The UIM domain of Hrs couples receptor sorting to vesicle formation

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Summary

Hepatocyte growth factor regulated tyrosine kinase substrate (Hrs), a main component of the 'bilayered' clathrin coat on sorting endosomes, was originally identified as a substrate of activated tyrosine kinase receptors. We have analysed Hrs phosphorylation in response to epidermal growth factor (EGF) stimulation and show that the evolutionary conserved tyrosines Y329 and Y334 provide the principal phosphorylation sites. Hrs is proposed to concentrate ubiquitinated receptors within clathrin-coated regions via direct interaction with its UIM (ubiquitin interaction motif) domain. We show that the same UIM domain is necessary for EGF-stimulated tyrosine phosphorylation of Hrs. Over-expression of wild-type Hrs or a double mutant, Y329/334F, defective in EGF-dependent phosphorylation, both substantially retard EGF receptor (EGFR) degradation by inhibiting internal vesicle formation and thereby preventing EGFR incorporation into lumenal vesicles of the multivesicular bodies. In contrast, mutation or deletion of the Hrs-UIM domain strongly suppresses this effect. In addition the UIM-deletion and point mutants are also observed on internal membranes, indicating a failure to dissociate from the endosomal membrane prior to incorporation of the receptor complex into lumenal vesicles. Our data suggest a role for the UIM-domain of Hrs in actively retaining EGFR at the limiting membrane of endosomes as a prelude to lumenal vesicle formation.

Key words: Hrs, Endocytosis, Clathrin, Ubiquitin, Phosphorylation

Introduction

Hepatocyte growth factor (HGF)-regulated tyrosine kinase substrate (Hrs) is a prominent target for tyrosine phosphorylation following activation of tyrosine kinase receptors (Komada and Kitamura, 1995). It was initially shown to lie downstream of the HGF/scatter factor receptor c-Met, but activation of other tyrosine kinase receptors and stimulation with cytokines such as IL-2 and GM-CSF also results in phosphorylation of Hrs (Asao et al., 1997). EGF-dependent phosphorylation of Hrs requires coincident localisation of activated receptor and Hrs at early endosomes, to which Hrs is targeted by interaction of its FYVE domain with PtdIns3*P* (Urbé et al., 2000).

A role for Hrs in receptor sorting was initially proposed by analogy with its yeast orthologue Vps27. Vps27 belongs to the class E set of Vps mutants, defective in transport from the sorting endosome to the vacuole, by virtue of an inability to generate cargo-laden vesicles that bud from the limiting membrane into the endosomal lumen (Bankaitis et al., 1986; Raymond et al., 1992). The *Drosophila* homologue of Hrs is required for invagination of endosomal membranes (Lloyd et al., 2002). Over-expression of mammalian Hrs leads to a block in lysosomal trafficking of receptors, retaining them in enlarged early endosomes that also accumulate mannose 6-phosphate receptor (Urbé et al., 2000).

Receptor sorting to lumenal vesicles frequently requires receptor ubiquitination (Hicke, 1999; Katzmann et al., 2001; Reggiori and Pelham, 2001; Urbanowski and Piper, 2001). Hrs contains a ubiquitin interaction motif (UIM), which was initially predicted on the basis of a bioinformatic analysis revealing homology with a 20 amino acid stretch of the S5 subunit of the proteasome that directly interacts with ubiquitin (Hofmann and Falquet, 2001). The UIM of Hrs is shared with a number of proteins known to be involved in endocytic trafficking. These include epsins, eps15 and eps15R that are believed to regulate receptor internalisation at the plasma membrane (Shih et al., 2002). A number of groups have now experimentally verified the ubiquitin binding properties of the UIM domain, including that of Hrs (Bilodeau et al., 2002; Bishop et al., 2002; Klapisz et al., 2002; Lloyd et al., 2002; Polo et al., 2002; Raiborg et al., 2002; Shih et al., 2002). A recent paper has revealed that the UIM domain does not only specify non-covalent binding to ubiquitin, but is also necessary for mono-ubiquitination of the UIM-containing proteins at a site lying outside the UIM. Eps15, Eps15R, Hrs and epsins 1 and 2 were each shown to be mono-ubiquitinated to varying degrees following activation of the EGFR (Klapisz et al., 2002; Polo et al., 2002; van Delft et al., 1997).

Hrs is concentrated in flat 'bilayered' clathrin coats that decorate the surface of predominantly early endosomes (Clague, 2002; Raiborg et al., 2002; Sachse et al., 2002). These coats also provide a site of concentration for receptors destined

for lysosomal degradation (EGFR and growth hormone receptor) but not for the recycling transferrin receptor (Sachse et al., 2002). It has been proposed that Hrs may act as an adaptor protein between ubiquitinated receptors and the clathrin coat (Clague, 2002). Indeed internalized transferrin can be retained within the early endosome if the cytoplasmic domain of the transferrin receptor is fused in frame to ubiquitin (Raiborg et al., 2002). In yeast, the UIM domain of Vps27 was shown to be required for efficient delivery of cargo to the lumen of the vacuole (Bilodeau et al., 2002; Shih et al., 2002).

So far, none of the identified components of the bilayered clathrin coat was found incorporated into lumenal vesicles, which often seem to form at the edges of coated areas (Sachse et al., 2002). This suggests a breaking of the connection between receptor cargo and the coat prior to vesicle incorporation, which may involve localised coat disassembly. Intriguing molecular explanations include competition for the Hrs UIM domain by other ubiquitinated factors at the endosome and translocation of phosphorylated Hrs to the cytosol (Clague, 2002; Sachse et al., 2002; Urbé et al., 2000).

We have identified the major sites of phosphorylation on Hrs following EGF stimulation and we show that the UIM domain is required for phosphorylation. Conversely, phosphorylation is not required for UIM-dependent ubiquitination. These observations have allowed us to examine the requirement for Hrs phosphorylation and Hrs-ubiquitin interaction with respect to its function in the sorting of EGFR. Together, fluorescence and electron microscopic studies suggest that the UIM-domain of Hrs plays an important role in the co-ordination of the sorting of EGFR from the clathrin-coated limiting endosomal membrane into the lumenal vesicles as well as in the concomitant dissociation of Hrs from the receptor complex.

Materials and Methods

Cell culture, plasmids and transfections

HeLa cells were cultured in a 5% CO_2 atmosphere in Dulbecco's modified Eagle medium supplemented with 10% FBS and 1% non-essential amino acids. All tissue culture reagents were purchased from Invitrogen Life Technologies, UK. For overexpression of GFP-tagged Hrs, the ORF of mouse Hrs was cloned into pEGFP-C (Clontech; details available on request). The UIM-deletion mutant (amino acids 257-278 deleted) was generated by PCR-based mutagenesis in pGEMT-Hrs, sequenced and the *Pml*I-*Xho*I fragment was subcloned into pEGFP-Hrs (GFP-ΔUIM). The point mutants GFP-Y329F, GFP-Y334F, GFP-Y329/334F and GFP-L269/S270A (GFP-LSAA) were generated by site directed mutagenesis of pGEMT-Hrs by Quickchange mutagenesis (Stratagene; primers available on request), sequenced and the *Pml*I-*Xho*I fragment subcloned into pEGFP-Hrs. HeLa cells were transfected according to a standard calcium phosphate precipitation method or with FuGENE 6 according to the manufacturer's instructions (Roche Diagnostics, UK). Typically 30-50% of cells expressed GFP-Hrs 22 hours post-transfection.

Antibodies and other reagents

Hrs polyclonal antibody generated against a C-terminal peptide has been described previously (Sachse et al., 2002). The rabbit polyclonal anti-phospho-Y334-Hrs antibody was generated against the peptide C-LNRN-Y(Pi)-WEKK. Monoclonal anti-ubiquitin antibody FK2 was purchased from Affiniti, UK. The anti-GFP antibody used in biochemical experiments was affinity-purified from sheep antiserum and has previously been described (Barr et al., 2001). Anti-transferrin receptor antibody was purchased from Roche Diagnostics, UK and the anti-EGFR antibody was a gift of Harry Mellor, Bristol, UK. The CI-M6PR antibody was a kind gift from Dr Paul Luzio, Cambridge, UK (Reaves et al., 1996). The anti-phosphotyrosine monoclonal antibody, PY20, was obtained from Transduction Laboratories. Purified mouse EGF was obtained from Dr J. Smith, Liverpool, UK. Fluorescent secondary antibodies were from Molecular Probes; HRP-coupled secondary antibodies, Protein A- and Protein G-agarose were purchased from Sigma. Antibodies used for immunogold labelling were as follows: Rabbit antiserum against GFP was a kind gift from Dr David Shima (Cancer Research, United Kingdom). Sheep antiserum against EGFR was obtained from Invitrogen (Leek, The Netherlands). Rabbit antiserum against sheep IgG was obtained from Nordica (Tilburg, The Netherlands). Rabbit antiserum against clathrin heavy chain was a kind gift from Dr S. Corvera (University of Massachusetts, MA).

Mass spectrometry

Proteins were extracted from Coomassie-Blue-stained gel slices and digested with trypsin using a protocol modified from that of Shevchenko et al. (Shevchenko et al., 1996). Excised gel pieces were cut into 1 mm cubes and transferred to a 1.5 ml microcentrifuge tube, then washed in 100 μl volumes of 50 mM NH_4HCO_3 for 10 minutes, and 100 μl acetonitrile for 10 minutes. This washing step was repeated twice. Acetonitrile was then removed by centrifugation in a vacuum centrifuge for 5 minutes at 40°C. To reduce the protein the gel particles were swelled in 10 μl of 10 mM DTT in 50 mM NH_4HCO_3 and incubated for 45 minutes at 56°C. Once cooled to room temperature excess liquid was removed and replaced immediately with 10 μl of freshly prepared 55 mM iodoacetamide in 50 mM NH_4HCO_3 for 30 minutes at room temperature in the dark. Gel particles were washed with 100 μl of 50 mM NH_4HCO_3 followed by 100 μl of acetonitrile for 10 minutes. Acetonitrile was then removed by centrifugation in a vacuum centrifuge for 5 minutes at 40°C, and the gel pieces rehydrated in 10 μl of 12.5 ng/μl sequencing-grade porcine trypsin (Promega) in 50 mM NH_4HCO_3 on ice. After 15 minutes, 10 μl of 50 mM NH_4HCO_3 were added and digestion allowed to proceed at 37°C for 16 hours. The peptide digests were analysed by peptide mass fingerprinting using a MALDI-TOF instrument (Reflex III, Bruker) and probability-based database searching (Perkins et al., 1999).

EGF stimulation and detection of phosphorylated Hrs and GFP-Hrs

Cells were starved for 16 hours in serum-free medium and then stimulated with 100 ng/ml EGF. The cells were washed twice with ice cold PBS and lysed for 20 minutes on ice in lysis buffer (25 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5% NP40, 50 mM NaF, supplemented with mammalian protease inhibitor cocktail and phosphatase inhibitor cocktail II; Sigma). Where indicated, 10 mM NEM was included in the lysis buffer. The lysate was pre-cleared by centrifugation and 0.7 to 1 mg of protein at 1 mg/ml was incubated with 5 μl of anti-Hrs or 1.5 μl anti-GFP and Protein A- or Protein G-sepharose respectively. Immunoprecipitates were washed three times with 25 mM Tris-HCl pH 7.5, 150 mM NaCl, supplemented with phosphatase inhibitor cocktail II and then once with 10 mM Tris pH 7.5 before preparation for SDS-PAGE (8% gels unless indicated otherwise). Following SDS-PAGE, proteins were either transferred to PVDF or nitrocellulose membranes (Millipore and Schleicher & Schuell, 0.45 μm) which were blocked overnight with blocking buffer, 1% BSA/0.1% Tween-20 in 10 mM Tris, pH 7.5, 100 mM NaCl or with 5% skimmed milk powder, 0.2% Triton X-100 in 10 mM Tris, pH 7.5, 100 mM NaCl. Primary and secondary antibody incubations were for 2 and 1 hours in the respective blocking buffer. Development of western blots was

Table 1. MALDI-TOF analysis of tryptic peptides derived from immunoprecipitated Hrs

Peptide sequence	Amino acids	Expected mass (Da)	Observed mass (Da)	Phosphate	EGF-treatment
YLNRNYWEK	329-337	1284.63	1284.57	0	No
NYWEKKQEEAR	333-343	1480.65	1480.65	0	No
YLNRNYWEK	329-337	1364.59	1364.56	+1	Yes
YLNRNYWEKK	329-338	1492.38	1492.39	+1	Yes

Hrs was immunoprecipitated from either non-stimulated or EGF-stimulated cells (8 minutes, 100 ng/ml) and proteins were separated by SDS-PAGE, extracted from gel pieces and digested with trypsin as described in Materials and methods. The peptide digests were analysed by MALDI-TOF and peptides corresponding to 26% of human Hrs sequence could be identified in both EGF-treated and untreated samples. Note that the pattern of trypsin cleavage is altered for the phosphorylated protein: for example peptide 329-338 is only seen in the EGF-treated sample.

by ECL using Pierce Supersignal. Blots were routinely stripped and reprobed to assess that equal amounts of protein had been immunoprecipitated in each sample.

Boiling SDS-lysis method

For the detection of ubiquitinated GFP-Hrs, transfected cells were lysed immediately, without prior washing, in 400 μl boiling lysis buffer (2% SDS, 1 mM EDTA, 50 mM NaF, supplemented with phosphatase inhibitor cocktail II and protease inhibitor cocktail, preheated at 110°C). The lysates were transferred to screw-cap tubes and incubated for a further 10 minutes at 110°C before being cooled to room temperature. The lysates were then diluted with 4 volumes of dilution buffer (2.5% Triton X-100, 12.5 mM Tris pH 7.5, 187.5 mM NaCl and phosphatase inhbibitor cocktail II and mammalian protease inhibitor cocktail) to a final concentration of 0.4% SDS, 2% Triton X-100. The lysates were precleared by centrifugation and subjected to immunoprecipitation overnight at 4°C with 1.5 μl anti-GFP antibody and Protein G-agarose. Immunoprecipitates were washed three times with Triton X-100/SDS wash buffer (2% Triton X-100, 0.4% SDS, 10 mM Tris pH 7.5, 150 mM NaCl) and then once with 10 mM Tris pH 7.5 before preparation for SDS-PAGE.

Preparation of membrane and cytosolic fractions

Cells were homogenised in homogenisation buffer (HB: 10 mM Hepes, 3 mM imidazole-HCl, pH 7.2, 250 mM sucrose, mammalian protease inhibitor cocktail) by repeated passage through a 23G needle at 4°C and then immediately supplemented with 50 mM NaF and phosphatase inhibitor cocktail II. Membrane/particulate and cytosolic fractions were prepared from post nuclear supernatants by ultracentrifugation for 15 minutes at 65,000 rpm in a Beckman TLA 100.2 rotor in an Optima-Max Ultracentrifuge.

Immunofluorescence

Transfected cells grown on cover slips were washed twice with PBS and fixed with 3% paraformaldehyde (PFA, TAAB, UK) in PBS. Residual PFA was quenched with 50 mM NH_4Cl/PBS. Cells were permeabilised with 0.2% Triton X-100/PBS and blocked with 10% goat serum in PBS. All antibody dilutions were in 5% goat serum and incubation times were 20-30 minutes at room temperature. Cover slips were mounted using Moviol and cells were viewed using a BioRad LaserSharp confocal microscope. Z-sections were taken at 260 nm steps and analysed with the accompanying software.

Immunogold electron microscopy on cryosections

Cells transfected with GFP-Hrs, GFP-Y329/334F, GFP-ΔUIM, GFP-LSAA or mocktransfected with FuGENE 6 (Roche Diagnostics) were starved for 16 hours in serum-free medium and stimulated 22 hours post-transfection with EGF (100 ng/ml). Cells were then fixed immediately with 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 2.5 hours at room temperature. The fixative was removed and free aldehydes were quenched with 50 mM glycine in PBS. Embedding in 12% gelatin and preparation for ultrathin cryosectioning and immunogold labeling was done as described previously (Raposo et al., 1997).

The relative distribution of EGFR label between the limiting membrane and internal membranes of endosomal vacuoles under different conditions was determined in the electron microscope on grids double labeled with rabbit anti-GFP (10 nm gold) and sheep anti-EGFR (15 nm gold). For each transfection at least 3 times 10 cell profiles, with a nucleus visible in the plane of the section, were analyzed. Gold particles representing EGFR within a distance of 20 nm of a membrane were considered as membrane-associated and assigned to the subdomain of the endosomal vacuole to which they localized. In parallel, the number of EGFR-positive vacuoles per cell profile was determined as well as the number of internal vesicles that they contained.

Results

Y334 is the major phosphorylated tyrosine residue of Hrs in response to EGF-stimulation

In order to identify tyrosine residues that are phosphorylated in response to EGF stimulation, Hrs was immunoprecipitated from HeLa cell lysates derived from cells that had been treated with or without EGF for 8 minutes (Urbé et al., 2000). After SDS-PAGE the Hrs band was excised and trypsin-digested in preparation for MALDI-TOF analysis as described in Materials and methods. Peptides corresponding to 26% of the human Hrs sequence could be identified by peptide mass fingerprinting. A peptide corresponding to amino acids 329-338 plus phosphate was detected in the EGF-treated sample (Table 1) and was observed to loose a characteristic mass of 80 Da on post source decay, indicative of phosphorylation. Two tyrosines (Y329, Y334) lie within this sequence, but the mass of the peptide suggests a single phosphorylation indicating that both sites are not simultaneously phosphorylated. Furthermore this peptide is unlikely to be generated if Y329 was phosphorylated, as trypsin cleavage occurs with reduced efficiency when arginine neighbours a phospho-amino acid. We can therefore conclude that Y334 is phosphorylated in response to EGF, but cannot exclude phosphorylation at Y329.

We generated a polyclonal antibody against an Hrs peptide containing phospho-Y334 to obtain antibodies specific to phosphorylated Hrs. Fig. 1A shows that the resultant antibody specifically recognises a band at the appropriate molecular mass only upon EGF stimulation leading to Hrs phosphorylation. This result was confirmed with a second antibody generated against the same peptide isolated from a different rabbit (data not shown).

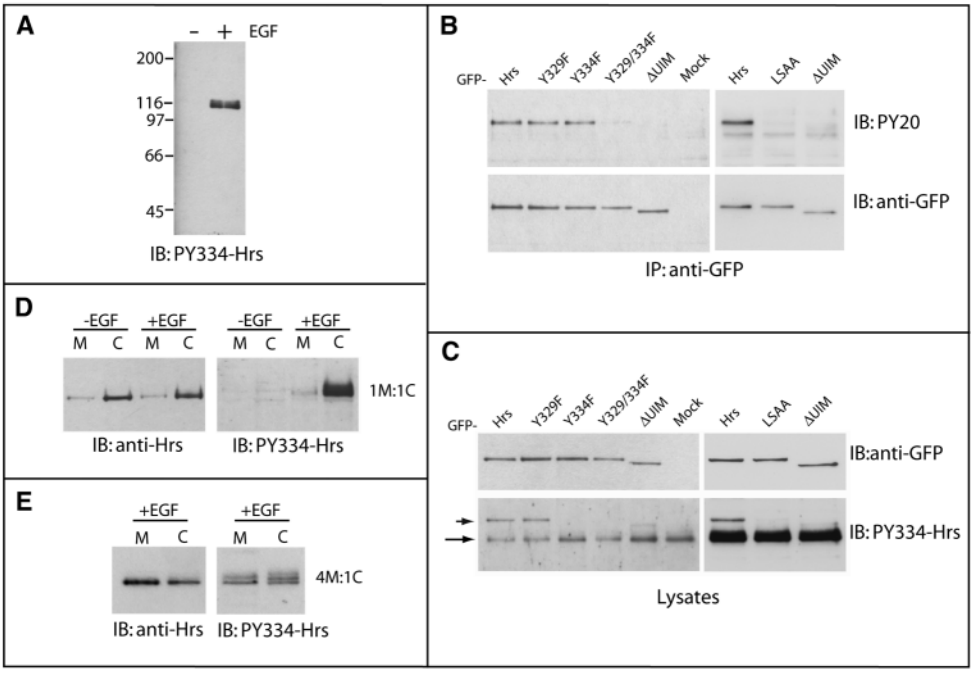

Fig. 1. Tyrosine phosphorylation of Hrs in response to EGF. (A) An antibody directed against an Hrs peptide containing phospho-Y334 recognizes Hrs specifically in response to EGF stimulation. HeLa cells were starved for 16 hours in serum-free medium and either not stimulated or stimulated for 8 minutes with EGF (100 ng/ml). Lysates were analysed by immunoblotting with anti-PY334-Hrs antibody. Molecular mass markers are indicated. (B,C) EGF-dependent tyrosine phosphorylation of Hrs is dependent on the presence of either Y329 or Y334 as well as on an intact UIM domain. (B) HeLa cells were transfected with GFP-Hrs, GFP-Y329F, GFP-Y334F, GFP-Y329/334F, GFP-ΔUIM, GFP-LSAA or mock-transfected, starved 16 hours in serum-free medium and then stimulated 22 hours post-transfection with EGF (100 ng/ml). Lysates were prepared and subjected for immunoprecipitation with anti-GFP antibody. Phosphorylation was assessed by immunoblotting with PY20 antibody. (C) Lysates were prepared as described in b and analysed by immunoblotting with anti-GFP and anti-PY334-Hrs antibodies respectively. The arrowhead indicates GFP-PY334-Hrs, the arrow indicates endogenous PY334-Hrs. (D) PY334-Hrs is enriched in the cytosol. HeLa cells were starved for 16 hours in serum-free medium and stimulated for 8 minutes with EGF (100 ng/ml) or left unstimulated. Membrane and cytosol fractions were prepared as described in the Materials and Methods. The relative distribution of tyrosine phosphorylated Hrs was assessed by analysing equal proportions of membrane and cytosolic fractions by immunoblotting with anti-Hrs and anti-PY334-Hrs antibodies. (E) Membrane and cytosol fractions were prepared from cells stimulated for 8 minutes with EGF, and the specific enrichment of Hrs phosphorylated at Y334 was analysed by loading four times more membrane fraction than cytosol fraction on SDS-PAGE followed by immunoblotting with anti-Hrs and anti-PY334-Hrs antibodies as in D.

The UIM-domain of Hrs is required for tyrosine phosphorylation downstream of EGFR

We made a deletion mutant (ΔUIM) and a double point mutant (L269A/S270A, LSAA) of the UIM domain as well as Y329F and Y334F mutants of GFP-Hrs and the corresponding double tyrosine mutant Y329/334F and expressed them in HeLa cells. Following EGF stimulation, each of the single tyrosine to phenylalanine mutants was phosphorylated with a similar efficiency to wild type as determined by blotting with PY20 antibody. However, phosphorylation of the double mutant was dramatically reduced (Fig. 1B) although some residual signal was evident at long exposures of the PY20 blot. Taken together these results suggest that in the absence of Y334, Y329 can serve as a phosphorylation site whilst phosphorylation at both sites simultaneously is unlikely to contribute substantially to the phospho-tyrosine signal observed. Deletion of the UIM domain, which lies 50 amino acids upstream of Y329, or mutation to alanine of L269 and S270 within this domain, ablated phosphorylation at both sites.

These mutant constructs also provided an additional control for our phospho-specific Hrs antibody. As expected, neither the Y334F point mutant nor the double point mutant protein were recognised by our antibody, in contrast to the Y329F mutant, which was as efficiently detected as the wild-type protein (Fig. 1C).

We next tested the specific activity of phospho-Y334 in membrane and cytosol fractions using our antibody (Fig. 1D). Previously, we have used immunoprecipitation of Hrs with a polyclonal antibody [raised against recombinant Hrs (Komada and Kitamura, 1995)] followed by PY20 blotting and observed a higher specific activity of phospho-Hrs in the cytosolic fraction indicative of translocation from membrane to cytosol (Urbé et al., 2000). We have now repeated these experiments with a polyclonal anti-peptide antibody (Sachse et al., 2002) and confirmed our results (data not shown). The availability of a specific anti-phospho-Hrs antibody allows us to directly probe for the relative amounts of total Hrs and phospho-Y334-Hrs in membrane and cytosol fractions without the need of immunoprecipitation. As shown in Fig. 1D, when equal proportions of membrane and cytosol fractions are analysed in parallel, the majority of phospho-Hrs is clearly found in the cytosol in agreement with our previous findings. When instead the loading of membrane and cytosolic proteins is roughly normalised to Hrs levels by comparing four parts of membranes to one part of cytosol, only a modest enrichment of phospho-Y334-Hrs-specific activity in the cytosol is evident (Fig. 1E). Differences in enrichment profiles associated with alternative experimental approaches may reflect the distribution of extra phosphorylation sites (e.g. Y329) recognised by the PY20 antibody, or the efficiency of immunoprecipitation of phospho-Hrs from cytosolic and particulate pools.

Association of Hrs with ubiquitinated proteins is independent of tyrosine-phosphorylation but requires an intact UIM-domain

Immunoprecipitation of GFP-tagged Hrs as well as GFP-

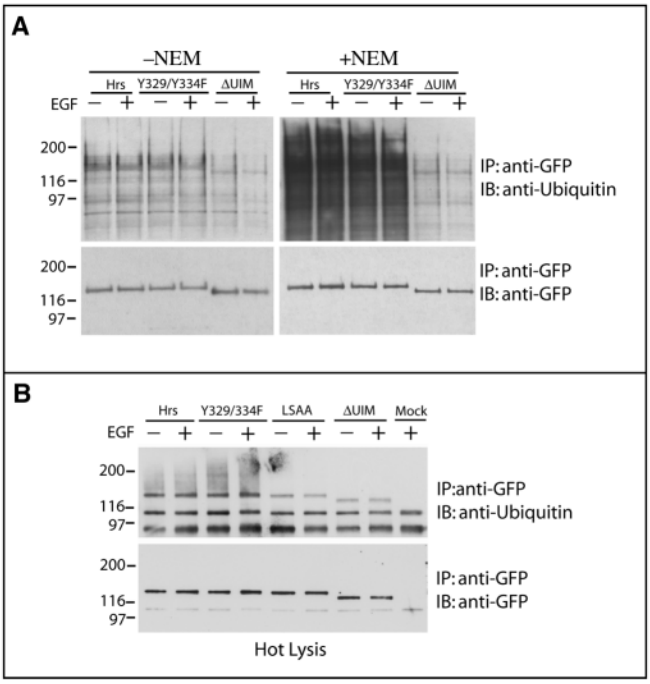

Fig. 2. Ubiquitination of overexpressed Hrs is constitutive and independent of tyrosine phosphorylation. (A) HeLa cells were transfected with GFP-Hrs, GFP-Y329F, GFP-Y334F, GFP-Y329/334F or GFP-ΔUIM, starved for 16 hours in serum-free medium and then stimulated 22 hours post-transfection for 8 minutes with EGF (100 ng/ml) or left unstimulated. Lysates were prepared in the presence or absence of 10 mM NEM and subjected to immunoprecipitation with anti-GFP antibody. Ubiquitination was assessed by immunoblotting with anti-ubiquitin antibody. Molecular mass markers are indicated on the left. (B) HeLa cells were transfected with GFP-Hrs, GFP-Y329F, GFP-Y334F, GFP-Y329/334F, GFP-LSAA, GFP-ΔUIM or mock transfected, and treated as in A. Lysates were prepared according to the 'boiling SDS-lysis' method (see Materials and Methods) and subjected to immunoprecipitation with anti-GFP antibody.

Y329/334F from transfected cell lysates followed by immunoblotting with anti-ubiquitin antibodies, revealed the presence of a large number of ubiquitinated proteins of both higher and lower molecular mass (Fig. 2A) indicating that Hrs participates in a network of interactions involving ubiquitinated proteins which does not require phosphorylation of Y334 or Y329. In concurrence, EGF stimulation did not have any significant effect on the level of ubiquitinated proteins associated with overexpressed GFP-Hrs and GFP-Y329/334F.

Deletion of the UIM domain resulted in a significant, but by no means complete, reduction in the ubiquitin signal when compared to wild-type Hrs. We reasoned that some of the ubiquitinated proteins might actually be lost during our experimental procedures as a result of de-ubiquitination activities in the cell lysates (Mimnaugh et al., 1999), which are active at low temperatures. Levels of ubiquitinated proteins co-immunoprecipitating with GFP-Hrs and GFP-Y329/334F were dramatically enhanced by inclusion of NEM in the lysis buffer to inhibit these enzymes, suggesting that the majority of the associating proteins may indeed be de-ubiquitinated during cell lysis. Significantly, the ubiquitin-signal detected in the GFP-ΔUIM immunoprecipitates was not affected by the presence of NEM and was substantially lower in comparison to GFP-Hrs and GFP-Y329/334F.

Ubiquitination of Hrs is independent of tyrosine-phosphorylation

Inclusion of NEM in the lysis buffer to inhibit de-ubiquitination does not allow clear visualisation of ubiquitinated Hrs due to the co-immunoprecipitating ubiquitinated proteins. We therefore next subjected transfected cells to 'Boiling SDS-lysis' (see Materials and Methods), which not only inactivates de-ubiquitinating enzymes but also destroys many (if not all) protein-protein interactions. Immunoprecipitations from GFP-Hrs transfected cell lysates prepared in this way, immunoblotted with anti-ubiquitin antibodies revealed a sharp band that is not found in mock-transfected cells, which we presume to be mono-ubiquitinated GFP-tagged Hrs. This band was present in similar amounts whether the cells were stimulated with EGF or not, and when comparing wild-type protein with phosphorylation defective mutants (Y329/Y334F) (Fig. 2B). In addition, we also noted the presence of a higher molecular mass smear that may represent poly-ubiquitinated forms of Hrs. Hrs phosphorylation is therefore not required for ubiquitination. In agreement with Polo et al. (Polo et al., 2002), we find a marked reduction in the degree of ubiquitination for both GFP-ΔUIM and GFP-LSAA mutants, that is most strikingly manifested by the complete absence of a poly-ubiquitin smear associated with these two mutants (Fig. 2B).

Phosphorylation-defective Hrs localises to early endosomes

We next used fluorescence microscopy to examine the distribution of the mutant and wild-type proteins and establish their effect on EGFR degradation (Figs 3, 4 and 5). Over-expression of wild-type protein leads to enlarged clusters of endosomes, which retain EGFR and prevent its degradation (Komada and Soriano, 1999; Urbé et al., 2000). All three transfected proteins (Hrs, Y329/334F and ΔUIM) colocalised to a large degree with transferrin receptor (Fig. 3A-C) and EEA1 (not shown). However, the ΔUIM mutant failed to form large Hrs-containing perinuclear endosomal clusters that are characteristic of wild-type Hrs overexpression: fluorescent punctae were smaller and more widely dispersed throughout the cell (Fig. 3C,F). In contrast, in cells overexpressing the Y329/334F mutant these large structures were accentuated and more frequently observed even at lower expression levels (Fig. 3B,E). The cation-independent mannose 6-phosphate receptor (M6PR), becomes entrapped in these large endosomal structures in cells expressing wild-type and Y329/334F Hrs, presumably because its trafficking itinerary through the endosomal pathway is inhibited (Fig. 3D,E). Accumulation of M6PR in Hrs positive compartments was not apparent with the ΔUIM mutant (Fig. 3F). None of the constructs colocalised with Lamp1 or had any effect on its distribution (data not shown).

Neither tyrosine phosphorylation, nor an intact UIM domain is necessary for colocalisation of Hrs with the EGFR in early endosomes

Illustrated in Fig. 4 are cells expressing low amounts of over-

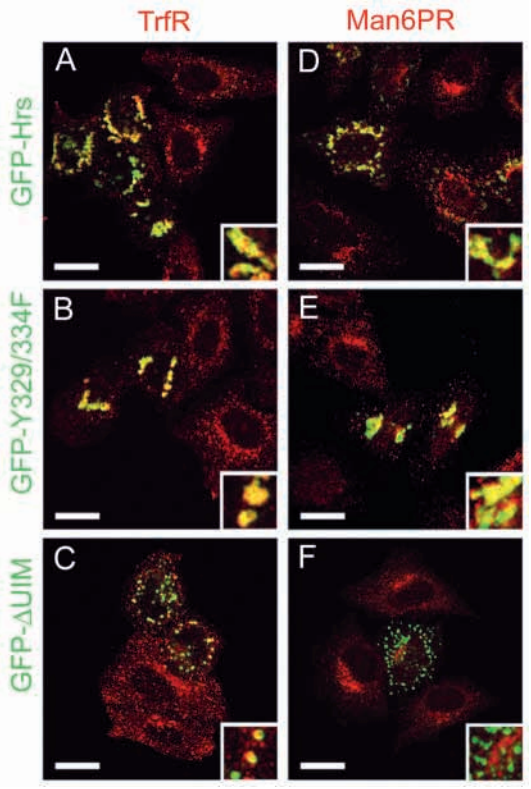

Fig. 3. Immunolocalisation of GFP-Hrs, GFP-Y329/334F and GFP-ΔUIM. HeLa cells were transfected with GFP-Hrs (A,D), GFP-Y329/334F (B,E) or GFP-ΔUIM (C,F) and fixed 22 hrs post-transfection with 3% PFA, permeabilised with Triton X-100 and stained with either anti-transferrin receptor (TrfR, A-C, shown in red) or anti-mannose-6-phosphate receptor (Man6PR, D-F, shown in red) antibodies followed by secondary antibodies coupled to Alexa Fuor 594. All panels show a single confocal section of a group of cells presenting the most typical staining pattern for each construct. Insets show a threefold enlargement of selected areas. Scale bars: 20 μm.

expressed protein, with minimal effect on endosomal morphology, which have been treated for 8 minutes with EGF. All three proteins can be seen to decorate endosomal compartments accessible to EGFR. The failure of the ΔUIM mutant to undergo EGF-dependent phosphorylation cannot therefore reflect a lack of coincident localisation between receptor and Hrs.

EGFR degradation is blocked by over-expression of wild-type and Y329/334F Hrs (Fig. 5). Note the intensity of EGFR in transfected cells compared to neighbouring untransfected cells (essentially at background levels) all treated for 4 hours with EGF. In the case of the ΔUIM mutant this effect is much less pronounced. A typical picture is seen in Fig. 5G-I wherein a significant fraction of cells expressing GFP-ΔUIM exhibit close to background levels of EGFR, whilst in other neighbouring cells EGFR can still be detected in dispersed Hrs-ΔUIM-positive punctae.

The UIM-domain of Hrs is required for efficient retention of the EGFR and Hrs at the limiting membrane of the early endosome

Next, we examined the distribution of the mutant proteins by immunoelectron microscopy (Fig. 6). As previously reported, over-expression of Hrs results in increased recruitment of the clathrin coat to vacuolar endosomes, such that many profiles are completely covered with clathrin (Raiborg et al., 2001). For this reason, relative enrichment of markers in coated regions cannot be quantified under conditions of over-expression. It is noteworthy that the coat recruited as a result of Hrs over-expression does not have the characteristic 'bilayered' appearance as in control cells. Over-expression of Hrs reduces the number of lumenal vesicles and the proportion of EGFR labelling (post-stimulation) associated with these vesicles (Fig. 7 and Table 2). 77±8.2% of EGFR is retained at the limiting membrane of endosomes when Hrs is over-expressed, compared with 16±5.6% in control cells. In addition, the number of lumenal vesicles in EGFR-positive endosomal vacuoles

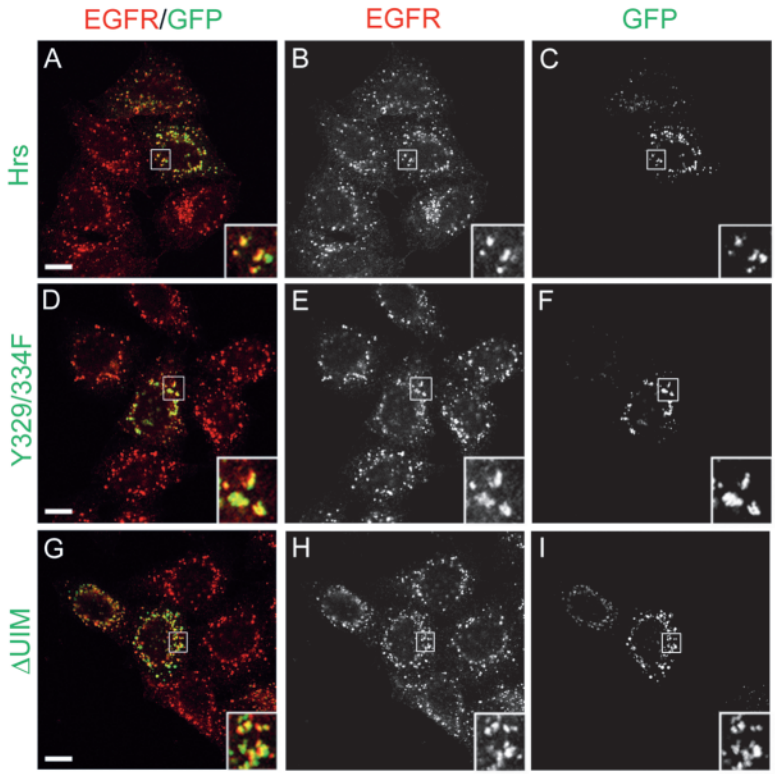

Fig. 4. Colocalisation of Hrs with activated EGFR does not depend on tyrosine phosphorylation of Hrs or on an intact UIM domain. HeLa cells were transfected with GFP-Hrs (A-C), GFP-Y329/334F (D-F) or GFP-ΔUIM (G-I), starved 16 hours in serum-free medium and stimulated 22 hours post-transfection for 8 minutes with EGF (100 ng/ml). The cells were then fixed with 3% PFA, permeabilised with Triton X-100 and stained with anti-EGFR (EGFR shown in red) followed by secondary antibody coupled to Alexa Fluor 594. All panels show a single confocal section of low-expressing cells. Insets show a threefold enlargement of selected areas. Scale bars: 10 μm.

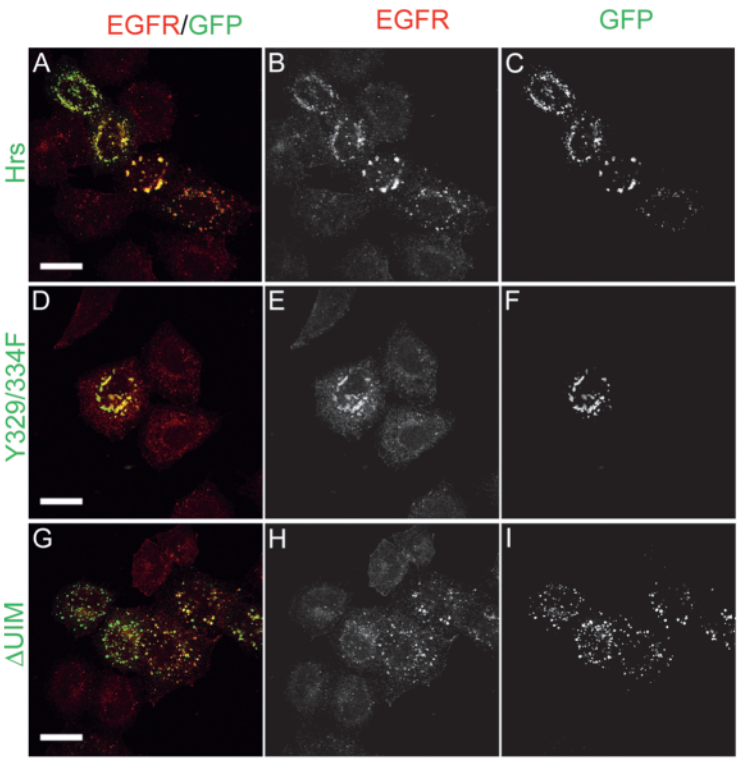

Fig. 5. EGFR downregulation is inhibited by over-expression of Hrs independently of tyrosine phosphorylation. HeLa cells were transfected with GFP-Hrs (A-C), GFP-Y329/334F (D-F) or GFP-ΔUIM (G-I), starved for 16 hours in serum-free medium and treated 22 hrs post-transfection for 4 hours with EGF (100 ng/ml). The cells were then fixed with 3% PFA, permeabilised with Triton X-100 and stained with anti-EGFR (EGFR shown in red) followed by secondary antibody coupled to Alexa Fuor 594. All panels show a single confocal section of cells presenting the most typical staining pattern for each construct. Scale bars: 20 μm.

decreases following Hrs over-expression (Table 2). The Y329/334F mutant accentuates this sorting defect: 90±3.5% of EGFR is at the limiting membrane although the number of internal vesicles is unchanged (Table 2). In contrast, the ΔUIM mutant (and the UIM-point mutant GFP-LSAA, data not shown) completely fails to suppress internal vesicle formation and allows significantly more EGFR to associate with lumenal vesicles, albeit less efficiently than in control cells. 57±2.2% of EGFR is at the limiting membrane in these cells. In cells overexpressing the ΔUIM mutant, lumenal vesicles also frequently labelled with anti-GFP antibodies indicating the presence of Hrs (ΔUIM) (Fig. 6C and Fig. 7D).

Discussion

Hrs is phosphorylated on Y334 in response to EGF

Hrs was originally identified as a major tyrosine phosphorylated substrate downstream of the Met receptor. The function of this phosphorylation is presently unclear and studies have been hampered by the fact that the target tyrosines were unknown. Human Hrs contains 30 tyrosines, 29 of which are conserved between mouse, rat and human and of these, six are in turn conserved in *Caenorhabditis elegans* and four in *Drosophila melanogaster*. We have previously shown that two of these 30 residues, Y197 and Y216 are not required for EGF-dependent tyrosine phosphorylation of Hrs (Urbé et al., 2000). In this study, we have used MALDI-TOF analysis of tryptic peptides derived from Hrs and have been able to identify the evolutionary conserved Y334 as the main tyrosine-phosphorylated residue downstream of EGFR in agreement with the findings by Steen et al. (Steen et al., 2002). We did not find any direct proof for a physiologically significant phosphorylation of Y329, however analysis of overexpressed mutant proteins lacking either Y334 or Y329 showed that Y329 can function as substrate in the absence of Y334 (Fig. 1). Phosphorylation on both residues simultaneously is unlikely to be a feature of the major proportion of phosphorylated Hrs, since mutation of either Y329 or Y334 alone does not substantially decrease the degree of Hrs phosphorylation compared to wild-type protein. We cannot rule out that other tyrosines within the molecule may be phosphorylated to a minor degree as we could still detect a low amount of signal with phospho-tyrosine antibodies in the Y329/334F mutant protein. In this respect, it should be noted that our MALDI-TOF analysis was limited to tryptic peptides and therefore did not include the 220 C-terminal amino acids of Hrs that do not contain a trypsin cleavage site. Phosphorylation at several sites is also suggested, firstly, by the fact that we observe multiple bands with our phospho-Hrs antibody (Fig. 1A,E), and secondly, by the study of Bache et al., who found three distinct spots whilst analysing Hrs immunoprecipitates by two-dimensional gel electrophoresis followed by immunoblotting with anti-phospho-tyrosine antibodies (Bache et al., 2002).

We have generated a polyclonal antibody directed against a peptide containing phospho-Y334 that specifically recognizes Hrs in response to EGF-stimulation thus confirming our MALDI-TOF analysis. We have used this antibody to confirm that the majority of tyrosine-phosphorylated Hrs is indeed found in the cytosol as we have previously described (Urbé et al., 2000). This observation suggests a scenario wherein Hrs first localises to the early endosome where the phosphorylation event takes place (Urbé et al., 2000) and

Table 2. Effect of wild-type and mutant Hrs over-expression on EGFR retention

	Distribution of EGFR over the endosomal vacuole (%)				
	Limiting membrane	Internal vesicles	Total number	Number of vacuoles	Number of internal vesicles per vacuole
Control	16±5.6	84±5.6	161	74	7±1.3
Hrs	77±8.2	23±8.2	401	159	3±1.1
Hrs-Y329/334F	90±3.5	10±3.5	291	141	3±0.3
Hrs-ΔUIM	57±2.2	43±2.2	294	107	10±3.0

The relative distribution of EGFR over endosomal vacuoles was determined by counting gold particles (total number indicated) for each transfection of at least 3×10 cell profiles (see Materials and Methods) and expressed as percentage of the total label present on vacuoles. For each EGFR-positive vacuole, the number of internal vesicles was counted. All values are mean±standard deviation.

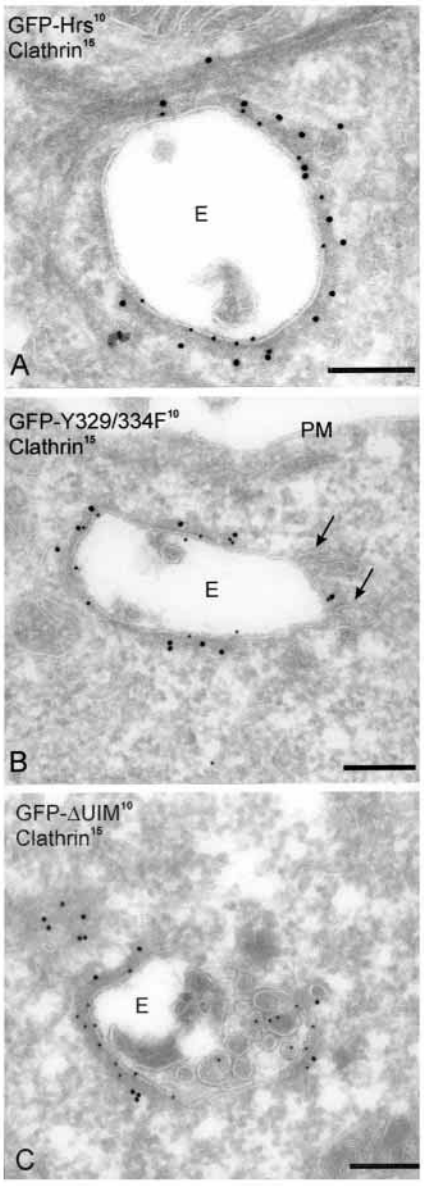

Fig. 6. Hrs-Y329/334F and Hrs-ΔUIM are present in clathrin coats on endosomes. HeLa cells were transfected with GFP-Hrs, GFP-Y329/334F or GFP-ΔUIM, processed for immunogold-labelling on cryosections as described in Materials and Methods. Cryosections were double labeled for GFP (10 nm gold) and clathrin (15 nm gold). (A-C) Overexpression of GFP-Hrs, GFP-Y329/334F and GFP-ΔUIM leads to an increased recruitment of clathrin to endosomal vacuoles (E). All three constructs colocalise with clathrin on the peripheral endosomal membrane but label is absent from tubular extensions that emerge from the vacuole (arrows in B and not shown). (C) In contrast to A and B, cells transfected with GFP-ΔUIM show GFP labelling at the limiting membrane as well as on internal vesicles of endosomal vacuoles. PM, plasma membrane. Scale bars: 200 nm.

second dissociates from the membrane in response to phosphorylation. The molecular basis for this dissociation is intriguing and may result from the disruption of any number of Hrs interactions: the availability of a phospho-specific Hrs antibody will now allow us to address this question directly in future studies.

Phosphorylation of Y334 is not required for ubiquitination of Hrs

Ubiquitination of tyrosine kinase receptors is frequently mediated by the E3 ligase c-Cbl (reviewed by Thien and Langdon, 2001). c-Cbl contains both a RING finger domain, characteristic of many E3-ligases and a PTB domain, which recognises phosphorylated receptor and provides for the stable association of the ubiquitination machinery that may be necessary for poly-ubiquitination to occur. Tyrosine phosphorylation on Y334 is clearly not a prerequisite for ubiquitination of overexpressed Hrs, since the non-phosphorylated GFP-Y329/334F mutant is ubiquitinated to the same degree as wild-type GFP-Hrs (Fig. 2B). Hence, phosphorylated Y334 does not seem to provide a docking site for the E3 ligase mediating UIM-dependent mono-ubiquitination of Hrs. In contrast to Polo et al. (Polo et al., 2002), we did not find any evidence for EGF-dependence of Hrs ubiquitination and we observed an additional ubiquitin smear characteristic of poly-ubiquitination, associated with both wild-type Hrs and phosphorylation-defective Y329/334F but not with the UIM deletion. This poly-ubiquitinated form of Hrs was also observed by Katz et al., in a stimulation-independent manner, in agreement with our study (Katz et al., 2002). Interestingly, the authors identified the HECT-family member Nedd4 as the Ubiquitin ligase that may be responsible for Hrs-ubiquitination in their study. One potential mechanism of recruitment of HECT-ligases involves a short PY motif (xPPxY) (Staub et al., 1996) that is indeed present in Hrs (^{234}PPEY^{237}) and may provide the docking site for Nedd4.

The UIM domain of Hrs is necessary for tyrosine phosphorylation

Whilst tyrosine phosphorylation is not required for ubiquitination, the UIM domain is necessary for efficient tyrosine phosphorylation of Hrs in response to EGF stimulation. We do not believe this reflects a requirement for mono-ubiquitination of Hrs (Polo et al., 2002) since Hrs-ubiquitination is independent of EGF stimulation in our hands. Our immunofluorescence studies show that the lack of tyrosine phosphorylation of the UIM-deletion mutant cannot be explained simply by mislocalisation: GFP-ΔUIM colocalises well with early endocytic markers and internalised EGFR. Failure of phosphorylation must therefore reflect failure of UIM-dependent molecular associations at the endosomal surface. This most likely reflects impaired association with ubiquitinated EGFR but could also be due to a more general impairment of interactions with ubiquitinated proteins at endosomes.

Formation of endosomal aggregates requires an intact UIM-domain

At the level of the light microscope, the morphology and distribution of the early endosomes in cells overexpressing GFP-ΔUIM (and GFP-LSAA, data not shown) is clearly different from that in cells overexpressing GFP-Hrs: the endosomal vacuoles do not form large clusters in the perinuclear area but rather remain distributed throughout the cell. This is not related to the fact that this mutant is not phosphorylated since the phosphorylation-defective

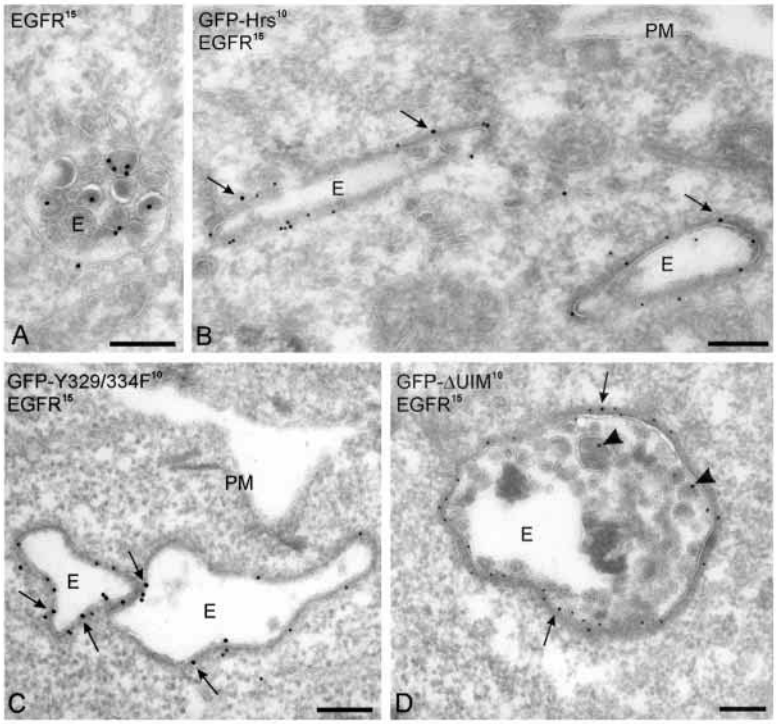

Fig. 7. The UIM domain of Hrs is required for efficient retention of the EGFR at the limiting membrane of the early endosome. HeLa cells were transfected with GFP-Hrs (B), GFP-Y329/334F (C) or GFP-ΔUIM (D), or mock-transfected (A), starved 16 hours in serum-free medium and stimulated 22 hrs post-transfection for 30 minutes with EGF. Cells were processed for immunogold-labelling on cryosections as described in Materials and methods and cryosections were double-labeled for GFP (10 nm gold) and EGFR (15 nm gold). (A) In control cells, EGFR is mainly localized on internal vesicles of endosomal vacuoles. (B) In cells overexpressing GFP-Hrs, EGFR is retained in the limiting membrane of endosomal vacuoles (arrows). (C) In HeLa cells expressing GFP-Hrs Y329/334F, EGFR is retained at the limiting membrane of endosomal vacuoles (arrows). (D) By contrast, in cells transfected with GFP-Hrs-ΔUIM, EGFR is localized at the limiting membrane (arrows) as well as on internal vesicles (arrowheads). E, endosomal vacuole; N, nucleus; PM, plasma membrane. Scale bars: 200 nm.

Y329/334F mutant forms clusters to an even greater extent than the wild-type protein. Bishop et al., noted enhanced binding of an anti-ubiquitin antibody to endosomes following Hrs over-expression (Bishop et al., 2002). It is conceivable that the clustering seen with the constructs containing a UIM-domain may result from the accumulation of poly-ubiquitinated proteins in the limiting endosomal membrane and the crosslinking of these endosomal vacuoles through ubiquitin:Hrs-UIM interactions.

The dominant-negative effect of Hrs overexpression on internal vesicle formation requires an intact UIM domain

Over-expression of wild-type Hrs inhibits the downregulation of EGFR, illustrated here by the persistence of EGFR staining in the early endosome after 4 hours of continuous stimulation with EGF. This dominant negative effect of Hrs over-expression may be due to titration of interacting factors that become limiting and are required downstream of Hrs for EGFR sorting and degradation [e.g. STAM/Hbp, Tsg101 (Babst et al., 2000; Bishop et al., 2002; Takata et al., 2000)] or for the release of Hrs from endosomal membranes. We have previously shown that only a small percentage of overexpressed Hrs is phosphorylated (Urbé et al., 2000). Since phosphorylation of Hrs may act as a trigger for membrane dissociation, it is not surprising that the phosphorylation-defective Y329/334F mutant recapitulates the dominant negative phenotype of the wild-type protein. The UIM-deletion mutant in contrast had a much less pronounced effect on EGFR downregulation.

Quantitative analysis by electron microscopy of EGFR sorting at the early endosome provides high-resolution support for this assessment. Expression of wild-type Hrs, as well as of both mutated forms of Hrs (Y329/334F and ΔUIM), led to the recruitment of a clathrin-based coat to the early endosome that lacks the characteristic striations seen in the Hrs/clathrin coat of non-transfected cells (Sachse et al., 2002). However, the UIM deletion mutant differs from the other two constructs in three important ways: (i) whilst wild-type Hrs, as well as the phosphorylation defective Y329/334F mutant causes a dramatic retention of EGFR in the limiting membrane compared to mock-transfected cells, this effect is suppressed by deletion of the UIM motif; (ii) whereas the wild-type GFP-Hrs and the Y329/334F mutant, just like the endogenous protein, are completely retained at the limiting membrane of the early endosome, the UIM-deletion mutant seems to escape that retention mechanism along with the EGFR and appears in the lumenal vesicles (Fig. 6C, Fig. 7D) and (iii) concomitantly with the retention of the EGFR, both wild-type Hrs and Y329/334F over-expression lead to a reduction in the observed number of internal vesicles whereas the UIM-deletion mutant appears without effect, in agreement with studies in yeast showing that deletion of the UIM domain of Vps27 does not impinge on the formation of lumenal vesicles (Bilodeau et al., 2002). In summary, deletion of the UIM domain suppresses the inhibitory effect of Hrs over-expression on lumenal vesicle formation and consequent internalisation of EGFR from the limiting membrane.

A role for the UIM-domain of Hrs in the co-ordination of receptor sorting and vesicle formation

We propose that the initial role of the UIM domain of Hrs at the sorting endosome is to recruit ubiquitinated receptors into the clathrin-coated microdomains by an active retention mechanism as a prelude to internalisation into lumenal vesicles. There must, however, be a release mechanism prior to incorporation of EGFR into inward budding vesicles, as Hrs and other coat components are normally not internalised. It is tempting to speculate that Hrs, or more precisely, the UIM domain of Hrs has to be displaced from the ubiquitinated receptor complex for internal vesicle formation to occur. Displacement of the Hrs-UIM by a competing Ubiquitin-binding protein, possibly a component of the downstream

acting ESCRT complex [e.g. Tsg101 (Babst et al., 2000; Bishop et al., 2002; Katzmann et al., 2001)], would then be able to release the clamp that Hrs imposes by recruiting further downstream elements of the ESCRT complexes (Babst et al., 2002a; Babst et al., 2002b), ultimately leading to vesicle formation. In this way Hrs dissociation from the Ubiquitin-receptor complex may be directly coupled to vesicle formation: besides allowing recruitment of the vesicle formation machinery to the ubiquitinated receptor, dissociation of Hrs could also serve to release the receptor complex from the clathrin-coated patch and allow (or facilitate) lateral diffusion out of this microdomain, which by nature of the rigid clathrin coat may be unable to invaginate. Indeed, profiles of inward budding vesicles are not observed underneath the clathrin coat but immediately adjacent to it (Sachse et al., 2002).

Based on this model, over-expression of wild type Hrs or Y329/334F Hrs accentuates the retention of receptors without effectively coupling to downstream factors mediating vesicle formation. In contrast, the UIM mutant of Hrs is unable to execute this clamp action and EGFR incorporation into vesicles proceeds albeit in a more stochastic and less efficient manner than in control cells presumably since overexpression of any endosome-localised, clathrin binding form of Hrs does not allow for local concentration of receptor complexes in clathrin-coated patches, which now frequently represent the entire vacuolar endosomal surface.

Why then is the UIM mutant of Hrs incorporated into internal vesicles? It is possible that the association between Hrs and the receptor complex is not solely mediated by the UIM domain and complete dissociation of Hrs from the receptor complex may yet require tyrosine phosphorylation of Hrs. This in turn, as we show here, requires an intact UIM domain, presumably to allow Hrs to engage in a network of ubiquitin-mediated interactions that may also serve to present Hrs to the relevant kinase.

Until now, high resolution data linking the Hrs UIM domain with receptor sorting into lumenal vesicles have not been available, although much has been made of circumstantial evidence. The domain binds ubiquitin and inhibits plasma membrane-directed recycling of a transferrin receptor-ubiquitin hybrid (Raiborg et al., 2002). Our data go beyond this to show that over-expression of Hrs inhibits formation of endosome lumenal vesicles that contain endogenous activated EGFR and that this inhibition requires the UIM domain of Hrs. This indicates a complex role for Hrs in lumenal vesicle formation, having both positive and negative aspects that may represent ‘checkpoint’ control of vesicle formation, ensuring that under physiological circumstances it is effectively coupled to cargo loading.

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