

Hrs regulates early endosome fusion by inhibiting formation of an endosomal SNARE complex

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Movement through the endocytic pathway occurs principally via a series of membrane fusion and fission reactions that allow sorting of molecules to be recycled from those to be degraded. Endosome fusion is dependent on SNARE proteins, although the nature of the proteins involved and their regulation has not been fully elucidated. We found that the endosome-associated hepatocyte responsive serum phosphoprotein (Hrs) inhibited the homotypic fusion of early endosomes. A region of Hrs predicted to form a coiled coil required for binding the Q-SNARE, SNAP-25, mimicked the inhibition of endosome fusion produced by full-length Hrs, and was sufficient for endosome binding. SNAP-25, syntaxin 13, and VAMP2

were bound from rat brain membranes to the Hrs coiled-coil domain. Syntaxin 13 inhibited early endosomal fusion and botulinum toxin/E inhibition of early endosomal fusion was reversed by addition of SNAP-25_(150–206), confirming a role for syntaxin 13, and establishing a role for SNAP-25 in endosomal fusion. Hrs inhibited formation of the syntaxin 13–SNAP-25–VAMP2 complex by displacing VAMP2 from the complex. These data suggest that SNAP-25 is a receptor for Hrs on early endosomal membranes and that the binding of Hrs to SNAP-25 on endosomal membranes inhibits formation of a SNARE complex required for homotypic endosome fusion.

Introduction

Organelles within the endocytic pathway are dynamic structures, resulting from continual rounds of fusion and fission of newly internalized vesicles with preexisting structures, initially the early endosome (Hopkins et al., 1985; Gruenberg and Maxfield, 1995; Ward et al., 1995; Ullrich et al., 1996; Gruenberg, 2001). The early endosome is a major decision point in the endocytic pathway in which cargo is sorted for transport to late endosomes for eventual degradation in the lysosome or for recycling to the plasma membrane (Hopkins et al., 1985; Gruenberg and Maxfield, 1995; Ward et al., 1995; Gruenberg, 2001). The early endosome is actually composed of at least two forms including the vacuolar or tubulovesicular sorting endosome, containing EEA-1 and rab 5, as well as the recycling endosome, containing rab 11 (Hopkins et al., 1985; Gorvel et al., 1991; Gruenberg and Maxfield, 1995; Ward et al., 1995; Ullrich et al., 1996; Trischler et al., 1999; Gruenberg, 2001). Recycling of cargo to the plasma membrane can take place from both early endosomal compartments as can transport to late endosomes

(Hopkins et al., 1985; Gorvel et al., 1991; Gruenberg and Maxfield, 1995; Ward et al., 1995; Ullrich et al., 1996; Trischler et al., 1999; Gruenberg, 2001). It is likely that a combination of maturation and vesicular transport mechanisms allows for tight control of the sorting, transport, and recycling functions in the early endosomal compartment (Gruenberg and Maxfield, 1995; Gruenberg, 2001).

Protein machinery is required to overcome the energy barrier for fusion of biological membranes. Interactions among proteins associated with donor membranes (e.g., VAMP–synaptobrevin) and acceptor membranes (e.g., syntaxin and SNAP-25) are thought to be essential for fusion (Sutton et al., 1998; Weber et al., 1998; Jahn and Sudhof, 1999; Chen and Scheller, 2001). These proteins are known as SNAREs (Sollner et al., 1993) and they are sufficient for membrane fusion in artificial membranes, suggesting that SNAREs are the core membrane fusion machinery (Weber et al., 1998). Botulinum and tetanus toxins are zinc endoproteases that the SNAREs, thus, inhibiting the formation of SNARE complexes, blocking fusion (Jahn and Sudhof, 1999; Lin and Scheller, 2000). The specificity of SNARE protein complex formation is likely dependent on protein localization and chaperone function (Jahn and Sudhof, 1999; Lin and Scheller, 2000; Chen and Scheller, 2001). SNAREs form cytoplasmic coiled-coil bundles that bridge

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two membranes to enable membrane fusion, perhaps via torsional forces produced during helical bundle formation that may deform the lipid bilayer (Sutton et al., 1998; Jahn and Sudhof, 1999; Chen and Scheller, 2001).

In vitro models of endosome fusion have been used to define the behavior of endosomal compartments after internalization of cargo. These assays have led to an understanding of some factors that influence the fate of internalized ligands and receptors and what components are required for homo- and heterotypic fusion (Braell, 1987, 1992; Diaz et al., 1988; Salzman and Maxfield, 1988; Gruenberg et al., 1989; Ward et al., 1989, 1990, 1997, 2000; Gorvel et al., 1991; Mullock et al., 2000). Endosomes and lysosomes can undergo homotypic fusion (e.g., early endosome with early endosome) and sequential compartments can fuse (e.g., early endosome with late endosome), although nonsequential compartments cannot (e.g., early endosomes do not fuse with lysosomes; Braell, 1987; Diaz et al., 1988; Salzman and Maxfield, 1988; Gruenberg et al., 1989; Ward et al., 1990, 1997; Mullock et al., 2000). These fusion reactions are dependent on SNARE proteins residing on the appropriate compartment (Prekeris et al., 1998, 1999; Mullock et al., 2000; Ward et al., 2000). For example, syntaxin 13 is found on early endosomes and a soluble fragment of syntaxin 13 inhibits homotypic early endosome fusion (Prekeris et al., 1998; McBride et al., 1999) without affecting homotypic lysosome fusion, whereas a soluble fragment of syntaxin 7, which is present on late endosome-lysosome membranes, inhibits homotypic lysosome fusion but not early endosome fusion (Ward et al., 2000). Thus, endosome fusion is dependent on SNARE protein complexes and is restricted, allowing orderly modification of ligand-receptor complexes and signaling in a sequential manner by altering the milieu (e.g., pH) in successive compartments.

Hepatocyte responsive serum phosphoprotein (Hrs)* is a mammalian protein predominantly localized on early endosomes (Komada et al., 1997; Tsujimoto et al., 1999). Hrs physically interacts with a number of proteins, including eps15 (Bean et al., 2000), SNX-1 (Chin et al., 2001), and SNAP-25 (Bean et al., 1997) that have been previously implicated in membrane trafficking. Hrs has homologues in fly (Lloyd et al., 2002) and yeast (Raymond et al., 1992). Deletion or mutation of Hrs results in an enlarged endosomal phenotype in mouse (Komada and Soriano, 1999), fly (Lloyd et al., 2002), and yeast (Raymond et al., 1992) without an obvious defect in lysosomal trafficking (e.g., the absence of Hrs does not result in increased expression of plasma membrane proteins and internalized proteins can be transported to lysosomes), suggesting that Hrs may affect endosome fusion.

Endocytosis of the EGF receptor (EGFR) is initiated by the binding of its ligand, EGF (Honegger et al., 1990; Barbieri et al., 2000; Carpenter, 2000; Burke et al., 2001; Schlessinger, 2002). The EGFR-ligand complex is transported through the

endocytic pathway where a choice about its fate, whether to be recycled or degraded, is made. The EGFR-ligand complex is then either recycled back to the cell surface or moves to late endosomes, and ultimately, the lysosome for degradation (Honegger et al., 1990; Barbieri et al., 2000; Carpenter, 2000; Burke et al., 2001). We have taken advantage of the well-characterized trafficking of the EGF-EGFR complex, the dependence of EGF receptor endocytosis on ligand binding, and previously developed cell-free endosome fusion assays, to develop a novel approach for measuring fusion of EGF-EGFR-containing compartments. By allowing different populations of HeLa cells to engage in receptor-mediated endocytosis of EGF linked to either Alexa₄₈₈ or tetramethylrhodamine (TMR), we are able to isolate donor and acceptor pools of endosomes and lysosomes. These compartments are used in fusion reactions that are analyzed by examining resonance energy transfer between the fluorophores to detect content mixing. This assay is dependent on temperature, time, energy, and cytosol. We have used our newly developed endosome fusion assay to examine the effect of Hrs on fusion of different populations of endosomal membranes. We observed that Hrs selectively inhibits the homotypic fusion of early endosomes and that the coiled-coil region of the protein mediates this effect as well as its endosomal membrane association. We determined that SNAP-25 is an endosomal receptor for Hrs, and that Hrs inhibits the formation of the early endosomal SNARE complex consisting of SNAP-25, syntaxin 13, and VAMP2, suggesting a mechanism by which Hrs inhibits early endosome fusion.

Results

A novel fluorescence resonance energy transfer (FRET)-based homotypic membrane fusion assay

We have taken advantage of the trafficking pattern of EGF-EGFR complexes and the dimerization of EGFR to design a cell-free assay to detect endosome fusion. By allowing different populations of HeLa cells to engage in receptor-mediated endocytosis of EGF linked to either Alexa₄₈₈ or TMR for various times, we are able to isolate donor and acceptor pools of endocytic intermediates. These compartments are used in fusion reactions analyzed by measuring resonance energy transfer between the fluorophores to detect content mixing.

After increasing periods of chase time EGF-TMR-labeled cells were immunolabeled with markers for the early endosome (EEA-1), late endosome (rab 7), and lysosome (LAMP1/2). By quantifying the amount of overlap between the two signals we generated a time course of EGF-TMR movement through the endocytic pathway that we used as the optimal labeling time for the various compartments. When HeLa cells are incubated with EGF-TMR for 15 min, the predominant localization of the EGF-TMR labeling was in an EEA-1-positive structure, putatively an early endosome (Fig. 1, A and B). If HeLa cells were labeled with a 15-min pulse of EGF-TMR, washed, and incubated in normal media for increasing incubation times the EGF-TMR moved from the early endosome to the late endosome (Fig. 1 C) and the lysosome (Fig. 1 D), as identified by colocalization of the EGF-TMR with markers for those compartments (rab 7 and LAMP 1/2, respectively).

*Abbreviations used in this paper: BoNT/E, botulinum neurotoxin E; DMEM, Dulbecco's minimum essential medium; EGFR, EGF receptor; FRET, fluorescence resonance energy transfer; Hrs, hepatocyte responsive serum phosphoprotein; TMR, tetramethylrhodamine; UIM, ubiquitin interacting motif.

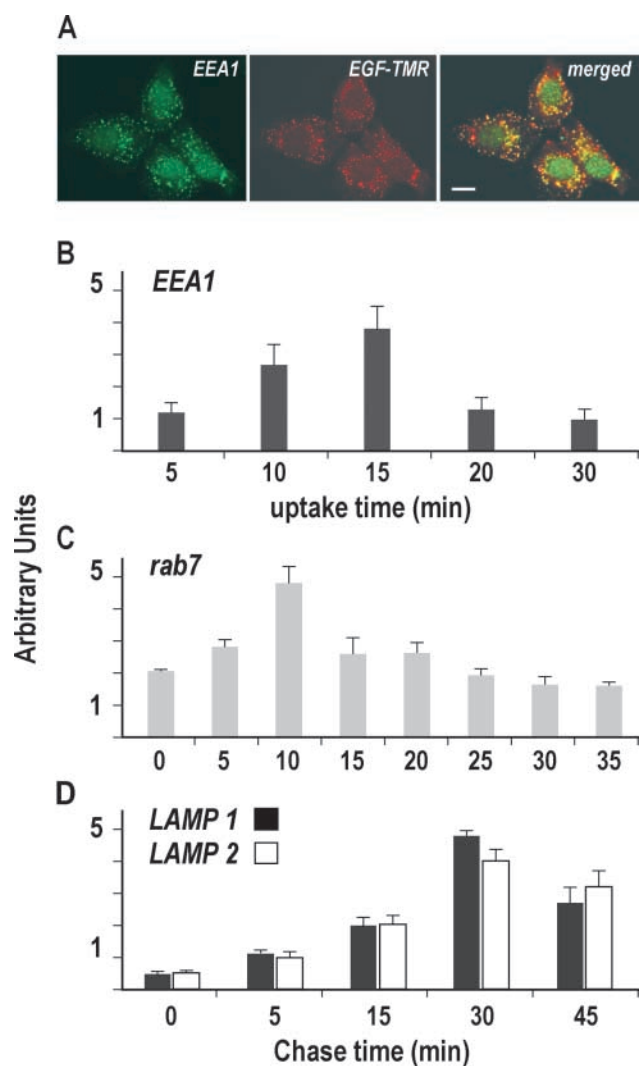


Figure 1. EGF is transported through endocytic compartments after internalization. (A) Colocalization of early endosomes with EGF-TMR in HeLa cells. The distribution of EEA1 (A, green), EGF-TMR (B, red), and the merged images (C) shows colocalization of EEA1-labeled early endosomes and EGF-TMR. (B) Percentage of EEA1-labeled early endosomes colocalized with EGF-TMR-containing vesicles after various incubation times. (C) Percentage of rab 7-labeled late endosomes colocalized with EGF-TMR-containing vesicles at various chase times after a 15-min pulse of EGF-TMR. (D) Percentage of LAMP 1/2-labeled lysosomes colocalized with EGF-TMR-containing vesicles at various chase times after a 15-min pulse of EGF-TMR. The error bars in B–D show SEM. Bar, 8 μ m.

To better understand the fate of EGF in our cells, we have examined the percentage of 125 I-EGF that is recycled, degraded, or within HeLa cells after a pulse of EGF. The amount of EGF recycled after a 15-min pulse is \sim 5–7% of the total cellular EGF, the amount degraded in 15 min is \sim 5% of the total, and the amount within the cells is \sim 90% of the total cellular EGF (unpublished data). These data are consistent with many reports in the literature for HeLa cells (Satin et al., 1997; Futter et al., 2001). The kinetics we have observed for ligand internalization is very similar to those for labeled EGFR internalization (Burke et al., 2001). Here, the movement of internalized labeled EGFR was examined in relation to its localization in the EEA-1 positive early endo-

some. The maximal colocalization of labeled receptor with EEA-1 occurred at 15 min after initiation of internalization. These data suggest that in HeLa cells the kinetics we have observed are consistent with many previously published reports for ligand and receptor internalization and trafficking to early endosomes. Moreover, very little of the ligand is being recycled or degraded at the time the endosomes are isolated and, therefore, the contribution of these steps to the fusion observed is likely minimal.

Based on the optimal labeling conditions for each compartment (Fig. 1), fluorescently labeled early and late endosomal populations, as well as lysosomes, were isolated and used in fusion reactions. In the absence of donor or acceptor membranes, ATP, or cytosol, compartment fusion did not occur as visualized because of a lack of a TMR emission signal (580 nm) that is significantly above baseline after excitation of the Alexa fluorophore at 495 nm (Fig. 2 A). These fusion reactions were also temperature dependent, as incubation at 0°C also resulted in a lack of TMR emission signal (Fig. 2 A). The fusion signal requires intact membranes because incubation of completed reactions with 1% Triton X-100 resulted in a signal that was not significantly different from the background (unpublished data). The fusion signal we observe is not likely due to the contribution of extra endosomal receptors because acid washing the plasma membrane before cell homogenization coupled with the addition of unlabeled EGF (300 μ g/ml) to the reactions did not alter the fusion-induced FRET signal (unpublished data). The energy transfer observed was either the result of heterodimerization of EGFRs that have bound EGF containing Alexa₄₈₈ and TMR or the exchange of differently tagged ligands on dimerized receptors (Schlessinger, 2002). We think it more likely that receptor heterodimerization is the mechanism because heterodimerization of EGFRs is known to occur in the plane of the plasma membrane (Muthuswamy et al., 1999; Wang et al., 1999; Saito et al., 2001). Moreover, EGF is generally thought to be stably associated with its receptor throughout the endocytic pathway, perhaps due to its high affinity (Futter et al., 1996). This would imply that after fusion of the endosomal membranes the ligands remain bound and the receptors can exchange partners, although receptor–receptor and receptor–ligand interactions are highly dynamic and it is not possible to distinguish among these possibilities within the scope of these studies. The FRET signal was not likely due to a high concentration of both fluorophores in the small volume of the endosome because as the fusion reactions progress the size of the fused compartment increases and the FRET signal did not decrease in proportion to the size of the fused compartment (unpublished data). Moreover, if donor compartments were labeled with EGF-Alexa₄₈₈ and the acceptor compartments were labeled by internalization of transferrin-TMR, no FRET signal significantly above background was obtained after a fusion reaction, which is consistent with the inability of EGF and transferrin receptors to dimerize (Fig. 2 B). Transferrin-TMR was localized in an EEA-1–positive compartment under these conditions in HeLa cells (unpublished data). Optimal fusion time was determined by examining the extent of fusion after incubating the reactions for various amounts of time at 37°C. After 20 min of incuba-

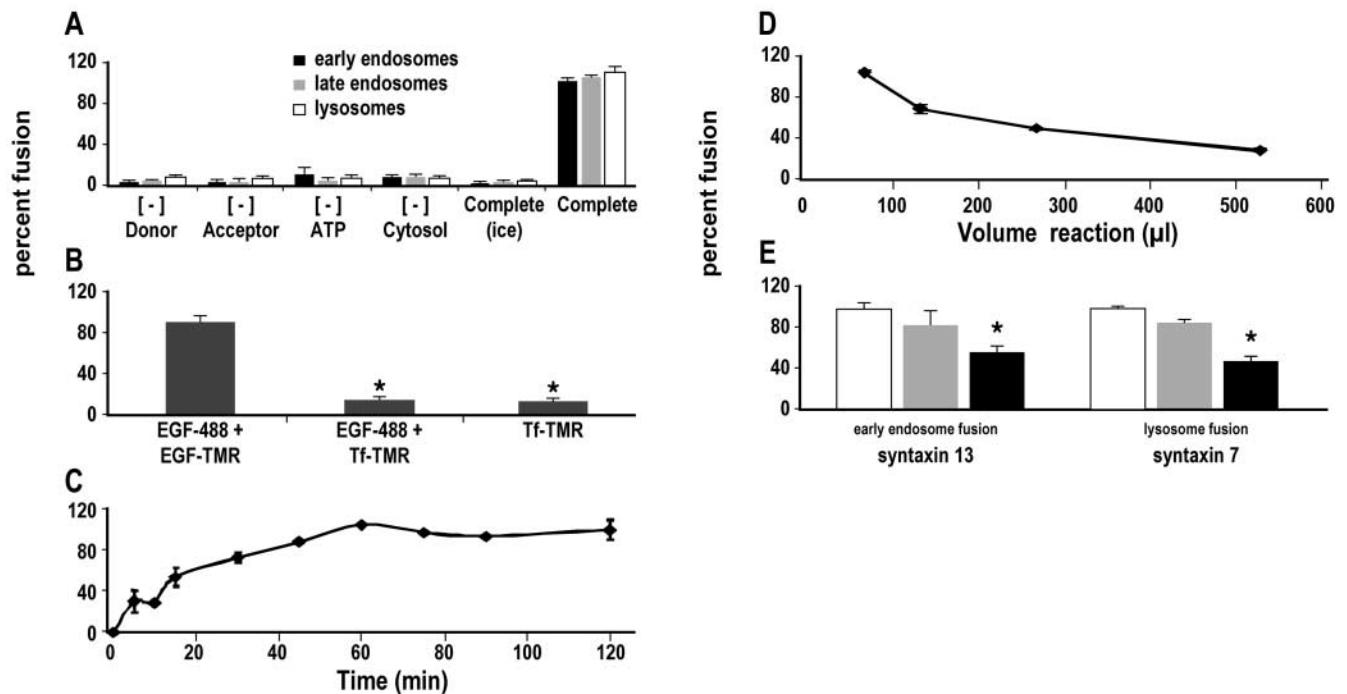


Figure 2. Characterization of a FRET-based assay measuring homotypic compartment fusion. (A) Fusion reactions were performed without the indicated component or on ice (0°C) to assess requirements for the reaction. There are significant differences between the complete homotypic fusion reactions and all other conditions in which a component has been omitted or altered (*, $P \leq 0.001$) by ANOVA and Fisher test. Raw data after background subtraction are as follows. Early endosome fusion: no donor, 14.1 ± 3.5 ; no acceptor, 15.4 ± 5.0 ; no ATP, 37.3 ± 16.0 ; no cytosol, 28.3 ± 3.6 ; ice, 10.7 ± 4.3 ; and complete homotypic reaction, 302.5 ± 7.7 . Late endosome fusion: no donor, 16.3 ± 3.4 ; no acceptor, 13.1 ± 12.8 ; no ATP, 15.6 ± 10.8 ; no cytosol, 26.6 ± 7.8 ; ice, 12.8 ± 4.8 ; and complete homotypic reaction, 312.1 ± 6.3 . Lysosome fusion: no donor, 28.4 ± 1.4 ; no acceptor, 21.0 ± 6.2 ; no ATP, 23.9 ± 8.2 ; no cytosol, 24.3 ± 7.0 ; ice, 13.3 ± 1.2 ; and complete homotypic reaction, 325.3 ± 16.0 . (B) Energy transfer is dependent on EGF-labeled donor and acceptor membranes. When both fluorescent tags are present on EGF and endosomes are labeled and fused a FRET signal occurs. However, labeling the acceptor population of endosomes with transferrin-TMR reduces the FRET signal to background levels (*, $P \leq 0.01$). The error bars show SEM. (C) The optimal incubation time for fusion reactions was determined by examining the extent of fusion after incubating the reactions for various amounts of time at 37°C . The amount of fusion increased until 60 min of incubation, after which no further significant increase in fusion is observed. (D) The fusion reaction volume was increased to dilute the concentration of donor/acceptor membranes while the concentrations of ATP and cytosol were maintained at a constant level. A decrease in FRET signal was observed concomitant with an incremental increase in the reaction volume. (E) Syntaxin 13 and 7 affect homotypic endosome fusion. Syntaxin 13 inhibited early endosome homotypic fusion. Syntaxin 7 inhibited homotypic lysosome fusion. These data are consistent with previously published reports (see Introduction and Results). *, $P \leq 0.01$.

tion, fusion was $\sim 50\%$ of the maximum and the amount of fusion increased until 60 min of incubation, after which no further significant increase in fusion was observed (Fig. 2 C). Therefore, the 60-min time point was chosen as the optimal fusion time for the assay.

To test the effect of membrane dilution, the reaction volume was increased to dilute the concentration of donor/acceptor membranes while the concentration of ATP and cytosol was maintained at a constant level. A decrease in FRET signal was observed concomitant with an incremental increase in the reaction volume (Fig. 2 D). This suggests that the concentration of donor and acceptor membranes has a critical threshold for optimal reconstitution of homotypic endosome fusion.

As a further confirmation that these fusion reactions are SNARE dependent and comparable to what has been previously observed in the literature (Prekeris et al., 1998, 1999; McBride et al., 1999; Ward et al., 2000), we examined the effect of the soluble fragments of syntaxin 13 and 7 on fusion of early and late endosomes as well as lysosomes (Fig. 2 E). The soluble syntaxin 13 protein specifically inhibited

early endosome fusion (Fig. 2 E) with no effect on late endosome or lysosome fusion (not depicted). The soluble syntaxin 7 protein specifically inhibited lysosome fusion (Fig. 2 E) with no effect of early or late endosome fusion (not depicted). As an additional control, we examined the effect of wild-type and mutant rab 15, a small GTPase that has been previously implicated as a regulator of early endosome fusion (Zuk and Elferink, 1999). Addition of lysate from cells overexpressing wild-type rab 15 significantly inhibited early, but not late, endosome fusion $46 \pm 4\%$ ($n = 3$, $P \leq 0.05$), whereas the Q67L mutant inhibited early endosome fusion by $65.6 \pm 4\%$ ($n = 3$, $P \leq 0.05$), which is consistent with previously published data (Zuk and Elferink, 1999).

Ultrastructural examination of the morphology of donor/acceptor membranes before a fusion reaction revealed the presence of consistently sized membrane-bound compartments (mean diameter, 58.3 ± 1.7 nm; Fig. 3 A), the majority of which were uncoated although an apparently clathrin-coated vesicle can be observed occasionally (Fig. 3 A). After fusion reactions, the mean diameter of membrane compartments was significantly enlarged to 188.5 ± 7.3 nm ($P \leq$

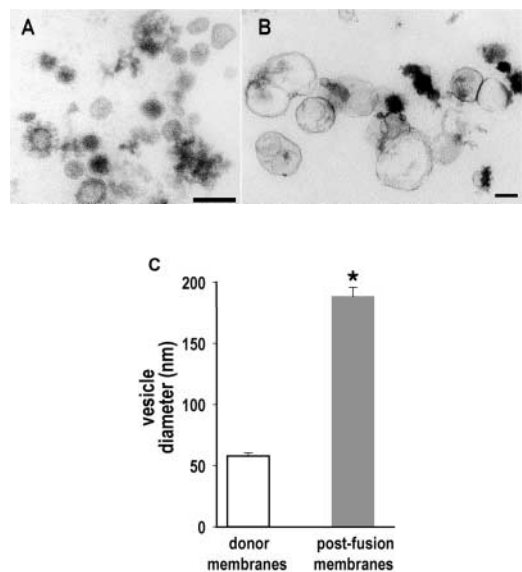


Figure 3. Morphology of endosomal membranes before and after fusion reactions. Membranes were obtained as described for homotypic reactions of early endosomes (see Materials and methods) and fixed in 3% glutaraldehyde (A, donor) or after incubation with acceptor membranes, cytosol, and ATP regenerating system (B, after fusion). The diameter of all membrane bound profiles was measured on images from donor and fused samples (C). The mean diameter of donor compartments was 58.3 ± 1.7 nm ($n = 123$) and postfusion compartment diameter was 188.5 ± 7.3 nm ($n = 96$; *, $P \leq 0.0001$). The error bars show SEM. Bars: (A and B) 100 nm.

0.05). Compartments containing membrane as well as membranes apparently docked and/or undergoing fusion could also be observed (Fig. 3, B and C).

Hrs inhibits homotypic fusion of early endosomes

We examined the effect of the Hrs protein on the three different homotypic fusion reactions. Hrs specifically inhibited early endosome fusion with no effect on late endosome or lysosome fusion (Fig. 4 A). The inhibition of early endosome fusion by recombinant Hrs was concentration dependent and saturable with half-maximal inhibition observed at ~ 30 nM. The total level of Hrs in HeLa cells is $\sim 2\text{--}4 \times 10^{-5}$ $\mu\text{g}/\text{cell}$. If the volume of a HeLa cell is ~ 4 nl (an average size for HeLa cells is $15\text{--}20$ μm in diameter for a suspended cell and, therefore, its volume $4/3\pi r^3 = 4,000$ nm^3 or 4×10^{-9} cm^3), and the rough estimation of a cytosolic intracellular Hrs concentration would be $0.5\text{--}1$ nM. The Hrs present in these cells is roughly 75% cytosolic and 25% membrane associated. Moreover, the localization of Hrs on endosomal membranes is patchy (Tsujimoto et al., 1999; Urbe et al., 2000; Raiborg et al., 2001a, 2002) with areas of apparently much higher concentration. Thus, it is very difficult to determine the local concentration of Hrs on the endosomal membrane and, therefore, what would be the physiologically relevant concentrations of Hrs for endosome fusion. We have observed a dose-dependent and saturable effect whose half-maximal value is ~ 30 nM and that saturates at ~ 100 nM. Given the caveats presented above, we believe this to be within the physiologically relevant range for the concentration of Hrs on the endosomal

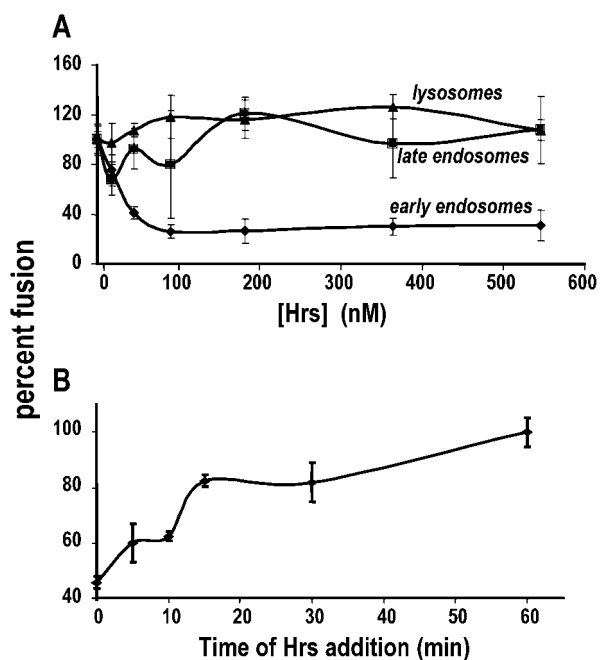


Figure 4. Hrs inhibits early endosome fusion at an early stage in the fusion reaction. (A) Hrs inhibits early endosome fusion in a dose-dependent and saturable manner (diamond), without having a significant effect on late endosome (square) or lysosome (triangle). (B) Hrs-induced inhibition of early endosome fusion occurs most prominently when Hrs is added to the reactions within the first 10 min of incubation. If Hrs is added after the first 10 min of the fusion reaction, its ability to inhibit the reaction reduced.

membrane. Hrs was required for an early event in the fusion reaction because the inhibition produced by Hrs was maximal if added within 10 min after the initiation of the reaction and diminished if Hrs was added after that time (Fig. 4 B). To examine the effect of Hrs depletion on early endosome fusion, we treated HeLa cells with RNAi duplexes targeted against Hrs in addition to immunodepleting Hrs from the cytosol. After treatment, Hrs was undetectable in whole cell lysates from which the donor/acceptor endosomes were isolated, as well as in the rat brain cytosol required for the assay. Under these conditions we observed a significant ($P \leq 0.05$), albeit modest, $16 \pm 4\%$ increase in endosome fusion, whereas the controls lacking cytosol or transfected with scrambled RNAi duplexes were not significantly different than the homotypic reaction (unpublished data).

To understand the mechanism by which Hrs inhibited early endosome fusion, we examined the effect of different domains of Hrs to determine whether a minimal fragment of Hrs was required for the effect. We examined a large NH_2 -terminal fragment of Hrs that contains the VHS, FYVE, and UIM domains, as well as the binding sites for eps15 (Bean et al., 2000) and STAM (Asao et al., 1997; Fig. 5 A). This domain did not significantly alter early endosome fusion (Fig. 5 B). However, a region of Hrs containing either both (Hrs₄₄₉₋₅₆₂) or just the second coiled-coil domain (Hrs₅₁₅₋₅₆₂) inhibited early endosome fusion with concentration dependence and saturability that was indistinguishable from the full-length protein (Fig. 5 C).

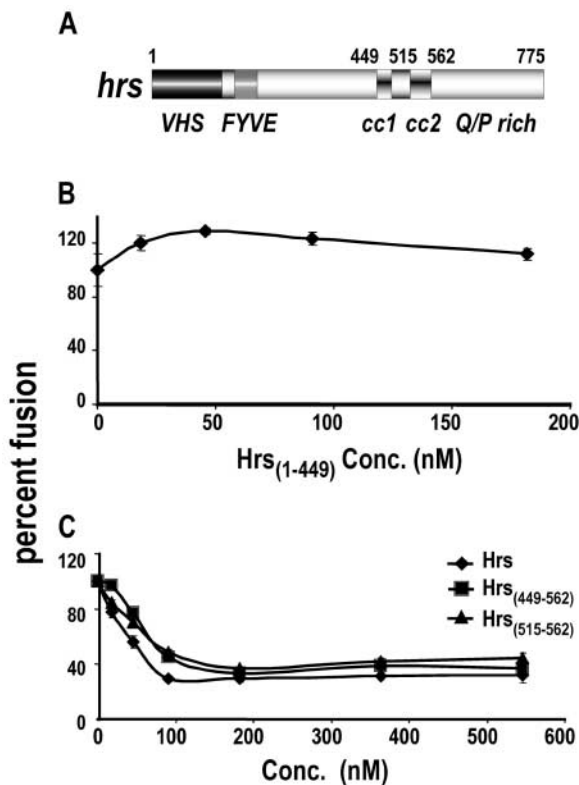


Figure 5. Determination of the domain of Hrs required for the inhibition of early endosome fusion. (A) The linear structure of Hrs highlighting some protein motifs. (B) The NH₂-terminal half of Hrs, including the VHS and FYVE domains, had no effect on early endosome fusion. (C) Although both helical domains of Hrs inhibit early endosome fusion with similar efficacy to the full-length protein, the minimal fragment of Hrs that is both necessary and sufficient for the recapitulation of the inhibition of early endosome fusion observed with full-length Hrs is the Q-SNARE domain Hrs₍₅₁₅₋₅₆₂₎.

Hrs binds to SNAP-25 on early endosomal membranes

To determine whether Hrs might exert its effect by binding to endosomal membranes, we incubated purified endosomes with increasing concentrations of recombinant Hrs (Fig. 6 A). We observed saturable binding of Hrs to EEA-1–positive early endosomes, suggesting that a finite number of binding sites were present on this membrane. Moreover, SNAP-25₍₁₅₀₋₂₀₆₎ inhibited the binding of Hrs to endosomal membranes (Fig. 6 B) suggesting that the Hrs–SNAP-25 interaction is responsible for endosomal binding of Hrs and that SNAP-25 is the endosomal Hrs receptor. We also observed that the coiled-coil domain Hrs₄₄₉₋₅₆₂ itself bound to endosomal membranes (Fig. 6 C). The binding of either Hrs or Hrs₄₄₉₋₅₆₂ was 80% complete after incubation with endosomal membranes for 15 min at 0°C (unpublished data).

Because Hrs bound saturably to endosomal membranes and this binding was inhibited by SNAP-25₍₁₅₀₋₂₀₆₎, we hypothesized the presence of a membrane receptor whose identity was likely SNAP-25. To identify potential membrane receptors, affinity chromatography was performed using immobilized Hrs₄₄₉₋₅₆₂ on a detergent-extracted rat brain membrane fraction. We detected SNAP-25, syntaxin13, and VAMP2 after salt elution from the affinity column, whereas none of these proteins were detected in the eluate from a

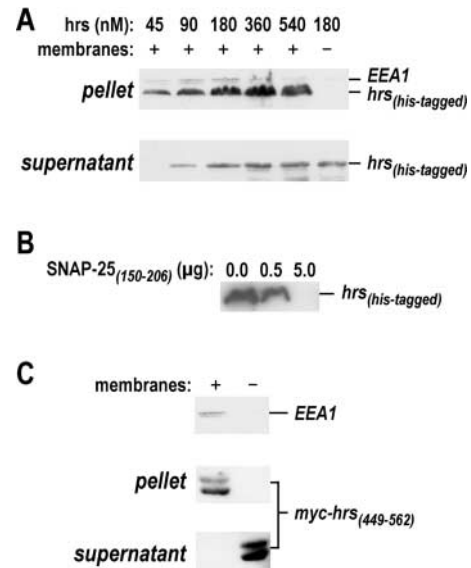


Figure 6. Hrs binds saturably to early endosomal membranes. Hrs and Hrs₍₄₄₉₋₅₆₂₎ bind to early endosomal membranes. Early endosomes were purified on a discontinuous sucrose gradient and contain the marker protein EEA-1. Full-length Hrs binds saturably to early endosomal membranes (A). SNAP-25₍₁₅₀₋₂₀₆₎ inhibited the binding of Hrs to endosomal membranes (B). Hrs₍₄₄₉₋₅₆₂₎ also binds to endosomal membranes (C), suggesting that the coiled-coil domain of Hrs is sufficient for endosomal membrane binding. Hrs or Hrs₍₄₄₉₋₅₆₂₎ is not found in the pellet in the absence of membranes.

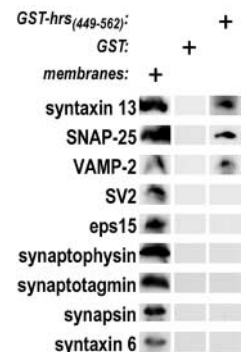
control (GST) column (Fig. 7). Neither SV2, eps15, synaptotagmin, synaptophysin, synapsin, syntaxin 6 (Fig. 7), rab 5, rab 15, nor EEA-1 (not depicted) were detected in the eluate from the affinity column. Because Hrs does not directly bind to VAMP or syntaxin (Tsujimoto and Bean, 2000), these data further suggested that the endosomal Hrs receptor is SNAP-25. This was consistent with the direct interaction of Hrs with SNAP-25 (Bean et al., 1997).

Hrs prevents the formation of a SNARE complex on early endosomes

Because we detected a SNAP-25–containing SNARE complex on endosomal membranes, we examined the effect of botulinum neurotoxin E (BoNT/E), a zinc endoprotease

Figure 7. Affinity chromatography of brain membranes using immobilized Hrs₍₄₄₉₋₅₆₂₎.

A SNARE complex consisting of syntaxin 13, SNAP-25, and VAMP2 has been previously reported to be present on early endosomal membranes (Prekeris et al., 1998). Hrs has been previously reported to bind to SNAP-25 (Bean et al., 1997) requiring aa 515–562 (Tsujimoto and Bean, 2000). Candidate proteins were examined by Western analysis on blots obtained from the affinity column eluate. SNAP-25, syntaxin 13, and VAMP2 bound to the column, whereas SV2, eps15, synaptophysin, synaptotagmin, synapsin, syntaxin 6, and rab 5, rab 15, and EEA-1 (not depicted) did not bind to Hrs₍₄₄₉₋₅₆₂₎.



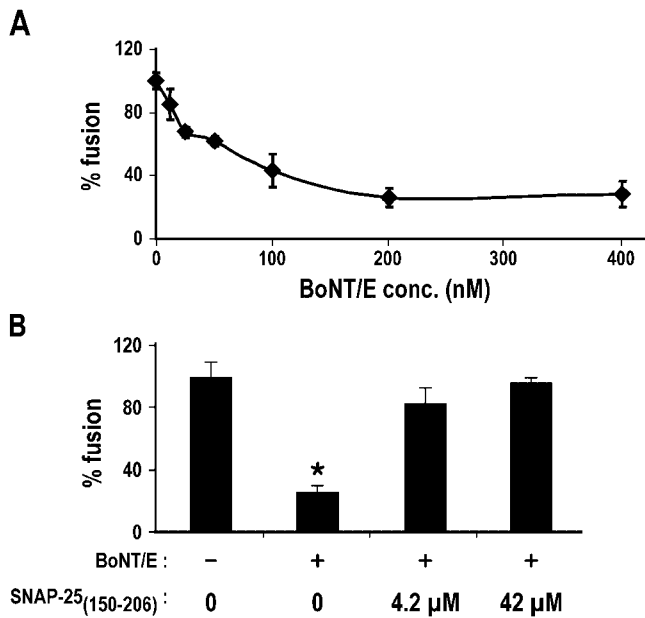


Figure 8. BoNT/E inhibits early endosomal fusion in a SNAP-25₍₁₅₀₋₂₀₆₎-dependent manner. (A) BoNT/E dose dependently and saturably inhibited early endosome fusion. (B) The inhibition of early endosome fusion by BoNT/E is reversed by addition of SNAP-25₍₁₅₀₋₂₀₆₎ supporting a role for SNAP-25 in endosome fusion.

that cleaves the COOH-terminal 26 aa of SNAP-25. BoNT/E blocks membrane fusion that requires a four-helical-SNARE complex containing SNAP-25 (Banerjee et al., 1996; Chen et al., 1999; Jahn and Sudhof, 1999). We observed that BoNT/E inhibited early endosome fusion in a concentration-dependent manner with a half-maximal inhibition of ~20 nM (Fig. 8 A). Moreover, the addition of SNAP-25₍₁₅₀₋₂₀₆₎ completely reversed the inhibition of fusion produced by BoNT/E (Fig. 8 B). These results suggest a previously unappreciated role for SNAP-25 in early endosome fusion.

To understand how Hrs might inhibit early endosome fusion, we examined the *in vitro* formation of an early endosomal 7S fusion complex containing syntaxin 13, SNAP-25, and VAMP2. Using immobilized syntaxin 13, we formed the 7S complex with SNAP-25 and VAMP2 (Fig. 9, lane 1). Increasing concentrations of Hrs inhibited the amount of VAMP2 inclusion in the 7S complex (Fig. 9). At saturating concentrations, Hrs completely inhibited VAMP from binding to the complex. These results suggested that Hrs could inhibit the formation of the 7S fusion complex by binding to SNAP-25, inhibiting VAMP incorporation into the complex (Fig. 10).

Discussion

We have shown that the Hrs protein specifically inhibits the homotypic fusion of early endosomes while having no effect on late endosome or lysosome fusion. Moreover, the coiled-coil region of Hrs binds to early endosomal membranes and is necessary and sufficient for the inhibition of endosome fusion. To identify a membrane receptor for Hrs, the coiled-coil region of Hrs was used to isolate proteins from rat brain

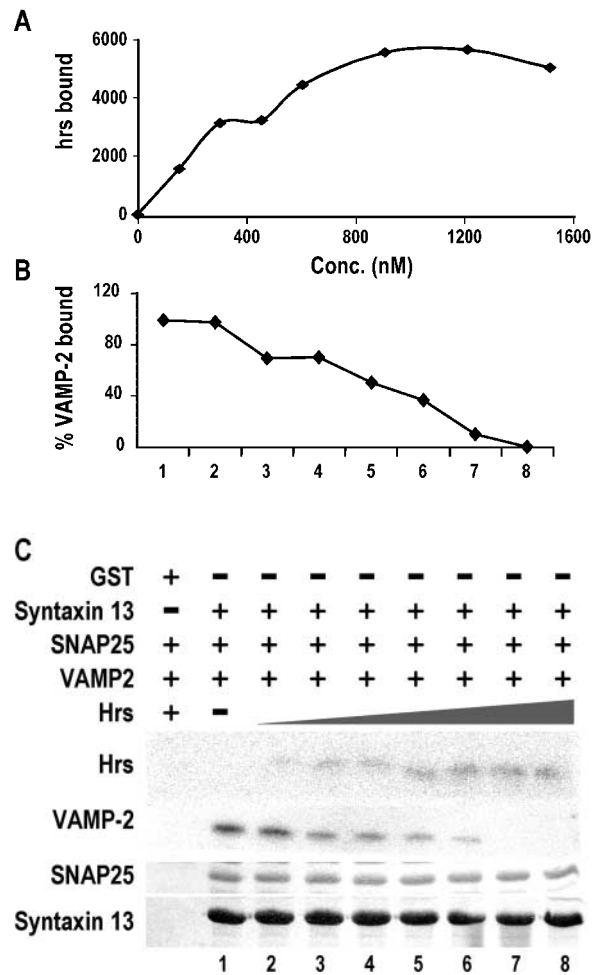


Figure 9. Hrs decreases the efficiency of early endosomal syntaxin13-SNAP-25-VAMP2 complex formation. Quantitation of the amount of Hrs (A, expressed in optical density units) or VAMP (B, expressed as a percentage of VAMP incorporated in the absence of Hrs) incorporated into the 7 S complex in the presence of increasing amounts of Hrs is shown. (C) Glutathione-immobilized GST or GST-syntaxin 13, SNAP-25, and VAMP2 were incubated in the absence (lane 1) and presence of increasing amounts of Hrs. Samples were processed, and immunoblots were visualized with ¹²⁵I-secondary antibodies and phosphorimaging.

membranes and identified a SNARE complex (SNAP-25, syntaxin 13, and VAMP2) thought to be present on early endosomes. The inhibition of early endosome fusion by BoNT/E and the rescue of that effect by the COOH-terminal coiled-coil region of SNAP-25, establishes a role for SNAP-25 in endosome fusion. Hrs inhibits the formation of the early endosome 7S SNARE complex, suggesting a mechanism by which Hrs inhibits early endosome fusion (Fig. 10). These data suggest a negative role for Hrs on endosome fusion that is mediated by its binding to SNAP-25 on endosomal membranes. The increase in endosome size observed after deletion of Hrs (Raymond et al., 1992; Komada and Soriano, 1999; Lloyd et al., 2002) is consistent with this observation.

By allowing different populations of HeLa cells to engage in receptor-mediated endocytosis of EGF linked to either Alexa₄₈₈ or TMR, we are able to isolate donor and acceptor

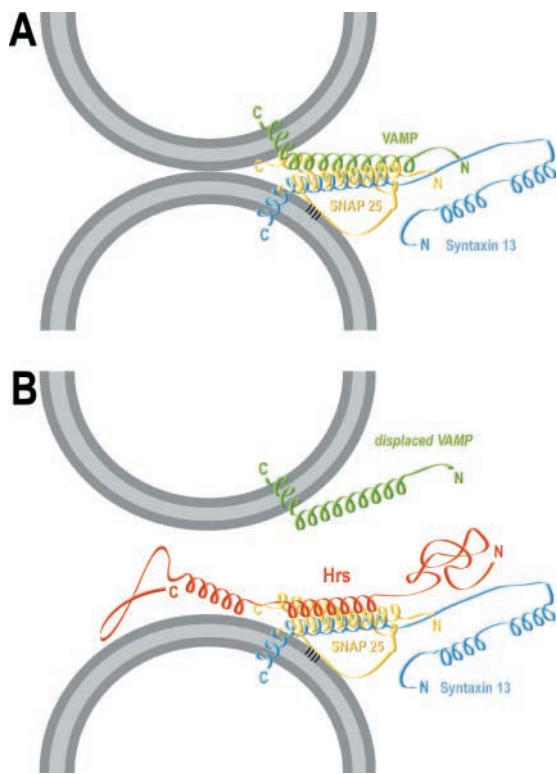


Figure 10. Model for the mechanism by which Hrs inhibits early endosome fusion. (A) Pairing of SNAREs (VAMP2, SNAP-25, and syntaxin 13) from opposing membranes results in helical bundle formation required for membrane fusion. (B) Hrs inhibits early endosome fusion by preventing SNARE complex formation. Hrs binds to SNAP-25 using its Q-SNARE-containing second coiled coil and inhibits VAMP2 from binding to the SNAP-25–syntaxin13 complex. N, NH₂; C, COOH.

pools of EGF-containing endosomes. These compartments were then used in fusion reactions that are analyzed by measuring resonance energy transfer between the fluorophores to detect content mixing. The ability to examine the fusion of compartments in which EGF–EGFR complexes reside, as well as the relative ease of measurement of the fusion signal, are advantages of this assay over previously developed fusion assays using immunoprecipitation of enzyme–substrate complexes (Gruenberg et al., 1989; Ward et al., 1990, 1997; Braell, 1992).

Hrs has been suggested to be involved in both exocytosis and endocytosis. Data implicating Hrs in exocytosis are that it binds to a protein, SNAP-25, that is essential for neurotransmitter secretion, and that the recombinant protein inhibits neurotransmitter secretion (Bean et al., 1997; Murai and Kitamura, 2000). However, the preponderance of evidence suggests that the Hrs protein is involved in endocytosis because it is localized on endosomal membranes (Komada et al., 1997; Tsujimoto et al., 1999), it interacts with proteins implicated in endocytosis (Bean et al., 2000; Chin et al., 2001), and it has homology with a yeast protein (Vps27p) required for endocytic protein sorting (Raymond et al., 1992; Komada and Kitamura, 1995). The effect of Hrs on secretion may result from promiscuous binding of Hrs to the 7S complex formed between the Golgi-derived secretory vesicle and the plasma membrane resulting from

the mistargeting of the exogenously added or highly overexpressed Hrs.

The mouse knockout of Hrs is embryonic lethal and the only observable phenotype is the presence of enlarged early endosomes (Komada and Soriano, 1999). The *Drosophila* knockout also possesses enlarged endosomes (Lloyd et al., 2002). The yeast homologue of Hrs is likely Vps27p, whose mutant phenotype is the presence of an enlarged “class E,” presumed prevacuolar/endosomal compartment (Raymond et al., 1992). A common thread among these phenotypes is the presence of an enlarged endosomal compartment and the hypothesis suggested to explain them is the inhibition of a sorting or trafficking step before the lysosome. In mammalian cells, the predominant localization of Hrs is on early endosomes, although a small percentage is found on the limiting membrane of late endosomes (Komada et al., 1997; Tsujimoto et al., 1999). The enlargement of early endosomes in mammalian cells suggests either an inhibition of trafficking out of these structures, an increase in the fusion of transport vesicles with early endosomes, or an increase in the homotypic fusion of early endosomes. We directly showed that Hrs inhibits the homotypic fusion of early endosomes. These data are consistent with a negative role of Hrs on early endosome fusion that would be absent in a null mutant. The adventitious expression of Hrs also produces an enlarged endosomal compartment in mammalian cells that appears similar to the null phenotype and is likely the result of a complex interaction of the binding and sequestration of the many Hrs binding partners due to its overexpression. The deletion of Hrs from mouse and fly results in enlarged early endosomes and, although the removal of the protein has its own limitations, the overexpression manipulation may provide less clarity due to the varied phenotypes observed with varying levels of expression (e.g., Raiborg et al., 2001a) that may be due to binding partner affinities or alterations in cellular localization.

The exact domain of Hrs required for membrane association has been unclear. For example, Komada et al. (1997) and Hayakawa and Kitamura (2000) have shown that deletion of the FYVE domain does not alter the membrane/endosomal localization of overexpressed Hrs. Urbe et al. (2000) have shown that overexpression of FYVE domain deletions results in a cytosolic localization and suggest that FYVE–PI3-P interactions cooperate with a second interaction domain located elsewhere in the protein to specify its membrane localization. Raiborg et al. (2001b) have suggested that the FYVE domain, in cooperation with the coiled-coil domain, contributes to the targeting of Hrs to endosomes. The difference between our work and the previously published work is that in the previous studies, the role of different domains in membrane association was determined by overexpression of Hrs or fragments. Potential mislocalization due to overexpression or oligomerization of fragments with the endogenous protein may be factors in the localization of various overexpressed fragments. The binding of purified Hrs protein to purified EEA-1–positive early endosomes shows that Hrs or Hrs_(449–562) can bind rapidly and in a saturable manner to early endosomal membranes. These data suggest that the coiled-coil domain of Hrs can bind to endosomal membranes in the absence of other domains and

that Hrs binds to a finite number of binding sites, thus implicating a membrane receptor. SNAP-25 is the likely membrane receptor as the region of SNAP-25 responsible for Hrs binding was able to completely inhibit the endosomal binding of Hrs, and the coiled-coil region of Hrs affinity isolated a SNAP-25 containing SNARE complex from brain membranes. Additionally, association of Hrs with endosomal membranes, even when the binding is performed at 0°C, suggests a high affinity interaction and rapid association rate. The domain of Hrs necessary and sufficient for the inhibition of early endosome fusion is the coiled-coil domain previously shown to be responsible for binding SNAP-25/23 (Tsujimoto et al., 1999; Tsujimoto and Bean, 2000). The NH₂-terminal region of Hrs containing the VHS and FYVE domains, as well as the eps15 (Bean et al., 2000) and STAM (Asao et al., 1997) binding sites, are not required for the inhibition of early endosome fusion, suggesting that activities ascribed to these domains are unrelated to endosome fusion and may be the basis for other functions of the Hrs protein.

An early endosomal SNARE complex has been purified using a syntaxin 13 affinity column that contains SNAP-25 and VAMP2 (Prekeris et al., 1998). Here, syntaxin 13 was localized to the tubulovesicular structures of early endosomes and shown to be present in a complex with β SNAP, VAMP 2/3, and SNAP-25 that binds α SNAP and NSF and dissociates in the presence of ATP, but not ATP γ S (Prekeris et al., 1998). Soluble fragments of syntaxin 13 or antibodies against syntaxin 13 inhibit endosome fusion, suggesting that its cognate SNARE complex is involved in early endosome fusion (Prekeris et al., 1998; McBride et al., 1999). Using a recombinant Hrs_(449–562) affinity column, we isolated SNAP-25, syntaxin 13, and small amounts of VAMP2. Hrs does not bind to either syntaxin 13 (unpublished observations) or VAMP2 (Tsujimoto and Bean, 2000), suggesting that Hrs_(449–562) binds to SNAP-25 in this complex. These data suggest that Hrs binds to this early endosomal SNARE complex and competes with VAMP2 for incorporation into this complex (Figs. 9 and 10), presumably due to a higher affinity for the SNAP-25–syntaxin 13 complex (Tsujimoto and Bean, 2000). Interestingly, the second coiled coil of Hrs has homology with the SNARE domain and contains Gln at the ionic 0 layer, suggesting that Hrs–SNAP-25–syntaxin 13 may form a four-helical bundle consisting of Q-SNAREs.

Although Hrs can completely inhibit the formation of the syntaxin 13–SNAP-25–VAMP2 complex, it does not completely inhibit early endosome fusion. Moreover, BoNT/E inhibits early endosome fusion to the same extent as Hrs. These data suggest that either there is a pool of endosomes whose fusion is independent of the Hrs-regulated, SNAP-25 containing, SNARE complex, or that preformed transcomplexes may be resistant to disassociation by Hrs or BoNT/E. VAMP8/endobrevin, another R-SNARE with a widespread distribution throughout the endocytic pathway, has been reported to function in the homotypic fusion of both early and late endosomes (Antonin et al., 2000). However, in the presence of VAMP8 antibodies, residual fusion of early endosomes is observed, although these antibodies completely prevented assembly of VAMP8 with Q-SNAREs in an in vitro assembly assay. Additionally, neurons do not express VAMP8 (Advani et al., 1998), although early endosomes are

involved in endosomal recycling in neurons, further suggesting the possible involvement of multiple R-SNAREs in early endosomal fusion.

SNAP-25 is considered a Q-SNARE, predominantly expressed on the plasma membrane, that is involved in exocytosis (Fasshauer et al., 1998; Sutton et al., 1998; Jahn and Sudhof, 1999; Lin and Scheller, 2000; Chen and Scheller, 2001). An endosomal localization of SNAP-25 (Tao-Cheng et al., 2000) and SNAP-23 (Chen and Whiteheart, 1999) implicate SNAP-25/23 to function in endosome fusion. Our results are the first to show that SNAP-25 is indeed required for early endosome fusion. The BoNT/E protease cleaves the COOH-terminal 26 aa from SNAP-25 rendering it unable to form a four-helical bundle and, therefore, unable to support synaptic vesicle fusion and neurotransmitter release (Banerjee et al., 1996; Chen et al., 1999; Jahn and Sudhof, 1999; Lin and Scheller, 2000). Treatment of early endosomal membranes with BoNT/E inhibited their ability to fuse. Importantly, addition of the COOH-terminal coiled-coil domain of SNAP-25 reverses this inhibition. These data provide clear biochemical evidence for the involvement of SNAP-25 in SNARE complex-driven early endosome fusion.

The role of Hrs in endosomal fusion is not inconsistent with a hypothesis suggesting that Hrs/Vps27p functions in endocytic protein sorting (Katzmann et al., 2001; Bilodeau et al., 2002; Lloyd et al., 2002). These studies have suggested that Vps27p is linked with proteins required for the ubiquitination and sorting of cargo (Katzmann et al., 2001; Bilodeau et al., 2002). These data suggest that Vps27p is localized to endosomal membranes by virtue of its FYVE domain binding to PI(3)P. Upon binding to endosomes, Vps27p may bind ubiquitin with its ubiquitin interacting motif (UIM) domain (Bilodeau et al., 2002; Polo et al., 2002; Shih et al., 2002). The UIM domain of Hrs is required for the cargo sorting function as mutation of that domain in Vps27p or Hrs blocks sorting of ubiquitinated cargo proteins, whereas other endosomal functions remain intact (Bilodeau et al., 2002; Shih et al., 2002). The endosomal sorting function has also been hypothesized to require a protein complex called ESCRT I (Katzmann et al., 2001). Hrs has been suggested to recruit the ESCRT 1 complex to early endosomes. Thus, Hrs-deficient endosomes probably fail to form intraluminal vesicles (Lloyd et al., 2002) because of the inability of Hrs-deficient endosomes to recruit ESCRT-I. The role of Hrs in recruiting sorting or signaling components to the endosomal membrane likely is a function of a number of factors including its phosphorylation and/or competition among binding proteins. Therefore, Hrs may bind to SNAP-25 using its Q-SNARE domain and inhibit endosomal fusion (Fig. 10) while it is involved in cargo sorting or endosome motility using NH₂-terminal VHS, FYVE, or UIM domains or via other protein interactions.

Several in vitro systems measuring endosome fusion have demonstrated that the majority of early endosomes are capable of fusion (Braell, 1987; Diaz et al., 1988; Gruenberg et al., 1989; Ward et al., 1990). However, the capability of early endosomes to fuse in vivo is restricted, suggesting that there are constraints on endosome association in vivo (Salzman and Maxfield, 1988; Ward et al., 1990). Why would it

be advantageous to inhibit fusion in situ? Perhaps after sorting cargo into different endosomes permitting them to fuse would allow the cargo to remix, producing a futile cycle. This would suggest that a sorting step might occur before, or coincident with, the inhibition of fusion. Moreover, if endosomes were tethered to cytoskeletal elements, the physical separation and vectorial restriction would provide a barrier to their interaction. In this regard, an interaction between Hrs and actinin-4 has been suggested to tether transferrin-containing early endosomes with actin filaments (unpublished data). This suggests that Hrs may provide a link between early endosomes and the actin cytoskeleton, as well as to directly inhibit their fusion (this paper). This dual function may be advantageous for endosomes that have sorted their cargo en route to another destination.

Materials and methods

Materials

Hrs was expressed in insect cells as described previously (Tsujimoto et al., 1999). Syntaxin 13 (a gift of R. Prekeris, University of Colorado Health Center, Denver, CO) and Syntaxin 7 (a gift of J. Pevsner, Johns Hopkins University, Baltimore, MD) and Hrs_(449–562) were expressed in *Escherichia coli* as described previously (Prekeris et al., 1998; Tsujimoto and Bean, 2000; Ward et al., 2000). Cell lysate from cells expressing rab 15 wt or Q67L were gifts of L. Elferink (University of Texas Medical Branch, Galveston, TX). The light chain of BoNT/E was expressed in *E. coli* (a gift of H. Niemann, Federal Research Center for Vital Diseases, Tübingen, Germany). Antibodies were obtained from the following sources: EEA1 and Eps15 (Santa Cruz Biotechnology, Inc.), SV2 (a gift of R. Janz, The University of Texas Health Science Center, Houston, TX), syntaxin 13 (a gift of R. Prekeris), SNAP-25 (Bean et al., 1997), synaptophysin (Boehringer), VAMP2, rab 7, and synaptotagmin (Transduction Labs), and syntaxin 6 (StressGen Biotechnologies).

Time course of EGF uptake

HeLa cells were cultured in Dulbecco's minimum essential medium (DMEM) containing 5% FBS on coverslips and starved for 1 h in DMEM containing 1% BSA. Cells were treated with 0.4 μ g/ml EGF-TMR for various times. At each time point, cells were washed with ice-cold PBS, acid washed (0.2 M glycine and 0.15 M NaCl) and again with ice-cold PBS, fixed with 4% PFA (10 min), and washed three times with ice-cold PBS. Cells were labeled with antibodies to EEA1 (early endosomes), rab 7 (late endosomes), and LAMP 1/2 (lysosomes).

To determine the length of uptake time necessary to label early endosomes, we incubated cells with EGF-TMR for 5, 10, 15, 20, and 30 min, fixed the cells, and labeled with anti-EEA1 antibody (1:100). We determined that 15 min was optimal for early endosome labeling (Results) and used this as the pulse time for labeling late endosomes and lysosomes. Thus, a 15-min pulse of EGF-TMR was applied and chased with DMEM containing 1% BSA for various times (0, 5, 10, 15, 20, 25, 30, 35, or 45 min) before fixation with 4% PFA.

We determined the optimal labeling time for early endosomes, late endosomes, and lysosomes by comparing the EGF-TMR labeling to that of compartment markers. Fixed cells were incubated with primary antibodies overnight at 4°C, and then with secondary antibodies conjugated to Alexa₄₈₈ for 30 min at 37°C. After three washes with PBS, coverslips were mounted with antifade medium (1 mg/ml paraphenylenediamine in 50% glycerol/PBS, pH 8.0). Images of internalized EGF-TMR and markers of early, late endosomes, and lysosomes were obtained using an Axiovert microscope (Carl Zeiss MicroImaging, Inc.) with a CCD camera (model ORCA; Hamamatsu) and optimized with MetaMorph software (Universal Imaging Corp.). Data were analyzed by first selecting threshold values for red and green signals, ensuring that colocalization of the like fluorophores was observed after thresholding. Subsequently, positive (nonbackground) pixels from red and green images were compared allowing determination of the overlap between green and red pixels. Data are presented as arbitrary units of colocalization that correspond to the percent overlap of red and green pixels at different time points after a 15-min pulse of TMR-labeled EGF. (Fig. 1) The data presented are representative of 10 experiments.

Cell-free fusion assay

HeLa cells were grown on 60-mm plates in DMEM containing 5% FBS and starved for 1 h (DMEM containing 1% BSA) before incubation (15 min at 37°C) with either EGF-488 or EGF-TMR. After washing and chasing for 0, 10, or 30 min, cells were harvested by scraping and centrifuged at 800 g for 5 min. Cells were resuspended in homogenization buffer (150 μ l containing 20 mM Hepes 7.4, 0.25 M sucrose, 2 mM EGTA, 2 mM EDTA, and 0.1 mM DTT) and homogenized by passage of the cell suspension 30 times through a 30-gauge needle. Membranes were collected by centrifugation (15,000 g for 10 min), and the resulting supernatant was further subjected to centrifugation at 100,000 g for 10 min to yield a crude endosomal fraction. The pellet was resuspended in homogenization buffer (150 μ l).

In the complete fusion reaction, donor membranes isolated from cells incubated with EGF-488 and acceptor membranes isolated from cells incubated with EGF-TMR were mixed on ice. The reactions (total volume 66 μ l) also contained rat brain cytosol (15 μ l, 6.9 mg/ml protein stock), an ATP regenerating system (2 mM MgATP, 50 μ g/ml creatine kinase, 8 mM phosphocreatine, and 1 mM DTT, final concentrations), and PEG 3350 (3.8%). The complete homotypic fusion reactions were incubated at 37°C for various times (0, 5, 10, 15, 30, 45, 60, 75, 90, and 120 min) to determine the optimal fusion time ($n = 6$). Other control conditions included reactions in the absence of donor or acceptor membranes, the ATP regenerating system, cytosol, or incubation on ice instead of 37°C ($n = 21$). We observed that PEG 3350 is neither required for endosome fusion to occur nor necessary for Hrs inhibition of early endosome fusion. The effect of detergent on the fusion reaction was determined by incubating the fusion reactions in reaction buffer containing 1% Triton X-100 for 10 min after the reaction was completed. To examine the dilution sensitivity of the fusion reactions, all reactions were performed with constant amounts of donor and acceptor membranes, constant concentrations of ATP (plus regeneration components), and cytosol while the total reaction volume was increased one-, two-, four-, and eightfold ($n = 9$). All reactions were stopped on ice by the addition of 550 μ l ice-cold PBS. The fluorescence in the entire reaction was quantified using a fluorimeter (PTI, Inc.). The excitation slit (2 nm) and the emission slits (4 nm) remained constant after optimization for best signal/noise. Data were acquired using the Felix software program (PTI, Inc.) and a single quartz cuvette by exciting samples at 495 nm and collecting emissions at 519 and 580 nm. We observed that the emission of Alexa₄₈₈ at 580 nm contributes <5% to the signal observed. Each sample was measured by sampling fluorescence at one data point per second for 10 s. Data are presented as a percentage of 580-nm signal obtained for a complete homotypic fusion reaction done in parallel for every experiment.

Expression and purification of fusion proteins

The His-tagged full-length Hrs, Hrs_(1–449) and His-tagged BoNT/E light chain fusion proteins, and all other GST fusion proteins including syntaxin 13, syntaxin 7, VAMP2, SNAP-25, and Hrs_(449–562) were prepared as described previously (Tsujimoto and Bean, 2000). We previously named our rat clone of Hrs, Hrs-2, because it was longer than the original mouse Hrs clone (Komada and Kitamura, 1995; Bean et al., 1997). We have reexamined our clones and we find a sequencing error in the original paper adding ~150 aa to the COOH-terminal end of the protein. Thus, the name Hrs-2 is a misnomer and, to our knowledge, there are no other isoforms of the Hrs protein. Importantly, this does not alter any of our conclusions or data from any published work as we have been using the same rat clones all along. His-tagged proteins were eluted in a batch format using 500 mM imidazole in PBST (PBS and 0.05% Tween 20). GST fusion proteins were cleaved from the GST moiety using thrombin (7.5 U/ml; Amersham Biosciences) in a buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 2.5 mM CaCl₂, and 0.1% β -mercaptoethanol. The cleavage reaction was stopped after end-over-end incubation at room temperature for 1 h (syntaxin 7), 2 h (VAMP2), or 4 h (SNAP-25) by the addition of 0.1 mM PMSF. His-tagged proteins were concentrated in concentrators (model YM30; Amicon) and resuspended in reaction buffer. Protein concentrations were estimated by comparing Coomassie blue staining of bands on SDS-PAGE gels against a BSA standard.

Effect of Hrs and other proteins on endosomal fusion

To examine the effect of Hrs on endosomal fusion, varying concentrations of recombinant Hrs (from 0 to 545 nM) were added to complete early endosome, late endosome, and lysosomal fusion reactions ($n = 12$). All reactions were incubated on ice for 15 min and further incubated at 37°C for 60 min. To examine at what stage of the fusion reaction Hrs was able to inhibit fusion, a constant concentration of Hrs (180 nM) was added (15 min

on ice) to complete fusion reactions at various time points (0, 5, 10, 15, 30, and 60 min) after the reactions had begun, and then transferred to 37°C for a total incubation time of 60 min ($n = 6$). Proteins that have been previously examined using other fusion assays were examined in our assay by incubating the recombinant soluble fragments of syntaxin 13 ($n = 3$), syntaxin 7 ($n = 9$), or cell lysates from cells overexpressing wild-type rab 15 or mutants (Q67L or K46L; $n = 3$) on homotypic fusion reactions with the complete fusion reactions on ice for 15 min and at 37°C for 60 min.

To identify the domain of Hrs responsible for the inhibition by Hrs on early endosome fusion, various concentrations of recombinant proteins full-length Hrs, Hrs_(449–562), Hrs_(515–562) (0–545 nM), and Hrs_(1–449) (0–180 nM) were added to complete early endosome fusion reactions on ice for 15 min and at 37°C for 60 min. The experiments presented are representative of 12 such determinations (Fig. 5).

Electron microscopy

Donor compartments or complete fusion reactions were fixed with 3% glutaraldehyde and washed three times in cacodylate buffer. Pellets were embedded in epon and sections were cut and viewed on a microscope (model 1010; Jeol). Images were captured directly with a camera (model Orca; Hamamatsu).

BoNT/E treatment

After isolating the early endosomal vesicles as described above, pellets were resuspended with 40 μ l of varying concentrations of BoNT/E (from 0 to 400 nM) and incubated at 37°C for 30 min. Membranes were collected by centrifugation (100,000 g for 10 min) and resuspended in 100 μ l of homogenization buffer for the fusion reactions. After BoNT/E treatment, the membranes were washed once in reaction buffer and resuspended in 50 μ l of reaction buffer containing either no SNAP-25_(150–206) or various concentrations of SNAP-25_(150–206) and incubated for 30 min at 37°C. Membranes were isolated again by centrifugation and pellets were resuspended in reaction buffer before assembling fusion reactions as described above. The experiments presented are representative of nine such experiments (Fig. 8).

Binding of Hrs to membranes

Endosomal membranes were purified from HeLa cells by centrifugation on a discontinuous sucrose gradient. In brief, one 10-cm plate (~80% confluent) was scraped in homogenization buffer (20 mM Hepes, pH 7.4, 0.25 M sucrose, 2 mM EGTA, 2 mM EDTA, 0.1 mM DTT, 0.4 ml total vol) and passed through a 30-gauge needle 30 times. The resulting lysate was centrifuged (100,000 g 10 min) and resuspended in 0.17 ml of homogenization buffer that was mixed with 61% sucrose to a final concentration of 46% sucrose (0.5 ml total). The 46% sucrose cushion was overlaid with two additional layers of sucrose 35% (0.65 ml) and 30% (0.45 ml) and with 0.4 ml of homogenization buffer. The gradients were subjected to centrifugation in a rotor (model TLS55; Beckman Coulter; 124,000 g for 60 min) and the early endosomes (interface between 30 and 35% sucrose, EEA-1 positive, and rab 7 negative by Western analysis) and late endosomes (upper part of 30%) were isolated. His-tagged Hrs or Myc-tagged Hrs_(449–562) were added to reactions containing purified endosomal membranes in the presence and absence of SNAP-25_(150–206) and incubated at 37°C for 60 min. Reactions were stopped by centrifugation (100,000 g for 10 min), and the amount of His-Hrs or myc-Hrs_(449–562) was determined in pellet and supernatant fractions by quantitative Western blotting using anti-His or anti-myc antibodies. The experiment shown is representative of 10 such experiments (Fig. 6).

Affinity chromatography with Hrs_(449–562)

20 rat brains (~18 g) per column were homogenized with eight strokes of a homogenizer (Dounce) in buffer A (20 mM Tris-HCl, pH 8.0, 0.1 M KCl, 250 mM sucrose, 2 mM MgCl₂, 1 mM DTT, and 1 mM PMSF; Sollner et al., 1993). The pellet resulting from centrifugation (SW28 rotor; Beckman Coulter; 60 min at 27,900 rpm) was washed once in buffer B (10 mM Hepes-KOH, pH 7.8, 100 mM KCl, 2 mM MgCl₂, and 1 mM DTT) and resuspended in 40 ml of buffer B with the addition of 4% (vol/vol) Triton X-100, and the suspension was incubated on ice with frequent mixing for 60 min. The lysate was centrifuged again (SW28 rotor 60 min at 27,900 rpm) and the supernatant was dialyzed overnight in buffer C (25 mM Tris-HCl, pH 7.8, 50 mM KCl, 1 mM DTT, 1% Triton). After dialysis, the lysate was centrifuged (SW28 rotor 60 min at 27,900 rpm) and the resulting supernatant was incubated with immobilized GST-Hrs_(449–562) or immobilized GST (control) in buffer D (20 mM Hepes-KCl, pH 7.0, 0.5% Triton X-100, 100 mM KCl, 1 mM DTT, and 2 mM EDTA) for 60 min at 4°C. After washing with 20 volumes of buffer D, proteins were eluted with three volumes of 1.5 M NaCl in buffer D. Eluted proteins were TCA precipitated, separated by

SDS-PAGE, and subjected to Western blotting using various antibodies. The experiment presented is representative of 18 such experiments (Fig. 7).

Western blotting

Proteins were resolved on SDS-polyacrylamide gels (12–17% acrylamide) and transferred to nitrocellulose. Blots were stained with Ponceau S to ensure accuracy of protein loading, blocked in blotto (5% dry milk in PBS), and incubated with primary antibody diluted in blotto. The following antibodies were used for detection of transferred proteins: 6 \times histidine (1:1,000; Sigma-Aldrich), anti-EEA1 (1:1,000; Transduction Laboratories), anti-SNAP-25 (1:1,000; Sternberger Antibodies), anti-syntaxin 13 (1:1,000), SV2 (1:1,000), and anti-VAMP2 (1:1,000, CHEMICON International, Inc.). Filters were washed, and antibody labeling was visualized using HRP-conjugated secondary antibody and chemiluminescence (Pierce Chemical Co.), or ¹²⁵I-conjugated secondary antibody and phosphorimaging (Molecular Dynamics).

In vitro protein binding

A constant amount of immobilized GST-syntaxin 13 (2 μ g/reaction) was incubated with constant amount of SNAP-25 (2 μ g), VAMP2 (2 μ g), and varying amounts of Hrs (from 0 to 6 μ g) in PBS binding buffer to a final reaction volume of 30 μ l. After an end-over-end incubation at 4°C for 1 h, samples were washed three times with 150 μ l of binding buffer, solubilized in SDS sample buffer, resolved by SDS-PAGE, and subjected to immunoblot analysis using anti-Hrs and anti-VAMP2 antibodies followed by appropriate ¹²⁵I-secondary antibodies. Immunoreactive bands were visualized and quantitated using phosphorimaging. The experiment shown is representative of seven such experiments (Fig. 9).

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