Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) is a mammalian homologue of yeast vacuolar protein sorting (Vps) protein Vps27p; however, the role of Hrs in lysosomal trafficking is unclear. Here, we report that Hrs interacts with sorting nexin 1 (SNX1), a recently identified mammalian homologue of yeast Vps5p that recognizes the lysosomal targeting code of epidermal growth factor receptor (EGFR) and participates in lysosomal trafficking of the receptor. Biochemical analyses demonstrate that Hrs and SNX1 are ubiquitous proteins that exist in both cytosolic and membrane-associated pools, and that the association of Hrs and SNX occurs on cellular membranes but not in the cytosol. Furthermore, endogenous SNX1 and Hrs form a ~550-kDa complex that excludes EGFR. Immunofluorescence and subcellular fractionation studies show that Hrs and SNX1 colocalize on early endosomes. By using deletion analysis, we have mapped the binding domains of Hrs and SNX1 that mediate their association. Overexpression of Hrs or its SNX1-binding domain inhibits ligand-induced degradation of EGFR, but does not affect either constitutive or ligand-induced receptor-mediated endocytosis. These results suggest that Hrs may regulate lysosomal trafficking through its interaction with SNX1.

Vesicular trafficking, the process by which a transport vesicle buds from a donor membrane and fuses with its target, is fundamental to the function of eukaryotic cells. For example, it is becoming increasingly clear that vesicular trafficking of ligand-activated receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) plays a critical role in controlling diversity, intensity, and duration of tyrosine kinase signaling (1, 2). Binding of EGF triggers the dimerization of EGFR and the activation of the tyrosine kinase at the cytoplasmic domain of the receptor, which then activates downstream signal transduction pathways (3). After ligand binding, the ligand-receptor complexes are recruited to clathrin-coated pits and internalized. Following endocytosis, the ligand-receptor complexes are transported to early endosomes, where a sorting decision must be made between recycling back to the cell surface or delivery to lysosomes for degradation. The internalized EGFR/EGF complexes are primarily transported to lysosomes, and their degradation represents a major mechanism for attenuating EGF signaling (4). Moreover, accumulating evidence indicates that the internalized EGF/EGFR continues to bind and phosphorylate downstream signaling proteins in pre-degradative intracellular compartments, leading to activation of signaling pathways that are distinct from those originated at the cell surface (2, 5). To ensure proper temporal and spatial signaling, the endocytic and lysosomal trafficking of EGF receptors is tightly regulated. Whereas the major events in endocytosis are fairly well understood, the molecular mechanisms underlying lysosomal trafficking of these receptors remain poorly characterized.

Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) was identified originally as a phosphoprotein whose tyrosine phosphorylation is induced upon stimulation by hepatocyte growth factor (6). Subsequent studies demonstrate that the tyrosine phosphorylation of Hrs is also induced by a variety of other growth factors and cytokines, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), interleukin 2, and granulocyte-macrophage colony-stimulating factor (6, 7). Hrs exists in cytosolic and membrane-associated forms, and appears to function in both signaling and vesicular trafficking (6–9). Hrs is thought to function in cell growth signaling by cytokines via its interaction with signal transducing adaptor molecule (STAM) (7). Recently, it was reported that Hrs also interacts with Hbp/STAM2, an STAM isoform involved in cytokine signaling and degradation of PDGF receptors (10, 11). In addition, our previous work has demonstrated that Hrs regulates the exocytotic fusion process via its interaction with SNAP-25, an essential component of the membrane fusion machinery (9). Hrs shares 20% sequence identity and similar domain structure with Vps27p, a yeast protein that is required for trafficking of proteins from a pre-vacuolar/endoosomal compartment to Golgi and vacuole, the yeast equivalent of the lysosome (8, 9, 12). Targeted disruption of Hrs gene in mice leads to abnormally enlarged early endosomes that are reminiscent of exaggerated “class E” compartment in yeast vps27 mutant, suggesting that Hrs may have an analogous function in vesicular trafficking through mammalian endosomes (8, 13). However, it has yet to be demonstrated whether Hrs actually acts in endosome-to-Golgi and endosome-to-lysosome trafficking in mammalian cells.
signaling, we performed a search for proteins that interact with Hrs using a yeast two-hybrid screen. We report here the isolation and characterization of a Hrs-interacting protein that is the rat counterpart of the human sorting nexin 1 (SNX1) (14). SNX1 was first identified as a protein that interacts with the lysosomal targeting signal-containing cytoplasmic region of EGFR (14). Overexpression of SNX1 accelerates degradation of EGFR, suggesting a role for SNX1 in endosome-to-lysosome trafficking (14). It remains controversial as to whether SNX1 interacts only with EGFR (14) or additionally with multiple types of other cell surface receptors, including the receptors for PDGF, insulin, leptin, and transferrin (15). Interestingly, SNX1 is homologous to Vps5p, a yeast protein that is required for endosome-to-Golgi trafficking (16–18). Recent evidence indicates that Vps5p is a molecular component of a multimeric membrane-associated protein complex termed the retromer complex, which serves as a novel membrane coat acting in the formation of vesicles for endosome-to-Golgi trafficking (18). It thus likely that SNX1 may function in a similar manner in mammalian cells, acting as a key component of the lysosomal sorting machinery by incorporating cargo proteins into a retromer-like membrane coat.

In the present study, we demonstrate that Hrs interacts with SNX1 both in vitro and in vivo. We define the structural requirement for this novel interaction and show that the Hrs-binding site of SNX1 overlaps with its EGFR-binding site. In addition, gel filtration analysis and coimmunoprecipitation studies reveal that SNX1 and Hrs form a ~550-kDa complex that excludes EGFR. We characterize the expression pattern and subcellular localization of SNX1 and show that it colocalizes with Hrs on early endosomes. Furthermore, we show that Hrs and SNX1 are involved in the regulation of the ligand-induced degradation of EGFR receptors, but not in the internalization of these receptors. Our data suggest that Hrs may regulate lysosomal sorting and trafficking pathways via its interaction with SNX1.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screens and Interaction Assays**—A bait plasmid, pPC97-Hrs, was constructed by subcloning the entire open reading frame of rat Hrs (9) into the pPC97 vector (19, 20). For the two-hybrid screen, the yeast strain CG-1945 (CLONTECH) was transformed sequentially with pPC97-Hrs and a rat hippocampal/cortical two-hybrid cDNA library (20). Positive clones were selected on 3-aminitopiazole-containing medium lacking leucine, tryptophan, and histidine, and confirmed by a filter assay for β-galactosidase activity (21). Prewashed positive colonies were rescued and re-transformed into fresh yeast cells with the Hrs bait or various control baits to confirm the specificity of the interaction. For analysis of Hrs-SNX1 interaction, deletion constructs of Hrs and SNX1 were made by polymerase chain reaction (PCR) and subcloned into the yeast two-hybrid vector pHIS3 and β-galactosidase as the reporter genes. Quantitative β-galactosidase assay was performed on the yeast extracts by using the substrate chlorophenol red β-galactopyranoside as described previously (21, 22).

**cDNA Cloning**—For cloning of the full-length rat SNX1, a rat hippocampal/cortical cDNA library in λZAPII (Stratagene) was screened using a partial SNX1 cDNA probe from the yeast two-hybrid prey clone, according to standard procedures (23). The cDNA inserts from positive SNX1 clones were sequenced multiple times on both strands, using an Applied Biosystems 373A DNA sequencer.

**Antibodies**—An anti-SNX1 antibody was raised in chicken against the COOH-terminal peptide of rat SNX1, CKYWEAFLEPARAIS. The NH2-terminal cysteine residue was added for the coupling purposes. The antibody was affinity-purified using the immunogen peptide coupled to a SulfoLink column (Pierce). Other antibodies that were used in these experiments are: anti-Hrs (9); anti-EGFR (1005 and 528, Santa Cruz Biotechnology, Inc.); anti-SNAP-25 (SMI 81, Sternberger Monoclonals, Inc.); anti-Rab5, anti-Rab11, and anti-EFA1 (Transduction Laboratories); anti-HA (3F10, Roche Molecular Biochemicals); anti-actin (C4, Roche Molecular Biochemicals); and secondary antibodies coupled with Texas Red (Jackson ImmunoResearch Laboratories). The anti-LAMP1 (HA3) and anti-LAMP2 (HB4) antibodies were obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa (Iowa City, IA).

**Western Blot Analyses**—Rat tissues were homogenized in 1% SDS and subjected to SDS-PAGE. The proteins were transferred onto nitrocellulose membranes, and probed with the anti-SNX1 and other antibodies. Antibody binding was detected by using the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

**Production of Recombinant Proteins**—The full-length rat SNX1 was subcloned into the prokaryotic expression vectors pET32c (Novagen) to obtain pCHA-SNX1. This plasmid encodes a fusion protein, S-tag-SNX1, which consists of (from amino to carboxyl terminus) the 109-aminio acid thioredoxin protein, a hexahistidine tag, and a 15-amino acid S-tag peptide fused in frame with the SNX1 protein. For the production of glutathione S-transferase (GST)-Hrs fusion proteins, rat Hrs (residues 225–776) was subcloned into the vector pGEX-SX-2 (Amersham Pharmacia Biotech). Fusion proteins were expressed in bacteria, and purified as described previously (21).

**In Vitro Binding Assays**—GST-Hrs fusion protein or GST control was immobilized on glutathione-agarose beads, and incubated with S-tag-SNX1 fusion protein for 3 h at 4 °C under gentle rocking in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Triton X-100. After extensive washes with the same solution, bound proteins were eluted by boiling in Laemmli sample buffer, subjected to SDS-PAGE, and immunoblotting with antibodies to rat Hrs or SNX1. Antibody binding was detected by using the enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech). For detection of the interaction of Hrs with endogenous SNX1, GST-Hrs fusion proteins immobilized on glutathione-agarose beads were incubated with rat brain homogenates for 2 h at 4 °C as described (21). Bound proteins were analyzed by SDS-PAGE and immunoblotting.

**Expression Constructs and Transfections**—Conventional molecular biological techniques (21) were used to generate the following expression constructs: pDNA3.1-SNX1 and pDNA3.1-Hrs, which direct the expression of full-length SNX1 and Hrs, respectively; pCHA-SNX1, pCHA-Hrs, and pCHA-Hrsp225-776, which direct the expression of NH2-terminal HA epitope-tagged, full-length SNX1, full-length Hrs, and a Hrs fragment (residues 225–776, respectively); and pEGFP-SNX1, pEGFP-Hrs, and pEGFP-Hrsp225-776, which direct the expression of NH2-terminal GFP-tagged, full-length SNX1, full-length Hrs, and a Hrs fragment (residues 225–776, respectively). Transfections of HEK293 and HeLa cells were performed using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions.

**Immunoprecipitation**—Extracts were prepared from HeLa cells transiently transfected with pMAX-EGFR alone (0.1 μg) or in combination with pCHA-SNX1 or pCHA-Hrs (1 μg), and immunoprecipitation was performed as described previously (24), using anti-HA antibody (3F10), anti-EGFR antibody (528), or corresponding control IgG. For coimmunoprecipitation of SNX1 and Hrs in cellular fractions, HA-SNX1-transfected cells were homogenized in buffer A (50 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μM leupeptin) and centrifuged at 15,000 g for 15 min. After removal of the supernatant (cytosolic) fraction, the particulate fraction was resuspended with buffer A containing 1% Nonidet P-40 and incubated at 4 °C for 30 min to solubilize membrane-bound proteins. The cytosol and membrane fractions were centrifuged at 100,000 × g for 20 min at 4 °C, and the supernatants were incubated with rat monoclonal anti-HA antibody (3F10) or control rat IgG for 1 h at 4 °C. The immunocomplexes were recovered by incubation with protein G-Sepharose beads (Sigma) for 1 h at 4 °C. After extensive washes with the same solution, the immunocomplexes were dissociated by boiling in the Laemmli sample buffer, and analyzed by SDS-PAGE and immunoblotting.

**Membrane Association Analyses**—Separation of PC12 cells into cytosolic fraction (100,000 × g supernatant) and membrane particulate fraction (100,000 × g pellet) were performed as described previously (21). The membrane fractions were subjected to extraction studies as described (21), using 1.5 mM NaCl or 4 M urea.

**Subcellular Fractionation**—HeLa cells were treated with 100 ng/ml EGF for 10 min at 37 °C and then washed twice with PBS at 4 °C. Cells were gently scraped from culture plates and collected by centrifugation. The supernatant was carefully removed by decantation. The cell pellet was homogenized in 1 ml of ICT (78 mM KCl, 4 mM MgCl2, 8.37 mM CaCl2, 10 mM EGTA, 50 mM HEPES/KOH, pH 7.0) plus 250 mM sucrose (25), and centrifuged at 1,000 × g for 5 min. The supernatant was placed on a 5–20% linear Optiprep (Nycomed) gradient formed in ICT, and centrifuged at 4 °C for 20 h at 125,000 × g in a SW40 rotor (Beckman). Following centrifugation, the gradient was harvested into 300-μl fractions using an Auto Densi-Flow gradient harvester
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**RESULTS**

Identification of Rat SNX1 as a Hrs-binding protein in Yeast Two-hybrid Screens—To identify proteins that interact with Hrs, we screened a rat hippocampal/cortical cDNA library using a full-length Hrs as bait in the yeast two-hybrid selection. Positive clones were rescued, and confirmed by re-transformation experiments. Of the 22 positive clones sequenced, 4 clones encoded STAM, a signal-transducing adaptor molecule that is known to interact with Hrs (7). Three independent overlapping clones encoded portions of a protein that is the rat homologue of the human sorting nexin 1 (Fig. 1A). The specificity of the interaction between Hrs and SNX1 was confirmed by yeast two-hybrid analysis demonstrating that SNX1 only interacts with Hrs, but not with a coiled-coil domain-containing protein SNAP-25 or the proline-rich cytoplasmic region of synaptophysin (data not shown).

By screening a rat hippocampal aZAP cDNA library, we isolated two full-length and four partial cDNA clones of rat SNX1. The full-length rat SNX1 cDNA (accession no. AF218916) contains an open reading frame encoding a 522-amino acid protein (Fig. 1B) that is 95% identical to human SNX1 (14, 15). Sequence analysis demonstrated that rat SNX1 is hydrophilic with a theoretical isoelectric point (pI) of 5.15 and a high percentage (30%) of charged amino acids distributed over the entire length. Like human sorting nexins, rat SNX1 contains a Phox homology (PX) domain (15, 26). The PX domain, whose function is as yet unknown, is an evolutionarily conserved sequence that is present in a number of proteins with diverse function, including proteins involved in vesicular trafficking (15–17, 26–29). By using the algorithm of Lupas et al. (30), we identified three regions with high probability (p = 0.94, 0.98, and 1.00) of forming a coiled-coil structure. In addition, rat SNX1 contains three putative SH3 domain binding (PX/PH) motifs (31). Thus, SNX1 could potentially interact with multiple proteins or be involved in the formation of multi-protein complexes via coiled-coil interactions, associations of its proline-rich motifs with the SH3 domain-containing proteins, and/or other types of protein-protein interactions through its PX domain.

**Tissue Distribution and Membrane Association of SNX1—To**
analyze SNX1 expression at protein level and biochemically characterize its association with Hrs, we raised a chicken polyclonal antibody against the COOH-terminal 14-amino acid peptide of rat SNX1. To characterize this antibody, HEK293 cells were transfected with pCHA-SNX1 or pCHA vector control. Cell lysates prepared from the transfected cells were immunoprecipitated (IP) with an anti-HA antibody. The precipitates were then analyzed by immunoblotting (IB) with anti-HA antibody and anti-SNX1 antibody. B, Western blot analysis of SNX1 expression in rat tissues. Equal amounts of homogenates (30 μg of protein/lane) from indicated rat tissues were analyzed by immunoblotting using the anti-SNIP antibody. Sk., Skeletal. C, nature of SNX1 association with cellular membranes. Postnuclear supernatant (T) from PC12 cells was separated into cytosol (C) and membrane (M) fractions. The membranes were extracted with 1.5 M NaCl or 4 M urea, and then separated into soluble (S) and pellet (P) fractions. Aliquots representing an equal percentage of each fraction were analyzed by SDS-PAGE and immunoblotting for SNX1 and EGFR.

Fig. 2. Characterization of expression and membrane association of SNX1. A, specificity of the anti-SNX1 antibody. HEK293 cells were transfected with pCHA-SNX1 or pCHA vector control. Cell lysates prepared from the transfected cells were immunoprecipitated (IP) with an anti-HA antibody. The precipitates were then analyzed by immunoblotting (IB) with anti-HA antibody and anti-SNX1 antibody. B, Western blot analysis of SNX1 expression in rat tissues. Equal amounts of homogenates (30 μg of protein/lane) from indicated rat tissues were analyzed by immunoblotting using the anti-SNIP antibody. Sk., Skeletal. C, nature of SNX1 association with cellular membranes. Postnuclear supernatant (T) from PC12 cells was separated into cytosol (C) and membrane (M) fractions. The membranes were extracted with 1.5 M NaCl or 4 M urea, and then separated into soluble (S) and pellet (P) fractions. Aliquots representing an equal percentage of each fraction were analyzed by SDS-PAGE and immunoblotting for SNX1 and EGFR.

anti-SNX1 antibodies were used on Western blots of various rat tissues. A doublet of protein bands with an apparent molecular masses of 66 and 61 kDa was detected (Fig. 2B). The size of the upper band agrees well with that of the endogenous SNX1 protein detected in murine NIH 3T3 and human HeLa cells (14). The lower band is likely to be the alternatively spliced isoform SNX1A, which has an NH2-terminal internal 65-amino acid deletion compared with SNX1 (15). As shown in Fig. 2B, SNX1 is abundantly expressed in pancreas, spleen, kidney, intestine, and lung, and moderately expressed in brain, liver, and ovary. Longer exposure of the same blot demonstrated that SNX1 is also present at low level in heart and skeletal muscle (data not shown). The additional immunoreactive bands of low molecular weights observed in pancreas and intestine are likely to be the degradation products of SNX1/1A since the relative intensity of these bands as compared with the SNX1/1A bands varied from preparation to preparation.

To examine the intracellular distribution of endogenous SNX1, postnuclear supernatant of PC12 cells was separated into cytosol and membrane particulate fraction, and then subjected to Western blot analysis with the anti-SNX1 antibody (Fig. 2C). SNX1 immunoreactivity was detected in both cytosol and membrane particulate fraction, although the relative amount of SNX1 in the cytosol fraction was severalfold more than that in the particulate fraction. To investigate the nature of SNX1 association with membranes, the membrane particulate fraction was extracted with 1.5 M NaCl or 4 M urea (Fig. 2C). Unlike the integral membrane protein EGFR, which was resistant to extraction by high salt and urea, SNX1 was extracted by these treatments, suggesting that SNX1 is peripherally associated with membranes.

Hrs and SNX1 Associate in Vitro—To obtain independent evidence for the interaction between Hrs and SNX1, GST-Hrs fusion protein or GST control was immobilized on glutathione-agarose beads, and incubated with S-tag-SNX1 fusion protein. Proteins that bound to the GST-Hrs or GST control were detected with the horseradish peroxidase-conjugated S protein against the S-tag (Fig. 3A). The results demonstrate that S-tag-SNX1 bound to GST-Hrs, but not to the GST control, indicating...
that recombinant Hrs and SNX1 are capable of interacting directly with each other. To determine whether recombinant Hrs is able to bind endogenous SNX1, GST-Hrs fusion protein immobilized on glutathione beads was used to affinity-purify (“pull-down”) endogenous SNX1 from brain homogenates. As shown in Fig. 3B, the GST-Hrs fusion protein was able to pull down endogenous SNX1. In contrast, control GST protein was unable to pull down SNX1. These results confirm the Hrs-SNX1 interaction detected in the yeast two-hybrid system.

**Association of Hrs and SNX1 Occurs on Cellular Membranes but Not in Cytosol—**Coimmunoprecipitation experiments were performed to determine whether Hrs interacts with SNX1 in vivo. Since both Hrs and SNX1 exist in a cytosolic pool and a membrane-associated pool (Fig. 2C) (9), we were interested to know whether the association of Hrs and SNX1 occurs in the cytosol or on membranes. To examine these possibilities, HA-SNX1-transfected HeLa cells were fractionated into the cytosol or on membranes. The interaction of these SNX1 deletion mutants with the full-length Hrs was analyzed by the ability to grow on histidine-deficient media and a β-galactosidase filter assay (data not shown) as well as a quantitative β-galactosidase assay (Fig. 4A). The results demonstrated that the SNX1 COOH-terminal region (residues 300–522) interacts strongly with Hrs, whereas neither the PX domain (residues 158–282) nor COOH-terminal fragments (residues 300–399 or residues 429–522) is able to bind Hrs. These data suggest that multiple domains and/or a complex folded structure of the SNX1 COOH-terminal region (residues 300–522) are required for binding Hrs. The Hrs-binding domain of SNX1 overlaps with its EGFR-binding site, which has been mapped to the COOH-terminal 58 amino acids (residues 465–522) of SNX1 (14).

**Identification of the Hrs-binding Domain in Hrs—**To further understand the structural requirements that underlie the interaction between Hrs and SNX1, we performed similar deletion analysis to map the specific region of Hrs involved in binding SNX1 (Fig. 4B). Deletions of the VHS domain, FYVE finger, and the COOH-terminal proline-rich domain had little effect on the ability of Hrs to bind SNX1, indicating that these domains are dispensable to the Hrs-SNX1 interaction. As shown in Fig. 4B, only the fusion proteins that contain the central region (residues 225–449) encompassing the two predicted coiled-coil domains H1 and H2 of Hrs were able to interact with SNX1. Unlike the interaction of Hrs with STAM, Hbp, or SNAP-25, which is mediated by the H2 domain (7, 8, 11), we found that the H2 domain (residues 443–541) by itself is not sufficient to bind SNX1. Moreover, a Hrs fusion protein
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(Fig. 5, C–H) and SNX1, HeLa cells were stained by Rab5, another marker for early endosomes (Fig. 7, I and J; data not shown). However, no colocalization was observed between the distribution of Hrs or SNX1 and that of LAMP1 and LAMP2, markers for late endosomes and lysosomes (data not shown). Together, these results suggest that Hrs and SNX1 primarily associate with early endosomes.

To further confirm the early endosomal localization of Hrs and SNX1, HeLa cells were stimulated for 10 min with EGF or Texas Red-conjugated EGF, and the internalized EGF-EGFR complexes were visualized by anti-EGFR antibodies (Fig. 7, E–H) or by Texas Red-EGF labeling (data not shown). It is known from previous studies using immunofluorescence and immunoelectron microscopy that, under similar conditions, the internalized EGF-EGFR complexes are almost exclusively localized to early endosomes (38, 39). As shown in Fig. 7, most of the Hrs- and SNX1-positive structures contained internalized EGF-EGFR complexes (Fig. 7, compare E and F, and G and H), in HeLa cells, and their intracellular distribution was analyzed by indirect immunofluorescence and confocal microscopy. The tagged Hrs and SNX1, when expressed at low levels, exhibited vesicular staining patterns (Fig. 6, A and B) which are indistinguishable from those observed for nontagged Hrs and SNX1 when visualized using the antibodies against Hrs and SNX1 (data not shown). These staining data are consistent with the results reported by Komada et al. (8) for Hrs and by Kurten et al. (14) for SNX1. The SNX1-positive vesicular compartment overlapped to a significant extent with Hrs, showing that at least a subpopulation of SNX1 colocalizes with Hrs (Fig. 6, compare A and B). Furthermore, recombinant Hrs, when expressed at high levels, caused the formation of enlarged vesicular structures (Fig. 6D), which are believed to be exaggerated early endosomes (8, 35). Although overexpression of recombinant SNX1 alone does not lead to formation of enlarged vesicular structures, SNX1 colocalized with Hrs to the exaggerated vesicular structures resulted from high level Hrs expression (Fig. 6, compare C and D). The colocalization of SNX1 with Hrs is in agreement with the results of subcellular fractionation studies (Fig. 5) and provides supporting evidence for an in vivo association of Hrs with SNX1.

To investigate the identities of the vesicular structures labeled by Hrs and SNX1, we performed double immunofluorescence experiments to compare the distribution of these proteins with early endosome antigen 1 (EEA1). EEA1, a core component of early endosome docking and fusion machinery, has been widely used as a marker for early endosomes (36, 37). Consistent with previous studies (8) and our results of subcellular fractionation (Fig. 5), a substantial overlap was observed between Hrs distribution and EEA1 immunoreactivity (compare Fig. 7, A and B). Similarly, a majority of SNX1-positive vesicular structures also contain EEA1 (compare Fig. 7, C and D). Moreover, many of the vesicular structures labeled by Hrs and SNX1 were stained by Rab5, another marker for early endosomes (Fig. 7, I and J; data not shown). However, no colocalization was observed between the distribution of Hrs or SNX1 and that of LAMP1 and LAMP2, markers for late endosomes and lysosomes (data not shown). Together, these results suggest that Hrs and SNX1 primarily associate with early endosomes.

Colocalization of Hrs and SNX1 on Early Endosomes by Immunofluorescence Microscopy—To further confirm the colocalization of Hrs and SNX1 within the cell, full-length, amino-terminally HA- or GFP-tagged Hrs and SNX1 were expressed (residues 225–449) containing the H1 domain and the proline-rich linker region was unable to bind SNX1. Taken together, these data suggest that multiple domains and/or a complex folded structure of the Hrs central region (residues 225–541) are involved in binding SNX1.

Cofractionation of SNX1 with Hrs and with Early Endosomal Markers on a Density Gradient—Since both Hrs and SNX1 exist in cytosolic and membrane-associated pools, we sought to determine whether Hrs and SNX1 associate with the same population of membranes by using subcellular fractionation. Postnuclear supernatants were prepared from HeLa cells and fractionated on a 5–20% linear Optiprep gradient as described under “Experimental Procedures.” The gradient was divided into 38 fractions, with fraction 1 corresponding to the top of the gradient. Equal volumes of each fraction were analyzed by SDS-PAGE, followed by immunoblotting for Hrs, SNX1, Rab5, Rab11, and LAMP2.
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...antibodies against HA tag (Hrs (A)) were directly visualized by the green fluorescence emitted by the GFP. The same cells were stained with primary antibodies against HA tag (B and D), followed by detection with secondary antibodies conjugated to Texas Red. The arrows indicate vesicular structure clearly visible in both panels (colocalized), whereas the arrowhead marks the vesicular structure visible in one panel but not the other (not colocalized). Scale bar = 10 μm.

providing further evidence for the early endosomal localization of Hrs and SNX1. To examine possible association of Hrs and SNX1 with recycling endosomes, HeLa cells were treated with Texas Red-conjugated transferrin for 30 min. It is well established that the internalized transferrin-receptor complexes accumulate in recycling endosomes, which are often concentrated at the microtubule organizing center (40) (Fig. 7L). The punctate staining pattern of SNX1 (Fig. 7K) or of Hrs (data not shown) exhibited limited overlap with the distribution of internalized transferrin (Fig. 7L), suggesting that most of Hrs and SNX1 does not associate with recycling endosomes. These data, together with the results of subcellular fractionation (Fig. 5), provide strong evidence for the colocalization of Hrs and SNX1 on early endosomes.

Hrs and SNX1 Coexist in a Large Protein Complex Within the Cell—To further characterize the in vivo association of Hrs and SNX1, we performed gel filtration analysis to assess the relative sizes of SNX1 and Hrs and their associated complexes in HeLa cells. As shown in Fig. 8, fractionation of HeLa cell extracts by size-exclusion chromatography on a Superose 6 high resolution column reveals that SNX1 exists in two distinct protein complexes that eluted from the column with apparent molecular masses of ~260 and ~550 kDa. In addition, a significant portion of SNX1 eluted in the void volume fractions. No SNX1 immunoreactivity was detected in the fractions corresponding to the size of monomeric form of SNX1 (predicted molecular mass of 59 kDa and apparent molecular mass of 66 kDa on SDS-PAGE), suggesting that SNX1 does not exist as monomers in vivo. The ~260-kDa complex may represent a SNX1 tetramer or a heteromeric complex of SNX1 with other sorting nexin isoforms. Consistent with this possibility, HA-tagged SNX2 has been shown to interact with itself and with SNX1, SNX1A, and SNX4 (15). Similarly, it appears that Hrs does not exist in the monomeric form (predicted molecular mass of 86 kDa and apparent molecular mass of 110 kDa on SDS-PAGE). The lowest apparent molecular weight of Hrs detected by the size-exclusion chromatography is ~185 kDa, which may correspond to a Hrs dimer or a heteromer of Hrs with another protein such as STAM (7) or STAM2/Hbp (10, 11). In contrast, monomeric form of EGFR was detected in the fractions corresponding to its expected size.

The ~550-kDa SNX1-containing complex seems to also include Hrs, as suggested by the coelution of Hrs with SNX1 in the same fractions (fractions 44–50). EGFR did not coelute with SNX1 and Hrs in these fractions, suggesting that EGFR is not part of the ~550-kDa complex. The size of ~550-kDa complex is significantly larger than a simple heterodimer of Hrs-SNX1, which has a combined molecular mass of ~175 kDa, suggesting that the ~550-kDa complex contains either multiple subunits of SNX1 and Hrs, or additional proteins such as mammalian homologue of Vps17p (18).

As shown in Fig. 8, SNX1, Hrs, and EGFR coeluted in the void volume of the Superose 6 column. This is reminiscent of the coelution of yeast retromer components (Vps5p, Vps29p, and Vps35p) in the void volume, which is thought to represent a very large (>1000 kDa) complex (18). Thus, one possible explanation of the data is that SNX1, Hrs, and EGFR may be components of an analogous high molecular weight complex in mammalian cells. Alternatively, the coelution of SNX1, Hrs, and EGFR in the void volume could be the result of nonspecific aggregation. The latter possibility seems to be more likely since no heterotrimeric Hrs-SNX1-EGFR complex could be detected in the coimmunoprecipitation experiments as shown in Fig. 9.

To further investigate the nature of Hrs- and SNX1-containing complexes, we performed coimmunoprecipitation experiments using antibodies against EGFR, HA-Hrs, and HA-SNX1 (Fig. 9). Immunoprecipitation of HA-Hrs with an anti-HA antibody was able to bring down endogenous SNX1 (Fig. 9, lane 3), further confirming the presence of a Hrs-SNX1 complex in vivo. However, EGFR did not coprecipitate with HA-Hrs and SNX1 (Fig. 9, lane 3), indicating that Hrs, SNX1, and EGFR do not coexist in a single complex. These results are consistent with the gel filtration data (Fig. 8) showing the presence of a ~550-kDa complex that contains Hrs and SNX1 but excludes EGFR. Immunoprecipitation of EGFR with an anti-EGFR antibody resulted in the coprecipitation of SNX1 but not Hrs (Fig. 9, lane 5), providing further evidence for the presence of the SNX1-EGFR complex but not the Hrs-SNX1-EGFR complex in vivo. As expected, immunoprecipitation of HA-SNX1 with an anti-HA antibody was able to bring down both Hrs and EGFR (Fig. 9, lane 1) due to the ability of the antibody to precipitate both SNX1-Hrs and SNX1-EGFR complexes. Together, these coimmunoprecipitation data demonstrate that there are two mutually exclusive complexes containing SNX1, one with Hrs and one with EGFR. This is in agreement with the results of deletion analysis (Fig. 4A) indicating that the Hrs-binding site of SNX1 overlaps with its EGFR-binding site (14). It is interesting to note that, although the SNX1-EGFR complex was first reported by Kurten et al. (14), and subsequently confirmed by Haft et al. (15) and this study, no such complex could be detected in the gel filtration analysis (Fig. 8). Thus, the relative abundance of the SNX1-EGFR complex seems to be very low compared with the abundance of the SNX1-Hrs complex in HeLa cells.

Hrs and SNX1 Are Involved in Degradation but Not in Internalization of EGFR—Since SNX1 has been shown to accelerate down-regulation of EGFR (14), the association and colocalization of Hrs with SNX1 raise the possibility that Hrs may also have a role in the down-regulation of EGFR. To test this possibility, we used a well established assay (14, 24, 41) to evaluate the effect of overexpression of Hrs on the down-regu-
In agreement with previous reports (14, 41), we observed that, in vector-transfected control cells, stimulation with EGF for 45 min led to a large decrease in the number of mature EGFR. The EGF-induced down-regulation of EGFR was significantly enhanced in cells overexpressing SNX1, which is consistent with the results of Kurten et al. (14).

In contrast, overexpression of Hrs resulted in a significant reduction in the EGF-induced down-regulation of EGFR. Similar extent of reduction in the down-regulation of EGFR was also observed in cells expressing a Hrs fragment (amino acids 225–776) containing the SNX1-interacting domain. These results suggest that Hrs is involved in the ligand-induced down-regulation of EGFR, perhaps via its interaction with SNX1.

To determine whether the effect of overexpressing SNX1 and Hrs on the down-regulation of EGFR is due to a change in the internalization of EGFR, HeLa cells overexpressing SNX1 or Hrs were tested for their capacity to internalize EGF-EGFR complexes in the presence of EGF or Texas Red-conjugated EGF. We found that, compared with untransfected cells, cells overexpressing SNX1, Hrs, or Hrs225–776 internalized similar amounts of EGFR and Texas Red-conjugated EGF (Fig. 7 (E–H) and data not shown), indicating that overexpression of these proteins has little effect on ligand-induced endocytosis/internalization of EGFR. Furthermore, no difference in the amount of internalized transferrin was observed between cells overexpressing SNX1, Hrs, or Hrs225–776 and untransfected cells (Fig. 7J, and data not shown), suggesting that Hrs and SNX1 are not involved in constitutive receptor-mediated endocytosis. Taken together, these data suggest that SNX1 and Hrs alter the down-regulation of EGFR by affecting lysosome trafficking of the receptor for degradation.

**FIG. 7.** Comparison of the distribution of Hrs and SNX1 with markers for early endosomes and recycling endosomes. HeLa cells were transiently transfected with either pEGFP-Hrs (A, B, E, and F) or pEGFP-SNX1 (C, D, and G–L). Transfected cells were identified by the green fluorescence emitted by the GFP (A, C, G, I, and K). Some of the cells were treated at 37 °C with either EGF for 10 min (E–H) or Texas Red-conjugated transferrin for 30 min (K and L). Internalized transferrin was visualized by the red fluorescence emitted by the Texas Red (L). Cells were stained with primary antibodies against EEA1 (B and D), EGFR (F and H), or Rab5 (J), followed by detection with secondary antibodies conjugated to Texas Red. The arrows indicate vesicular structure clearly visible in both panels (colocalized), whereas the arrowhead marks the vesicular structure visible in one but not the other panel (not colocalized). Scale bar = 10 μm.

**FIG. 8.** Gel filtration analysis of protein complexes containing Hrs and SNX1. HeLa cell extracts were prepared as described under “Experimental Procedures” and fractionated by size-exclusion chromatography using a Superose 6 high resolution analytical gel filtration column. Equal volumes of each fraction were analyzed by SDS-PAGE, followed by immunoblotting for SNX1, Hrs, and EGFR. Standards used for column calibration are blue dextran (2000 kDa), thyroglobulin (670 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), and carbonic anhydrase (29 kDa).
DISCUSSION

Trafficking of EGFR from endosome to lysosome plays a key role in attenuating EGF signaling. However, little is known at the molecular level about the mechanisms that regulate the lysosomal trafficking pathway. Previous studies have defined the lysosome-targeting signals within the cytoplasmic domain of EGFR that are responsible for the EGF-induced lysosomal degradation (42, 43). The first sorting nexin, SNX1, which recognizes the EGFR lysosome-targeting signals, has recently been identified and shown to function in lysosomal trafficking of EGFR (14). In this paper, we describe a novel interaction between SNX1 and Hrs, a protein that is implicated in both vesicular trafficking and cell growth signaling. The interaction of SNX1 with Hrs was demonstrated in the yeast two-hybrid system and confirmed by in vitro binding studies and coimmunoprecipitation experiments. Deletion analysis reveals that the Hrs-SNX1 interaction involves multiple coiled-coil domains and complex folded structures of the Hrs central region (residues 225–541) and of the SNX1 COOH-terminal region (residues 300–522). Several lines of evidence support a physiological significance of the observed interaction between SNX1 and Hrs. 1) Hrs and SNX1 are ubiquitously expressed proteins that exist in both cytosolic and membrane-associated pools. 2) Coimmunoprecipitation experiments demonstrate that the association of Hrs and SNX occurs on cellular membranes but not in the cytosol. 3) Gel filtration analysis reveals the presence of an endogenous ~550-kDa protein complex containing SNX1 and Hrs. 4) Subcellular fractionation studies show that SNX1 cofractionates with Hrs and early endosomal markers on an Optiprep density gradient. 5) Double immunofluorescence analysis demonstrates that Hrs and SNX1 colocalize on early endosomes. 6) Overexpression of Hrs or its SNX1-binding domain inhibits ligand-induced degradation of EGFR, but has no effect on EGFR internalization. Together, these data suggest that the interaction between SNX1 and Hrs may be involved in the regulation of endosome-to-lysosome trafficking of EGFR.

Our results indicate that SNX1 and Hrs share similar properties with their yeast homologues, Vps5p and Vps27p. In yeast, both Vps5p and Vps27p are localized to the prevacuolar/endosomal compartment, although it is not known whether they colocalize with each other (17, 18, 44). In mammalian cells, we found that Hrs and SNX1 colocalize on the early endosome, a sorting compartment where membrane proteins destined for degradation are sorted away from proteins that are recycled back to the cell surface. The involvement of SNX1 and Hrs in lysosomal trafficking of EGFR is consistent with the role of Vps5p and Vps27p in yeast vesicular trafficking. Recently, it was reported that Vps5p assemblies with Vps17p, Vps26p, Vps29p, and Vps35p to form a novel coat complex called the retromer complex (18). The presence of mammalian homologues of other retromer components Vps26p, Vps29p, and Vps35p suggest that SNX1 may function in a manner that is analogous to Vps5p by forming a retromer-like coat complex in mammalian cells (18). Since SNX1 directly interacts with lysosome-targeting signals on cargo proteins such as EGFR (14), it is likely that SNX1 performs its sorting function by selectively recruiting specific cargo proteins into the retromer-like coat complex.

The mutually exclusive interaction of Hrs and of EGFR with SNX1 indicates that the association of Hrs with SNX1 is likely to interfere with the ability of SNX1 to bind and recruit EGFR into a functional coat complex for delivery to lysosomes. Supporting this view, overexpression of Hrs or its SNX1-binding domain in HeLa cells leads to an inhibition of lysosomal trafficking of EGFR for degradation. Based upon these data, a model for the role of Hrs in lysosomal trafficking can be envisaged. Hrs, by interacting with SNX1, might serve as a regulator for the assembly of functional sorting machinery. The association of Hrs with SNX1 keeps SNX1 in an inactive state,
unavailable to interact with cargo proteins and/or with other components of the retromer-like coat complex. Disruption of this association by protein phosphorylation (6, 7) or interaction with signaling proteins such as STAM and STAM2/Hbp (7, 10, 11) would then increase the availability of SNX1 and promote cargo recruitment and assembly of functional coat complexes, and hence facilitate lysosomal sorting and trafficking. Future studies will test this model and determine the molecular mechanisms by which Hrs and SNX1 regulate vesicular trafficking.

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