Hrs-2 Regulates Receptor-mediated Endocytosis via Interactions with Eps15*

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Hrs-2, via interactions with SNAP-25, plays a regulatory role on the exocytic machinery. We now show that Hrs-2 physically interacts with Eps15, a protein required for receptor-mediated endocytosis. The Hrs-2/Eps15 interaction is calcium dependent, inhibited by SNAP-25 and α-adaptin, and results in the inhibition of receptor-mediated endocytosis. Immunoelectron microscopy reveals Hrs-2 localization on the limiting membrane of multivesicular bodies, organelles in the endosomal pathway. These data show that Hrs-2 regulates endocytosis, delineate a biochemical pathway (Hrs-2-Eps15-AP2) in which Hrs-2 functions, and suggest that Hrs-2 acts to provide communication between endo- and exocytic processes.

Initial attempts to understand the molecular basis of exocytosis involved the characterization of proteins present on synaptic vesicles and on the presynaptic plasma membrane (1–3). These studies revealed a series of protein-protein interactions that have been suggested to mediate events leading to the fusion of vesicles with the plasma membrane (1–4). SNAP-25

1 is associated with the plasma membrane and binds to both syntaxin (found on the plasma membrane) and VAMP (found on the vesicle) (4–7). Through these interactions SNAP-25 is a critical component of a protein complex (7 S) that is proposed to be necessary for fusion of synaptic vesicles with the plasma membrane (4, 6–9).

Hrs-2 is an ATPase that physically associates with SNAP-25 in a calcium-regulated manner (10). Hrs-2 binds to SNAP-25 through its second coiled-coil domain and does not directly interact with either syntaxin or VAMP (11). When Hrs-2 is bound to the complex of SNAP-25 and syntaxin, VAMP binding to SNAP-25 is inhibited, reducing the amount of 7 S complex formed (11). Thus, Hrs-2, via interaction with SNAP-25, may play a negative regulatory role on the exocytic machinery that is alleviated when calcium concentrations are elevated, dissociating Hrs-2 from the Hrs-2-SNAP-25-syntaxin complex and allowing VAMP to bind to SNAP-25-syntaxin.

Ultrastructural and physiological experiments have identified at least two endocytic pathways: a rapid clathrin-independent pathway, and a slower clathrin-dependent pathway (12–21). Clathrin triskelia form cages that capture vesicle membrane for retrieval during the endocytic process (14, 22, 23). Clathrin and its adapter proteins (AP2 and AP180 are recruited to the plasma membrane and link clathrin to the membrane) along with other binding partners (e.g. dynamin and amphiphysin) appear to be involved in early events in the endocytic pathway related to vesicle budding and maturation (14, 22–29). Other proteins necessary for endocytosis have been found in genetic screens as well as by using biochemical methods. Eps15 is the prototypical member of a family of proteins containing EH motifs that bind to NPF domains in target proteins (30–37). Eps15 contains three amino-terminal EH domains, a central coiled-coil region, and 15 COOH-terminal DPF repeats (32, 33). Eps15 is localized to components of the endocytic pathway (38) and has been shown to be essential for receptor-mediated endocytosis through interactions with α-adaptin (35, 39). The yeast homolog of Eps15, Pan1p, is an EH domain-containing protein found in Saccharomyces cerevisiae that is required for endocytosis (31, 40). Additionally, Pan1p binds to another EH-containing protein, END3p, as well as the yeast homolog of AP180, and has genetic interactions with synaptojanin and a ubiquitin-protein ligase (31, 40).

Exocytosis and endocytosis are closely linked and temporally coordinated during the vesicle cycle. These two distinct events must be regulated precisely such that they occur sequentially in order to maintain the fidelity of vesicle-mediated secretion and cellular architecture. However, the molecular mechanisms that coordinate these events remain elusive. The protein machinery involved in endocytosis and exocytosis is a likely substrate for their interaction. We have found that Hrs-2, a protein implicated in regulation of the formation of the SNAP-25-syntaxin-VAMP complex, interacts with Eps15, a protein necessary for clathrin-mediated endocytosis. The interaction of Hrs-2 with Eps15 is calcium dependent, is inhibited by SNAP-25 and α-adaptin, and results in the inhibition of receptor-mediated endocytosis. Moreover, the inhibition of endocytosis produced by expression of Hrs-2 is rescued by coexpression of α-adaptin. These data show that Hrs-2 has a regulatory role in endocytosis, delineate a biochemical pathway (Hrs-2-Eps15-AP2) in which Hrs-2 functions, and suggest a mechanism by which exocytosis and endocytosis are linked.

EXPERIMENTAL PROCEDURES

Two-hybrid Assay—Full-length rat Hrs-2 was subcloned into the pGBlc vector and used to screen a human brain cDNA library inserted...
FIG. 1. Characterization of Hrs-2 interaction with Eps15. A, recombinant Hrs-2 binding to immobilized Eps15. Eps15 (0.3 μM) was immobilized on glutathione beads and incubated with increasing concentrations of Hrs-2. Hrs-2 did not bind to GST bound to glutathione-agarose (see graph). The EC50 (affinity is reported as the 50% effective concentration (EC50), the concentration of protein at which half-maximal binding occurs, instead of Kd, since the washing phase of these in vitro binding assays is conducted under non-equilibrium conditions) of Hrs-2 for immobilized Eps15 is 1.8 μM, and the stoichiometry is 0.1–0.4:1 (Eps15:Hrs-2) depending on which protein is immobilized. B, communoprecipitation of Eps15 and Hrs-2. Brain post-nuclear supernatant (PNS) containing Eps15 and Hrs-2 (lane 1) was incubated with anti-Hrs-2 antibodies (lane 2) or purified mouse IgG (lane 3). The co-precipitation of Eps15 and Hrs-2 from brain confirms the interaction observed using the two-hybrid system and binding of recombinant fusion proteins (in A). C, five GST-Hrs-2 fusion proteins encompassing residues 1–478 (A), 1–449 (B), 216–449 (C), 450–478 (D), and 515–562 (E, depicted in the schematic diagram, top) were generated by bacterial expression, immobilized on glutathione-agarose beads, and assayed for binding interactions with full-length Eps15. Eps15 binds to Hrs-2 truncation mutants that contain the region between the FYVE finger and the first coiled-coil domain (216–449, lanes A-C). Neither of the two coiled-coil regions (lanes D and E) were able to
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downstream of the GAL4 activation domain in the pGAD10 vector (CLONTECH). Yeast strains (SFY526, HF7c) used herein have been previously characterized (S. Fields, CLONTECH). All constructions were verified by sequencing (Sequenase). 3.06 \times 10^6 independent clones were screened by sequential transformation with pGBT/Hrs-2 and pGAD/Adh acting as reporters, respectively. Both were plated on yeast synthetic minimal agar containing yeast extract base (6.7 g/liter, Difco), dextrose (2%), and an amino acid mixture lacking histidine, leucine, tryptophan, and uracil and stored at 30 °C in the dark. Five-12 days after plating, large colonies (>3 mm diameter, n = 190) were replica plated onto new plates and \( \beta \)-galactosidase activity was assessed on filter lifts. Single colonies from clones that turned blue within 1 h (n = 40) were grown overnight in SD medium lacking leucine, tryptophan, and histidine and DNA was extracted. DNA was electrophoreted into HB101 Escherichia coli cells, and DNA was isolated from single colonies. After restriiction digests confirmed the presence of the activation domain plasmid, multiple co-transformations were performed with the candidate DNA and the pGBT9/Hrs-2, as well as pGBT9/p53. Clones that reacted positively for \( \beta \)-galactosidase activity with the pGPTW/Hrs-2 but not either by themselves or with the control plasmids containing the binding domain alone or the binding domain fused to the tumor suppressor gene p53, were considered for further study (n = 36). Sequencing revealed a single clone whose sequence was identical to SNAP-25b and a single clone identical to human Eps15 (amino acids 416–858). Other clones remain to be analyzed.

Production of Recombinant Proteins—Full-length Hrs-2 was subcloned into Hta baculovirus vector (Life Technologies, Inc.), and Hrs-2 virus was produced according to the manufacturer's protocol. Hrs-2 protein was produced by infecting a 500-mI culture of SF21 cells at a multiplicity of infection of 0.1. Post-infection (72–96 h), cells were harvested by centrifugation, and pellets were frozen. Proteins were extracted by incubation (60 min at 4 °C) with 5% betaine in 10 mM Tris, pH 7.5, 1 mM EGTA, 1 mM EDTA, and a mixture of protease inhibitors including aprotinin, pepstatin, and phenylmethylsulfonyl fluoride, affinity isolated using Ni-NTA-agarose (Qiagen), and eluted from the resin when necessary, using 250 mM imidazole. Hrs-2 truncations were produced as described (11). GFP fusion proteins were co-expressed in pEGFP (CLONTECH) by direct transfer from the GST vector. Hrs-2 was produced by partial digestion of pEGFP-Hrs-2 with PstI removing amino acids 258–484. Using the two-hybrid technique we have observed that the Hrs-2/eps proteins do not bind to Eps15 although it does bind to SNAP-25 and full-length Hrs-2 (data not shown).

SNAP-25 was subcloned into pGEX-KG and grown at 37 °C in the AB1899 strain of E. coli. Full-length GST-Eps15 was a kind gift of A. E. Salcini and P. DiFiore, while deletion constructs of Eps15 were a kind gift of A. Benmerah and N. Cerf-Bensussan. All constructs were grown in BL21 cells at 37 °C. After reaching mid-log phase, isopropyl-1-thio-

\[ \beta \]-\( \delta \)-galactosidase (300 \mu M) was added, and cells were incubated for an additional 3 h. Cells were harvested by centrifugation, lysed in a French Press, and protein was affinity isolated using glutathione-agarose.

In Vitro Binding—Eps15/GST (0.3 \mu M) bound to glutathione beads was incubated with the indicated amount of recombinant Hrs-2 protein for 60 min at 4 °C in either: 10 mM Hepes-KOH, pH 7.5, 140 mM KCl, 1 mM MgCl\(_2\), 0.1 mM EGTA, 0.1% gelatin, 0.05% Tween 20 or the same buffer containing either 2 mM EGTA and various concentrations of Ca\(^{2+}\), Ba\(^{2+}\), or Sr\(^{2+}\). Beads were washed 3 times with PBS-T, and the proteins remaining on the beads were solubilized in 15 \mu l of sample buffer. Samples were separated using SDS-polyacrylamide gels and transferred to nitrocellulose. Western blotting was performed using anti-Hrs-2 antibodies followed by \( ^{125}\)I-labeled secondary antibody and quantitated by phosphorimaging. The EC\(_{50}\) is defined as half-maximal binding of each soluble protein based on optical density obtained by phosphorimaging (Molecular Dynamics model 300A).

For saturation binding, various concentrations of Hrs-2 were incubated (4 °C for 60 min) with glutathione-agarose beads containing either glutathione S-transferase or Eps15. To determine interacting domains, various Hrs-2 or Eps15 fragments were purified and incubated (4 °C for 60 min) with either Eps15 or Hrs-2, respectively. For complex formation studies Hrs-2 bound to Ni-agarose was incubated with Eps15 alone or Eps15 and various concentrations of SNAP-25 at 4 °C for 60 min. To determine whether Ca\(^{2+}\) had an effect on Hrs-2/Eps15 interactions, single concentrations of Hrs-2 were incubated with Eps15 bound to glutathione-agarose (4 °C for 60 min) in the presence of various concentrations of free Ca\(^{2+}\) (buffered with 2 mM EGTA and calculated using WebMAXCalc version 1.1). Free Ba\(^{2+}\) or Sr\(^{2+}\) (0.5 mM) were used to examine selectivity. To examine the effect of \( a \)-adaptin on Hrs-2/Eps15 binding, Eps15 was immobilized on glutathione-agarose and then incubated with \( a \)-adaptin bacterial lysate. The Eps15/agarose, with or without with \( a \)-adaptin, was then incubated with increasing concentrations of Hrs-2. Following binding incubations, reactions were washed three times with PBS containing 0.05% Tween 20, and SDS sample buffer was added to the beads. Proteins bound to the beads were separated by SDS-PAGE and subjected to immunoblot analysis using anti-Hrs-2 antibodies and either horseradish peroxidase- or \(^{125}\)I-labeled secondary antisera.

Immunohistochemistry—For electron microscopic immunohistochemical localization, rats were perfused with 3% paraformaldehyde, 0.1% glutaraldehyde, 0.2% picric acid in 0.1 M phosphate buffer. Brains were post-fixed \( \textit{in situ} \) and sectioned on a vibratome. Tissues were embedded in Lowecryl and thin sectioned. Embedded tissues were incubated with Hrs-2 antibody (10) (1:200), anti-rabbit secondary antibody conjugated to 1.4 nm gold particles (nanogold 2004, Nanoprobes, Stony Brook, NY), and silver intensified (Nanoprobes). Cells were examined on an electron microscope (Jeol) and photographed.

Receptor-mediated Endocytosis—HeLa cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and penicillin/streptomycin. Cells were plated onto coverslips and transiently transfected (Qiagen, effectene transfection reagent) with pEGFP, pEGFP-Hrs-2, pEGFP-Hrs-2Aeps15, pEGFP-Hrs-2Aeps15 fragments, pCDNA3-a-adaptin or pEGFP-Hrs-2Aeps15-a-adaptin 24 h prior to

![Figure 2: Effect of Ca\(^{2+}\) and SNAP-25 on the interaction of Hrs-2 with Eps15. Ca\(^{2+}\) inhibits Hrs-2 binding to Eps15. A single concentration of Hrs-2 (0.6 \mu M) was incubated in the presence of increasing concentrations of free Ca\(^{2+}\) and GST-Eps15 (0.22 \mu M) immobilized on glutathione-agarose. As the Ca\(^{2+}\) concentration was increased the amount of Hrs-2 bound to Eps15 decreased. The half-maximal inhibition was approximately 100 nM. This concentration approaches the resting level of Ca\(^{2+}\) in the cell.](http://www.jbc.org/Downloaded from)
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RESULTS

Hrs-2 Interacts with Eps15—Using full-length Hrs-2 as bait in a two-hybrid screen, we recovered a cDNA clone identical to amino acids 416–858 of human Eps15. The interaction appeared specific as it was not observed using either p53 or GAL4 as bait (data not shown). To confirm the two-hybrid result we obtained the rat Eps15 clone, produced the recombinant protein, and performed binding assays using purified Hrs-2 and Eps15 (Fig. 1A). Hrs-2 binding to immobilized Eps15 approached saturation with an apparent EC50 for binding of \( \sim 1.8 \mu M \). These data suggest that Hrs-2 interacts with Eps15 directly in the absence of other protein components.

Eps15 from brain postnuclear supernatant was found to coimmunoprecipitate with Hrs-2 (Fig. 1B). Thus, while Eps15 was bound to protein A-Sepharose to which Hrs-2 antibody had been cross-linked (lane 2), Eps15 was not detected on protein A-Sepharose containing a control antibody, mouse IgG (lane 3). These data support the two-hybrid data and confirm that Hrs-2 can interact with Eps15 from brain.

We used truncated forms of the recombinant proteins to determine the domains of Hrs-2 and Eps15 that mediate the interaction between these molecules. To determine the domain of Hrs-2 that binds to Eps15, we constructed a set of GST fusion proteins containing regions of Hrs-2 predicted to form structural motifs (Fig. 1C, top). These truncation mutants were expressed, bound to glutathione-agarose beads, and then examined for interaction with full-length soluble Eps15 during an in vitro binding assay. As shown in Fig. 1C (bottom), Eps15 bound to Hrs-2 truncations that contained the region between the FYVE zinc finger region and the first coiled-coil domain, and not to GST beads alone or to either of the coiled-coil regions. Since Eps15 was able to bind to the Hrs-2 truncation containing amino acids 216–449, we conclude that this region of Hrs-2 is both necessary and sufficient for the binding of Eps15.

In a similar manner, we determined the domain of Eps15 that binds to Hrs-2 (Fig. 1D). GST fusion proteins containing regions of Eps15 were expressed, immobilized on glutathione-agarose, and then assayed for interaction with full-length Hrs-2. Hrs-2 bound to both the coiled-coil domain and the carboxyl-terminal domain of Eps15 that contains NPFF repeats (Fig. 1D). Moreover, the region in the carboxyl-terminal domain necessary for Hrs-2 binding was delineated using additional deletion constructs of that region and found to correspond to the region required for \( \alpha \)-adaptin binding to Eps15 (Fig. 1D). The amino-terminal EH domain construct was unable to interact with Hrs-2. Thus, we conclude that Eps15 contains two domains capable of interaction with Hrs-2, the coiled-coil motif, as well as the \( \alpha \)-adaptin binding region in the COOH-terminal domain.

The Interaction of Hrs-2 with Eps15 Is Dependent on Calcium—Since Hrs-2 is known to have calcium-sensitive interactions with SNAP-25, we examined whether the interaction with Eps15 is altered by calcium. Recombinant Eps15 was immobilized, and a single concentration of Hrs-2 was added in the presence of increasing free Ca\(^{2+}\). As the concentration of Ca\(^{2+}\) was increased, less Hrs-2 bound to Eps15 (Fig. 2). The half-maximal inhibition of Hrs-2 binding produced by Ca\(^{2+}\) was \( \sim 100 \) nm. Ba\(^{2+}\) and Sr\(^{2+}\) (0.5 mM concentrations) did not affect the binding of Hrs-2 to Eps15 (data not shown). These data suggest that Ca\(^{2+}\) regulates the ability of Hrs-2 and Eps15 to interact.

Eps15 and SNAP-25 Compete for Hrs-2 Binding—The calcium concentration necessary for half-maximal inhibition of Hrs-2/Eps15 binding is \( \sim 1000 \) lower than that needed to inhibit Hrs-2/SNAP-25 binding, suggesting that Eps15 and SNAP-25 do not interact with Hrs-2 simultaneously. Additionally, since SNAP-25 is involved in regulation of the exocytic machinery and Eps15 in the endocytic machinery, it seems unlikely that they would bind to Hrs-2 simultaneously. To examine whether SNAP-25 and Eps15 interact with Hrs-2 simultaneously, we incubated immobilized Hrs-2 with Eps15 and increasing concentrations of SNAP-25 (Fig. 3). As the concentration of SNAP-25 increased, less Eps15 bound to Hrs-2. At 3 \( \mu \)M SNAP-25, Eps15 binding was reduced by 90% indicating that while SNAP-25 and Eps15 can both bind to Hrs-2, SNAP-25, and Eps15 do not bind to Hrs-2 simultaneously.

Hrs-2 and Eps15 Are Present on Endocytic Structures—The demonstration of a direct interaction between Hrs-2 and Eps15 suggests that Hrs-2 may play a role in the endocytic machinery. Eps15 is localized to components of the endocytic pathway (38, 42), although biochemical studies in-
Hrs-2 binds to Eps15 and regulates transferrin uptake (Fig. 5). Expression of the coil region of Hrs-2 necessary for SNAP-25 interaction (Hrs-2 cc2) did not significantly affect transferrin uptake (Fig. 5, G and H). These data suggest that Hrs-2 can inhibit endocytosis and that its interaction with Eps15 is necessary for this function.

Hrs-2 Inhibits Receptor-mediated Endocytosis by Competing with α-Adaptin for Eps15—Hrs-2 binds to Eps15 through two domains, one of which is also necessary for α-adaptin binding (Fig. 1). Since Eps15 regulates endocytosis through an interaction with α-adaptin, and Hrs-2 can regulate this process, we examined whether Hrs-2 regulates endocytosis by altering the Eps15/α-adaptin interaction. Immobilized Eps15 was incubated with increasing concentrations of Hrs-2 in the presence and absence of α-adaptin (Fig. 6A). In the absence of α-adaptin, Hrs-2 bound to Eps15 as we had observed in Fig. 1. In the presence of α-adaptin the binding of Hrs-2 to Eps15 was markedly reduced (Fig. 6A, lanes 2–5 compared with 6–9). These data suggest that Hrs-2 and α-adaptin compete for binding to Eps15.

If Hrs-2 inhibits endocytosis by competition with α-adaptin for Eps15 binding, then expression of α-adaptin may rescue the inhibition of endocytosis produced by expression of Hrs-2. Expression of Hrs-2 significantly inhibited, while α-adaptin had no significant effect on transferrin uptake (Fig. 6B). However, transferrin uptake was not significantly different than control in the cells coexpressing α-adaptin and Hrs-2 (Fig. 6B). Since Hrs-2 apparently competes with α-adaptin for Eps15 binding, these data suggest a mechanism in which Hrs-2 regulates endocytosis by binding to Eps15, reducing its availability for α-adaptin binding.

**DISCUSSION**

Using the two-hybrid approach we have found that Hrs-2 associates with Eps15. We have confirmed a direct physical interaction using recombinant fusion proteins and coimmunoprecipitation. Additionally, we have shown that SNAP-25 competes with Eps15 for binding to recombinant Hrs-2. The binding of the recombinant Hrs-2 protein to Eps15 is calcium-dependent as the addition of calcium inhibits the Hrs-2/Eps15 interaction. Furthermore, overexpression of full-length Hrs-2 or only the region necessary for Eps15 binding, but not a mutant Hrs-2 lacking the Eps15-binding domain, inhibits the endocytosis of transferrin in HeLa cells. Moreover, both Eps15 and Hrs-2 are localized to components of the endocytic pathway. Thus, Hrs-2 is involved in endocytic processes through interactions with Eps15. Since Hrs-2 is involved in regulation of exocytic complex assembly and is unable to interact with SNAP-25 and Eps15 simultaneously, these data suggest that Hrs-2 may provide communication between endo- and exocytic processes by regulated interactions with the molecular machinery underlying both functions.

The in vitro binding of recombinant Hrs-2 and Eps15 demonstrates a direct interaction in the absence of other protein components. The EC_{50} of the Hrs-2/Eps15 interaction is approximately 1.8 μM. Thus, the interaction of Hrs-2 with Eps15 in vitro is of lower apparent affinity than that of the Hrs-2 with SNAP-25. This may reflect a more transient in vivo Hrs-2/ Eps15 interaction or may be related to the ability of SNAP-25 to displace Eps15 from Hrs-2 (see below).

The binding site of Eps15 on Hrs-2 is in a region between the FYVE finger and the first coiled-coil domain, a different region from that involved in SNAP-25 binding (11). The inhibition of Eps15 binding to Hrs-2 by SNAP-25 was therefore unexpected based on the difference in their binding site localization. Conformational changes due to SNAP-25 binding may result in an altered affinity of Hrs-2 for Eps15. The ability of SNAP-25 to

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2 A. J. Bean, unpublished observations.

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**Fig. 4. Ultrastructural localization of Hrs-2 in neurons.** Immuno-gold labeling of cerebellar neurons using an Hrs-2 antibody that was detected using silver-intensified gold and revealed labeling associated with MVBs (A, B, and C) along with some cytoplasmic and endosomal labeling (A). Scale bar = 110 nm in A, and 220 nm in B and C.
inhibit Eps15 binding to Hrs-2 suggests that Hrs-2 may toggle between SNAP-25 and Eps15, perhaps based on local calcium concentrations (see below). Hrs-2 binds to two distinct regions of Eps15, the coiled-coil and the α-adaptin-binding region in the COOH-terminal of Eps15. The coiled coil region of Eps15 is responsible for homologimerization (45) which has been shown to result in increased affinity of Eps15 for α-adaptin (46). These data suggest that the Hrs-2/Eps15 interaction may alter the oligomeric state of Eps15 and/or its association with α-adaptin, or that Hrs-2 may not interact with the fraction of Eps15 that is in a complex with α-adaptin. Since the Eps15/α-adaptin Association is required for endocytosis (35, 39), the inhibition of this interaction by Hrs-2 would be expected to inhibit endocytosis and may underlie the inhibition of endocytosis produced by overexpression of Hrs-2 (see below).

Calcium inhibits the binding of Eps15 to Hrs-2. Secondary structure predictions reveal that Hrs-2 may form multiple helix-loop-helix structures and could coordinate divalent cations in its dual zinc finger region (10). Additionally, Eps15 may form helix-loop-helix structures and has been suggested to bind calcium (33). Neither Hrs-2 nor Eps15 has been formally shown to bind calcium. However, both proteins have potential divalent cation binding sites and are altered in their ability to interact with other proteins when calcium is present. The half-maximal calcium concentration for the inhibition of Hrs-2/Eps15 binding is approximately 100 nm, which approaches the resting level of calcium in the cell. Thus, either compartmentalization within the cell produces low calcium concentrations near endocytic structures or, perhaps more likely, the binding of Hrs-2 to Eps15 is finely regulated by very small alterations in intracellular calcium. There is a 1000-fold difference in the calcium sensitivity of the binding of Hrs-2 to Eps15 compared with the Hrs-2/SNAP-25 interaction. The mechanism of the differential sensitivity to calcium may involve differences in Hrs-2, SNAP-25, and/or Eps15 protein conformation due to the presence of calcium. Alternatively, calcium may alter the oligomeric state of Hrs-2 or Eps15.

Eps15 has been shown to be associated with components of the endocytic pathway by biochemical fractionation as well as electron microscopy (38, 42). Initially, Eps15 was suggested to be present only on the edges of membrane invaginations (42), although recent data suggest that it is associated with both early and late endosomes during epidermal growth factor receptor-stimulated endocytosis (38). We have observed Hrs-2 immunoreactivity on the limiting membrane of MVBs in nerve terminals in the brain. MVBs are a sorting organelle in the late endocytic pathway that function to separate proteins destined for degradation in the lysosome from those that recycle back to the Golgi (e.g. mannose 6-phosphate receptor) or to the plasma membrane (e.g. transferrin receptor) (47–50). Examination of wild type and kinase mutant epidermal growth factor receptor internalization suggests that mutant receptors are found on the MVB limiting membrane and are recycled back to the plasma membrane, while wild type receptors are found in luminal MVB vesicles and are degraded (48). We have not observed labeling for Hrs-2 on the internal vesicles of MVBs. These data suggest that Hrs-2 is involved in functions related to the trafficking of the MVBs themselves or to sorting in the MVB compartment.

In addition to its localization on endocytic structures, we expected to find Hrs-2 immunoreactivity on the plasma membrane or components of the exocytic pathway due to its interaction with SNAP-25. We have observed Hrs-2 immunoreactivity in nerve terminals and cell bodies that appeared not to be associated with membranous structures. Negative immunohistochemical data is difficult to interpret. It is possible that the binding of Hrs-2 to SNAP-25, as would likely occur on the plasma membrane, masks the epitope for our antibody resulting in a lack of labeling on this structure.

Expression of Hrs-2 or the region of Hrs-2 that is necessary for Eps15 binding (Hrs-2(216–449)) inhibits receptor-mediated endocytosis in HeLa cells. Hrs-2 lacking the Eps15-binding domain (Hrs-2ΔEps) or the Hrs-2 fragment (cc2) necessary for binding to SNAP-25 do not inhibit receptor-mediated endocytosis in HeLa cells. Thus, the mechanism by which Hrs-2 inhibits endocytosis involves Eps15 or its downstream effectors. Eps15 is known to bind to α-adaptin, an interaction required for receptor-mediated endocytosis (35, 39). We observed
Fig. 6. α-Adaptin competes with Hrs-2 for binding to Eps15 and suppresses Hrs-2 inhibition of receptor-mediated endocytosis. Immobilized Eps15 was incubated with increasing concentrations of Hrs-2 in the absence and presence of α-adaptin (A). In the absence of α-adaptin (lanes 2–5), Hrs-2 bound to Eps15 as we had observed in Fig. 1. In the presence of α-adaptin (lanes 6–9), the binding of Hrs-2 to Eps15 was reduced (A). The concentration of immobilized Eps15 was held constant at 0.3 μM. Lane 1 contains GST (0.6 μM) as a negative control. The Ponceau-stained blot is shown as a loading control. Hrs-2 concentrations and integrated density values (×1000) were: lane 1, 4 μM, 1.75; lane 2, 1 μM, 2.08; lane 3, 2 μM, 7.58; lane 4, 3 μM, 9.62; lane 5, 4 μM, 18.07; lane 6, 1 μM, 2.95; lane 7, 2 μM, 2.11; lane 8, 3 μM, 2.56; lane 9, 4 μM, 2.51. These data suggest that Hrs-2 and α-adaptin likely do not bind to Eps15 simultaneously. B, expression of Hrs-2 (n = 15) significantly inhibited, while α-adaptin (n = 20) had no significant effect on transferrin uptake (B). Transferrin uptake in cells coexpressing α-adaptin and Hrs-2 (n = 15) was not significantly different than that found in untransfected cells. * denotes significance using ANOVA with multiple comparisons p < 0.005.

that Hrs-2 and α-adaptin compete for binding to Eps15 and that the Hrs-2-induced disruption of endocytosis is rescued by α-adaptin. The interaction of Hrs-2 with Eps15 might be an upstream event (prior to Eps15/α-adaptin binding) in the biochemical pathway that results in endocytosis and Hrs-2 would therefore be well positioned to act on this process in a regulatory manner. Local calcium concentration, lipid binding, or post-translational modifications may regulate Hrs-2 itself. Endocytosis is an energy-dependent process with at least two ATP-dependent steps (51). Thus, the ATPase activity of Hrs-2 may provide for ATP-dependent alterations in protein conformation or protein complex assembly/disassembly that are necessary for endocytosis. The molecular mechanism by which Hrs-2 inhibits endocytosis appears to be similar to that in which it uses exocytosis; Hrs-2 inhibits protein complex formation (Eps15/α-adaptin or syntaxin-SNAP-25/VAMP) by competing with one of the complex constituents.

Hrs-2 can regulate both exo- and endocytosis and the process regulated by Hrs-2 at any one time likely depends on the protein machinery with which it is currently interacting. The regulation of these interactions may depend on local calcium concentrations that could switch Hrs-2 from interacting with SNAP-25 to Eps15. Another potential role for Hrs-2 may be in the regulation of Q-SNARE sorting by binding to and excluding SNAP-25 and/or the SNAP-25-syntaxin complex from the endocytic pathway when it is being used for recycling synaptic vesicle membrane and constituent proteins. This would enable sorting of plasma membrane Q-SNAREs away from vesicle constituents following fusion, allowing for segregation of compartmental proteins and membrane composition. Although neuronal cells rely heavily on exo- and endocytosis for synaptic transmission, these processes must be coupled in all eukaryotic cells. Hrs-2 is found in neurons as well as in non-neuronal cells where it interacts with SNAP-23, a SNAP-25 homolog (11). The localization of Hrs-2 on endocytic structures as well as its ability to interact with SNAP-25 (presumably on the plasma membrane) suggests that it may be in a position to regulate both exo- and endocytosis on the membranes involved in these trafficking events. Hrs-2 may act in endocytosis during two stages: an early stage involving coordination through calcium-dependent protein interactions with essential components of both processes and a later stage that is non-Eps15/α-adaptin-dependent and involves trafficking of MVBs.

Efficient linkage of the exocytic and endocytic machinery requires that the coupling signal be transient, able to interact with both processes, and able to act at a critical stage in either process. Hrs-2, through its interactions with SNAP-25 and Eps15, may play a regulatory function on the molecular machinery underlying both exo- and endocytosis. The role of Hrs-2 is likely dependent on the local calcium, lipid, and nucleotide microenvironment. The possibility that Hrs-2 toggles between SNAP-25 and Eps15 suggests a mechanism by which this coupling signal may occur.

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REFERENCES
1. Rothman, J. E. (1994) Nature 372, 55–63
2. Scheller, R. H. (1995) Neuron 14, 893–897
3. Sudhof, T. C. (1995) Nature 375, 645–653
4. Sollner, T., Bennett, M., Whiteheart, S. W., Scheller, R. H., and Rothman, J. E. (1993) Cell 75, 409–418
5. Oyler, G. A., Higgins, G. A., Hart, R. A., Battenberg, E., Billingse, M., Bloom, F. E., and Wilson, M. C. (1989) J. Cell Biol. 109, 3629–3632
6. Chapman, E. K., An, S. H., Barton, N., and Jensen, R. (1994) J. Biol. Chem. 269, 27427–27432
7. Faasshauer, D., Buerger, A. T., and Jahn, R. (1998) Biochemistry 37, 10354–10362
8. Sutton, R. B., Faasshauer, D., Sollner, T. H., and BRUNGER, A. T. (1998) Nature 395, 347–353
9. Weber, T., Zemelman, B. V., McNew, J. A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T. H., and Rothman, J. E. (1998) Cell 92, 759–772
10. Bean, A. J., Seifert, R., Chen, Y. A., Sacks, R., and Scheller, R. H. (1997) Nature 385, 826–829
11. Tsujimoto, S., and Benaner, J. A. (2000) J. Biol. Chem. 275, 2983–2982
12. Artalejo, C. R., Henley, J. R., McNiven, M. A., and Palfrey, H. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8328–8332
13. Burgoyne, R. D. (1995) J. Physiol. Arch. 430, 213–219
14. De Camilli, P., and Takei, K. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1177–1194
Pelicci, P. G., and Di Fiore, P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9530–9534
31. Tang, H. Y., Munn, A., and Cai, M. (1997) Mol. Cell. Biol. 17, 4294–4304
32. Paoluzi, S., Castagnoli, L., Lauri, I., Salcini, A. E., Coda, L., Fre, S., Confalonieri, S., Pelicci, P. G., Di Fiore, P. P., and Cesareni, G. (1998) EMBO J. 17, 6541–6550
33. Fazioli, F., Minichiello, L., Matoskova, B., Wong, W. T., and Di Fiore, P. P. (1993) Mol. Cell. Biol. 13, 5814–5828
34. Chen, H., Fre, S., Slepnev, V. I., Capua, M. R., Takei, K., Butler, M. H., Di Fiore, P. P., and De Camilli, P. (1998) Nature 394, 793–797
35. Carbone, R., Fre, S., Iannolo, G., Belludi, F., Mancini, P., Pelicci, P. G., Torrisi, M. R., and Di Fiore, P. P. (1997) Cancer Res. 57, 5498–5504
36. Salcini, A. E., Confalonieri, S., Doria, M., Santolini, E., Tassi, E., Minenkova, O., Cesareni, G., Pelicci, P. G., and Di Fiore, P. P. (1997) Genes Dev. 11, 2239–2249
37. Sengar, A. S., Wang, W., Bishay, J., Cohen, S., and Egan, S. E. (1999) EMBO J. 18, 1159–1171
38. Torrisi, M. R., Lotti, L. V., Belludi, F., Gradini, R., Salcini, A. E., Confalonieri, S., Pelicci, P. G., and Di Fiore, P. P. (1999) Mol. Biol. Cell 10, 417–434
39. Benmerah, A., Lamaze, C., Begue, B., Schmid, S. L., Dautry-Varsat, A., and Cerf-Bensussan, N. (1998) J. Cell Biol. 140, 1055–1062
40. Wendland, B., and Emr, S. D. (1998) J. Cell Biol. 141, 71–84
41. Tsujimoto, S., Pelto-Huikko, M., Aitola, M., Meister, B., Vik-Mo, E. O., Duvvanger, S., Scheller, R. H., and Bean, A. J. (1999) Eur. J. Neurosci. 11, 3047–3063
42. Tebar, F., Sorkina, T., Sorkin, A., Ericsson, M., and Kirchhausen, T. (1996) J. Biol. Chem. 271, 28727–28730
43. Komada, M., Masaki, R., Yamamoto, A., and Kitamura, N. (1997) J. Biol. Chem. 272, 20538–20544
44. Benmerah, A., Bayrou, M., Cerf-Bensussan, N., and Dautry-Varsat, A. (1999) J. Cell Sci. 112, 1303–1311
45. Cupers, P., ter Haar, E., Boll, W., and Kirchhausen, T. (1997) J. Biol. Chem. 272, 33430–33434
46. Tebar, F., Confalonieri, S., Carter, R. E., Di Fiore, P. P., and Sorkin, A. (1997) J. Biol. Chem. 272, 15413–15418
47. Hirst, J., Futter, C. E., and Hopkins, C. R. (1998) Mol. Biol. Cell 9, 809–816
48. Felder, S., Miller, K., Moehren, G., Ullrich, A., Schlessinger, J., and Hopkins, C. R. (1998) Cell 81, 623–634
49. Futter, C. E., Pearse, A., Hewlett, L. J., and Hopkins, C. R. (1996) J. Cell Biol. 132, 1011–1023
50. Odorizzi, G., Babet, M., and Emr, S. D. (1998) Cell 95, 847–858
51. Schmid, S. L., and Smythe, E. (1991) J. Cell Biol. 114, 869–880
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