Hrs recruits clathrin to early endosomes

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The hepatocyte growth factor-regulated tyrosine kinase substrate, Hrs, has been implicated in intracellular trafficking and signal transduction. Hrs contains a phosphatidylinositol 3-phosphate-binding FYVE domain that contributes to its endosomal targeting. Here we show that Hrs and EEA1, a FYVE domain protein involved in endocytic membrane fusion, are localized to different regions of early endosomes. We demonstrate that Hrs co-localizes with clathrin, and that the C-terminus of Hrs contains a functional clathrin box motif that interacts directly with the terminal β-propeller domain of clathrin heavy chain. A massive recruitment of clathrin to early endosomes was observed in cells transfected with Hrs, but not with Hrs lacking the C-terminus. Furthermore, the phosphatidylinositol 3-kinase inhibitor wortmannin caused the dissociation of both Hrs and clathrin from endosomes. While overexpression of Hrs did not affect endocytosis and recycling of transferrin, endocytosed epidermal growth factor and dextran were retained in early endosomes. These results provide a molecular mechanism for the recruitment of clathrin onto early endosomes and suggest a function for Hrs in trafficking from early to late endosomes.
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Introduction

The uptake of macromolecules by way of endocytosis serves many important cellular functions, including the acquisition of extracellular nutrients, regulation of cell surface receptor expression, maintenance of cell polarity and antigen presentation (Gagescu *et al*., 2000; Mostov *et al*., 2000; Watts, 2001). A complex molecular machinery regulates vesicle-mediated transport of proteins and lipids between organelles in the endocytic pathway (Gu and Gruenberg, 1999; Marsh and McMahon, 1999; Sorkin, 2000). The phosphatidylinositol 3-kinase (PI 3-kinase) product, phosphatidylinositol 3-phosphate [PtdIns(3)P], is an example of a lipid that regulates endocytic trafficking in yeast and higher eukaryotes (Wurmser *et al*., 1999; Gillooly *et al*., 2001). Potential effectors of PtdIns(3)P

have emerged through the observation that a conserved domain, the FYVE zinc finger, binds specifically to this lipid (Burd and Emr, 1998; Gaullier *et al*., 1998; Patki *et al*., 1998). Several FYVE domain proteins have functions in endocytic membrane trafficking and receptor signalling (Stenmark and Aasland, 1999; Gillooly *et al*., 2001).

We previously have studied the subcellular localization of two FYVE domain proteins. Membrane targeting of the early endosomal autoantigen EEA1 is thought to be mediated via a cooperative binding of its FYVE and Rab5-binding domains to PtdIns(3)P and Rab5, respectively (Simonsen *et al*., 1998b; Gaullier *et al*., 2000). Recently, we found that FYVE and coiled-coil domains of the hepatocyte growth factor-regulated tyrosine kinase substrate Hrs target this protein to early endosomes (Raiborg *et al*., 2001). Thus, like EEA1, the endosomal targeting of Hrs is mediated by a PtdIns(3)P-binding FYVE domain in cooperation with an additional domain. This raises the questions as to whether EEA1 and Hrs share the same localization on the early endosomal membrane, and whether both proteins function in the same biochemical pathways.

While the function of EEA1 as a tethering molecule involved in the homo- and heterotypic fusion of early endosomes is well characterized (Mills *et al*., 1998; Simonsen *et al*., 1998b; McBride *et al*., 1999; Rubino *et al*., 2000), little is known about the role of Hrs in endocytic trafficking. Hrs was identified originally as a tyrosine-phosphorylated protein in melanoma cells stimulated with hepatocyte growth factor (Komada and Kitamura, 1995). The protein is ubiquitously expressed and is also tyrosine phosphorylated upon stimulation of cells with epidermal growth factor (EGF), platelet-derived growth factor, interleukin-2 and granulocyte–macrophage colony-stimulating factor (Komada and Kitamura, 1995; Asao *et al*., 1997). The phosphorylation of Hrs is prevented when endocytosis is inhibited, suggesting that this post-translational modification takes place at the endosome (Urbé *et al*., 2000). In addition to the FYVE domain, Hrs contains two coiled-coil domains, a VHS domain and proline/glutamine-rich regions. Through its various domains, Hrs has been shown to bind the signal transducing adaptor molecules STAM (Asao *et al*., 1997) and STAM2/Hbp (Endo *et al*., 2000; Takata *et al*., 2000), the SNARE protein SNAP-25 (Kwong *et al*., 2000) and Smad2, which is known to transmit signals by activated transforming growth factor-β/activin receptors (Miura *et al*., 2000). Hrs is required for ventral folding morphogenesis, and Hrs^{−/−} mouse embryos accumulate enlarged endosomes and die at the 11-day stage (Komada and Soriano, 1999). A putative yeast homologue of Hrs, Vps27p, is involved in vacuolar protein sorting (Piper *et al*., 1995; Odorizzi *et al*., 1998). Given the essentiality of Hrs and its implication in

both signalling and membrane trafficking, it will be of great importance to study the function of Hrs on early endosomes. Here we show that the C-terminal domain of Hrs recruits the membrane coat protein clathrin to early endosomes. Moreover, we provide evidence for a function of Hrs in trafficking from early to late endosomes.

Results

The two PtdIns(3)P-binding proteins EEA1 and Hrs localize to distinct regions of Rab5^{Q79L}-induced giant early endosomes

In order to study the relative distribution of the two PtdIns(3)P-binding proteins EEA1 and Hrs on the early endosomal membrane, we transfected BHK cells with a GTPase-deficient mutant form of Rab5 (Rab5^{Q79L}) that leads to the formation of enlarged early endosomes due to increased homo- and heterotypic fusion (Stenmark *et al.*, 1994). The enlarged perimeter of the Rab5^{Q79L}-positive endosomes provides the opportunity to study the relative localization of proteins in the vesicle membrane. As expected, EEA1 was non-uniformly located in regions of the limiting membrane of giant early endosomes (Figure 1A, inset). These 'hot-spots' have been demonstrated previously by video microscopy to be involved in membrane fusion (McBride *et al.*, 1999; Roberts *et al.*, 1999). In contrast, while Hrs was also detected on the surface of the enlarged endosomes (Figure 1B, inset), this protein localized to regions different from EEA1. These results indicate that EEA1 and Hrs are associated with distinct molecular complexes in the endosomal membrane.

Clathrin localizes to Hrs-containing regions on endosomes

The lack of precise co-localization between EEA1 and Hrs suggests that these two proteins are involved in distinct biochemical pathways. Since EEA1 regulates membrane fusion, we reasoned that Hrs might regulate some other endosomal trafficking events, such as receptor sorting or vesicle budding. We therefore investigated the possible co-localization between Hrs and molecules implicated in the latter events. Previous studies have identified clathrin on early endosomes (Stoorvogel *et al.*, 1996; Sorkina *et al.*, 1999; Raposo *et al.*, 2001). In order to detect endosomal clathrin by confocal immunofluorescence microscopy, we transfected BHK cells with Rab5^{Q79L} and stained the cells with an anti-clathrin antibody. As expected, the anti-clathrin antibody stained numerous punctae that presumably correspond to coated pits in the plasma membrane and coated vesicles throughout the cytoplasm of the cell (Figure 1D). In addition, a subset of the clathrin was found on the enlarged Rab5^{Q79L}-positive endosomes (Figure 1D, arrows), confirming the existence of an endosomal pool of clathrin. We did not detect the clathrin adaptors AP1 and AP2 on the enlarged endosomes, and endosomal AP3 was not found to co-localize with clathrin (not shown). Like EEA1 and Hrs, clathrin was detected on regions of the enlarged endosomes (Figure 1D, inset). Significantly, whereas clathrin localized to regions different from EEA1 (Figure 1C and D, inset), it was found to co-localize strongly with Hrs-containing regions on the endosomal membrane (Figure 1E and F, inset).
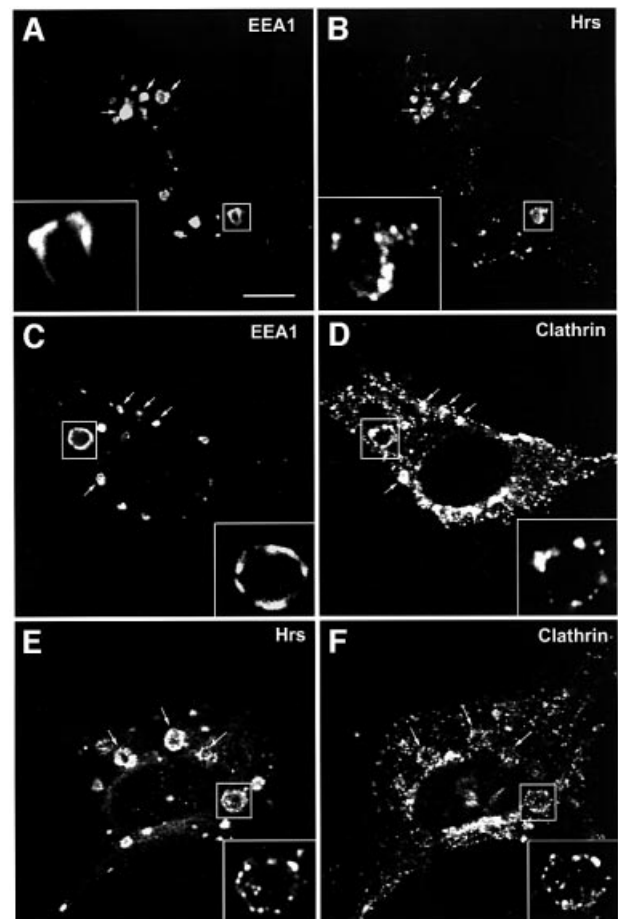

Fig. 1. Localization of Hrs, EEA1 and clathrin in Rab5^{Q79L}-over-expressing BHK cells. BHK cells were transfected with Rab5^{Q79L} and permeabilized with 0.05% saponin prior to fixation. They were then stained with anti-EEA1 (**A** and **C**), anti-Hrs (**B** and **E**) or anti-clathrin (**D** and **F**) and studied by confocal immunofluorescence microscopy. Arrows indicate examples of co-localization. Bar, 5 μm.

Having established that Hrs co-localizes with clathrin on Rab5^{Q79L}-expanded early endosomes, we set out to determine whether the same is the case in non-transfected cells. Hrs was identified originally in melanoma cells (Komada and Kitamura, 1995), and melanoma cells recently have been shown by electron microscopy to contain clathrin-coated endosomes involved in melanosome formation (Raposo *et al.*, 2001). To investigate the co-localization of Hrs and clathrin further, we therefore triple stained non-transfected melanoma cells with anti-EEA1, anti-Hrs and anti-clathrin antibodies (Figure 2). In agreement with the data obtained with Rab5^{Q79L}-transfected BHK cells, we observed an extensive co-localization between Hrs and clathrin on EEA1-positive endosomes of melanoma cells (Figure 2A–D). Moreover, when magnifying these early endosomes (Figure 2E–I), we could observe that EEA1 and Hrs had a differential localization, and that clathrin co-localized with the Hrs-containing regions. The same observation was also made in non-transfected HEp-2 cells (not shown), although these cells had less clathrin on endosomes. The striking co-localization between Hrs and clathrin suggests that these proteins may be found in the same molecular complexes on early endosomes.
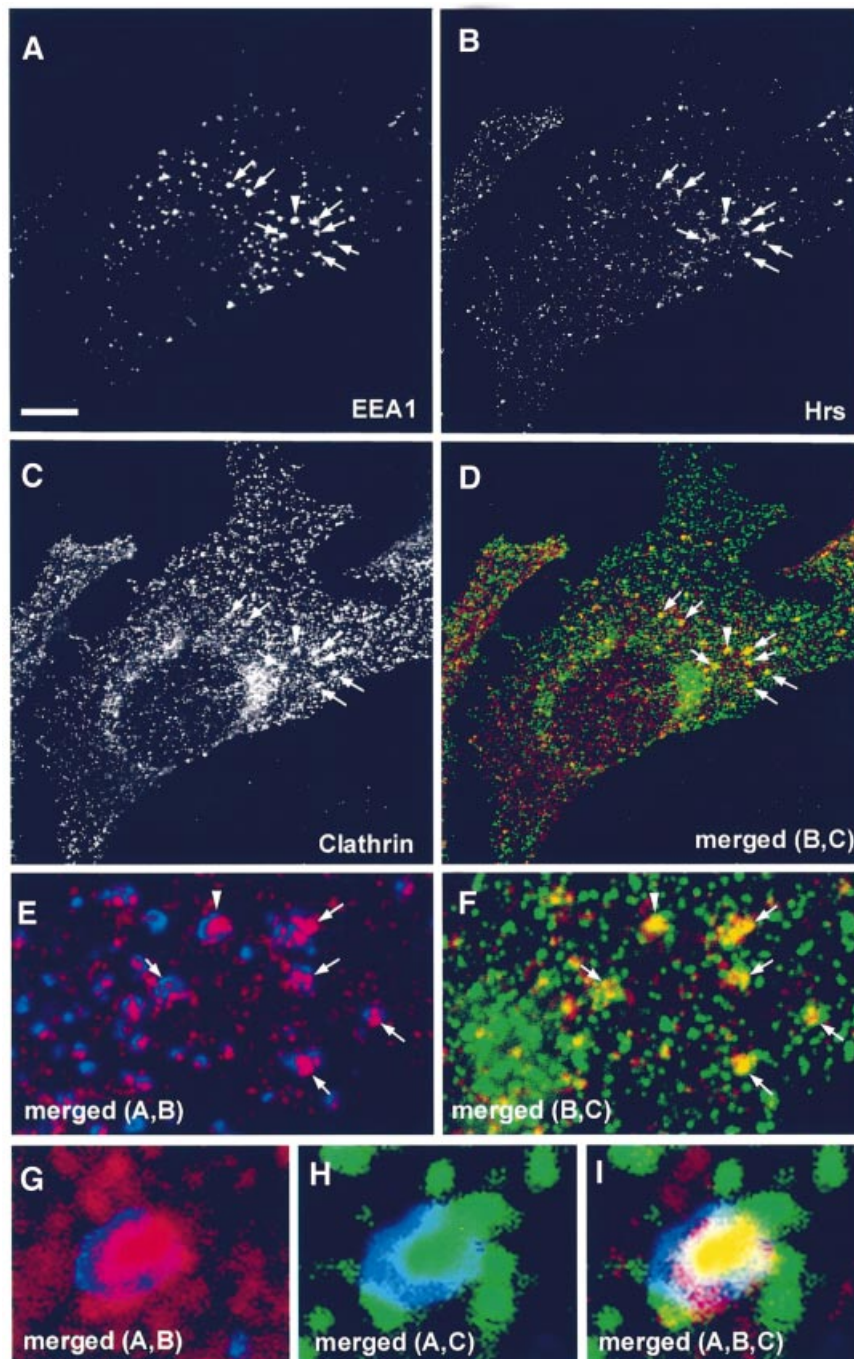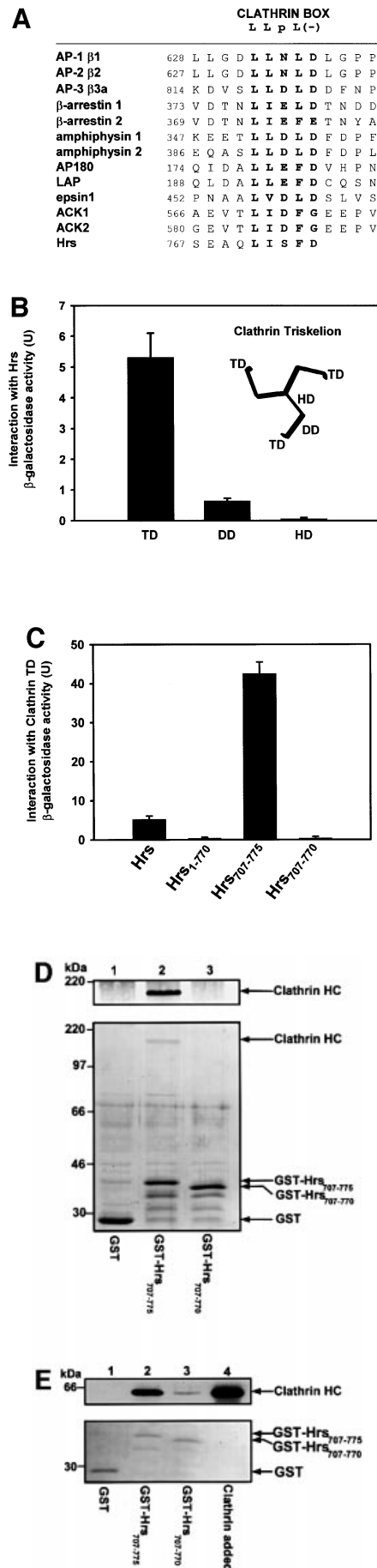
Fig. 2. Localization of Hrs, EEA1 and clathrin in melanoma cells. A melanoma cell, permeabilized with 0.05% saponin prior to fixation, was stained with anti-EEA1 (**A**), anti-Hrs (**B**) and anti-clathrin (**C**). Arrows indicate co-localization between EEA1, Hrs and clathrin. Yellow indicates co-localization between Hrs and clathrin (**D**). The arrowhead points to an endosome that is shown magnified in (G–I). Bar, 5 μm. (**E** and **F**) Magnification of the endosomes highlighted by arrows in (A–D). The arrowhead points to an endosome that is shown magnified in (**G–I**). (**G–I**) Merged images of a triple-stained magnified endosome. Note the differential localization of EEA1 in blue and Hrs in red, and the co-localization (yellow) between clathrin in green and Hrs.

Hrs binds directly to the terminal domain of clathrin heavy chain

When we compared the amino acid sequence of Hrs with those of different clathrin-binding proteins such as amphiphysins, β-arrestins and adaptor proteins, we detected a cluster of five conserved residues in the very C-terminus of Hrs. In the other clathrin-binding proteins, this sequence, which consists of one polar amino acid flanked by hydrophobic and acidic amino acids [LLpL(–)],

has been described as the 'clathrin box' and interacts specifically with the terminal domain (TD) of clathrin heavy chain (HC) (Goodman *et al.*, 1997; Dell'Angelica *et al.*, 1998; ter Haar *et al.*, 2000). An alignment of the clathrin box motif from different clathrin-binding proteins and that of Hrs is shown in Figure 3A.
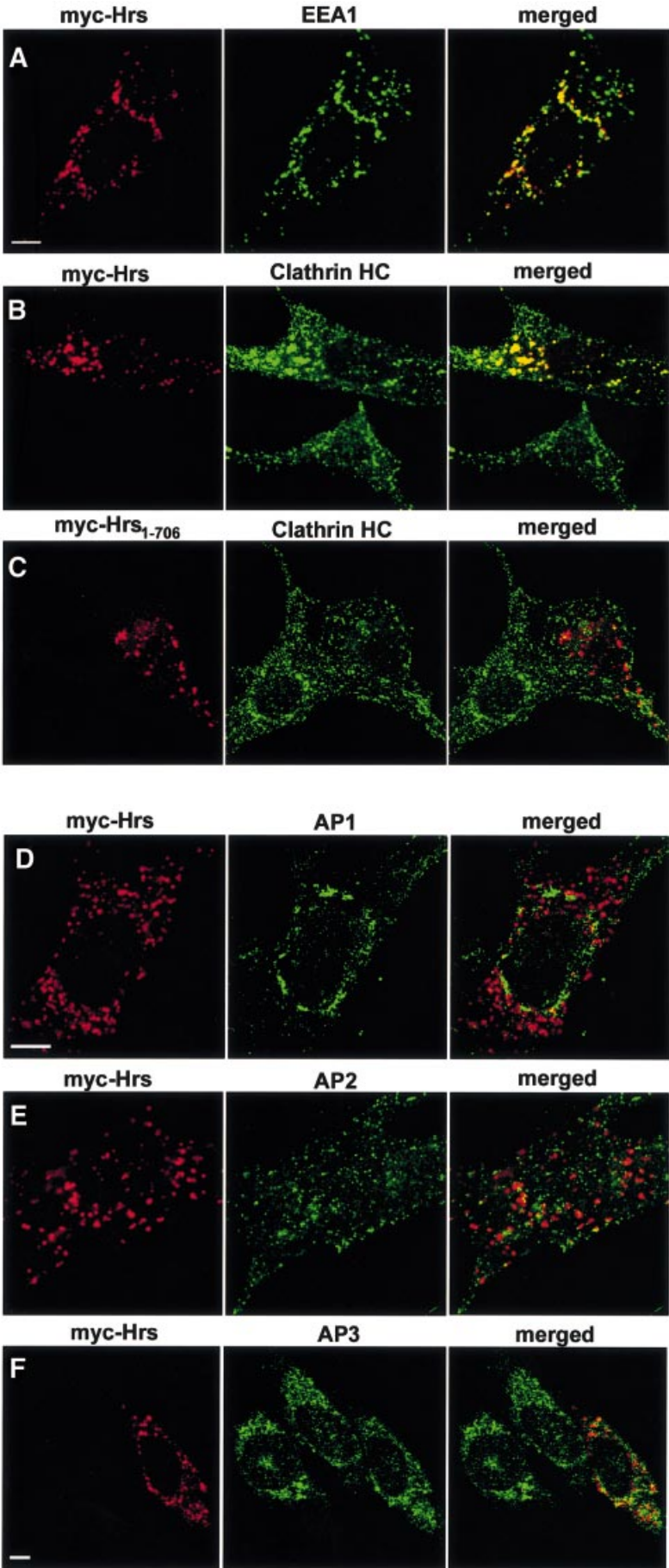
Based on these findings, we wanted to investigate whether Hrs could interact with clathrin. Using the yeast two-hybrid system, we first tested whether there was an

A

CLATHRIN BOX
L L p L (-)

AP-1 β1	628	L L G D	**L L N L D**	L G P P
AP-2 β2	627	L L G D	**L L N L D**	L G P P
AP-3 β3a	814	K D V S	**L L D L D**	D F N P
β-arrestin 1	373	V D T N	**L I E L D**	T N D D
β-arrestin 2	369	V D T N	**L I E F E**	T N Y A
amphiphysin 1	347	K E E T	**L L D L D**	F D P F
amphiphysin 2	386	E Q A S	**L L D L D**	F D P L
AP180	174	Q I D A	**L L E F D**	V H P N
LAP	188	Q L D A	**L L E F D**	C Q S N
epsin1	452	P N A A	**L V D L D**	S L V S
ACK1	566	A E V T	**L I D F G**	E E P V
ACK2	580	G E V T	**L I D F G**	E E P V
Hrs	767	S E A Q	**L I S F D**	

B

C

D

E

Fig. 3. The C-terminus of Hrs binds clathrin TD. (**A**) Alignment of sequences found in proteins that bind clathrin (ter Haar *et al.*, 2000; Teo *et al.*, 2001; Yang *et al.*, 2001). This illustrates the existence of a potential clathrin-binding motif within residues 770–775 of Hrs. Hrs is the only protein that has the clathrin box motif at the very C-terminus. (**B–E**) Interaction of Hrs with clathrin. (**B** and **C**) L40 reporter yeast cells were transformed with bait constructs in pLexA and prey constructs in pGAD. Reporter β-galactosidase activities (in arbitrary units) indicate binding and are represented as mean values of two independent experiments performed in duplicate. Error bars denote ± SEM. In (B), a clathrin triskelion (consisting of three heavy chains) is illustrated, with the terminal domain (TD), distal domain (DD) and hub domain (HD) indicated. (**D**) Recombinant GST (lane 1), GST–Hrs$_{707-775}$ (lane 2) or GST–Hrs$_{707-770}$ (lane 3) were immobilized on glutathione–Sepharose beads and incubated with pig brain cytosol. The beads were recovered by centrifugation and washed. Pellet fractions were resolved by SDS–PAGE and transferred to nitrocellulose. The blot was stained with Ponceau S (lower panel) prior to detection of clathrin with anti-clathrin heavy chain antibodies (upper panel). (**E**) Recombinant GST (lane 1), GST–Hrs$_{707-775}$ (lane 2) or GST–Hrs$_{707-770}$ (lane 3), were immobilized on glutathione–Sepharose beads and incubated with purified recombinant clathrin terminal domain (TD$_{1-579}$). The beads were recovered by centrifugation and washed. Pellet fractions were resolved by SDS–PAGE and transferred to nitrocellulose. The blot was stained with Ponceau S (lower panel) prior to detection of clathrin-TD$_{1-579}$ with anti-clathrin heavy chain (upper panel). Lane 4 represents the total amount of recombinant clathrin-TD$_{1-579}$ added to the beads.

interaction between Hrs and three different domains of the clathrin HC (Figure 3B). While we found no significant interaction between full-length Hrs and the distal domain (residues 493–1072) or the hub domain (residues 1066–1675) of clathrin HC, we observed a strong interaction between Hrs and the terminal domain (TD) of clathrin HC (residues 1–579) (Figure 3B). An interaction was also found with a shorter fragment of clathrin HC (residues 1–330) (not shown), constituting the β-propeller domain of the clathrin TD (ter Haar *et al.*, 2000). These results suggest that Hrs binds specifically to clathrin TD. While a deletion mutant lacking the 102 most C-terminal amino acids of Hrs (Hrs$_{1-673}$) did not bind (not shown), a small construct consisting of the 69 most C-terminal amino acids (Hrs$_{707-775}$) showed a strong interaction with clathrin TD (Figure 3C), indicating that the C-terminal part of Hrs is an autonomous clathrin-binding domain. Further, deletion of the clathrin box motif of full-length Hrs (Hrs$_{1-770}$) or the clathrin-binding domain (Hrs$_{707-770}$) largely abolished the interaction with clathrin TD (Figure 3C), demonstrating the importance of these five amino acids for clathrin binding.

To confirm the two-hybrid data biochemically, we studied the interaction between Hrs and clathrin-TD in a GST pull-down assay. When GST–Hrs$_{707-775}$ coupled to glutathione–Sepharose was incubated with pig brain cytosol, clathrin HC bound to the fusion protein and was pelleted with the beads (Figure 3D, lane 2). No detectable clathrin was pulled down with GST alone or with GST–Hrs$_{707-770}$, confirming the importance of the clathrin box motif (Figure 3D). To investigate whether Hrs binds directly to the clathrin TD, we examined the interaction between these proteins by using purified recombinant clathrin TD (clathrin-TD$_{1-579}$). When incubated with GST–Hrs$_{707-775}$ immobilized on glutathione–Sepharose, clathrin-TD$_{1-579}$ was retained in the bead fraction

myc-Hrs   EEA1   merged

A

myc-Hrs   Clathrin HC   merged

B

myc-Hrs$_{1-706}$ Clathrin HC   merged

C

myc-Hrs   AP1   merged

D

myc-Hrs   AP2   merged

E

myc-Hrs   AP3   merged

F

(Figure 3E, lane 2), whereas no binding was observed with GST alone. It is worth noting that a small fraction of clathrin-TD$_{1-579}$ bound to GST–Hrs$_{707-770}$ (Figure 3E, lane 3), indicating that the deletion of the clathrin box reduces, but does not completely abolish, the affinity of Hrs for clathrin.

Hrs recruits clathrin to early endosomes

In order to study whether Hrs is able to recruit clathrin to early endosomes *in vivo*, we expressed myc epitope-tagged Hrs constructs in BHK cells and examined their co-localization with clathrin. As previously described (Urbé *et al*., 2000; Raiborg *et al*., 2001), overexpressed Hrs co-localized with EEA1 on clustered early endosomes (Figure 4A). Significantly, a massive recruitment of both clathrin HC (Figure 4B) and clathrin light chain (LC) (not shown) was observed on these structures, as compared with non-transfected cells (Figure 4B). Co-localization with clathrin was also observed with a deletion mutant of Hrs lacking the N-terminal VHS domain (not shown). Hrs still showed some co-localization with clathrin after deletion of the five amino acids constituting the clathrin box (not shown), indicating that this motif is not absolutely essential for clathrin recruitment, and that additional residues in the C-terminus of Hrs may participate in the interaction. This is consistent with results with β-arrestin (ter Haar *et al*., 2000) and epsin 1 (Drake *et al*., 2000) and in agreement with our results obtained with the GST pull-down assay (Figure 3E). However, a deletion mutant lacking the 69 most C-terminal amino acids (Hrs$_{1-706}$) did not show any recruitment of clathrin to the Hrs-positive endosomes (Figure 4C). These findings indicate that the C-terminal part of Hrs recruits clathrin to early endosomes.

The clathrin adaptor proteins AP1 and AP2 are found in the *trans*-Golgi network (TGN) and in the plasma membrane, respectively, whereas AP3 is found in the TGN and on endosomes (Dell'Angelica *et al*., 1997; Hirst and Robinson, 1998; Kirchhausen, 1999). Because these adaptor complexes interact directly with clathrin through a clathrin box sequence (Shih *et al*., 1995; Dell'Angelica *et al*., 1998), we investigated their possible recruitment to Hrs-overexpressing endosomes. As expected, AP1 was found in the *trans*-Golgi region (Figure 4D), while AP2 was detected on the plasma membrane and in coated vesicles (Figure 4E), and AP3 had mainly a perinuclear distribution (Figure 4F). In contrast to clathrin, no recruitment of AP1, AP2 or AP3 onto Hrs-overexpressing endosomes was observed (Figure 4D–F). AP1 and AP2 have been previously reported to co-localize with EGF receptors and clathrin on endosomes in A431 cells stimulated with EGF (Sorkina *et al*., 1999), whereas very little AP2 was found on endosomes in Cos-1 cells (Sorkina *et al*., 1999). Since we also did not detect the presence of AP1 or AP2 on endosomes, the endosomal localization of

these clathrin adaptors may be cell type dependent. Unlike the adaptor proteins, the coated vesicle-associated GTPase, dynamin, was found to be recruited by Hrs onto early endosomes (Figure 5A), together with Eps15 (Figure 5B), which has recently been shown to bind directly to the N-terminal half of Hrs (Bean *et al*., 2000). Similarly to clathrin, dynamin did not co-localize with Hrs lacking the C-terminus (Hrs$_{1-706}$), suggesting that it may be recruited via clathrin (not shown). In contrast, Eps15 was recruited to endosomes by Hrs$_{1-706}$ (Figure 5C), consistent with Eps15 binding to a region of Hrs different from clathrin.

Early endosomes in BHK cells have been well characterized by electron microscopy (Griffiths *et al*., 1989) and typically consist of isolated cisternae as exemplified in Figure 6A. When cells overexpressing Hrs were analysed, a clustering of bovine serum albumin (BSA)–gold-containing Hrs-positive endosomes was observed (Figure 6B), in agreement with the effect seen by confocal microscopy. The Hrs-positive vesicles contained an electron-dense flat coat, which gives these structures a characteristic morphology (Figure 6B). Consistent with the immunofluorescence data, the coat contained clathrin (Figure 6C), Eps15 (Figure 6D) and dynamin (Figure 6E). The Hrs-induced coat was slightly thicker than a typical clathrin coat observed on clathrin-coated pits (Figure 6C, inset). Moreover, a coat could also be observed in cells transfected with a mutant deficient in clathrin binding, Hrs$_{1-706}$ (not shown), indicating that it consists of additional proteins.

Overexpressed Hrs does not interfere with transferrin endocytosis and recycling

Due to the strong recruitment of clathrin to Hrs-positive early endosomes, we wanted to assess the role of Hrs in clathrin-mediated endocytosis. Under our transfection conditions, BHK cells co-transfected with transferrin (Tf) receptor and Hrs or various fragments of Hrs (Hrs$_{707-775}$, Hrs$_{707-770}$ and Hrs$_{1-706}$, not shown) showed no reduction in the endocytosis of Tf when compared with cells transfected with Tf receptor alone (Figure 7A). Moreover, the cytosolic clathrin-binding fragment of Hrs (Hrs$_{707-775}$) did not behave as a dominant-negative mutant for clathrin recruitment on endosomes (not shown). Overexpression of Hrs also did not affect the recycling of Tf (Figure 7B). These results indicate that Hrs does not play a role in the endocytic internalization or recycling pathways.

Overexpressed Hrs inhibits trafficking from early to late endosomes

Recently, overexpressed Hrs was shown to inhibit degradation of the EGF receptor (Chin *et al*., 2001), and it was suggested that Hrs may regulate the sorting of this receptor for transport to late endocytic compartments. We

Fig. 4. (**A–C**) Overexpressed Hrs recruits clathrin to early endosomes. BHK cells were transfected with myc-tagged Hrs (A and B) or Hrs$_{1-706}$ (C) and permeabilized with 0.05% saponin prior to fixation. They were stained with anti-myc (left panels), anti-EEA1 (A, middle panel) or anti-clathrin (B and C, middle panels) and studied by confocal immunofluorescence microscopy. (**D–F**) Overexpressed Hrs does not recruit AP1, AP2 or AP3. BHK cells or HEp-2 cells (in the case of AP3) were transfected with myc-tagged Hrs and permeabilized with 0.05% saponin prior to fixation. The cells were stained with anti-myc (left panels), anti-AP1 (D, middle panel), anti-AP2 (E, middle panel) or anti-AP3 (F, middle panel) and studied by confocal immunofluorescence microscopy. The right panels show the merged images, with yellow indicating co-localization. Bar, 5 μm.
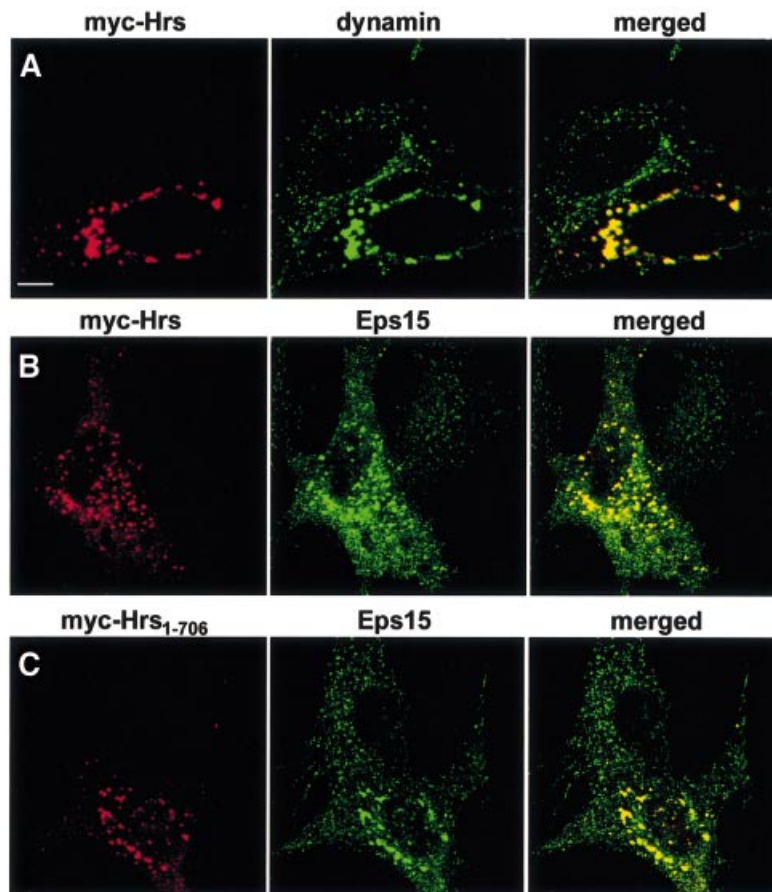
Fig. 5. Overexpressed Hrs recruits dynamin and Eps15 to endosomes. BHK cells were transfected with myc-tagged Hrs (**A** and **B**) or Hrs$_{1–706}$ (**C**) and permeabilized with 0.05% saponin prior to fixation. They were stained with anti-myc (left panels), anti-dynamin (A, middle panel) or anti-Eps15 (B and C, middle panels) and studied by confocal immunofluorescence microscopy. The right panels show the merged images, with yellow indicating co-localization. Bar, 5 μm.

investigated whether this inhibition is specific to EGF or whether overexpression of Hrs could also affect transport of molecules taken up by fluid-phase endocytosis. By internalizing rhodamine-labelled EGF for 1 h, followed by chasing EGF for different time periods, we indeed observed a strong reduction of EGF degradation in cells transfected with Hrs compared with non-transfected cells (Figure 7C). Rhodamine-labelled EGF co-localized strongly with Hrs after both 0 (Figure 8A, B and D) and 3 h of chase (Figure 8G, H and J). However, similar results were obtained with internalized biotin–dextran (Figure 9), demonstrating that overexpression of Hrs leads to a general inhibition of transport. The inhibitory effect was observed in cells expressing both high and low levels of Hrs. Somewhat surprisingly, the non-clathrin-binding deletion mutant of Hrs (Hrs$_{1–706}$) also caused a general inhibition of transport (not shown). While there was a strong co-localization between overexpressed Hrs and dextran after both 0 and 3 h of chase (Figure 9A and B), endogenous Hrs in non-transfected cells only co-localized with internalized dextran after 0 h and not after the chase period (Figure 9C and D). This is consistent with the localization of Hrs to early endosomes.

Remarkably, in Hrs-overexpressing cells, the EGF-containing endosomes (Figure 8) or dextran-containing endosomes (not shown) were still positive for EEA1 after

3 h of chase, whereas they were negative for the late endosome/lysosome marker LAMP-1 (not shown), indicating that internalized EGF and dextran are retained in early endosomes in cells transfected with Hrs. Thus, under conditions in which Tf uptake and recycling are unaffected, Hrs overexpression inhibits the trafficking of both a receptor-bound and a fluid-phase marker from early to late endosomes. We next studied this effect at the ultrastructural level. In contrast to non-transfected cells, in which the majority of the internalized BSA–gold was found within multivesicular bodies after 1 h of internalization, BSA–gold accumulated in myc-Hrs-positive structures (not shown), whereas labelling of multivesicular bodies and lysosomes was never observed. Taken together, our data suggest that Hrs is a specific regulator of membrane trafficking from early to late endosomes.

Wortmannin removes Hrs and clathrin from transferrin-positive endosomes

The association of Hrs with early endosomes requires PI 3-kinase activity (Komada and Soriano, 1999; Urbé *et al.*, 2000). To test our conclusion that Hrs recruits clathrin to endosomes, we therefore investigated whether the PI 3-kinase inhibitor wortmannin could disrupt the endosomal localization of clathrin. In order to study the presence of Hrs and clathrin on endosomes, internalized Tf was used

as an endosomal marker. In control cells, we observed a co-localization between Hrs and clathrin on Tf-positive endosomes (Figure 10A, arrows). As expected, a 30 min wortmannin treatment led to the dissociation of Hrs from endosome membranes (Figure 10B) and caused a tubulation of endosomes (Shpetner *et al.*, 1996). Significantly, wortmannin also disrupted the association of clathrin with Tf-positive endosomes (Figure 10B), while clathrin could still be detected in the presumed TGN region (arrowhead) and on the plasma membrane. These results confirm the role of Hrs in the recruitment of clathrin to endosomes.
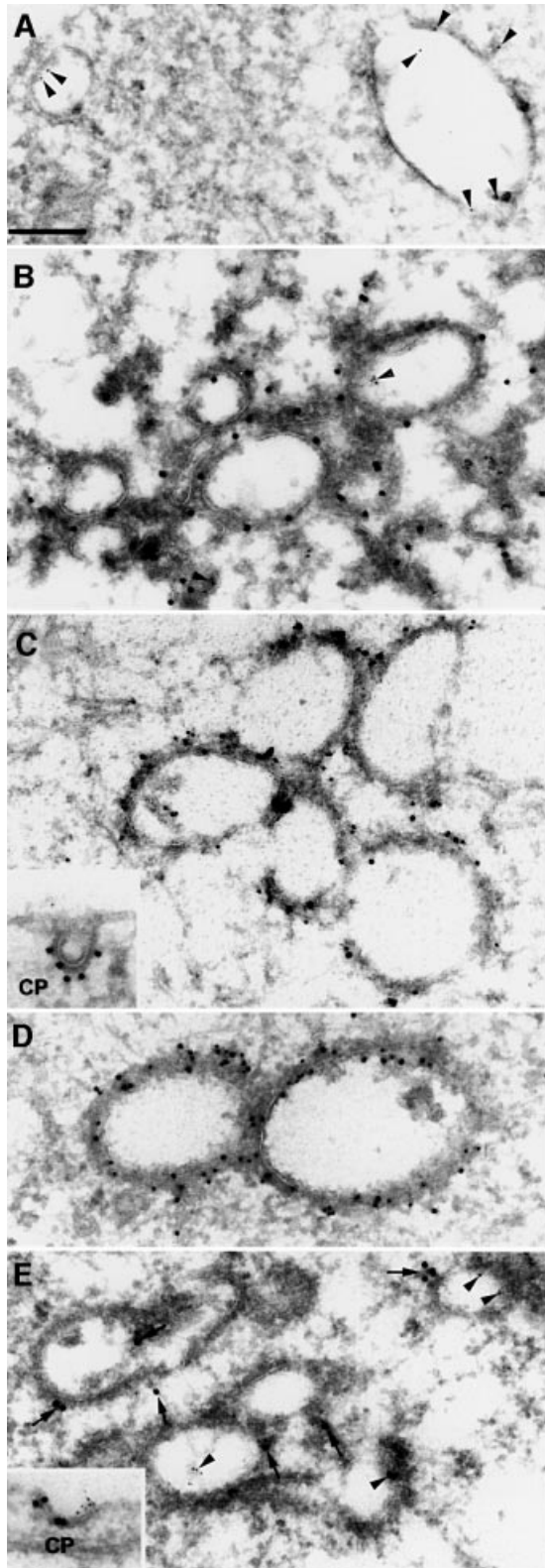
Discussion

In this report, we have shown that the two PtdIns(3)P-binding proteins, EEA1 and Hrs, localize to distinct regions of early endosomes, and that the C-terminal 69 residues of Hrs constitute a novel clathrin-binding domain. This provides a mechanism for the specific recruitment of clathrin onto early endosomes and suggests that Hrs may function as an endosomal clathrin adaptor. By recruiting clathrin, Eps15 and other proteins into restricted domains of early endosomes, Hrs appears to regulate trafficking (or maturation) from early to late endosomes.

Differential localization of Hrs and EEA1 on early endosomes

The mechanisms that define distinct membrane domains of early endosomes are not known, but it has been suggested that the differential localization of endosomal Rab GTPases may contribute to define membrane identity (Sönnichsen *et al.*, 2000). Even though most FYVE domain proteins studied so far have been localized to early endosomes, their relative distribution on these organelles has not been investigated. Our finding that EEA1 and Hrs localize to distinct regions of early endosomes now shows that the precise localization of FYVE domain proteins is not only determined by the FYVE–PtdIns(3)P interaction. This is consistent with the fact that a Rab5-binding domain of EEA1 and a coiled-coil domain of Hrs are also involved in the subcellular targeting of these proteins (Simonsen

Fig. 6. Electron microscopy of Hrs-overexpressing endosomes. (**A**) Early endosomes from a non-transfected BHK cell that prior to fixation had endocytosed 5 nm BSA–gold (arrowheads) for 10 min. (**B**) Localization of Hrs in a myc-Hrs-transfected BHK cell that prior to fixation had endocytosed 5 nm BSA–gold (arrowheads) for 1 h. Thawed cryosections were labelled with anti-myc antibodies followed by rabbit anti-mouse antibodies and 15 nm protein A–gold. Note the coated appearance and clustering of the Hrs-positive compartments. (**C**) Immunoelectron microscopic localization of clathrin in myc-Hrs-transfected cells. Thawed cryosections were double labelled using rabbit anti-clathrin LC (10 nm gold) followed by mouse anti-myc and rabbit anti-mouse antibodies (15 nm gold). The labelling showed that clathrin localized to the characteristic myc-Hrs-induced endosomal coat. (C, inset) A clathrin-coated pit labelled with goat anti-clathrin followed by rabbit anti-goat and 15 nm protein A–gold. (**D**) Immunoelectron microscopic localization of Eps15 in myc-Hrs-transfected cells. Thawed cryosections were double labelled with anti-Eps15 (10 nm protein A–gold) followed by labelling for the myc epitope (15 nm protein A–gold). There is a strong Eps15 labelling on the characteristic Hrs-positive endosomal coat. (**E**) Immunoelectron microscopic localization of dynamin in myc-Hrs-transfected cells. Thawed cryosections of cells that had endocytosed 5 nm BSA–gold for 1 h (arrowheads) were labelled with mouse anti-dynamin 1 antibody (Hudy 1) followed by rabbit anti-mouse antibodies and 15 nm protein A–gold (arrows). In cells showing the characteristic clustering of coated endosomes due to Hrs overexpression, labelling of dynamin is localized to the endosomal coat as well as to typical clathrin-coated pits at the plasma membrane (CP, inset). Bar, 200 nm.

et al., 1998b; Raiborg *et al.*, 2001). While the EEA1-containing regions are clearly involved in membrane tethering and fusion, our present results suggest that the Hrs-containing regions may play a role in protein sorting.

Endosomal clathrin

The molecular machinery responsible for active sorting of integral membrane proteins in early sorting endosomes has so far not been identified. Such sorting can be expected to employ cytoplasmic coats analogous to the different types of coats identified for Golgi cisternae, the endoplasmic reticulum, the TGN and the plasma membrane (Rothman and Wieland, 1996; Hirst and Robinson, 1998; Gu and Gruenberg, 1999; Scales *et al.*, 2000). There is indeed evidence for coats on endosomes (Aniento *et al.*, 1996; Stoorvogel *et al.*, 1996; Futter *et al.*, 1998; Sorkina *et al.*, 1999), and our detection of clathrin on the enlarged Rab5^{Q79L}-positive endosomes, and on endosomes in non-transfected melanoma and HEp-2 cells, confirms the presence of clathrin on restricted regions of endosomes (Stoorvogel *et al.*, 1996; Raposo *et al.*, 2001). In melanoma cells, the endosomal clathrin coat consists of planar or invaginated lattices without budding profiles (Raposo *et al.*, 2001) while, in A431 cells, clathrin-coated buds have been detected, suggesting the formation of endosome-derived clathrin-coated vesicles (Stoorvogel *et al.*, 1996). Thus, there are probably at least two different clathrin coats on endosomes, and our present results are most consistent with a function of Hrs in the formation of the flat clathrin coat previously identified on endosomes in melanoma cells. Our finding that wortmannin-induced dissociation of Hrs from early endosome membranes in HEp-2 cells is accompanied by the disappearance of endosomal clathrin further suggests that Hrs plays an important role in clathrin recruitment to endosomes in these cells. Whether the dissociation of clathrin from endosomes may explain some of the effects of wortmannin on endosome morphology and function (Shpetner *et al.*, 1996) remains to be investigated.

Hrs as a regulator of endocytic trafficking

Our finding that overexpression of Hrs did not affect Tf endocytosis contradicts a previous report in which Hrs was found to inhibit Tf endocytosis when expressed at high level (Bean *et al.*, 2000). The reason for this discrepancy is not clear, but one possibility is that the reported inhibition was caused by a higher level of overexpression, which might cause a redistribution of clathrin from the plasma
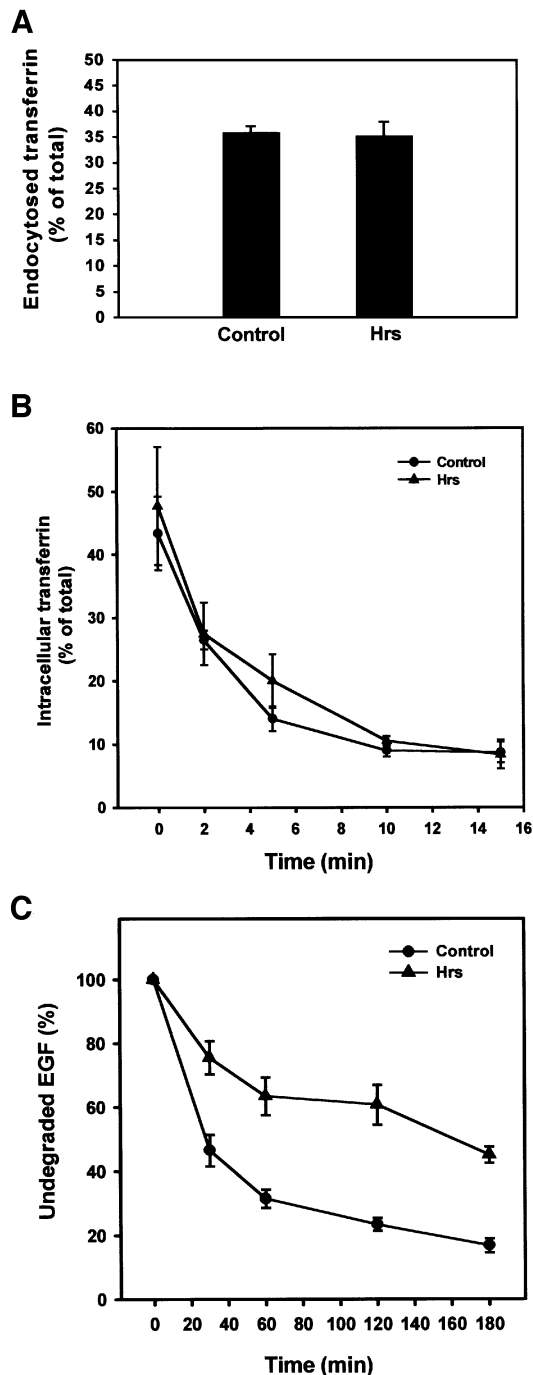

Fig. 7. Overexpressed Hrs does not interfere with transferrin endocytosis or recycling, but inhibits EGF degradation. (**A**) Effect of Hrs on transferrin endocytosis. BHK cells were transfected with transferrin receptor alone (control) or co-transfected with transferrin receptor and Hrs for 6 h. The cells were incubated with Ru-tag-labelled transferrin for 15 min at 37°C and surface-bound transferrin was removed by pronase. The amount of cell-associated transferrin was measured using an ORIGEN analyser, and endocytosed transferrin is presented as a percentage of total cell-associated transferrin. Error bars represent the SEM of three independent experiments performed in duplicate. (**B**) Effect of Hrs on transferrin recycling. BHK cells were transfected with transferrin receptor alone (control) or co-transfected with transferrin receptor and Hrs for 6 h. The cells were incubated with Ru-tag-labelled transferrin for 30 min at 37°C and surface-bound transferrin was removed in a portion of the wells by MESNA. The MESNA-treated cells were incubated further for the times indicated. The amount of cell-associated transferrin was measured using an ORIGEN analyser, and intracellular transferrin is presented as a percentage of total cell-associated transferrin. Error bars represent the SEM of two independent experiments performed in duplicate. (**C**) Effect of Hrs on EGF degradation. HEp-2 cells were transfected with myc-tagged Hrs for 6 h, and rhodamine-EGF was internalized for 1 h at 37°C. The cells were washed and incubated further for different times up to 3 h. The cells were permeabilized with 0.05% saponin prior to fixation. They were stained with anti-myc and studied by confocal immunofluorescence microscopy. The mean intensity of the rhodamine-EGF signal from 10 transfected and 10 non-transfected cells from each time point was quantified and is presented as the percentage of undegraded EGF compared with the rhodamine-EGF signal before chase. Error bars denote± SEM.
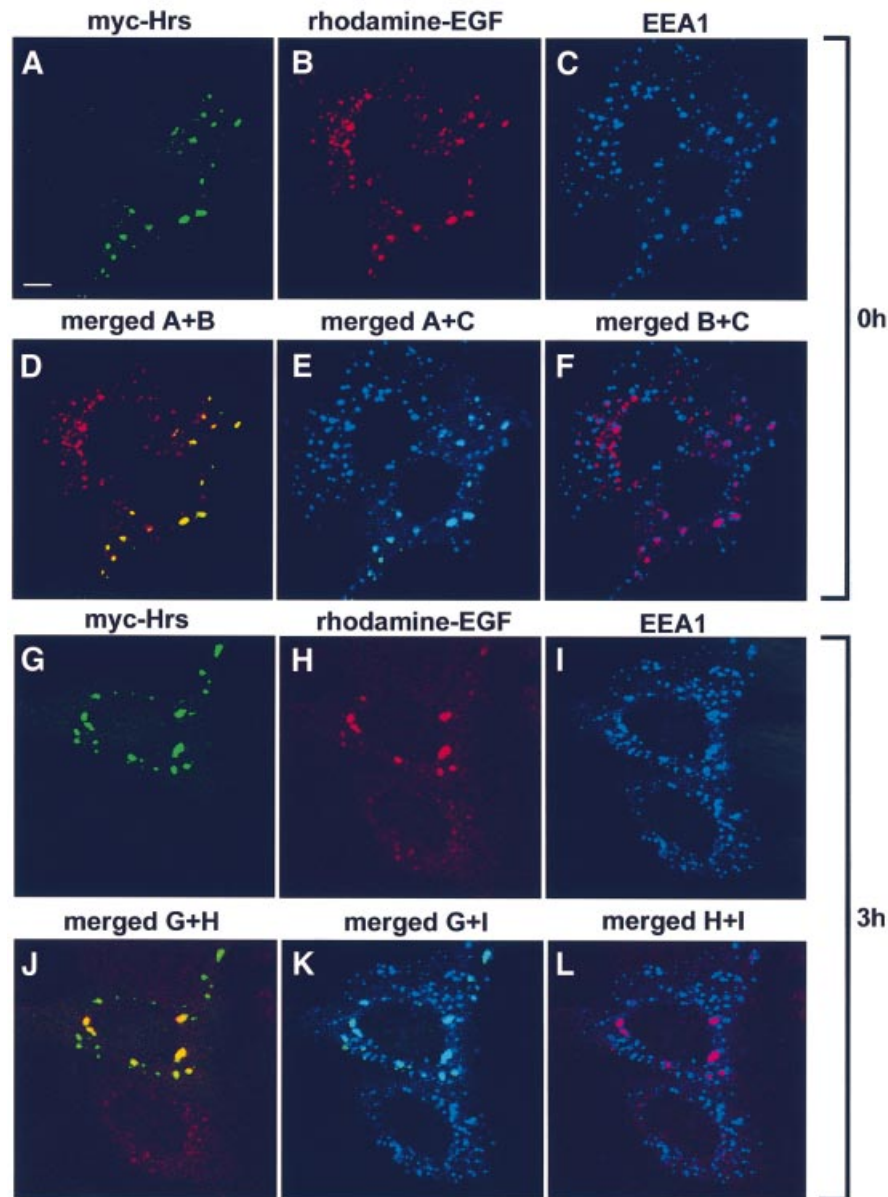
Fig. 8. Overexpressed Hrs inhibits EGF trafficking from early to late endosomes. HEp-2 cells were transfected with myc-tagged Hrs for 6 h and incubated with rhodamine-EGF for 1 h at 37°C (**A–F**). A portion of the cells was washed and incubated further for 3 h (**G–L**). The cells were permeabilized with 0.05% saponin prior to fixation. They were stained with anti-myc (A and G) or anti-EEA1 antibodies (C and I) and studied by confocal immunofluorescence microscopy. Turquoise indicates co-localization between Hrs and EEA1 (E and K). Purple indicates co-localization between EGF and EEA1 (F and L). Yellow indicates co-localization between Hrs and EGF (D and J). Bar, 5 μm.

membrane to endosomes. Importantly, under our conditions in which Tf uptake and recycling were unaffected, Hrs overexpression was found to inhibit the trafficking of both a receptor-bound and a fluid-phase marker from early to late endosomes. This indicates that Hrs functions in the trafficking from early to late endosomes rather than between the plasma membrane and early endosomes.

Somewhat surprisingly, the inhibitory effect of Hrs overexpression did not seem to be due to the massive recruitment of clathrin to early endosomes, as a non-clathrin-binding deletion mutant of Hrs (Hrs$_{1-706}$) also caused a general inhibition of transport. The overexpression of endosome-targeted Hrs constructs leads to a clustering of early endosomes (see Figure 6B), irrespective

of the presence of the clathrin-binding C-terminus (Raiborg *et al.*, 2001). We therefore speculate that the overexpression of these constructs may lead to a hyper-recruitment or sequestering of specific proteins that regulate endosome docking or motility.

Hrs as a possible clathrin adaptor

Clathrin adaptors are recruited from the cytosol to specific membranes through a cooperative binding to receptor proteins and phosphoinositides (Gaidarov and Keen, 1999; Kirchhausen, 1999). Here we demonstrate that Hrs, which binds PtdIns(3)P and is localized to early endosomes, contains a C-terminal clathrin-binding domain, and that overexpression of Hrs recruits clathrin to early endosomes.
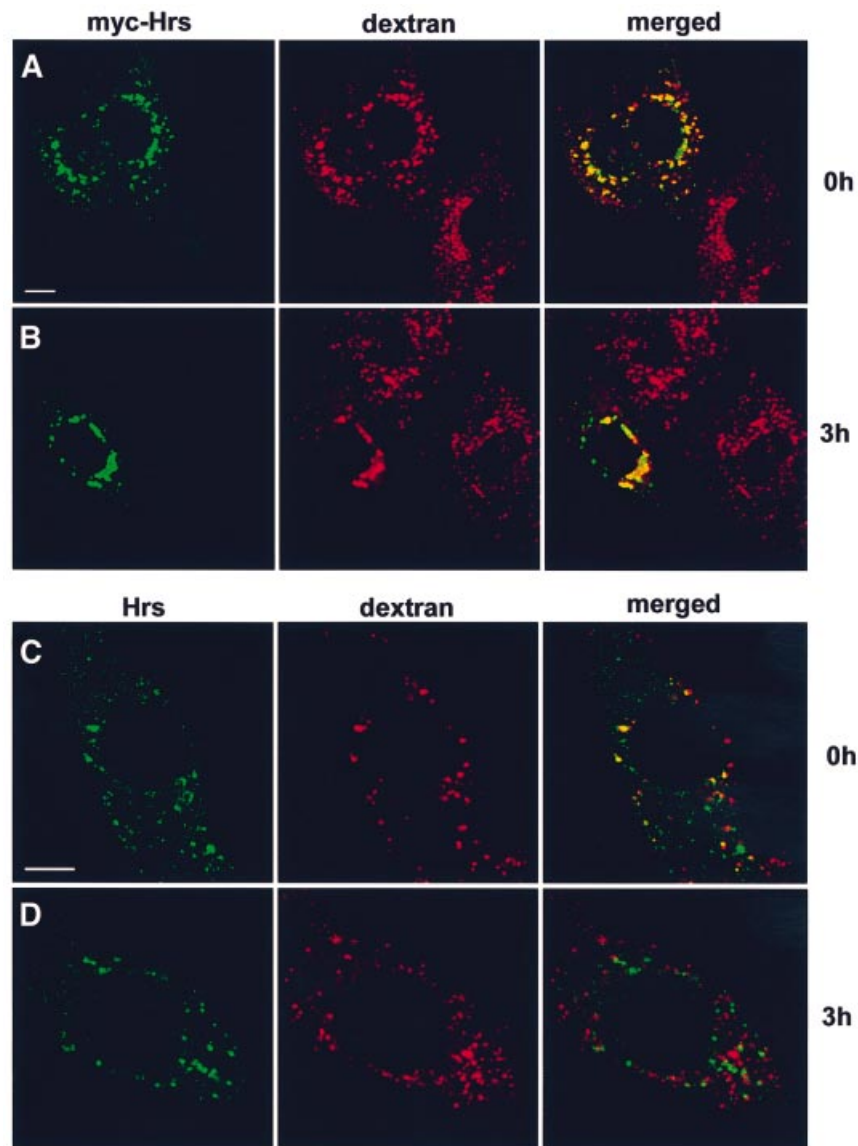
Fig. 9. The transport of endocytosed dextran from early to late endosomes is inhibited in Hrs-overexpressing cells. HEp-2 cells were transfected with myc-tagged Hrs for 6 h and incubated with biotin–dextran for 1 h at 37°C (**A**). The cells were washed and incubated further for 3 h (**B**). (**C**) A non-transfected cell incubated with biotin–dextran for 1 h at 37°C. (**D**) A non-transfected cell after 3 h of chase. The cells were stained with anti-myc (left panels A and B), anti-Hrs (left panels C and D) or streptavidin–Cy3 (middle panels) and studied by confocal fluorescence microscopy. The right panels show the merged images, with yellow indicating co-localization. Bar, 5 μm.

Hrs therefore is a possible candidate for an endosomal clathrin adaptor. A well-established function of adaptors is to recognize the sorting signals present in the cytosolic tails of those transmembrane proteins that are recruited to coated pits and vesicles. So far, we have been unable to detect any complex between Hrs and receptors, but it is interesting to note that EAST, the chicken homologue of the tightly Hrs-associated protein STAM, has been found to interact with the EGF receptor in a ligand-dependent fashion (Lohi *et al.*, 1998). Moreover, Hrs has been found to interact with sorting nexin-1 (Snx1), which recognizes the lysosomal sorting motif of the EGF receptor (Chin *et al.*, 2001). Even more intriguing are the recent findings that GGA proteins, which contain VHS domains, appear to function as monomeric clathrin adaptors in the TGN, and

that the VHS domain interacts with receptor tails (Nielsen *et al.*, 2001; Puertollano *et al.*, 2001). Since both Hrs and STAM contain VHS domains, we speculate that the Hrs–STAM complex may function as a dimeric clathrin adaptor on early endosomes. Our findings that Hrs co-localizes with clathrin but not with the adaptor proteins AP1, AP2 and AP3 on endosomes, and that wortmannin causes the dissociation of clathrin from endosomes, support the idea that Hrs may itself act as an endosomal clathrin adaptor.

A recruitment of receptors into flat clathrin lattices may be a mechanism to sort cargo that is destined for late endosomes. The endosomal clathrin coat would then have to dissociate before the formation of endosomal invaginations, and a possible mechanism is suggested by
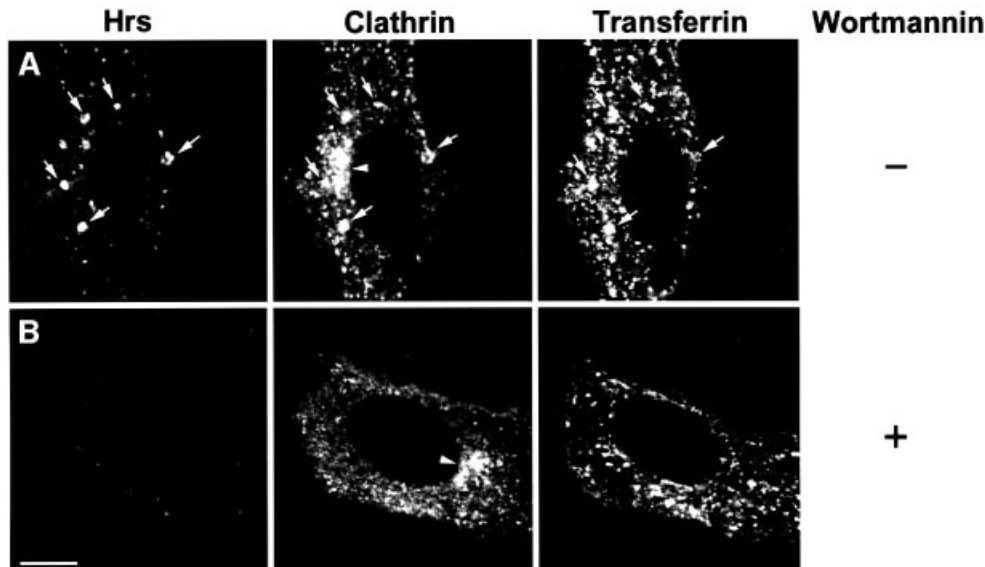
Fig. 10. Wortmannin disrupts the association of Hrs and clathrin with transferrin-positive endosomes. HEp-2 cells were incubated with Alexa[488]–transferrin for 30 min at 37°C in the absence (**A**) or presence (**B**) of 100 nM wortmannin. The cells were permeabilized with 0.05% saponin prior to fixation in order to remove cytosolic proteins. They were stained with anti-Hrs (left panels) and anti-clathrin (middle panels) and studied by confocal fluorescence microscopy. Arrows indicate examples of co-localization between Hrs, clathrin and internalized transferrin. Arrowheads point at the presumed TGN region stained with anti-clathrin. Bar, 5 µm.

the finding that phosphorylated Hrs dissociates from endosomes (Urbé *et al.*, 2000). In order to test these hypotheses it will be necessary to identify receptors that are recognized by Hrs–STAM.

Materials and methods

Antibodies and labelled proteins

Anti-myc antibodies were from the 9E10 hybridoma (Evan *et al.*, 1985). Human anti-EEA1 serum was a gift from Ban-Hock Toh. For immunoblotting, an antiserum against Hrs was prepared by injecting two rabbits with a synthetic peptide corresponding to residues 242–256 of mouse Hrs, coupled to keyhole limpet haemocyanin. The antiserum was affinity purified on recombinant Hrs_{1-289} (Gaullier *et al.*, 1998) immobilized on Affi-Gel (Bio-Rad, Hercules, CA). For immunocytochemistry, an antiserum against recombinant maltose-binding protein (MBP)–Hrs was raised in two rabbits. The antiserum was first adsorbed against immobilized MBP and then affinity purified on Affi-Gel beads containing immobilized MBP–Hrs. Mouse anti-clathrin heavy chain (HC) was from Research Diagnostics, Inc. (Flanders, NL) and goat anti-clathrin HC (C-20) was from Santa Cruz Biotechnology, Inc. Rabbit anti-clathrin LC (Liu *et al.*, 2001) was a gift from Frances Brodsky. Anti-AP1 (γ-adaptin) was purchased from Sigma, while anti-AP2 (α-adaptin) was from Affinity Bioreagents, Inc. Anti-AP3 (δ-adaptin) was provided by Stefan Höning. Rabbit anti-Eps15 (C-terminus) was from Berkeley Antibody Company (Berkeley, CA). Anti-dynamin (Hudy 1) was from Upstate Biotechnology. Rhodamine-, fluorescein isothiocyanate-, Cy3-, Cy5- and horseradish peroxidase-labelled secondary antibodies were from Jackson Immunoresearch (West Grove, PE). Rhodamine-labelled EGF and biotinylated dextran were from Molecular Probes, Inc. Rabbit-anti mouse IgG and rabbit-anti goat IgG antibodies used for electron microscopy were from Cappel.

Plasmid constructs

The Hrs constructs indicated were generated by PCR with mouse Hrs (Komada and Kitamura, 1995) as the template. Synthetic oligonucleotides were from MedProbe (Oslo, Norway). For expression in mammalian cells with the T7 RNA polymerase vaccinia virus system, constructs were cloned behind the myc epitope of pGEM-myc4 (Simonsen *et al.*, 1998a). For use in the two-hybrid system, constructs were cloned into pLexA/pBTM116 (Vojtek *et al.*, 1993) as bait and pGAD GH (Clontech) as prey. For expression of GST fusion proteins in *Escherichia coli*, pGEX-Hrs or

the deletion constructs indicated were obtained by subcloning the respective cDNAs into the polylinker sites of pGEX-6P (Amersham Pharmacia). $pGEX-2T-clathrin_{1-579}$ was a gift from Dr Jim Keen (Goodman *et al.*, 1997). For expression of a fusion protein between MBP and Hrs (MBP–Hrs), Hrs was cloned into the polylinker of pMAL-c2 (New England Biolabs).

Transient expression in BHK or HEp-2 cells

pGEM and pcDNA3 constructs were expressed in cells using modified Ankara T7 RNA polymerase recombinant vaccinia virus and lipofection as decribed (Stenmark *et al.*, 1995). Cells were analysed 6 h after transfection.

Confocal fluorescence microscopy

BHK, HEp-2 or FEMX-III melanoma cells (Fodstad *et al.*, 1988) grown on coverslips were fixed with 3% (w/v) paraformaldehyde (PFA) and stained for fluorescence microscopy as described (Simonsen *et al.*, 1998a). In some experiments, cells were permeabilized with 0.05% saponin prior to fixation. When indicated, cells were incubated with biotinylated dextran, rhodamine-labelled EGF, Alexa[488]–Tf (25 µg/ml) or wortmannin (100 nM) prior to fixation. Coverslips were examined using a Leica TCS NT confocal microscope equipped with a Kr/Ar laser and a PL Fluotar 100×/1.30 oil immersion objective. Appropriate emission filter settings and controls were included to exclude bleed-through effects.

Yeast two-hybrid methods

The yeast reporter strain L40 (Vojtek *et al.*, 1993) was co-transformed (Schiestl and Gietz, 1989) with the indicated pLexA and pGAD plasmids, and β-galactosidase activities of transformants were determined as previously described (Guarente, 1983).

Protein expression and purification

GST and various GST fusion proteins were produced in *E.coli* BL21 (DE3) cells as described previously (Callaghan *et al.*, 1999). The recombinant proteins were purified on glutathione–Sepharose 4B (Amersham Pharmacia Biotech AB, Uppsala, Sweeden) after lysis of the bacteria in B-PER™ reagent (Pierce). GST was cleaved from clathrin-TD_{1-579} by digestion with thrombin protease (Amersham Pharmacia Biotech), using the conditions recommended by the manufacturer. MBP–Hrs was expressed in *E.coli* BL21 (DE3) cells as described previously (Callaghan *et al.*, 1999) and purified on amylose resin (New England Biolabs) according to the manufacturer's instructions. Pig brain cytosol was prepared as previously described (Garred *et al.*, 2001).

GST pull-down assay

Aliquots (50 or 25 µl) of glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech) were washed three times with assay buffer (25 mM HEPES–KOH pH 7.2, 125 mM potassium acetate, 2.5 mM magnesium acetate, 5 mM EGTA and 1 mM dithiothreitol) before incubation with 0.4 or 0.1 nmol of GST, GST–Hrs$_{707-775}$ or GST–Hrs$_{707-770}$ in 200 µl of assay buffer for 30 min at room temperature. The beads were then washed twice in assay buffer before incubation with either 200 µl of pig brain cytosol or with 25 pmol recombinant clathrin-TD$_{1-579}$ [in assay buffer containing 10% fetal calf serum (FCS)] for 1 h at 4°C. Finally, the beads were washed four times with assay buffer and resuspended in SDS–PAGE sample buffer. Cytosolic clathrin HC associated with the beads was detected by SDS–PAGE, followed by immunoblotting with the goat anti-clathrin HC polyclonal antibody from Santa Cruz Biotechnology, Inc. and a SuperSignal chemiluminescence kit from Pierce. Recombinant clathrin-TD$_{1-579}$ associated with the beads was detected with the mouse anti-clathrin HC monoclonal antibody from Research Diagnostics, Inc.

Electron microscopy

Cells were either fixed immediately or incubated with 5 nm BSA-coated colloidal gold (Slot and Geuze, 1985) in the medium at 37°C for 1 h or 10 min to identify endosomal compartments. At the end of the incubation with BSA–gold, the cells were washed with phosphate-buffered saline (PBS) and immediately fixed with 0.1% glutaraldehyde/4% PFA in Soerensen phosphate buffer. Following fixation, the cells were scraped from the culture dish, pelleted, infused with 2.3 M sucrose, mounted, frozen and stored in liquid nitrogen. Immunocytochemical labelling was performed on thawed cryosections as described (Griffiths *et al.*, 1984), using different primary antibodies followed by 10 or 15 nm protein A–gold (purchased from G.Posthuma and J.Slot, Utrecht, The Netherlands) either directly or after incubation with secondary antibodies. The labelled cryosections were viewed in a Philips CM120 electron microscope.

Transferrin endocytosis and recycling

In order to study the effect of overexpressed Hrs on Tf endocytosis and recycling, BHK cells, which have low endogenous levels of Tf receptors, were co-transfected with human Tf receptor and the Hrs constructs indicated. With the vaccinia system, the level of co-transfection was found to be >95%, and non-transfected cells only contributed to a minor extent to the measured endocytosis. Endocytosis and recycling of Tf were measured using the ORIGEN analyser (IGEN Inc., Rockville, MD), which is based on electrochemiluminescence detection. Human holo-Tf (Sigma, St Louis, MO) was labelled with *N*-hydroxysuccinimide ester-activated tris (bipyridine)-chelated ruthenium(II) (Ru-tag) (IGEN Inc.) according to the manufacturer's instructions, and simultaneously labelled with the reducable NHS-SS-Biotin (Pierce) for recycling measurements or with Biotin-LC-Sulpho NHS Ester (IGEN Inc.) for the study of endocytosis. For the measurement of Tf recycling, transfected BHK cells were washed twice with HEPES medium and incubated with Ru-tag-labelled Tf (50 ng/ml) in the presence of BSA (2 mg/ml) for 30 min at 37°C. A portion of the cells were then treated with 0.1 M 2-mercaptoethanesulfonic acid (MESNA) for 1 h to reduce the SS-linked biotin in the cell surface-bound Tf. Only Tf that is Ru-tag-labelled and still biotinylated is detected in the cell lysate using streptavidin beads (Dynal, Oslo, Norway) and the ORIGEN analyser. Cells treated with MESNA correspond to the amount of endocytosed Tf, whilst untreated cells correspond to the total amount of Tf associated with the cells. A portion of the MESNA-treated cells were incubated for a further 2–15 min at 37°C for recycling measurements, and re-treated with MESNA to remove signal from any surface-bound Tf. For endocytosis experiments, cells were incubated with Ru-tag-labelled Tf for 15 min at 37°C for the measurement of total cell-associated Tf. Endocytosed Tf was measured after removal of surface-bound Tf by 2 mg/ml pronase (Sigma).

Degradation and transport of EGF and dextran

Transfected HEp-2 cells were washed twice in HEPES medium and incubated with rhodamine-labelled EGF (200 ng/ml) for 1 h at 37°C in the presence of BSA (2 mg/ml). In the case of dextran, cells were pre-incubated with 10% FCS on ice for 30 min followed by internalization of biotinylated dextran (5 mg/ml) for 1 h at 37°C. The cells were then washed three times in HEPES medium and incubated for various times up to 3 h in HEPES medium containing cycloheximide (10 µg/ml). The cells were permeabilized with 0.05% saponin prior to fixation in 3% PFA, and stained for confocal microscopy. For the quantitation of EGF degradation, images from 10 transfected and 10 non-transfected cells from each time point were taken, each without changing intensity settings for the laser

and photomultiplier. The mean intensity of the rhodamine-EGF signal from each cell was quantified with the software provided with the Leica TCS NT confocal microscope.

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