Ca²⁺ and N-Ethylmaleimide-sensitive Factor Differentially Regulate Disassembly of SNARE Complexes on Early Endosomes*

Qing Yan‡, Wei Sun‡, James A. McNew§, Thomas A. Vida¶, and Andrew J. Bean‡||

The endosome-associated protein Hrs inhibits the homotypic fusion of early endosomes. A helical region of Hrs containing a Q-SNARE motif mediates this effect as well as its endosomal membrane association via SNAP-25, an endosomal receptor for Hrs. Hrs inhibits formation of an early endosomal SNARE complex by displacing VAMP-2 from the complex, suggesting a mechanism by which Hrs inhibits early endosome fusion. We examined the regulation of endosomal SNARE complexes to probe how Hrs may function as a negative regulator. We show that although NSF dissociates the VAMP-2-SNAP-25-syntaxin 13 complex, it has no effect on the Hrs-containing complex. Whereas Ca²⁺ dissociates the Hrs-containing complex but not the VAMP-2-containing SNARE complex. This is the first demonstration of differential regulation of R/Q-SNARE and all Q-SNARE-containing SNARE complexes. Ca²⁺ also reverses the Hrs-induced inhibition of early endosome fusion in a tetanus toxin-sensitive manner and removes Hrs from early endosomal membranes. Moreover, Hrs inhibition of endosome fusion and its endosomal localization are sensitive to baflomycin, implying a role for luminal Ca²⁺. Thus, Hrs may bind a SNARE protein on early endosomal membranes negatively regulating trans-SNARE pairing and endosomal fusion. The release of Ca²⁺ from the endosome lumen dissociates Hrs, allowing a VAMP-2-containing complex to form enabling fusion.

Endocytosis is a fundamental process essential for all eukaryotic cells. It functions in nutrient uptake, regulation of the protein and lipid composition in the plasma membrane, and modulation of cellular responses by affecting exocytosis and receptor signaling. Molecules transit through the endocytic pathway by passing from one compartment to another through a series of membrane fission and fusion reactions. The ultimate role of this pathway is to allow the sorting of molecules to be recycled from those to be degraded. To function correctly, this system must regulate both the sorting events and the fusion events that promote proper targeting of internalized small molecules, lipids, and proteins.

Protein machinery is required for fusion of biological membranes. Interactions among SNARE proteins (1–4) associated with donor membranes (e.g. VAMP/synaptobrevin) and acceptor membranes (e.g. syntaxin and SNAP-25) are thought to be essential for fusion (1–5). SNAREs are sufficient for membrane fusion in artificial membranes, suggesting that they form the core membrane fusion machinery (6). SNAREs form cytoplasmic helical bundles that bridge two membranes (trans-SNARE complex) to enable membrane fusion (1, 4, 7). SNAREs are characterized by a helical “SNARE” motif that contains a glutamine or arginine at its center (4, 8). Botulinum and tetanus toxins are zinc endoproteases that cleave the SNAREs, inhibit the formation of SNARE complexes, and block fusion (1, 2, 9). Once membrane fusion has occurred, the cytoplasmic adapter protein NSF binds to the SNARE complex and recruits N-ethylmaleimide-sensitive factor (NSF) from the cytoplasm (1, 2, 7). The ATPase activity of NSF dissociates the cis-SNARE complex, allowing the proteins to be available for trans-pairing and subsequent fusion events (1, 2, 7).

The trans-SNARE complex may be the catalyst for membrane fusion, although regulatory events or molecules may influence this process. For example, intraorganellar Ca²⁺ release from the yeast vacuole, mammalian endosome, or nuclear vesicles is required for fusion events involving those compartments (10–13). A model that has emerged from these studies is that an unknown event triggers Ca²⁺ release from the organelle. This local pool of Ca²⁺ is required for the fusion event, although the nature of its effector is unclear. Evidence from studies of homotypic vacuole fusion suggest that calmodulin may be a Ca²⁺ target because it binds to vacuoles upon Ca²⁺ release and appears to promote bilayer mixing of vacuoles and endosomes (11–13), although the effect of calmodulin may be via regulation of SNARE complexes (14, 15).

The endosome-associated protein Hrs (hepatocyte-growth factor-regulated tyrosine kinase substrate) has been shown to bind the Q-SNARE SNAP-25 (16) and inhibits the homotypic fusion of early endosomes (17). A heptad repeat region of Hrs containing a Q-SNARE motif mediates this effect as well as its endosomal membrane association (17). SNAP-25 is an endosomal receptor for Hrs, and Hrs inhibits the formation of an early endosomal SNARE complex by disallowing VAMP incorporation into the complex (17). Hrs is also likely involved in cargo sorting at the level of the early/late endosome by recruiting sorting or signaling components to the endosomal membrane. Therefore, Hrs may bind to SNAP-25 using its Q-SNARE domain and inhibit endosomal fusion while it is involved in cargo sorting or endosome motility using NH₂-terminal VHS (Vps27, sensitive factor attachment protein receptors; SNAP-25, synaptosomal associated protein of 25 kDa; VAMP, vesicle-associated membrane protein; NSF, N-ethylmaleimide-sensitive factor; GST, glutathione S-transferase; ERA1, early endosomal antigen-1; TeTX, tetanus toxin; ATPγS, adenosine 5′-(thiotriphosphate); ER, endoplasmic reticulum.

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‡ The abbreviations used are: SNARE, soluble N-ethylmaleimide-

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Hrs, STAM, a domain found in a number of proteins involved in trafficking that, in some cases, binds to GGA proteins; FYVE (Fab 1, YotB, Vac1, EE1), a dual zinc finger domain found in a number of proteins involved in trafficking some of which bind to phosphatidylinositol 3-phosphate, phosphatidylinositol 3-phosphate; or ubiquitin-interacting motif, a domain found in a number of proteins that binds ubiquitin with low affinity dominance. Ref. 17 and references therein) or via other protein interactions. Interestingly, the binding of Hrs to SNAP-25 is negatively regulated by Ca$^{2+}$ such that Ca$^{2+}$ inhibits the bind-
of a VAMP-2/SNAP-25-syntaxin 13 SNARE complex and that in the presence of Ca\(^{2+}\) VAMP-2 is able to displace Hrs for SNAP-25-syntaxin 13 binding.

Ca\(^{2+}\) Relieves the Hrs-dependent Inhibition of Early Endosome Fusion—The ability of Ca\(^{2+}\) to displace Hrs from the SNARE complex and allow complex assembly with VAMP-2 suggests that calcium might reverse the inhibition of early endosome fusion produced by Hrs. We previously developed an assay for endosome fusion in which different populations of HeLa cells engage in receptor-mediated endocytosis of epidermal growth factor linked to either Alexa 488 or tetramethylrhodamine, allowing isolation of donor and acceptor pools of endosomes. 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 (17). Fusion reactions containing 180 nM Hrs were inhibited 70.2 ± 1.4% compared with control reactions (Fig. 2). However, when Ca\(^{2+}\) was added to these reactions in the range of 3 mM to 30 mM, fusion became more efficient in a concentration-dependent fashion reaching a maximum of 84.0 ± 5.4% of control reactions (Fig. 2). This effect had a half-maximal value of −90 μM Ca\(^{2+}\) and was specific to calcium because other divalent cations such as Cu\(^{2+}\), Ba\(^{2+}\), and Mn\(^{2+}\) (all at 1 mM) were unable to relieve the inhibition (Fig. 2). Although not shown, Cu\(^{2+}\), Ba\(^{2+}\), and Mn\(^{2+}\) did not prevent Hrs from binding in SNARE complexes formed in vitro as performed earlier (Figs. 1 and 2). The concentration of calcium required and the cation specificity necessary for relieving the Hrs-dependent inhibition of early endosome fusion correlated well with the block in formation of the Hrs/SNAP-25-syntaxin 13 SNARE complex. This provided evidence to suggest that calcium might function to dissociate a “nonfusogenic” SNARE complex (containing Hrs) and allow a “fusogenic” SNARE complex (containing VAMP-2) to assemble on early endosomes.

Calcium Blocks Hrs from Binding to Early Endosomes—To further examine the notion that calcium might block binding of Hrs to SNARE complexes on membranes, we examined the effect of divalent cations on the binding of Hrs to purified early endosomes. When Hrs was added (180 nM) to early endosomes (purified as described under “Experimental Procedures”) in the presence of increasing Ca\(^{2+}\), a concentration-dependent decrease of Hrs binding was observed (Fig. 3a, lanes 1–7). The half-maximal value for calcium in this inhibition was −30 μM. As seen previously (Fig. 2), Cu\(^{2+}\), Ba\(^{2+}\), and Mn\(^{2+}\) had no effect (Fig. 3a, lanes 8–10). Importantly, Ca\(^{2+}\) did not affect the binding of an early endosomal protein, EEA1, suggesting specificity in its effect on Hrs (Fig. 3a, lanes 1–7). The inhibition of Hrs binding to early endosomes and the SNAP-25-syntaxin 13 SNARE complex suggested that Ca\(^{2+}\) might regulate how Hrs interacts with a receptor on membranes such as SNAP-25 (17). SNAP-25 is considered to be a strictly neuroendocrine or neuronal protein (although see Ref. 25). Our use of HeLa cells for not only purified early endosomes but also homotypic endosome fusion assays and the dependence of these assays on SNAP-25 as suggested by their sensitivity to botulinum toxin E suggests that SNAP-25 resides in this non-neuronal cell. To demonstrate that this is indeed the case, we attempted to detect SNAP-25 in HeLa cell lysates and purified early endosomes. As expected, markers for the ER (calnexin), plasma membrane (Na/K ATPase), lysosomes (LAMP1), and early endosomes (EEA1) were detectable in crude lysates from HeLa cells (Fig. 4, lane 1). The HeLa cell lysate also contained SNAP-25 (Fig. 4, lane 1). Most importantly, purified membranes from HeLa cells containing the early endosomal marker, EEA1, also contained SNAP-25 (Fig. 4, lane 2). These membranes did not contain detectable amounts of the Na/K ATPase, calnexin, or LAMP1, suggesting the lack of plasma membrane, ER, and lysosomes, respectively (Fig. 4, lane 2). This demonstrated that the membranes were not contaminated with other organelles and allowed us to conclude that SNAP-25 was indeed located on early endosomes from HeLa cells.

VAMP-2 Is Required for Calcium to Efficiently Relieve Hrs Inhibition of Early Endosome Fusion—Certain bacterial toxins are valuable tools for dissecting the molecular details of SNARE-dependent membrane fusion. These toxins are proteases with highly specific cleavage recognition sites (9, 24). For example, botulinum toxin E (BoNT/E) is a zinc endoprotease.
tease that cleaves the COOH-terminal 26 amino acids of SNAP-25 and thereby blocks membrane fusion that requires a four helical SNARE complex containing SNAP-25 (9). Likewise, TeTx cleaves the COOH-terminal 41 amino acids of VAMP-2 (24) and blocks membrane fusion that requires a four helical SNARE complex containing VAMP-2 (1, 7, 9).

Pretreatment of early endosomes with TeTx inhibited membrane fusion in a dose-dependent manner (Fig. 5a). Treatment of early endosomal membranes resulted in 56.2 ± 9.7% maximal fusion efficiency (Fig. 5, a and b). The half-maximal value
of TeTx for this inhibition was ~80 nM, comparable with the concentrations required to inhibit neurotransmitter secretion. This result suggested that a TeTx substrate, presumably VAMP-2, played a role in early endosome fusion. The combination of TeTx and Hrs was no more efficacious than Hrs alone in inhibiting early endosome fusion (Figs. 5b or 3). When Ca\textsuperscript{2+} was added along with Hrs, the fusion efficiency increased to 85.6 ± 5.6% (Figs. 5b and 2). However, if membranes were first treated with TeTx and then incubated in the presence of Hrs and free Ca\textsuperscript{2+}, the fusion efficiency was just 57.4 ± 4.8% (Fig. 5b). This suggested that a TeTx substrate, presumably VAMP-2, was in part required for calcium to reverse the Hrs-dependent inhibition of early endosome fusion, accounting for a loss of 28.3% (85–57%; Fig. 5b).

**Release of Calcium from Luminal Stores Affects Hrs Function and Localization**—An experiment was performed to test whether release of Ca\textsuperscript{2+} from luminal stores could reverse Hrs-dependent inhibition of early endosome fusion. The steady-state level of Ca\textsuperscript{2+} in HeLa cells is estimated to be about 100 nM (26), which is below the concentration that affects Hrs-mediated block in early endosome fusion. During isolation, endosomes lose their Ca\textsuperscript{2+} in 5–10 min through a vacuolar-type ATPase (27). To overcome this, we loaded HeLa cells with extracellular calcium in the presence of bafilomycin, an inhibitor of vacuolar-type ATPases (28, 29). Bafilomycin was maintained at a constant level during the lysis and isolation of endosomal membranes used in the fusion assay, which would block release of luminal Ca\textsuperscript{2+}. In control cells, Hrs inhibited early endosome fusion by 74.5 ± 3.7% when it was present throughout the fusion reaction (Fig. 6a, left panel). In the continued presence of bafilomycin, fusion was inhibited by 79.3 ± 5.6% when Hrs was incubated with the endosomes and washed out prior to the beginning of fusion reactions (Fig. 6a, right panel). However, in the absence of bafilomycin, the effect of Hrs addition and removal prior to the fusion reactions was significantly decreased (74.5 ± 3.7% versus 20.7 ± 5.6% of control; Fig. 6a, right panel). This nearly 4-fold increase suggested that luminal Ca\textsuperscript{2+} loss though a bafilomycin-sensitive V-ATPase can decrease the Hrs-dependent inhibition of early endosome fusion. To examine whether Ca\textsuperscript{2+} release from luminal stores could dissociate Hrs from endosomal membranes, we performed a similar experiment and quantitatively examined the amount of Hrs present on the resulting endosomes. Similar amounts of Hrs were found on control endosomes and those in which bafilomycin was present throughout the fusion reaction (lanes 1 and 3). In the absence of bafilomycin the amount of Hrs on the endosomal membranes decreased to 15.4 ± 5.3% of control values. The results shown are representative of three experiments.

**DISCUSSION**

The SNARE hypothesis offers a molecular explanation for how membranes can overcome the energy barrier for fusion. Pairing of SNARE proteins on opposing membranes is required for, and may underlie the specificity of, the fusion reaction (1, 7). Regulation of SNARE complex formation/dissociation is crit-
ical for the control of whether and when membrane fusion occurs. However, the control of SNARE complex assembly/disassembly is poorly understood. The endosomal SNAREs, SNAP-25 and syntaxin 13, exist in at least two distinct heterotrimeric complexes; one is fusogenic and includes VAMP-2, and the other is nonfusogenic and includes Hrs (17, 20). The Hrs-containing complex appears to predominate in vitro and prevents formation of the VAMP-2-containing complex (17). This suggests a mechanism by which Hrs can inhibit endosome fusion. We have examined the regulation of the Hrs- and VAMP-2-containing complexes and find that although the VAMP-2-containing complex is dissociated by the action of the NSF ATPase, NSF does not dissociate the Hrs-containing complex. In contrast, Ca$^{2+}$ dissociates the Hrs-containing complex without affecting the VAMP-2-containing complex. The molar concentrations of Ca$^{2+}$ required for the dissociation of the Hrs-containing complex are similar to those that remove Hrs from endosomal membranes and that reverse the Hrs-induced inhibition of VAMP-2-dependent homotypic endosome fusion. This is the first demonstration of differential regulation of R/Q-SNARE and all Q-SNARE containing SNARE complexes. Furthermore, these data suggest a mechanism by which the regulation of Hrs- and VAMP-2-containing complexes allows for the regulation of homotypic endosome fusion. Thus, Hrs binding to SNAP-25 on early endosomal membranes negatively regulates trans-SNARE pairing, and once Ca$^{2+}$ dissociates Hrs, a VAMP-2-containing complex forms, allowing fusion to occur.

The ATPase activity of NSF has been shown to dissociate SNARE complex protein interactions (1, 3, 6, 7) and recently to dissociate other protein complexes (30–33), suggesting a conserved function as a dissociating factor. NSF binds indirectly to syntaxin family members via interactions with αSNAP and in the presence of Mg/ATP disrupts syntaxin/SNAP-25-VAMP complexes. The simplest explanation for the NSF resistance of the Hrs-containing complex is steric hindrance. Perhaps Hrs, being relatively larger than the SNAREs, blocks syntaxin from binding to SNAP and NSF. Steric hindrance would likely not block calcium from dissociating an Hrs-SNAP-25-syntaxin 13 complex. The effect of Ca$^{2+}$ is specific because other divalent cations such as barium, copper, and manganese have no effect on the Hrs-containing complex. The role of Ca$^{2+}$ in fusion of organelle membranes has been recognized for years, although there are many possible effector proteins to which Ca$^{2+}$ binds (1, 7, 11–13, 34), making understanding of the mechanism of Ca$^{2+}$ action unclear. Ca$^{2+}$ has been suggested to play a role in conformational/structural changes in proteins, actions that could lead to protein complex assembly or dissociation.

SNAP-25 has been recently shown to have a role in early endosome fusion (17) in accord with its presence on endosome membranes (37). However, SNAP-25 has been almost exclusively associated with a function in exocytosis. Moreover, SNAP-25 has been thought to be present solely in neuronal and neuroendocrine tissues and cell lines (although see Ref. 25). We have presented evidence that SNAP-25 is present in HeLa cell lysate and on purified early endosomes isolated from HeLa cells, a cervical tumor-derived cell line. The presence of SNAP-25 on endosomes in these cells is a further suggestion that SNAP-25 may be present in various cells from different lineages where it may be involved in membrane fusion events (25).

The mechanism of the membrane association of Hrs has been

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**Fig. 7.** A model of how calcium may regulate the formation of fusogenic and nonfusogenic SNARE complexes on early endosomes. **a**, the concentration of calcium near two early endosomes is below that amount required to release Hrs from a Q-SNARE complex with SNAP-25 and syntaxin 13. Fusion of the endosomes is blocked. **b**, an unknown trigger activates a calcium channel in an early endosome (one endosome shown for simplicity). The concentration of calcium rises locally to the point that causes Hrs to dissociate from the Q-SNARE complex, most likely because of a putative conformational change. **c**, once calcium removes Hrs, VAMP-2 (or other R-SNAREs) can then form a fusogenic SNARE complex with SNAP-25 and syntaxin 13, facilitating fusion of early endosomes. This model is based in part on a model describing how calcium might regulate vacuole fusion in yeast (11).
the subject of some debate (Ref. 17 and references therein). Although the FYVE domain may provide a link to the membrane through an interaction with phosphatidylinositol 3-phosphate (38), the Q-SNARE domain of Hrs interacts with SNAP-25 and SNAP-25 can act as a saturable binding site for Hrs on endosomal membranes (17). Because the FYVE domain may partially penetrate membranes and regulate residence time (38), it is possible that SNAP-25 is the protein receptor and that the FYVE-lipid interaction may be a regulatory influence for Hrs binding to endosomes. Ca\(^{2+}\) dissociates Hrs from early endosomal membranes at concentrations similar to that required for the dissociation of the Hrs-SNAP-25 proteins complex. How relevant are these Ca\(^{2+}\) concentrations for membrane fusion in the endocytic pathway? The luminal concentration of Ca\(^{2+}\) in endosomes has been estimated at 1 mM (35), 360 \(\mu M\) in the Golgi, 350 \(\mu M\) in the ER (26), and 2 mM in yeast vacuole (11). Resting Ca\(^{2+}\) in HeLa cells is ~100 mM creating a large driving force for Ca\(^{2+}\), and the concentration of Ca\(^{2+}\) at the endosome surface directly outside of the pore could likely reach 100 \(\mu M\) or greater (36). Ca\(^{2+}\) in the micromolar range can reverse the effect of chelators and support endosome fusion (12, 13). For homotypic vacuolar fusion, 100 \(\mu M\) Ca\(^{2+}\) may partially penetrate membranes and regulate residence time (38), it is possible that SNAP-25 is the protein receptor to which luminal Ca\(^{2+}\) is required to establish the normal luminal Ca\(^{2+}\) required for the reversal of chelators and support endosome fusion (12, 13). For homotypic vacuolar fusion, 100 \(\mu M\) Ca\(^{2+}\) may reverse the effect of chelators and support endosome fusion (12, 13). For homotypic vacuolar fusion, 100 \(\mu M\) Ca\(^{2+}\) may partially penetrate membranes and regulate residence time (38), it is possible that SNAP-25 is the protein receptor to which luminal Ca\(^{2+}\) is required to establish the normal luminal Ca\(^{2+}\) required for the reversal of chelators and support endosome fusion (12, 13).

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