HeLa cDNA (CLONTECH Laboratories, Inc.) and verified by sequencing. They were cloned into the EcoRI–SalI sites of pGAD GH (CLONTECH Laboratories, Inc.) for use as a prey in the yeast two-hybrid system, into the EcoR–SalI sites of pGEM-myc4 (Simonsen et al., 1998) for in vitro transcription, and into the EcoRI–SalI sites of pMAL-c2 (New England Biolabs, Inc.) for bacterial expression. Full-length and truncated mouse Hrs constructs in pLexA were subcloned as EcoRI–SalI fragments

from the respective pGEM plasmids (Raiborg et al., 2001b) and used as baits in the yeast two-hybrid system. Hrs^{P351A} was generated from wild-type Hrs using the Quikchange site-directed genesis kit (Stratagene). For expression as GST fusion proteins in *Escherichia coli*, $Hrs_{287–573}$ and $Hrs_{287–573}^{P351A}$ were amplified by PCR and cloned into the EcoRI–SalI sites of pGEX-6P-1 (Amersham Biosciences).

Antibodies

Affinity-purified rabbit antibodies against recombinant Hrs have been described previously (Raiborg et al., 2001a). Antibodies against a recombinant maltose binding protein, hVps28 fusion protein, were prepared in a similar manner. These antibodies recognized endogenous hVps28 by Western blotting but not by immunocytochemistry. Mouse mAbs against Tsg101 were obtained from GeneTex. Human anti-EEA1 antiserum (Mu et al., 1995) was a gift from B. Toh (Monash University, Melbourne, Australia). Mouse mAbs against human LAMP-1 were from the Developmental Studies Hybridoma Bank of the University of Iowa. Mouse mAbs against LAMP-2 were provided by G. Griffiths (University of Oxford, Oxford, UK). Mouse mAbs against LBPA (Kobayashi et al., 1998) were provided by J. Gruenberg (University of Geneva, Geneva, Switzerland). Cy2-, Cy3-, and Cy5-labeled secondary donkey antibodies were from Jackson ImmunoResearch Laboratories. The Zenon® mouse IgG labeling kit was from Molecular Probes. It was used to label anti-Tsg101 antibodies with Alexa-588–conjugated Fab fragments according to the manufacturer's instructions.

Expression of GST fusion proteins in *E. coli*

GST fusion of $Hrs_{287–573}$ and the P351A mutation of the same Hrs fragment was produced in *E. coli* BL21 (DE3) cells transformed with the respective pGEX constructs as described previously (Raiborg et al., 2001a). The recombinant protein was purified on glutathione-Sepharose 4B (Amersham Biosciences) after lysis of the bacteria in B-PER™ reagent (Pierce Chemical Co.), according to the manufacturer's instructions.

In vitro transcription and translation

pGEM-Tsg101 was linearized with SalI, and in vitro transcribed with T7 RNA polymerase (Promega), according to the manufacturer's protocol. The resulting mRNA was translated in the presence of [^{35}S]methionine (Amersham Biosciences) in rabbit reticulocyte lysate according to the manufacturer's instructions. The lysate was dialysed overnight against assay buffer (20 mM Hepes, pH 7.2, 140 mM NaCl, and 1 mM dithiothreitol).

GST pull-down assay

Purified GST or GST fusion protein (100 pmol) was bound to 20-μl aliquots of glutathione-Sepharose (Pharmacia Biosciences) at 4°C for 60 min. The beads were washed with assay buffer and 50 μl of in vitro-translated Tsg101 was added. After rotation at 4°C for 60 min, the beads were washed three times with assay buffer and analyzed by SDS-PAGE and fluorography. Films were scanned (model ScanJet 6100C; Hewlett Packard), and images were processed with Adobe Photoshop 7.0.

Cell culture and transfection

HeLa cell cultures were maintained as recommended by the American Type Culture Collection. For expression in mammalian cells, we used the FuGENE system according to the manufacturer's instructions (Roche Diagnostic Corporation). Constructs to be expressed were cloned behind the myc epitope of pcDNA3 (Invitrogen). Cells were analyzed 48 h after transfection. Transfection of HeLa cells with siRNA was performed as described previously (Elbashir et al., 2002). The cells were first transfected with siRNA for 3 d, then the cells were replated, and the transfection was repeated for another 3 d.

Two-hybrid methods

For use in the two-hybrid system, constructs were cloned into pLexA/pBTM116 (Vojtek et al., 1993) as bait and pGAD GH (CLONTECH Laboratories, Inc.) as prey. The yeast reporter strain *L40* (Vojtek et al., 1993) was cotransformed (Schiestl and Gietz, 1989) with the indicated pLexA and pGAD plasmids, and β-galactosidase activities of transformants were determined as described previously (Guarente, 1983).

Preparation of membrane and cytosolic fractions of HeLa cells treated with siRNA against Hrs

HeLa cells were transfected with siRNAs against Hrs or a scrambled RNA duplex as control (Bache et al., 2003). The cells were washed three times with ice-cold PBS, and homogenized in homogenization buffer (10 mM Hepes, 3 mM imidazole, pH 7.2, 250 mM sucrose, and mammalian protease inhibitor cocktail [used according to the manufacturer's instructions]; Sigma-Aldrich) by repeated passages through a 22-gauge needle at 4°C. Membrane particulate and cytosolic fractions were prepared from postnuclear supernatants by ultracentrifugation for 15 min at 65,000 rpm in a TLA-100 rotor using a table top ultracentrifuge (Beckman Coulter).

Confocal immunofluorescence microscopy

Transfected or nontransfected HeLa cells grown on coverslips were permeabilized with 0.05% saponin, fixed with 3% PFA, and stained for fluorescence microscopy as described previously (Simonsen et al., 1998). Coverslips were examined using a META microscope (model LSM 510; Carl Zeiss MicroImaging, Inc.) equipped with a Neo-Fluar 100×/1.45 oil immersion objective. Image processing was done with Adobe Photoshop version 7.0.

Electron microscopy

To label lysosomes, we incubated cells with BSA gold (final OD_{520} value of 2–3) for 3 h followed by an overnight chase. Cells were fixed in a mixture of 4% formaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, washed, and scraped in 1% gelatin/PBS. After embedding with 12% gelatin/PBS, tissue blocks were infused with 2.3 M sucrose overnight at 4°C, mounted, and frozen in liquid nitrogen (Peters et al., 1991). Ultrathin cryosections were cut at −110°C on a microtome (Ultracut; Leica) and collected with a 1:1 mixture of 2% methyl cellulose and 2.3 M sucrose. Sections were transferred to formvar/carbon-coated grids and labeled with primary antibodies followed by protein A–gold conjugates essentially as described previously (Slot et al., 1991). For plastic embedding, the cells were fixed in 2% glutaraldehyde in cacodylate buffer, fixed after in 2% osmium tetroxide, and stained with uranylacetate. After dehydration and embedding, the cells were sectioned and examined in an electron microscope (model CM100; Philips).

We thank Eva Rønning for technical assistance, Gillian Griffiths for providing anti–LAMP-2 antibodies, Jean Gruenberg for providing anti-LBPA antibodies, and Scott Emr and Wes Sundquist for sharing results before publication.

This work was supported by the Top Research Programme, the Research Council of Norway, the Norwegian Cancer Society, the Novo-Nordisk Foundation, and the Anders Jahre's Foundation for the Promotion of Science.

Submitted: 24 February 2003
Accepted: 11 June 2003

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