Early Endosomal Localization of Hrs Requires a Sequence within the Proline- and Glutamine-rich Region but Not the FYVE Finger*

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Hrs is an early endosomal protein that is tyrosine-phosphorylated in cells stimulated with growth factors. Hrs is thought to play a regulatory role in endocytosis of growth factor-receptor complexes through early endosomes. Early endosomal localization of Hrs seems to be essential for Hrs to exert its function in the endocytosis. Hrs has a FYVE finger domain that binds specifically to phosphatidylinositol 3-phosphate in vitro. The FYVE finger is a likely domain that mediates membrane association of endosomal proteins. In this study, we examined whether the FYVE finger participates in early endosomal targeting of Hrs. Hrs with a zinc binding-defective FYVE finger was still localized to early endosomes. In addition, the N-terminal FYVE finger-containing fragment of Hrs showed a cytosolic distribution in mammalian cells. These results indicate that the FYVE finger is not required for the localization of Hrs to early endosomes. Furthermore, by analyzing a series of deletion mutants of Hrs, we identified a sequence of about 100 amino acids within the C-terminal proline- and glutamine-rich region as a domain essential for the targeting of Hrs to early endosomes.

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A Sequence Required for Early Endosomal Localization of Hrs

5'-GGGGCGGCCGCTCACCCTGTCATGTCCGCTCTTCGCTGAAAAGGGATAGCTTAATGTTAAGGAGGATT TTACAGAGGACTCC-3' and mouse Hrs cDNA as a template. The cDNA encoding C-terminally HA-tagged Hrs mutant, Hrs604–775, was constructed by PCR using oligonucleotide primers, 5'-AGGAGGGAAACTT-3', 5'-GGGGCGGCCGCGTCGACGCCATGGAGCAGAAGCTCATTGCAGTGGGAAAATC-3', and mouse Hrs cDNA as a template. The PCR products were digested with HindIII and NotI and inserted into the HindIII and NotI sites of an expression vector pmiw-HA. The cDNA encoding N-terminally HA-tagged Hrs was described previously (2). To construct an expression vector with the HA tag sequence, two oligonucleotides, 5'-GGGGCGGCCGCGTCGACGCCATGGAGCAGAAGCTCATTGCAGTGGGAAAATC-3' and 5'-GGGGCGGCCGCGTCGACGCCATGGAGCAGAAGCTCATTGCAGTGGGAAAATC-3', were annealed and inserted into the HindIII and NotI sites of an expression vector pmiwHA. The cDNA encoding N-terminally HA-tagged Hrs was constructed by PCR using oligonucleotide primers, 5'-GGGGCGGCCGCGTCGACGCCATGGAGCAGAAGCTCATTGCAGTGGGAAAATC-3' and mouse Hrs cDNA as a template. The PCR product was digested with NotI and inserted into the NotI site of the expression vector pmiw-HA. The cDNA encoding N-terminally myc-tagged EEA1 was constructed by PCR using oligonucleotides primers, 5'-GGGGCGGCCGCGTCGACGCCATGGAGCAGAAGCTCATTGCAGTGGGAAAATC-3' and mouse Hrs cDNA as a template. The PCR product was digested with NotI and XhoI and inserted into the NotI and XhoI sites of an expression vector pcdNA3 (Invitrogen).

Indirect Immunofluorescence Staining—HeLa cells were transfected with 1 μg of plasmid DNA. At 48 h after transfection, cells were incubated with 50 μg/ml of Alexa 594-conjugated transferrin (Molecular Probe) for 15 min at 37 °C. After incubation, cells were washed twice with cold PBS, fixed, and permeabilized in 100% methanol for 5 min at −20 °C. The primary antibody was a mouse anti-FLAG monoclonal antibody (Sigma) or a mouse anti-myc monoclonal antibody (Santa Cruz Biotechnology, Inc.). The secondary antibody was an Alexa 488-conjugated anti-mouse immunoglobulin G antibody (Molecular Probe). Cells were examined by confocal immunofluorescence microscopy.

Liposome Binding—Liposome binding was performed as described previously (15) with a slight modification. Liposomes were prepared by mixing phosphatidylserine, phosphatidylinositol, and PtdIns(3)P at the proportions indicated, drying the mixture under nitrogen, and resuspending to a final concentration of 1 mg of total phospholipid/ml in 200 μl of a suspension buffer (50 mM Hepes, pH 7.2, 100 mM NaCl, and 0.5 mM EDTA). Resuspended lipids were sonicated in a bath sonicator until a homogenous suspension was formed (5 min). Liposomes were collected by centrifugation at 35,000 × g for 10 min and resuspended in 100 μl of PBS. Near confluent HeLa cells in a 100-mm dish were homogenized by a Teflon glass homogenizer in 1 ml of PBS containing protease inhibitors, Complete® EDTA-free (Roche Molecular Biochemicals), and then centrifuged at 1,000 × g for 5 min at 4 °C. The supernatant was centrifuged at 105,000 × g for 1 h at 4 °C. A 400-μl volume of the supernatant was incubated with 50 μl of liposome at room temperature for 15 min. The samples were centrifuged at 35,000 × g for 10 min, and 20 μl of each supernatant was collected and mixed with 20 μl of 2 × sample buffer (25 mM Tris-HCl, pH 6.5, 1% SDS, 1% 2-mercaptoethanol, and 5% glycerol). The pellets were washed with 1 ml of PBS and centrifuged at 35,000 × g for 10 min. The pellets were suspended with 50 μl of PBS and mixed with 50 μl of 2 × sample buffer. Samples were boiled for 5 min, and 20 μl of each sample was subjected to immunoblotting using an anti-FLAG antibody. C, structures of the transfected constructs. P and Q are proline and glutamine, respectively.

FIG. 1. Immunofluorescence staining of the wild-type Hrs and mutant Hrs with the zinc binding-defective FYVE finger (C215S) in transfected HeLa cells. A, HeLa cells expressing the FLAG-tagged wild-type (a–c) or mutant Hrs (d–f) were incubated with Alexa 594-conjugated transferrin. After incubation, cells were fixed, permeabilized, and incubated with a mouse anti-FLAG antibody followed by Alexa 488-conjugated anti-mouse immunoglobulin G antibody (a and d). The internalized transferrins are shown in b and e. The yellow stain indicates co-localization (c and f). B, expression of the proteins was verified by immunoblotting using an anti-FLAG antibody. C, structures of the transfected constructs. P and Q are proline and glutamine, respectively.
to SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting.

**Immunoprecipitation and Immunoblotting**—Immunoprecipitation and immunoblotting were performed as described previously (1). For immunoprecipitation, 5 μg of anti-FLAG epitope monoclonal antibody (Roche Molecular Biochemicals) was used. For immunoblotting, 200-fold diluted anti-Hrs antiseraum (1), 1 μg/ml of anti-EEA1 antibody (Santa Cruz), anti-FLAG monoclonal antibody, or anti-HA monoclonal antibody 12CA5 (Roche Molecular Biochemicals) was used.

**RESULTS**

**Early Endosomal Localization of Hrs with a Zinc Binding-defective FYVE Finger**—Bacterially expressed glutathione S-transferase-Hrs1–289 protein containing the FYVE finger binds specifically to PtdIns(3)P in vitro, but a zinc binding-defective mutant glutathione S-transferase-Hrs1–289 in which Cys-215 in the FYVE finger is replaced with a serine (C215S) does not (11). Further, the EEA1 protein in which the FYVE finger is replaced with the mutant FYVE finger (C215S) of Hrs lacks the ability to associate with endosomes (11). To examine whether the FYVE finger of Hrs is involved in early endosomal targeting, the FLAG-tagged Hrs and HA-tagged Hbpl was analyzed directly by immunoblotting (lane 1) or immunoprecipitated with an anti-FLAG antibody (lane 2) first and then analyzed by immunoblotting (IB). For a positive control, lysate of HeLa cells co-expressing the FLAG-tagged Hrs and HA-tagged Hbpl was analyzed directly by immunoblotting (lane 3), or immunoprecipitated with an anti-FLAG antibody (lane 4) first and then analyzed by immunoblotting. Antibodies used for immunoblotting were an anti-HA antibody (A) and an anti-FLAG antibody (B). Molecular mass standards are indicated in kDa. Bands a and b are nonspecific, and band c corresponds to the heavy chain of antibodies.

**No Dimerization or Oligomerization of Hrs**—HeLa cells contain endogenous Hrs that is localized to early endosomes (2). Thus, there is a possibility that the exogenously expressed zinc binding-defective Hrs associates with endogenous Hrs and is detected on early endosomes. To examine this possibility, the FLAG-tagged Hrs and HA-tagged Hrs were co-expressed in HeLa cells, and the association of these proteins was analyzed by co-immunoprecipitation using an anti-FLAG antibody. As a positive control, the FLAG-tagged Hrs was co-expressed with the HA-tagged Hbpl, a Hrs-binding protein, in HeLa cells, and a similar analysis was performed. The HA-tagged Hrs was detected in the lysate from HeLa cells expressing both the FLAG-tagged and HA-tagged Hrs (Fig. 2, A and B, lane 1). But it was not detected in the fraction containing the FLAG-tagged receptor complexes. These results, together with our previous result that the deletion mutant of Hrs lacking the entire FYVE finger was localized to early endosomes (2), suggest that the FYVE finger in Hrs is not required for early endosomal targeting of Hrs.
Hrs that was immunoprecipitated with an anti-FLAG antibody (Fig. 2, A and B, lane 2), indicating that the FLAG-tagged and HA-tagged Hrs did not associate with each other. The HA-tagged Hbp was co-immunoprecipitated with the FLAG-tagged Hrs by an anti-FLAG antibody (Fig. 2A, lane 4), indicating a tight association of the FLAG-tagged Hrs and HA-tagged Hbp. These results suggest that Hrs does not dimerize or oligomerize and that the exogenously expressed Hrs does not associate with endogenous Hrs.

Failure of the Full-length Hrs to Bind to PtdIns(3)P—The bacterially expressed truncated form of Hrs (Hrs1–289) has been shown to bind specifically to liposomes containing PtdIns(3)P (11). The full-length Hrs with the zinc binding-defective FYVE finger was still localized to early endosomes. Thus, the full-length Hrs may not bind to PtdIns(3)P, even though it contains the wild-type FYVE finger. To determine whether the full-length Hrs binds to PtdIns(3)P, endogenous Hrs in HeLa cells was assayed for binding to liposomes containing PtdIns(3)P. Endogenous EEA1 in HeLa cells was also assayed as a positive control, because it has been shown to bind to liposomes containing PtdIns(3)P (13). Neither protein bound to the liposome matrix, which was composed of 50% phosphatidylserine and 50% phosphatidylinositol (Fig. 3). Endogenous EEA1 bound to the liposomes containing 1% PtdIns(3)P, whereas endogenous Hrs did not (Fig. 3). These results suggest that the FYVE finger in the full-length Hrs expressed in mammalian cells cannot function as a PtdIns(3)P binding domain.

Early Endosomal Localization of Hrs in Cells Treated with Wortmannin—PtdIns(3)P is produced through the phosphorylation of phosphatidylinositol by phosphatidylinositol-3-OH kinase (PI(3)K) (16). If PtdIns(3)P is involved in early endosomal localization of Hrs through the FYVE finger, treatment of cells with PI(3)K inhibitors would result in change of the localization. Thus, the FLAG-tagged Hrs was expressed in HeLa cells, and the cells were treated with wortmannin, a PI(3)K inhibitor. As a positive control, the Myc-tagged EEA1 was expressed in HeLa cells, and the cells were treated with wortmannin. Then their intracellular localization was analyzed by immunofluorescence staining with an anti-FLAG or anti-Myc antibody. Treatment with wortmannin changed early endosomal localization of EEA1 to be cytosolic (Fig. 4, c and d), whereas it did not affect the localization of Hrs (Fig. 4, a and b). These results suggest that PtdIns(3)P is not involved in endosomal membrane association of Hrs.

Early Endosomal Localization of the C-terminal Half of Hrs—Because bacterially expressed Hrs1–289 binds specifically to liposomes containing PtdIns(3)P, it is possible that this truncated form of Hrs is localized to early endosomes when expressed in mammalian cells. Thus, the FLAG-tagged Hrs1–289 was expressed in HeLa cells, and its intracellular...
localization was analyzed by immunofluorescence staining with an anti-FLAG antibody. The localization of the expressed proteins was cytosolic and punctate staining was not observed in the cells (Fig. 5Aa–c), indicating that the FYVE domain is not sufficient for early endosomal targeting of Hrs in mammalian cells. Instead, the remaining C-terminal region may contribute to the targeting. Thus, the FLAG-tagged truncated form of Hrs (Hrs391–775) was expressed in HeLa cells, and its intracellular localization was analyzed. The expressed protein was co-localized with the internalized transferrin (Fig. 5Ad–f), suggesting that the sequence responsible for early endosomal localization of Hrs is located within the region 391–775. It should be noted that early endosomes were of normal size in cells expressing Hrs391–775 (Fig. 5Ad–f), whereas larger early endosomes were observed in cells expressing the full-length of Hrs (Fig. 1A).

Early Endosomal Localization of Hrs Lacking the STAM or Hbp-binding Site—The C-terminal half of Hrs (Hrs391–775) contains a coiled-coil motif that mediates the binding of Hrs to the interacting proteins, STAM and Hbp (4, 5). Thus, it is possible that these proteins associate with early endosomal membrane, and Hrs is localized to the same compartment by binding to STAM or Hbp through the coiled-coil motif. To examine this possibility, the FLAG-tagged deletion mutant of Hrs lacking the coiled-coil motif (HrsΔCC) was expressed in HeLa cells, and its intracellular localization was analyzed by immunofluorescence staining. The expressed protein was co-localized with the internalized transferrin (Fig. 5Aa–c), suggesting that the sequence responsible for early endosomal localization of Hrs is located within the region 391–775. It should be noted that early endosomes were of normal size in cells expressing Hrs391–775 (Fig. 5Aa–f), whereas larger early endosomes were observed in cells expressing the full-length of Hrs (Fig. 1A).

Identification of the Sequence Required for Early Endosomal Localization of Hrs—To identify the sequence required for early endosomal localization of Hrs, various FLAG-tagged deletion mutants were expressed in HeLa cells, and their intracellular localization was analyzed by immunofluorescence staining. The deletion mutant lacking the C-terminal 172 amino acid residues (Hrs1–603) showed punctate staining (Fig. 7Aa) and was co-localized with the internalized transferrin (data not shown). However, further deletion mutations (Hrs1–502 and Hrs1–465) caused a cytosolic distribution of the expressed proteins (Fig. 7Ab and c). Reciprocally, the deletion mutant lacking the N-terminal 502 amino acid residues (Hrs604–775) showed punctate staining (Fig. 7Ad) and was co-localized with the internalized transferrin (data not shown). The mutant of Hrs with a further 101-amino acid deletion (Hrs604–775) did not show punctate staining (Fig. 7Ae) and was not co-localized with the internalized transferrin (data not shown), although it did not show a cytosolic distribution. Furthermore, the deletion mutant of Hrs lacking the N-terminal 104 amino acids in the proline- and glutamine-rich region (ΔPQ) showed a cytosolic distribution (Fig. 7Af). These results
indicate that an N-terminal sequence of about 100 amino acids within the proline- and glutamine-rich region is required for Hrs to be localized to early endosomes. Larger early endosomes were observed in cells expressing Hrs containing the N-terminal region (Hrs1–603), whereas the size of early endosomes was normal in cells expressing the C-terminal region of Hrs (Hrs503–775). These results, together with the results in Figs. 1A, 5A, and 6A, suggest that overexpression of Hrs that contains the N-terminal region results in larger early endosomes.

DISCUSSION

In this study, we found that Hrs with the zinc binding-defective FYVE finger is still localized to early endosomes and also that the N-terminal FYVE finger-containing fragment of Hrs shows a cytosolic distribution in mammalian cells. These findings led us to conclude that the FYVE finger is not required for the localization of Hrs to early endosomes. Furthermore, the deletion mutant of Hrs that lacked about 100 amino acid residues within the proline- and glutamine-rich region showed a cytosolic distribution. Thus, the sequence essential for the early endosomal targeting of Hrs was identified outside the FYVE finger.

The bacterially expressed truncated form of Hrs containing the FYVE finger has been shown to bind specifically to liposomes containing PtdIns(3)P (11). Furthermore, the FYVE finger of Hrs can replace that of EEA1 with respect to the early endosomal targeting of EEA1 (11). However, in the present study, we found that the full-length Hrs expressed in mammalian cells does not bind to liposomes containing PtdIns(3)P. We also found that treatment with a PI(3)K inhibitor wortmannin does not change the early endosomal localization of Hrs. These findings suggest that unlike EEA1, the FYVE finger of Hrs...
does not interact with PtdIns(3)P in mammalian cells. The conformation of the full-length Hrs may interfere with the interaction of the FYVE finger with PtdIns(3)P. Alternatively, a protein associated with Hrs may prevent the FYVE finger from interacting with PtdIns(3)P.

The function of FYVE fingers of several proteins has been investigated. The FYVE finger of EEA1 is required for both PtdIns(3)P and Rab5 binding, and this dual interaction is critical for endosomal localization of EEA1 and for regulation of endocytic membrane fusion (17, 18). Because an excess of Rab5 increases the endosomal association of EEA1 and decreases its dependence on PtdIns(3)P, the levels of Rab5 seem to be rate-limiting for the recruitment of EEA1 to endosomal membranes, and PtdIns(3)P may modulate the interaction of EEA1 with Rab5 through the FYVE finger (17, 18). Mutations in the FYVE finger of yeast Vps27p do not affect its membrane association, but these mutant proteins do not complement the phenotype of Rab5 through the FYVE finger (17, 18). Mutations in the FYVE finger limiting for the recruitment of EEA1 to endosomal membranes, dependence on PtdIns(3)P, the levels of Rab5 seem to be rate-limiting for endosomal localization of EEA1 and for regulation of other protein(s). Rab5 would be a candidate molecule that interacts with the FYVE finger of Vac1p required for its function but not for its membrane association. Vac1p membrane association is not dependent on PtdIns(3)P or P(3)K (Vps34p), and Vac1p FYVE finger mutant missorting phenotypes are suppressed by a defective allele of VPS34, indicating that PtdIns(3)P may play a regulatory role, possibly involved in mediating Vac1p protein-protein interactions through the FYVE finger (19). In the present study, we demonstrated that the FYVE finger of Hrs is not required for early endosomal localization of Hrs. We also showed that Hrs membrane association is not dependent on PtdIns(3)P. Like EEA1 and Vac1p, the FYVE finger of Hrs may be required for its interaction with other protein(s). Rab5 would be a candidate molecule that interacts with the FYVE finger of Hrs. However, no interaction between Hrs and the wild-type Rab5 or constitutively activated Rab5b (Rab5bQ79L) was detected by yeast two-hybrid analysis (data not shown). Thus, protein(s) other than Rab5 may interact with Hrs through the FYVE finger and may modulate the function of Hrs.

A domain required for early endosomal targeting of Hrs was identified within the proline- and glutamine-rich region. The sequence of the domain did not show any similarity with those of other proteins. Proline-rich regions and glutamine-rich regions of several proteins have been shown to be involved in multiple protein interactions (21–23). Thus, the newly identified domain may mediate interaction of Hrs with a protein that is localized on early endosomes. Rab5 is one of the early endosomal proteins, but, as described above, Hrs does not seem to bind to Rab5. Two Hrs-binding proteins, STAM and Hbp, were identified (4, 5), but Hrs binds to them through the coiled-coil motif outside the proline- and glutamine-rich region. Thus, it is not known at present whether the domain involved in endosomal targeting of Hrs mediates interaction of Hrs with an endosomal protein. Alternatively, it is possible that Hrs associates directly with endosomal membranes through the domain. Further characterization is required to understand the mechanism by which the domain mediates interaction of Hrs with endosomal membranes.

Overexpression of Hrs causes the aggregation of early endosomes, which is observed by immunoelectron microscopy (2). The aggregation is observed as large sized early endosomes when analyzed by immunofluorescence staining (2). In the present study, we also observed large early endosomes in cells expressing some Hrs mutants. Expansion of early endosomes is observed in Hrs knockout cells (24). These observations suggest that Hrs regulates docking and/or fusion of endosomal membranes. Overexpression of the truncated form of Hrs that consists of only the C-terminal half (Hrs(391–727)) did not affect the size of early endosomes. Thus, the remaining N-terminal half appears to be involved in the aggregation of early endosomes. This region contains the N-terminal VHS domain, the FYVE finger, and the C-terminal proline-rich region. Overexpression of Hrs lacking the entire FYVE finger still results in large early endosomes (2), indicating that the FYVE finger does not mediate the aggregation of early endosomes. VHS domains are present in proteins involved in endocytosis and vesicular trafficking (25, 26). Thus, the VHS domain of Hrs may be involved in the aggregation of early endosomes. Although the three-dimensional structure of the VHS domain has been revealed (27), its role in the function of Hrs is not known. Identification of the domain involved in the aggregation of early endosomes may reveal the regulatory role of Hrs in docking and/or fusion of endosomal membranes.

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