Hrs, a Tyrosine Kinase Substrate with a Conserved Double Zinc Finger Domain, Is Localized to the Cytoplasmic Surface of Early Endosomes*

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Hrs is a 115-kDa double zinc finger protein that is rapidly tyrosine phosphorylated in growth factor-stimulated cells. However, its function remains unknown. Here we show that Hrs is localized to early endosomes. Intracellular localization of endogenous Hrs and exogenously expressed Hrs tagged with the hemagglutinin epitope was examined by immunofluorescence staining using anti-Hrs and anti-hemagglutinin epitope antibodies, respectively. Hrs was detected in vesicular structures and was colocalized with the transferrin receptor, a marker for early endosomes, but only partially with CD63, a marker for late endosomes. A zinc finger domain deletion mutant of Hrs was also colocalized with the transferrin receptor, suggesting that the zinc finger domain is not required for its correct localization. Immunoelectron microscopy showed that Hrs was localized to the cytoplasmic surface of these structures. By subcellular fractionation, Hrs was recovered both in the cytoplasmic and membrane fractions. The membrane-associated Hrs was extracted from the membrane by alkali treatment, suggesting that it is peripherally associated with early endosomes. These results, together with our finding that Hrs is homologous to Vps27p, a protein essential for protein traffic through a prevacuolar compartment in yeast, suggest that Hrs is involved in vesicular transport through early endosomes.

Hrs (hepatocyte growth factor (HGF)4-regulated tyrosine kinase substrate) was originally identified as a tyrosine phosphorylated protein in cells stimulated with HGF (1). In the cells expressing the HGF receptor tyrosine kinase, c-Met, which was mutated in the cytoplasmic domain and could not mediate the cellular responses triggered by HGF, Hrs was not tyrosine phosphorylated in response to ligand stimulation (1). This result suggested that Hrs plays an important role in the signaling pathway of HGF.

Hrs was purified from HGF-treated B16-F1 mouse melanoma cells by anti-phosphotyrosine immunofinity chromatography, and its cDNA was subsequently cloned based on the partial amino acid sequences obtained from the purified protein (2). The cDNA sequence revealed that mouse Hrs is a novel 775-amino acid protein with a double zinc finger domain that is structurally conserved in several other proteins. Northern blotting showed that Hrs mRNA is ubiquitously expressed in mouse tissues. In addition, experiments using anti-Hrs antibody, which was raised against bacterially expressed Hrs, showed that 1) tyrosine phosphorylation of Hrs reaches a maximal level 5 min after exposing cells to HGF and disappears within 2 h; 2) Hrs does not form a stable complex with the HGF receptor, c-Met, even when tyrosine phosphorylated; and 3) tyrosine phosphorylation of Hrs is also induced in cells stimulated with epidermal growth factor or platelet-derived growth factor (2).

There was no domain in the Hrs sequence that allowed its function to be predicted. However, the double zinc finger domain of Hrs was 40–50% identical to those of several other proteins including FGD1, EEA1, Fab1p, Vps27p, and ZK632.12. The consensus sequence is Cys-Xaa3-Cys-Xaa4-Phe-Xaa4-Arg-(Arg/Lys)-His-His-Cys-(Arg/Lys)-Xaa3-Cys-Gly-Xaa-(Val/Ile)-Val/Phe-Cys-Xaa3-Cys-Ser-Xaa3-14-16Arg-Val-Cys-Xaa3-Cys-(Tyr/Phe). FGD1 is a product of the human faciogenital dysplasia gene and is considered to be a guanine nucleotide exchange factor of small G proteins (3). EEA1 (early endosome antigen 1) is a hydrophilic human protein that is peripherally associated with early endosomes (4). Fab1p is a phosphatidylinositol 4-phosphate 5-kinase required for normal vacuole morphology and function in Saccharomyces cerevisiae (5). Vps27p is required for vacuolar and endocytic traffic through a prevacuolar compartment in S. cerevisiae (6). The function of the Caenorhabditis elegans protein ZK632.12 (Swiss Prot accession number P94657) is not known. In addition, the N- terminal half of the zinc finger domain of Hrs was 57% identical to that of Vac1p, a protein required for vacuolar inheritance and vacuolar protein sorting in S. cerevisiae (7). These facts suggest that the zinc finger domains of these proteins play the same or similar important role(s) in vesicular functions in cells and, more importantly, that Hrs is also involved in vesicular function.

In this study, we showed that the amino acid sequence of Hrs is 23% identical to that of Vps27p, a protein required for vesicular traffic in yeast. Moreover, we showed by immunofluorescence and immunoelectron microscopy that Hrs is localized to the cytoplasmic surface of early endosomes. These results suggested that, like Vps27p in yeast, Hrs is important for vesicular transport through early endosomes in mammalian cells.

EXPERIMENTAL PROCEDURES

Construction of Hrs Expression Vectors—To construct the cDNA encoding Hrs that is COOH-terminally tagged with the human influenza virus hemagglutinin (HA) epitope (Hs-Hs-HA), four oligonucleotide prim-
ers were synthesized: 5'-ACAGACCAGCAACATAGGCT-3' (primer 1), 5'-CCCTCTAGAGTCAAAAGGATGACGCT-3' (primer 2), 5'-GGGTTCTAGATCTAGCTATC-3' (primer 3), and 5'-GGGGAGCTCAAGCCTTTATCCTTCGCCGAT-3' (primer 4). Using primers 1 and 2 and mouse Hrs cDNA as a template, a 274-base pair fragment was amplified by polymerase chain reaction, digested by SmaI and XbaI, and purified by polyacrylamide gel electrophoresis (PAGE) (fragment 1). Using primers 3 and 4, and pCG-HA, which contains the HA epitope sequence (8), a 72-base pair fragment was amplified, digested by XbaI and SacI, and purified by PAGE (fragment 2). In addition, the pBluescript including the full-length mouse Hrs cDNA at the HindIII site was digested by SmaI and SacI, and the fragment containing the vector sequence and the partial Hrs sequence (nucleotides 1–2193) was purified from an agarose gel (fragment 3). Fragments 1, 2, and 3 were ligated together to construct the cDNA for Hrs-HA. The cDNA was excised from the vector by digestion with HindIII and cloned into the HindIII site of the mammalian expression vector pmw (9).

The sequence of the zinc finger domain of the Hrs-HA cDNA was deleted using the Transformer Site-Directed Mutagenesis Kit (CLONTECH) according to the manufacturer's instructions. We used 5'-CTTGTTCAGCTGCTCATATTCCTCAGCATCCACCCA-3' as a mutagenic primer and 5'-CTTCCTTTTTCGATATCATTGAAGCATT-3' as a selection primer.

Cell Culture and DNA Transfection—HeLa cells were provided by Dr. T. Kimura (Kansai Medical University) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. DNA transfection was performed by the standard calcium phosphate precipitation method. Cells were examined 2 days after transfection.

Immunoprecipitation and Immunoblotting—Immunoprecipitation and immunoblotting were performed as described previously (2). For immunoprecipitation, 7.5 μl of anti-Hrs antiserum (2), 2 μg of anti-HA epitope monoclonal antibody 12CA5 (Boehringer Mannheim), 2 μg of anti-phospholipase C-γ (Santa Cruz), or 2 μg of anti-c-Met (Santa Cruz) or 1 μg/ml of anti-phospholipase C-γ, or 1 μg/ml of anti-c-Met was used. For immunoblotting, 100 times diluted anti-Hrs antiserum, 1 μg/ml of anti-phospholipase C-γ, or 1 μg/ml of anti-c-Met was used.

**FIG. 1.** Alignment of the amino acid sequence of Hrs with that of Vps27p. Identical amino acid residues are shown by asterisks, and gaps in the sequences are indicated by hyphens. The zinc finger domains are boxed, and the amino acid numbers are indicated on the right.
**Indirect Immunofluorescence Staining**—Immunofluorescence staining was performed as described previously (10) except for the fixation conditions for staining with anti-Hrs. In this case, cells were fixed and permeabilized in 100% methanol for 5 min at -20°C. The primary antibodies were the rabbit anti-Hrs polyclonal antiserum (1:2,000) (2), a mouse anti-HA polyclonal antibody (5 μg/ml) (MBL), a mouse antihuman transferrin receptor monoclonal antibody (10 μg/ml) (Oncogene Science), and a mouse anti-human CD63 monoclonal antibody (1:50) (Immunotech S.A.). The secondary antibodies were the Cy3-conjugated goat anti-rabbit immunoglobulin G antibody (1:1,000) (Amer sham Corp.), rhodamine-conjugated anti-rabbit immunoglobulin G antibody (1:100) (Protos Immunoresearch), and fluorescein-conjugated anti-mouse immunoglobulin G antibody (1:100) (American Qualex Antibodies and Immunoc hemicals). Cells were examined by confocal immunofluorescence microscopy.

**Immunoelectron Microscopy**—Immunoelectron microscopic detection was performed as described by Mrini et al. (11) with a slight modification. Cells were transfected with or without the Hrs-HA cDNA on collagen-coated plastic sheets (Sumitomo Bakelite), fixed in 4% paraformaldehyde for 30 min, and treated with 1% sodium borohydride for 20 min. The cells were cryo-protected in 35% sucrose and 14% paraformaldehyde for 30 min, and treated with 1% sodium borohydride for 20 min. The cells were incubated with rabbit anti-HA epitope antibody (1:2,000) (Amer sham Corp.), rhodamine-conjugated anti-rabbit immunoglobulin G antibody (1:100) (Protos Immunoresearch), and fluorescein-conjugated anti-mouse immunoglobulin G antibody (1:100) (American Qualex Antibodies and Immunoc hemicals). Cells were examined by confocal immunofluorescence microscopy.

**RESULTS**

**Homology between Hrs and Vps27p**—The S. cerevisiae gene, VPS27, which is required for the normal morphology and function of a prevacuolar compartment has recently been identified (6). In VPS27-deficient cells, transport of endocytosed proteins and newly synthesized vacuolar proteins to vacuoles was disrupted, and these proteins accumulated in a prevacuolar compartment. The gene encoded a novel protein with a double zinc finger domain (Fig. 2). This is in accord with the fact that Hrs-HA is 15 kDa slower on a SDS gel than the endogenous mouse and human Hrs (Fig. 2, control), indicating that the zinc finger domain of Hrs is homologous to those of several proteins that are involved in vesicular functions, we also constructed a zinc finger domain deletion mutant (ΔZF) to determine whether the zinc finger domain is required for its intracellular localization. A region from Cys^166^ to Cys^215^ (Fig. 1) was deleted in the mutant. The Hrs-HA and Hrs-HAΔZF cDNAs were inserted into the mammalian expression vector, pmw, then transfected into HeLa cells. When the cells transfected with the Hrs-HA cDNA were lysed, immunoprecipitated by anti-HA antibody, and immunoblotted by anti-Hrs, a single 115-kDa band was detected (Fig. 2, mouse Hrs). It had the same apparent molecular mass as mouse Hrs immunoprecipitated from B16-F1 cells (Fig. 2, mouse Hrs). Immunoblotting with anti-phosphotyrosine antibody showed that Hrs was tyrosine phosphorylated in HeLa cells transfected with the respective cDNAs. Immunoprecipitated proteins were separated by SDS-PAGE under reducing conditions and immunoblotted with anti-Hrs. Molecular mass standards are indicated in kDa.

**Indirect Immunofluorescence Staining of Hrs**—To elucidate the intracellular localization site of Hrs, we performed indirect immunofluorescence staining of Hrs. When HeLa cells were fixed with methanol and stained by anti-Hrs, vesicular structures in the cytoplasmic and perinuclear regions were stained (Fig. 3, A and B). These structures were not stained by a preimmune serum or in the presence of excess of the bacterially expressed Hrs that was used as an immunogen to raise the antibody (data not shown). To identify these structures, the cells were double stained with anti-transferrin receptor or anti-CD63. The transferrin receptor is a marker of early endosomes and plasma membrane (12, 13), whereas CD63 is that of late
endosomes and early lysosomes (14). Most of the vesicles that were stained with anti-Hrs were also stained with anti-transferrin receptor, although some of the small vesicles that were positive for transferrin receptor were not stained with anti-Hrs (Fig. 3, A’, A, and A’). In addition, the plasma membrane, which was stained with anti-transferrin receptor, was not stained with anti-Hrs (Fig. 3, A, A’, and A’). In contrast, most of the Hrs-positive vesicles were not stained with anti-CD63 (Fig. 3, B, B’, and B’). These results indicated that Hrs is localized to early endosomes.

**Indirect Immunofluorescence Staining of Hrs-HA**—To confirm the early endosomal localization of Hrs by examining the localization of exogenously expressed Hrs, HeLa cells were transfected with the Hrs-HA cDNA, fixed with paraformaldehyde, and stained with anti-HA. Similar to the staining with anti-Hrs, anti-HA also detected vacuolar or vesicular structures in transfected cells (Fig. 4, A and B). In addition, Hrs-HA was completely colocalized with the transferrin receptor (Fig. 4, A’, A, and A’), but only partially with CD63 (Fig. 4, B, B’, and B’), further confirming that Hrs is localized to early endosomes. In this case, the plasma membrane was not stained with anti-transferrin receptor (Fig. 4A’), as observed in Fig. 3A’. This may be due to the difference of the conditions to fix cells. It should be noted that the early endosomes in Hrs-HA-transfected cells were larger than those in untransfected cells (Fig. 4A’, arrow and arrowheads).

It was possible that the structurally conserved double zinc finger domains in several proteins involved in vesicular function play a role in their intracellular localization. However, Hrs-HAΔZF was also colocalized with the transferrin receptor (Fig. 4, C, C’, and C’), suggesting that the zinc finger domain is not required for the correct localization of Hrs.

**Immunoelectron Microscopic Detection of Hrs-HA**—To investigate the more detailed localization of Hrs in early endosomes, we detected Hrs-HA in transfected cells by means of immunoelectron microscopy. Unfortunately, we could not use anti-Hrs for immunoelectron microscopic detection of Hrs, because this antibody could detect Hrs only in cells fixed with methanol and methanol-fixed cells cannot be adapted for immunoelectron microscopy. HeLa cells transfected with the Hrs-HA cDNA were fixed, permeabilized, and incubated with rabbit anti-HA epitope antibody. The cells were then successively incubated with biotin-labeled anti-rabbit immunoglobulin G antibody and horseradish peroxidase-conjugated avidin, and localization of Hrs was visualized using diaminobenzidine and H2O2. Consistent with the results of immunofluorescence staining shown in Fig. 4, vesicular structures were again stained (Fig. 5A, arrow). No dense background staining was detected in untransfected cells (Fig. 5A, cells without arrows). When the ultrathin sections of the Hrs-HA-expressing cells were examined by an electron microscope, Hrs-HA was detected on the cytoplasmic surface of the vesicular structures (Fig. 5B). The size of these structures (roughly 0.2–0.5 μm in diameter) was similar to that of early endosomes (15). This immunoreactivity was not detected on vesicular structures of untransfected cells (Fig. 5A and not shown), indicating that this staining is specific for transfected Hrs. Together with the results of immunofluorescence staining, this finding indicated that Hrs is localized to the cytoplasmic surface of early endosomes. The Hrs-HA-positive endosomes in transfected cells often aggregated with each other, which was not detected in untransfected cells (Fig. 5B and not shown).

**Subcellular Fractionation**—In the case of Vps27p, it has been shown by subcellular fractionation that about 60% of the protein in whole yeast cell lysates sediments with the membrane fraction only in the presence of divalent cations (6). We therefore performed the subcellular fractionation of endogenous Hrs, Hrs-HA, and Hrs-HAΔZF in untransfected or transfected HeLa cells in the presence of EDTA or Ca2+. The cells were homogenized in the presence or the absence of 1 mM EDTA and 1 mM Ca2+, and then the post-nuclear supernatants were separated into cytoplasmic and membrane fractions. When endogenous Hrs was immunoprecipitated from each fraction by anti-Hrs and immunoblotted by the same antibody, the protein was detected in both the cytoplasmic and membrane fractions irrespective of the presence of EDTA or Ca2+ (Fig. 6A, top panel). The amount of Hrs that was recovered in the cytoplasmic fraction was severalfold more than that recovered in the membrane fraction. When Hrs-HA and Hrs-HAΔZF were fractionated from transfected cells, these proteins were equally detected in both of the fractions (Fig. 6A, second and third panels). In contrast, the cytoplasmic protein, phospho-
lipase C-\(\gamma\), and the transmembrane protein, c-Met receptor, were recovered in the cytoplasmic and membrane fractions, respectively, under all conditions, verifying the integrity of the fractions (Fig. 6A, fourth and bottom panels).

To characterize the nature of the association of Hrs, Hrs-HA, and Hrs-HA\(\Delta\)ZF, with the membrane, we treated the membrane fractions with 100 mM Na\(2\)CO\(_3\), pH 11.5, which extracts peripherally associated membrane proteins from membranes (16). As shown in Fig. 6B, the membrane-associated portions of the three proteins were extracted into the supernatants of the Na\(2\)CO\(_3\)-treated membranes (M-1) but were undetectable in the treated membranes (M-2), suggesting the peripheral association of Hrs with early endosomes.

These results suggest that the membrane association of Hrs, which was observed by immunofluorescence and immunoelectron microscopy, was not so tight and the proteins easily dissociated from the membrane during fractionation. This was in agreement with the fact that Hrs has no hydrophobic region that may act as a membrane spanning domain (2). A similar type of peripheral membrane association has also been reported for EEA1, a hydrophilic early endosomal protein with a zinc finger domain highly homologous to that of Hrs (4). In addition, the membrane association of Hrs depended on neither Ca\(^{2+}\) nor its zinc finger domain.

**DISCUSSION**

In this study, we found that Hrs is localized to early endosomes in HeLa cells. In addition, the amino acid sequence of Hrs was 23% identical to that of Vps27p throughout the sequences. VPS27 is one of the genes whose disruption in *S. cerevisiae* interrupts traffic of endocytosed proteins and newly synthesized vacuolar proteins to vacuoles (a counterpart of mammalian lysosomes) and causes the accumulation of these proteins in a prevacuolar compartment termed the “class E” compartment (6), which is considered to be a counterpart of mammalian endosomes (17). Moreover, Vps27p is localized to the class E compartment (6). Considering from these observations, it is possible that Hrs plays a role in mammalian cells that is similar to that of Vps27p in yeast. Namely, Hrs may be involved in protein traffic through early endosomes. Although it is not known whether Hrs is a mammalian homolog of Vps27p, it is of interest to examine whether Hrs complements the phenotype of the VPS27 mutation in yeast.

The zinc finger domain of Hrs is highly homologous not only to that of Vps27p but also to those of EEA1 (4), Fab1p (5), and Vac1p (7), which are all involved in the function of endosomes or vacuoles. Thus, the zinc finger domains in these proteins must be important for their function. However, the zinc finger domain of Hrs was not required for its correct localization to early endosomes. It is reported that mutations in the zinc finger domain of Vps27p also do not affect its membrane association, although these mutant proteins do not complement the phenotypes of the VPS27 mutation, indicating that the zinc finger domain is essential for its function but not for its membrane localization (6). In contrast to these observation, Sten-
mark et al. (18) recently reported that the zinc finger domain of EEA1 is essential for its early endosomal localization and membrane association. In the case of Fab1p, it is known that the zinc finger domain is not required to complement the phenotypes of FAB1 mutation such as enlargement of vacuoles and the formation of aploid and binucleate cells (5). Therefore, further investigation is necessary to clarify the function of the zinc finger domain in each of these proteins.

Immunoelectron microscopy showed that in cells expressing Hrs-HA, early endosomes often aggregated with each other. This may be the reason why Hrs-HA-positive endosomes that were observed by immunofluorescence staining looked exaggerated. In addition, the ratio of the amounts of Hrs-HA and Hrs-HAΔZF that were subcellularly fractionated into the membrane fractions was higher than that of endogenous Hrs. This may also be due to the aggregation of early endosomes in cells expressing Hrs-HA or Hrs-HAΔZF. It remains unknown whether it is a functional consequence of overexpression of Hrs or simply an artificial structure caused by overexpression irrespective of its function. However, the latter case is likely because it was also observed in cells expressing Hrs lacking the structurally conserved double zinc finger domain.

After binding of growth factors, the growth factor-receptor tyrosine kinase complexes are internalized and targeted to lysosomes through early endosomes and late endosomes (19, 20). In this pathway, they are delivered to early endosomes within a few minutes after growth factor binding and receptor tyrosine kinases remain active after delivery to early endosomes (21–25). Considering that the tyrosine phosphorylation of Hrs is induced in a few minutes after exposing cells to growth factors (2) and that Hrs is localized on the cytoplasmic surface of early endosomes, it is most likely that Hrs is tyrosine phosphorylated on early endosomes by internalized receptor tyrosine kinases that are exposed on the cytoplasmic surface of early endosomes (Fig. 7). Taken together, we propose the following hypothesis for the function of Hrs (Fig. 7). The internalized receptor tyrosine kinase phosphorylates Hrs on early endosomes. Tyrosine phosphorylation activates Hrs and, as speculated from the function of Vps27p in yeast, it stimulates vesicular transport of the growth factor-receptor complex from early endosomes to late endosomes and lysosomes for degradation.

Tyrosine kinase activity of growth factor receptors is required for the internalization and/or degradation of growth factor-receptor complexes (26). However, the mechanism of the process is not well understood. Several proteins are considered to be implicated in the process. A platelet-derived growth factor receptor mutant that cannot associate with phosphatidylinositol 3-kinase is not endocytosed into and not degraded in cells, suggesting a role for phosphatidylinositol 3-kinase in the downregulation of the receptor (27). Annexin I and annexin II may also be involved in the process, because they are localized to the endosomal compartments, promote vesicle fusion, and are phosphorylated by receptor tyrosine kinases (28, 29). Benmerah et al. (30) and Okabayashi et al. (31) have reported that the receptor tyrosine kinase substrates, eps15 and Shc, are constitutively associated with the plasma membrane adaptor, AP-2. Because AP-2 participates in the recruitment of growth
Hrs Is Localized to Early Endosomes

FIG. 7. Hypothetical model for the function of Hrs. The growth factor-bound receptor tyrosine kinase is internalized and delivered to the early endosome, where it phosphorylates Hrs residing on the cytoplasmic surface of the early endosome. Tyrosine phosphorylation activates Hrs, and the activated Hrs stimulates the vesicle transport of the growth factor-receptor complex to the late endosome and lysosome.

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