FYVE and coiled-coil domains determine the specific localisation of Hrs to early endosomes

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SUMMARY

Hrs, an essential tyrosine kinase substrate, has been implicated in intracellular trafficking and signal transduction pathways. The protein contains several distinctive domains, including an N-terminal VHS domain, a phosphatidylinositol 3-phosphate (PtdIns(3)P)-binding FYVE domain and two coiled-coil domains. Here we have investigated the roles of these domains in the subcellular localisation of Hrs. Hrs was found to colocalise extensively with EEA1, an established marker of early endosomes. While the membrane association of EEA1 was abolished in the presence of a dominant negative mutant of the endosomal GTPase Rab5, the localisation of Hrs to early endosomes was Rab5 independent. The VHS-domain was nonessential for the subcellular targeting of Hrs. In contrast, the FYVE domain as well as the second coiled-coil domain, which has been shown to bind to SNAP-25, were required for targeting of Hrs to early endosomes. A small construct consisting of only these two domains was correctly localised to early endosomes, whereas a point mutation (R183A) in the PtdIns(3)P-binding pocket of the FYVE domain inhibited the membrane targeting of Hrs. Thus, like EEA1, the endosomal targeting of Hrs is mediated by a PtdIns(3)P-binding FYVE domain in cooperation with an additional domain. We speculate that binding to PtdIns(3)P and a SNAP-25-related molecule may target Hrs specifically to early endosomes.

Key words: Endocytosis, FYVE domain, Membrane traffic, Phosphoinositide, Rab5

INTRODUCTION

Accumulating evidence indicates that endocytic membrane trafficking regulates signalling by extracellular ligands (Ceresa and Schmid, 2000). An interesting candidate for a molecule that coordinates endocytosis and signalling is the hepatocyte growth factor-regulated tyrosine kinase substrate, Hrs (Komada and Kitamura, 1995). The tyrosine phosphorylation of Hrs is induced by several growth factors and cytokines (Komada and Kitamura, 1995; Komada et al., 1997; Asao et al., 1997), and this phosphorylation appears to require endocytosis of the receptor/ligand complex (Urbé et al., 2000). Mice that lack Hrs die during early embryogenesis, and the embryos show severe defects in ventral folding morphogenesis and contain enlarged transferrin receptor-positive structures (Komada and Soriano, 1999). Hrs has been shown to interact with the signal-transducing adaptor molecule, STAM, implicated in Jak kinase signalling (Asao et al., 1997), and with the SNARE protein, SNAP-25, which is involved in membrane fusion (Kwong et al., 2000). Given the essential function of Hrs, and its implications in signal transduction and membrane trafficking pathways, it will be important to identify the mechanisms that target this protein to endosomes.

We have previously characterised the subcellular targeting of the early endosomal autoantigen EEA1. This large protein, which regulates endocytic membrane fusion (Simonsen et al., 1998b; Mills et al., 1998), is found exclusively on early endosomes (Mu et al., 1995; Wilson et al., 2000). Targeting of EEA1 to early endosomes has been found to rely on a cooperative lipid and protein interaction. The FYVE zinc finger domain of EEA1 binds to the phosphatidylinositol 3-kinase (PI 3-kinase) product, phosphatidylinositol 3-phosphate (PtdIns(3)P), whereas an adjacent domain binds to the endosomal GTPase, Rab5 (Simonsen et al., 1998b). Even if the two individual interactions are of low affinity, the dual interaction is sufficiently strong to efficiently recruit EEA1 to early endosomal membranes. Early endosome membranes are presumably the only cellular membranes that contain the combination of PtdIns(3)P and Rab5 (Gillooly et al., 2000), and this may explain the highly specific localisation of EEA1.

Like with EEA1, the FYVE domain of Hrs binds to PtdIns(3)P (Gaullier et al., 1998; Gillooly et al., 2000), and the membrane association of Hrs is regulated by PI 3-kinase (Komada and Soriano, 1999; Urbé et al., 2000). This raises the question whether Hrs has the same mechanism of membrane targeting as EEA1. In addition to the FYVE domain, Hrs also contains an N-terminal VHS-domain (Lohi and Lehto, 1998), a proline-rich domain, two coiled-coil domains (CC1 and CC2, respectively), and a C-terminal proline- and glutamine-rich domain (Komada and Kitamura, 1995) (see Fig. 1). Based on the recently solved X-ray structure of the N terminus of Hrs, a model has been proposed for its membrane association (Mao et al., 2000). According to this model, a dimeric FYVE domain of Hrs binds to two molecules of PtdIns(3)P in the membrane,

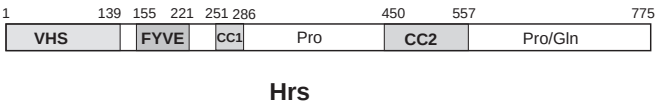

Fig. 1. Schematic representation of Hrs. The boundaries of the different domains are indicated by their corresponding amino acid numbers. Putative coiled-coil domains (CC1 and CC2) were identified with the Coils program (Lupas et al., 1991), whereas the VHS and FYVE domains were identified with SMART (Schultz et al., 1998). Domains rich in proline or proline and glutamine are indicated.

and the membrane interaction is further stabilized by the interaction of the VHS domains with the cytoplasmic face of the membrane. To date, this model has not been tested experimentally. Here we have investigated the roles of the various Hrs domains in its subcellular targeting. We find that targeting of Hrs to early endosomes is Rab5 independent and is executed by the FYVE domain in cooperation with the SNAP-25-binding CC2 domain.

MATERIALS AND METHODS

Antibodies and fluorescently labelled proteins

Anti-myc antibodies were from the 9E10 hybridoma (Evan et al., 1985). 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), immobilised on Affi-Gel (Bio-Rad, Hercules, CA, USA). Human anti-EEA1 serum was a gift from Ban-Hock Toh. Fluorochrome- or horseradish peroxidase-conjugated secondary antibodies were from Jackson Immunoresearch (West Grove, PE, USA). Human holo-transferrin (Sigma, St Louis, MO, USA) was labelled with Alexa^{488} (Molecular Probes, Eugene, OR, USA) according to the instructions from the manufacturer.

Plasmid constructs

Hrs or the deletion constructs indicated were generated by PCR with mouse Hrs (Komada and Kitamura, 1995) as a template. Synthetic oligonucleotides were from MedProbe (Oslo, Norway). The FYVE and CC2 domain constructs used consisted of residues 147-223 and 420-573 of Hrs, respectively. The Hrs^{C215S} mutation has been described before (Gaullier et al., 1998). The Hrs^{R183A} mutant was prepared by PCR mutagenesis. PCR errors were excluded by sequencing. For expression in mammalian cells with the T7 RNA polymerase vaccinica virus system, constructs were cloned behind the myc-epitope of pGEM-myc4 (Simonsen et al., 1998a). For expression in mammalian cells with Fugene$^{(R)}$ (Roche) transfection, we constructed a myc-eptiope-tagged variant (pcDNA3-myc) of pcDNA3 (Invitrogen). Constructs were cloned in-frame behind the myc-epitope of this plasmid. 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 as GST fusions in *E. coli* BL-21(DE 3) cells, the FYVE domains (residues 147-223) of Hrs, Hrs^{R183A} and Hrs^{C215S} were cloned into pGEX-6P-3 (Pharmacia Amersham). Expression and purification were performed as described (Gaullier et al., 2000).

Transient expression in BHK cells

In order to minimise possible artefacts associated with transient protein expression, we used two different transient expression systems. The pGEM constructs were expressed in cells using the modified Ankara T7 RNA polymerase recombinant vaccinia virus system and lipofection as described (Stenmark et al., 1995a; Sutter et al., 1995), and the cells were analyzed 6 hours after transfection. The pcDNA3 constructs were expressed in cells using Fugene$^{(R)}$ (Roche) according to the manufacturer's instructions. In these cases, cells were analysed 24 hours after transfection. SDS-PAGE of transfected cells followed by immunoblotting with anti-myc antibodies indicated that the various constructs were expressed at comparable levels.

Confocal fluorescence microscopy

BHK cells grown on coverslips were transiently transfected, fixed with 3% paraformaldehyde and stained for fluorescence microscopy as described (Simonsen et al., 1998a). In some experiments, cells were permeabilised with 0.05% saponin (Simonsen et al., 1998a) prior to fixation. When indicated, cells were incubated with Alexa^{488}-transferrin (25 μg/ml) prior to fixation. Toto-3 (Molecular Probes) was used for the labelling of nuclei. 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 in order to exclude bleed-through effects.

Electron microscopy

To identify early endosomal compartments cells were incubated with 3-7 nm BSA-coated colloidal gold (Slot and Geuze, 1985) in the medium at 37°C for 10 minutes. At the end of the incubation with BSA-gold the cells were washed with PBS and immediately fixed with 0.1% glutaraldehyde/4% paraformaldehyde 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 mouse anti-myc antibodies followed by rabbit-anti mouse IgG antibodies and 15-nm protein A-gold (purchased from G. Posthuma and J. Slot, Utrecht, The Netherlands). The labelled cryosections were viewed in a Phillips CM120 electron microscope.

Circular dichroism (CD) spectroscopy

CD spectra of wild-type and mutant GST-FYVE fusion proteins were recorded using a Jasco J-810 spectropolarimeter calibrated with ammonium d-camphor-10-sulfonate. Measurements were performed at 20°C using quartz cuvettes with a path length of 0.1 cm. All the measurements were performed with a protein concentration of 0.15 mg/ml in 10 mM sodium phosphate, pH 7.0. Samples were scanned 5 times at 50 nm/minute over the wavelength range 200-240 nm. The data were averaged and the spectrum of a protein-free control sample was subtracted. The resultant spectra were then smoothed with the binominal method. All measurements were conducted at least twice.

Surface plasmon resonance

Surface plasmon resonance was recorded at 25°C on a BiaCore X (BiaCore, Sweden). The liposomes used contained 63% phosphatidyl-choline, 20% phosphatidylserine, 15% phosphatidylethanolamine and 2% PtdIns(3)P (Echelon) (Gaullier et al., 2000). Liposomes (0.35 mg/ml) were loaded onto a Biacore L1 chip by three successive injections of 80 μl liposomes at a flow rate of 5 μl/minute. The reference cell was loaded with similar liposomes lacking PtdIns(3)P. Sensorgrams were recorded upon the injections of 0.1-2 μg protein at a flow rate of 20 μl/minute. The lipid surface was regenerated using 10 mM NaOH.

Two-hybrid methods

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

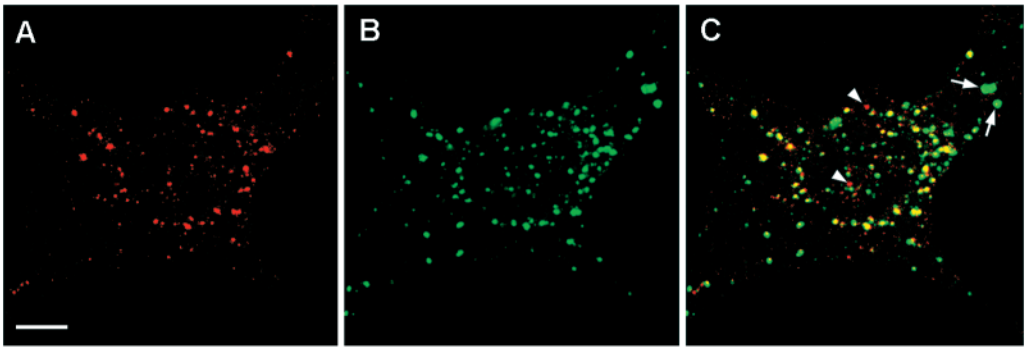

Fig. 2. Localisation of Hrs and EEA1 by confocal immunofluorescence microscopy. A BHK cell stained with anti-Hrs (A) and anti-EEA1 (B) was studied by confocal immunofluorescence microscopy. Yellow colour in the merged image (C) indicates colocalisation. The arrows indicate examples of structures that are strongly positive for EEA1 but negative for Hrs. The arrowheads indicate structures that are positive for Hrs but negative for EEA1. Bar, 5 μm.

Subcellular fractionation

Cells grown in 10-cm plastic dishes were collected with a rubber policeman, and post-nuclear supernatants, membrane and cytosol fractions were prepared as described (Stenmark et al., 1994). Equal samples of the post-nuclear supernatant, cytosol and membrane fractions were analysed by SDS-PAGE and immunoblotting with anti-myc antibodies. For detection, we used horseradish peroxidase-conjugated goat anti-mouse IgG antibodies and the SuperSignal chemoluminescence system (Pierce), according to the instructions from the manufacturer.

RESULTS

Hrs colocalises with EEA1 on early endosomes

Previous studies have revealed that endogenous Hrs partially colocalises with the transferrin receptor on vesicular structures (Komada et al., 1997). To compare its localisation with that of a more specific early endosome marker, we prepared an affinity-purified anti-Hrs antibody and studied the colocalisation of endogenous Hrs with EEA1 by confocal immunofluorescence microscopy. The anti-Hrs antibody stained vesicular structures throughout the cytoplasm (Fig. 2A), and there was a strong (>50%) colocalisation with EEA1 (Fig. 2B). However, we could also detect some structures strongly positive for EEA1 and negative for Hrs (arrows) and vice versa (arrowheads). This indicates that Hrs is mainly but not exclusively localised to early endosomes.

The subcellular targeting of Hrs, in contrast to that of EEA1, is Rab5 independent

The subcellular targeting of 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). To study if the targeting of Hrs is Rab5-dependent as well, we examined the intracellular localisation of EEA1 and Hrs in cells that expressed a myc-epitope-tagged, dominant-negative (Stenmark et al., 1994) Rab5^{S34N} mutant. In order to detect membrane-associated proteins only, we permeabilised the cells with saponin prior to fixation and immunofluorescence staining (Fig. 3). While EEA1 was abundant on early endosomes of untransfected cells (Fig. 3B), almost no membrane-associated EEA1 was detected in myc-Rab5^{S34N}-transfected cells (Fig. 3A,B, arrow). This confirms the importance of Rab5 for the recruitment of EEA1 to endosome membranes. In contrast to EEA1, Hrs remained membrane associated even upon the expression of myc-Rab5^{S34N} (Fig. 3C,D, arrow), indicating that Hrs is not recruited by Rab5. Even if Hrs is not regulated by Rab5, it might interact with another endosomal Rab GTPase. We therefore studied the localisation of Hrs upon the transfection of cells with inhibitory mutants of the endosomal Rab GTPases, Rab4, Rab7 and Rab22. However, neither Rab4^{S22N}, Rab7^{S22N} nor Rab22^{S19N} had any effect on the localisation of Hrs (not shown).

To further investigate whether Hrs is able to interact with Rab4, Rab5, Rab7 or Rab22 we employed the yeast two-hybrid

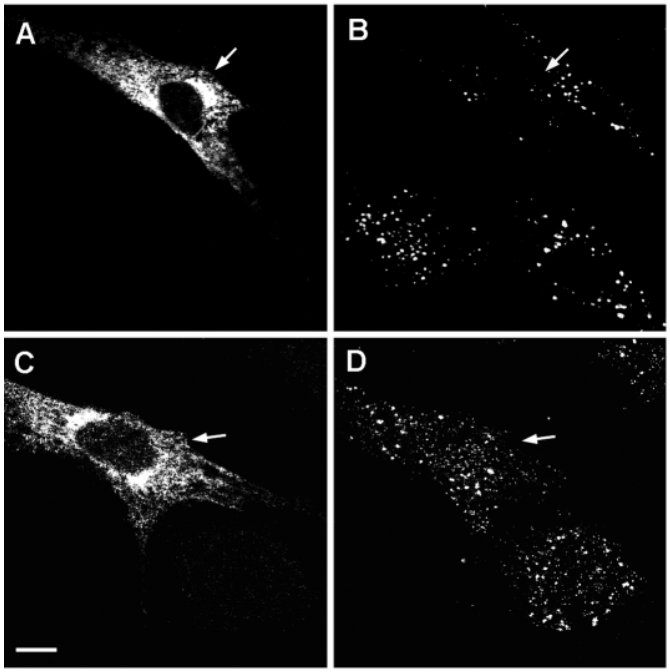

Fig. 3. The endosomal localisation of EEA1 but not Hrs is abolished upon expression of a dominant-negative Rab5 mutant. BHK cells were transfected with myc-Rab5^{S34N} and permeabilized with 0.05% saponin (Simonsen et al., 1998a) prior to fixation. They were then stained with anti-myc (A,C), anti-EEA1 (B) or anti-Hrs (D). Transfected cells are indicated by arrows. Bar, 5 μm.

Table 1. Interactions of EEA1 and Hrs with GTPase-deficient Rab mutants in the two-hybrid system

Bait	Prey	β-galactosidase activity
EEA1	$Rab5^{Q79L}$	13.2±2.8
Hrs	$Rab5^{Q79L}$	0.09±0.00
Hrs	$Rab4b^{Q67L}$	0.18±0.03
Hrs	$Rab7^{Q67L}$	0.09±0.03
Hrs	$Rab22^{Q64L}$	0.13±0.06

The values indicate β-galactosidase activities in arbitrary units.
Neither of the bait constructs showed any significant reporter activation in the absence of a prey construct.

system, which we have previously used successfully to demonstrate the interactions of several Rab GTPases with their effectors (Stenmark et al., 1995b; Gournier et al., 1998; Vitale et al., 1998; Simonsen et al., 1998b). GTPase-deficient ('active') mutants of Rab4, Rab5, Rab7 and Rab22 were used as 'prey' constructs and Hrs was used as 'bait' (Table 1). While we detected a strong interaction between $Rab5^{Q79L}$ and EEA1, as shown previously (Simonsen et al., 1998b), we detected no interaction between Hrs and $Rab5^{Q79L}$, $Rab4^{Q67L}$, $Rab7^{Q67L}$ and $Rab22^{Q64L}$. Collectively, our results indicate that the targeting of Hrs to early endosomes is not mediated by any of the endosomal Rab GTPases tested.

Multiple domains are required for the localisation of Hrs to early endosomes

In order to identify the region(s) of Hrs responsible for its targeting to early endosomes, we expressed myc-epitope-tagged deletion constructs of Hrs in BHK cells and studied their intracellular localisation by confocal immunofluorescence microscopy. The full-length Hrs protein (Fig. 4A) colocalised extensively with EEA1 when overexpressed, as expected from the localisation of endogenous Hrs. As previously reported (Komada et al., 1997), the overexpression of Hrs led to a clustering of endocytic structures (compare the EEA1 labelling in the transfected versus the untransfected cell in Fig. 4A). The N-terminal VHS domain has been proposed to play a role in the membrane localisation of Hrs (Mao et al., 2000). However, a deletion mutant lacking this domain, $Hrs_{\Delta VHS}$, showed an intracellular localisation similar to the full-length protein (Fig. 4B), indicating that the VHS domain is unimportant for the endosomal targeting of Hrs. Since the FYVE domain binds to PtdIns(3)P, which is present on early endosomes (Gillooly et al., 2000), we investigated whether this domain is sufficient to target Hrs to these organelles. However, the FYVE domain as such was shown to be cytosolic (Fig. 4C), and the same was the case with the N-terminal part, Hrs_{1-289}, consisting of the VHS, FYVE and CC1 domains (not shown). Likewise, the reciprocal C-terminal part, $Hrs_{287-775}$, containing the proline-rich, CC2 and proline/glutamine-rich domains, was cytosolic (not shown). This suggests that several, spatially distant, domains are required for the subcellular targeting of Hrs.

In order to define the endosomal targeting domains closer, we prepared a series of new deletion mutants of Hrs. While a construct consisting of residues 1-500 was mainly cytosolic (Fig. 4D), a slightly longer construct containing the entire CC2 region (Hrs_{1-573}), colocalised with EEA1 (Fig. 4E). On the other hand, the C-terminal part of Hrs ($Hrs_{500-775}$) did not colocalise with EEA1 (Fig. 4F). This indicates that the CC2 region is necessary but not sufficient for correct localisation of Hrs. Since coiled-coil domains are frequently involved in protein homodimerisation (Lupas, 1996), we considered the possibility that this domain may mediate a homodimerisation of Hrs required for efficient membrane binding. However, we detected no interaction of the CC2 domain with itself in the

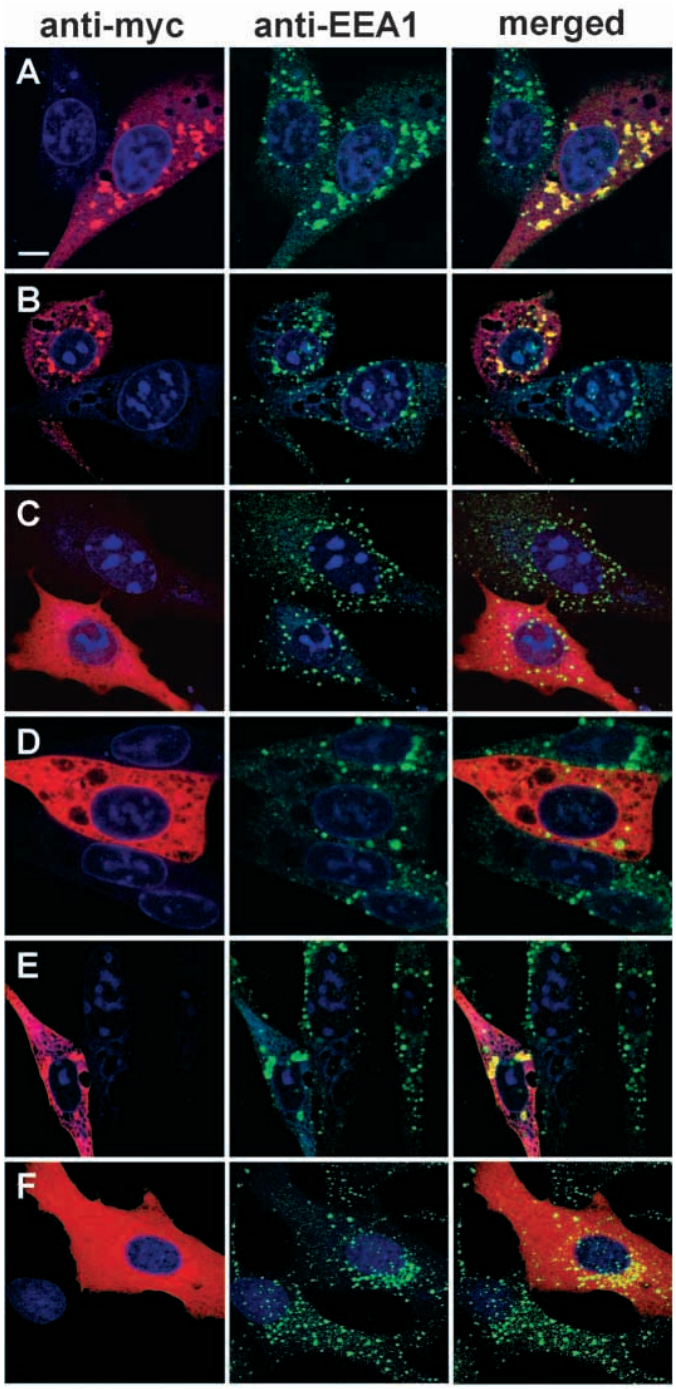

Fig. 4. Colocalisation of Hrs constructs with EEA1. BHK cells were transfected with myc-tagged Hrs (A), $Hrs_{\Delta VHS}$ (B), Hrs_{FYVE} (C), Hrs_{1-500} (D), Hrs_{1-573} (E) or $Hrs_{500-775}$ (F) and stained with anti-myc (left) or anti-EEA1 (middle). (Right) The merged images, with yellow colour indicating colocalisation. Nuclei are visualised with Toto-3 (blue colour). Bar, 5 μm.

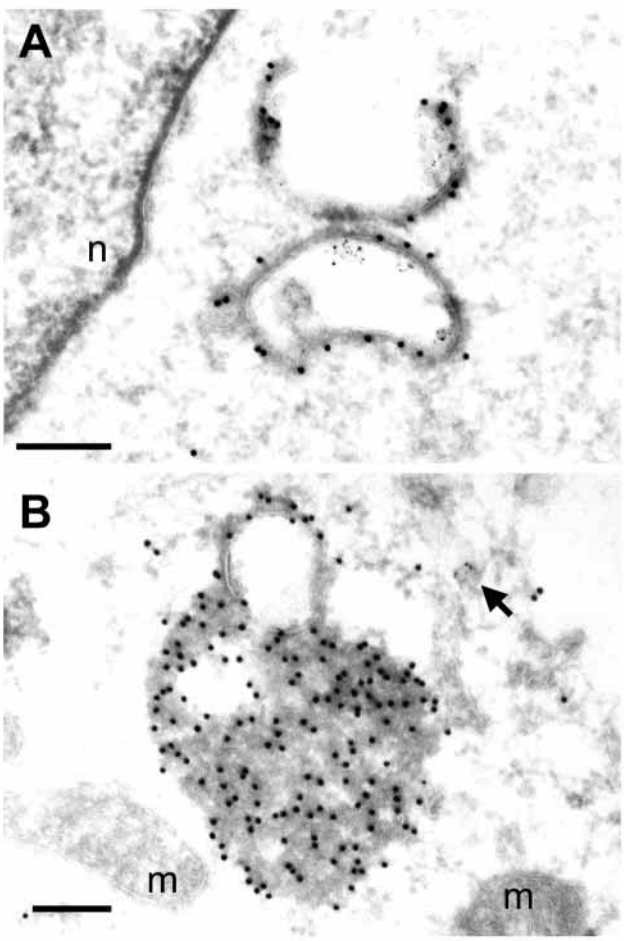

Fig. 5. Electron microscopy of structures containing myc-tagged wild-type Hrs (A) and Hrs^{C215S} (B). Transfected BHK cells were incubated in the presence of 3-7-nm BSA-gold for 10 minutes at 37°C prior to fixation and immunostaining for anti-myc (15-nm gold). Endocytosed BSA-gold is visible in the two myc-Hrs-positive structures (A). The arrow in (B) indicates a small endocytic profile that contains encocytosed BSA-gold, whereas the Hrs^{C215S}-containing structure is negative. n, nucleus; m, mitochondrion. Bars, 200 nm.

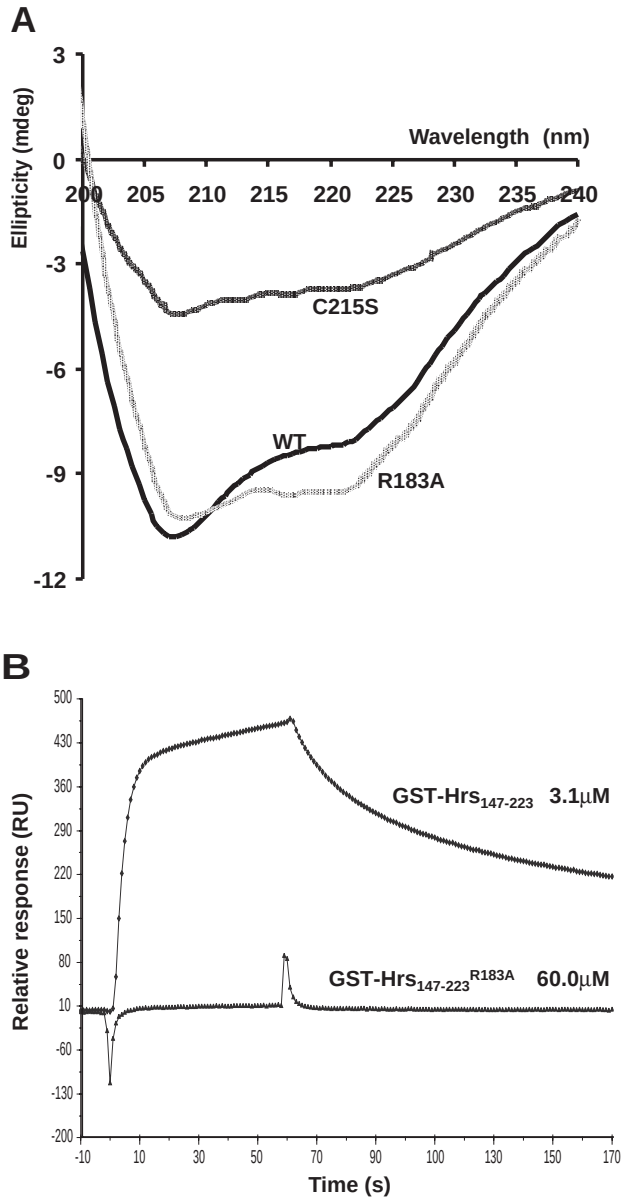

Fig. 6. Biophysical and biochemical properties of wild-type and mutant Hrs FYVE domains. The α-helical content (in millidegrees) of GST-fusions of wild-type and mutant FYVE domains was studied by CD spectroscopy (A), and the binding of GST-Hrs$_{FYVE}$ and GST-Hrs$_{FYVE}^{R183A}$ to PtdIns(3)P was analysed by surface plasmon resonance (B), as described in Materials and Methods. RU, resonance units.

two-hybrid system (data not shown), suggesting that this domain does not function in homodimer formation.

A functional FYVE domain is important for the subcellular targeting of Hrs

Even though incubation of cells with wortmannin causes the dissociation of endogenous Hrs from endosomes (Komada and Soriano, 1999; Urbé et al., 2000), conflicting results have been obtained regarding the importance of the FYVE domain (Hayakawa and Kitamura, 2000; Urbé et al., 2000). Of two different studies employing the PtdIns(3)P-binding deficient FYVE mutant Hrs^{C215S}, one concluded that this mutation abolishes the endosomal targeting of Hrs (Urbé et al., 2000), whereas the other one found no effect (Hayakawa and Kitamura, 2000). When we expressed Hrs^{C215S} and analysed the cells by electron microscopy, we noticed that this protein, when expressed at high level, accumulates in proteinaceous aggregates devoid of membranes (Fig. 5B), which were never observed with wild-type Hrs (Fig. 5A). These aggregates were often observed close to endocytic profiles (see arrow in Fig. 5B), and since they resemble endosomes when examined by fluorescence microscopy, they may complicate the interpretation of the intracellular localisation of Hrs^{C215S}. The C215S mutation affects zinc binding of the FYVE domain, and the corresponding mutation in EEA1 has been shown to cause a distortion of the FYVE structure (Stenmark et al., 1996; Gaullier et al., 2000). We therefore sought to introduce a mutation in the PtdIns(3)P binding basic pocket of Hrs, R183A, which is predicted to abolish PtdIns(3)P binding without causing any structural rearrangements (Gaullier et al., 2000; Misra and Hurley, 1999). Indeed, when the wild-type and

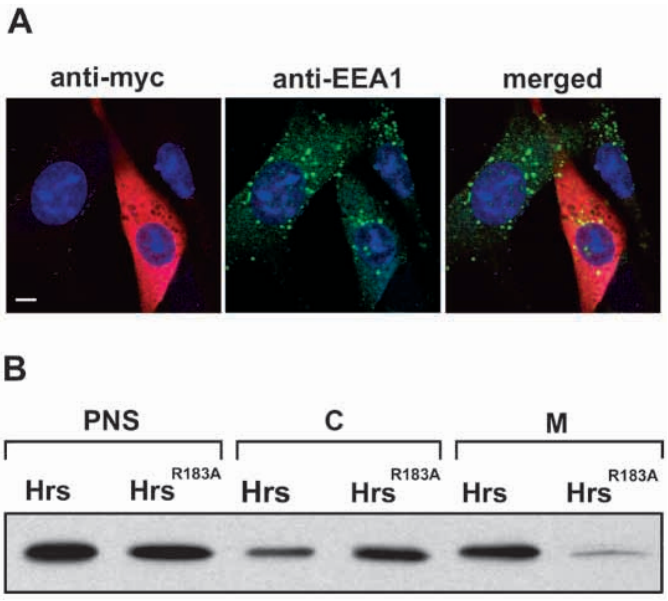

Fig. 7. The FYVE mutation R183A inhibits membrane targeting of Hrs. (A) BHK cells were transfected with myc-tagged Hrs^{R183A}. The cells were stained with anti-myc (left) and anti-EEA1 (middle). Yellow colour in the merged images (right) indicates colocalisation. Nuclei are stained with Toto-3 (blue). Bar, 5 μm. (B) BHK cells were transfected with myc-tagged wild-type Hrs or Hrs^{R183A}. Equivalent samples of the post-nuclear supernatant (PNS), cytosol (C) and membrane (M) fractions were analysed by SDS-PAGE and immunoblotting with anti-myc antibodies.

mutant FYVE domains were analysed by circular dichroism (CD) spectroscopy, the spectrum of the R183A mutant was similar (albeit not identical) to that of the wild-type protein, whereas the spectrum of the C215S mutant indicated a much less ordered structure (Fig. 6A). The structural distortion caused by the C215S mutation presumably explains the propensity of Hrs^{C215S} to aggregate when expressed in cells. To study if the R183A mutation causes a reduced affinity for PtdIns(3)P as predicted, we analysed the wild-type and R183A mutant FYVE domains by surface plasmon resonance (Fig. 6B). While we could detect the binding of the GST-tagged wild-type Hrs FYVE domain to PtdIns(3)P by surface plasmon resonance even at low concentrations, we were unable to detect any binding of GST-$FYVE^{R183A}$ even at high protein concentrations. Thus, the R183A mutation causes a >100-fold loss of affinity for PtdIns(3)P.

We next expressed Hrs^{R183A} in BHK cells in order to investigate the role of the FYVE domain in the subcellular targeting of Hrs. In contrast to wild-type Hrs (see Fig. 4A), Hrs^{R183A} accumulated in the cytosol in the majority of the cells, indicating that the mutation inhibits membrane association of Hrs (Fig. 7A). In order to verify the result from the confocal microscopy, we analysed the transfected cells by subcellular fractionation. As shown in Fig. 7B, only a small fraction of Hrs^{R183A} was found on membranes, in marked contrast to wild-type Hrs. This is consistent with the microscopic analysis and demonstrates that a functional FYVE domain is crucial for the membrane targeting of Hrs.

The FYVE and CC2 domains cooperatively target Hrs to early endosomes

The logical implication of the above findings was that the targeting of Hrs to early endosomes is mediated by the FYVE and CC2 domains in cooperation. In order to test this hypothesis directly, we prepared a small construct ($Hrs_{FYVE+CC2}$) that consists only of these two domains. Like the FYVE domain (see Fig. 4C), the CC2 domain alone (Hrs_{CC2}) was cytosolic and did not colocalise with EEA1 (Fig. 8A). In contrast, $Hrs_{FYVE+CC2}$ colocalised extensively with EEA1 in clustered vesicular structures (Fig. 8B). To verify the localisation of $Hrs_{FYVE+CC2}$ with another endocytic marker, we studied the extent of colocalisation of $Hrs_{FYVE+CC2}$ with endocytosed $Alexa^{488}$-transferrin. Like wild-type Hrs (Fig. 9A), $Hrs_{FYVE+CC2}$ colocalised extensively with the endocytosed $Alexa^{488}$-transferrin (Fig. 9B). This confirms the specific localisation of this construct to early endosomes.

In order to quantify the degree of colocalisation of the key constructs (wild-type Hrs, Hrs_{FYVE}, Hrs_{CC2}, $Hrs_{FYVE+CC2}$ and Hrs^{R183A}) with EEA1, we randomly selected 20 cells transfected with each construct and counted the number of profiles positive for both EEA1 and the respective Hrs constructs. To minimise any possible effects due to protein overexpression, we transfected cells for 24 hours using $Fugene^{(R)}$, which gives a slower expression than the T7 vaccinia system. To detect membrane associated protein only, we permeabilised the cells with saponin prior to fixation. As shown in Fig. 10, both wild-type Hrs and $Hrs_{FYVE+CC2}$ showed a >50% colocalisation with EEA1, similar to endogenous Hrs. It should be noted that this high degree of colocalisation between Hrs/$Hrs_{FYVE+CC2}$ and EEA1 was observed even in individual cells expressing barely detectable levels of the myc-

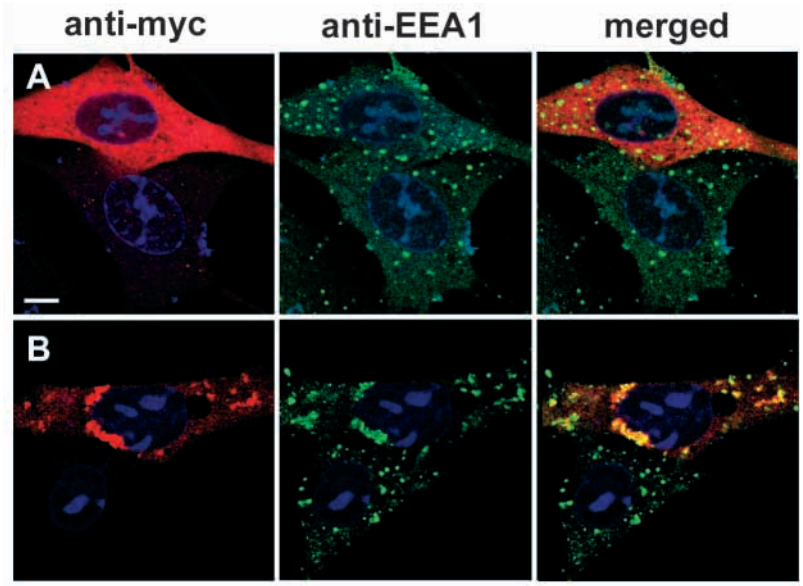

Fig. 8. The FYVE and CC2 domains of Hrs determine its colocalisation with EEA1. BHK cells were transfected with myc-tagged Hrs_{CC2} (A) or with $Hrs_{FYVE+CC2}$ (B). The cells were stained with anti-myc (left) and anti-EEA1 (middle). Yellow colour in the merged images (right) indicates colocalisation. Nuclei are stained with Toto-3 (blue). Bar, 5 μm.

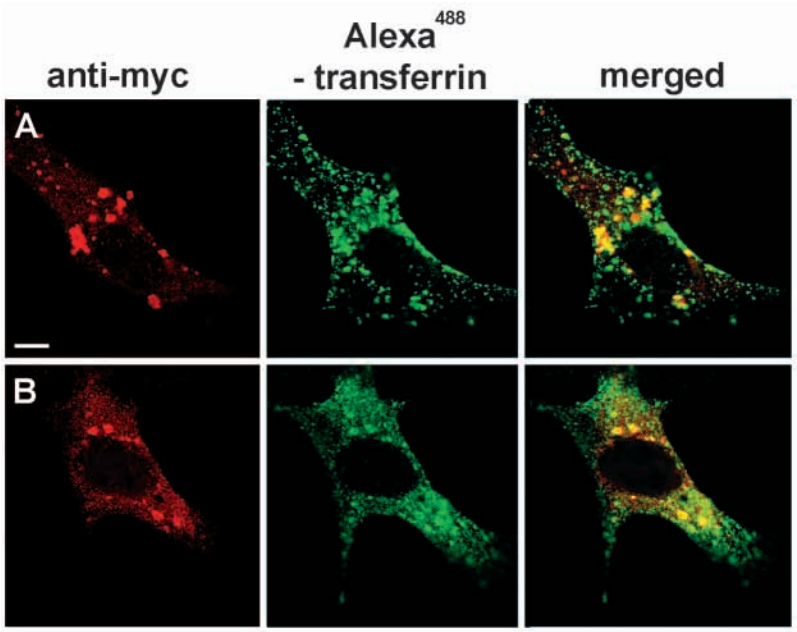

Fig. 9. Colocalisation of Hrs constructs with endocytosed Alexa^{488}-transferrin. BHK cells were cotransfected with the human transferrin receptor and myc-tagged Hrs (A) or with Hrs$_{FYVE+CC2}$ (B) and incubated with Alexa^{488}-transferrin at 37°C for 15 minutes. The fixed cells were stained with anti-myc antibodies (left); (middle) the internalized Alexa^{488}-transferrin; (right) the merged images. Yellow colour indicates colocalisation. Bar, 5 μm.

tagged proteins (not shown), excluding the possibility of artificial targeting as a consequence of mass action. Even though Hrs^{R183A} was mainly cytosolic (see Fig. 7), the remaining membrane-associated fraction of this protein showed a significant (20%) colocalisation with EEA1. Thus, impairment of FYVE function does not completely abolish the targeting of Hrs to endosomes. A possible explanation for this is provided in the Discussion. In contrast, Hrs$_{FYVE}$ and Hrs$_{CC2}$ exhibited essentially no colocalisation with EEA1. Similar results were obtained with HeLa, Hep2 and NIH 3T3 cells, demonstrating that the subcellular targeting of Hrs is not strictly cell-type dependent (not shown). Altogether, our results (summarised in Fig. 11) indicate that the FYVE and CC2 domains cooperatively mediate the specific targeting of Hrs to early endosomes.

DISCUSSION

In this report we have shown that Hrs has a highly specific localisation to early endosomes, and that its FYVE and CC2 domains are responsible for this subcellular targeting. In contrast to EEA1, the endosomal localisation of Hrs is Rab5-independent, indicating that Rab5 binding is not a general feature of FYVE-finger proteins.

According to the crystal structure of the N terminus of Hrs, the VHS domain is likely to interact with the membrane surface (Mao et al., 2000). However, since deletion of the VHS domain did not interfere with the ability of Hrs to localise to early endosomes, the putative membrane interaction of the VHS domain may have a regulatory function rather than a role in the subcellular targeting of Hrs. In contrast, since the endosomal localisation of Hrs requires PI 3-kinase activity (Komada and Soriano, 1999; Urbé et al., 2000) as well as a functional FYVE domain, the interaction of the FYVE domain with PtdIns(3)P appears to be crucial for the intracellular targeting of Hrs. In addition, the CC2 domain is required, and the most straightforward interpretation of our results is that the binding of the CC2 domain to an endosomal membrane molecule contributes to the targeting of Hrs to endosomes, in cooperation with the FYVE-PtdIns(3)P interaction. On the other hand, at present we cannot rule out the possibility that one of these domains confers an allosteric regulation to the other, rather than binding directly to the endosome membrane.

What is the identity of the CC2-interacting molecule that contributes to the endosomal targeting of Hrs? From our previous studies of EEA1, we had expected to find an interaction between Hrs and Rab5, but we were unable to detect any interaction with Rab5 and other endosomal Rab GTPases, suggesting that the mechanism of targeting of Hrs to early endosomes is principally different to that of EEA1. It is interesting to note that the CC2 domain of Hrs binds to the the SNARE molecule SNAP-25, which regulates membrane fusion (Kwong et al., 2000). SNAP-25 is present mainly at the plasma membrane, but related molecules have been found on endosomes (Chen and Whiteheart, 1999). Thus, SNAP-25-related SNARE molecules may be regarded as possible candidates for targeting Hrs to early endosomes.

Conflicting results have been obtained regarding the

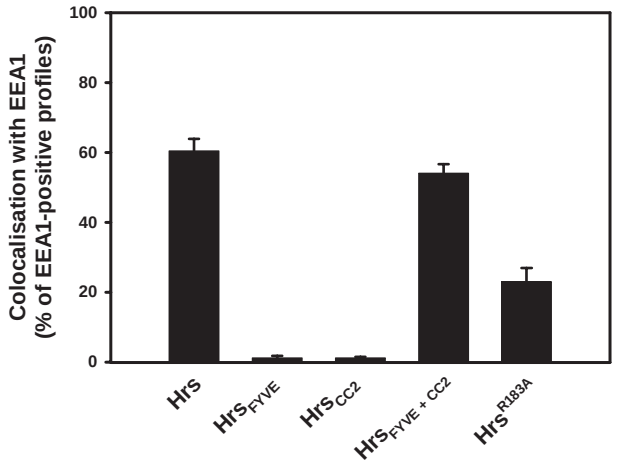

Fig. 10. Quantitation of the extent of colocalisation between Hrs constructs and EEA1. BHK cells were transfected with the indicated myc-tagged Hrs constructs and permeabilised with saponin prior to fixation and immunolabelling with anti-EEA1 and anti-myc antibodies. Images of randomly selected transfected cells were recorded by confocal microscopy. Profiles positive for both EEA1 and the respective Hrs construct were counted manually, and these numbers were expressed as a percentage of the total number of EEA1-positive structures. For each Hrs construct, 20 cells (10 cells each from two independent transfections) were analysed. In total, about 12,000 profiles were counted in this analysis. Values are means ± s.e.m.

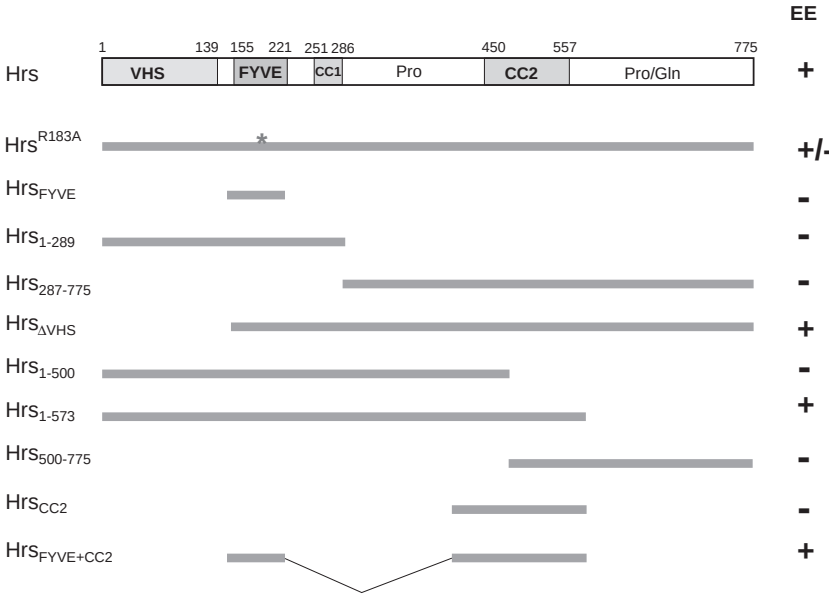

Fig. 11. Properties of Hrs and its different domains. The various Hrs constructs are represented as thick lines, and their localisation (+) or not (–) to early endosomes (EE) is indicated to the right. The asterisk indicates the R183A mutation.

importance of the FYVE domain for the subcellular targeting of Hrs (Hayakawa and Kitamura, 2000; Urbé et al., 2000). In order to clarify this issue we used two early endosome markers (EEA1 and endocytosed transferrin) and tested four different cell lines. Furthermore, we performed a quantitative analysis of confocal micrographs and employed a novel FYVE construct with a mutation in the PtdIns(3)P-binding pocket. All our results indicate that the FYVE domain is essential for the targeting of EEA1 to early endosomes. This is perhaps best illustrated by the quantitation in Fig. 10, which shows that a 'minimal' construct consisting of the FYVE and CC2 domain is targeted to early endosomes, whereas the individual FYVE and CC2 domains are not.

Even though the PtdIns(3)P-binding-defective Hrs^{R183A} mutant was mainly cytosolic, a fraction of this protein did localise to early endosomes. If PtdIns(3)P-binding by the FYVE domain is involved in the subcellular targeting of Hrs, how can this result be explained? One possibility is that overexpressed Hrs constructs may dimerise with endogenous Hrs on the membrane. This interpretation is supported by the fact that the N terminus of Hrs crystallises as a dimer (Mao et al., 2000), and that coexpression of wild-type Hrs increases the amount of Hrs^{R183A} found on endosome membranes (C. Raiborg and H. Stenmark, unpublished results).

The ability of overexpressed Hrs to cause the clustering of endosomal structures is intriguing and may give a clue about the function of Hrs. We see two possible explanations for this clustering effect. First, Hrs overexpression may cause an increased docking between endosomes, without increasing their fusion. Second, it may affect the motility of endosomes. Further work is needed in order to distinguish between these possibilities, but it is interesting to note that even the construct consisting only of the FYVE and CC2 domains, Hrs$_{FYVE+CC2}$, caused the endosomal clustering effect. One of these domains is therefore likely to interact with the endosomal docking or motility apparatus.

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