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Inhibition of early endosome fusion by Rab5-binding defective Ras interference 1 mutants

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ABSTRACT

Rin1 has been shown to play an important role in endocytosis. In this study we demonstrated that depletion of Rin1 from the cytosol blocked the fusion reaction. More importantly, endosome fusion was rescued by the addition of Rin1 proteins depending on the presence of Rab5, and its effector EEA1. Furthermore, we found that Syntaxin 13, but not Syntaxin 7, was required by Rin1 to support endosome fusion. We also identified six mutations on the Vps9 domain of Rin1 that failed to rescue the fusion reaction. Two of them, Rin1: D537A and Rin1: Y561F mutants showed dramatic inhibitory effect on the fusion reaction, which correlate with their inability to properly activate Rab5 or to bind endosomal membranes. Taken together, our results suggest that specific residues on the Vsp9 domain of Rin1 are required for its interaction with Rab5, binding to the endosomal membranes and subsequent regulation of the fusion reaction.

Introduction

Endocytosis of the epidermal growth factor (EGF)-receptor is initiated by the binding of EGF at the cell surface. The EGF-receptor-ligand complex is then transported through the endocytic pathway, in which the EGF-receptor/EGF complex is either recycled to the cell surface or directed into lysosome for degradation [1–5].

EGF-receptor endocytosis is facilitated, in part, by members of the small GTP binding protein superfamily, including Ras, Rac/Rho and Rab5, and is also tightly regulated [6–11]. Upon ligand interaction, the activated receptor dimerizes and auto-phosphorylates in several tyrosine residues within the cytoplasmic tail. These phosphorylated residues mediate the recruitment of multiple downstream effectors, including Shc, Grb2/mSOS, PI3-kinase, PLC-γ [12–14], as well as cytoplasmic factors required for the activation of Rab5 proteins (i.e., Rabex-5 and Rin1[1]) [15,16]. Interestingly, these two factors are recruited onto the activated EGF-receptor tail via two different mechanisms. Rabex-5 required ubiquitination [15], while Rin1 required tyrosine phosphorylation of the EGF-receptor [16].

Rin1, a Rab5-guanine exchange factor (GEF), affected the internalization of EGF-receptor as well as the uptake of HRP, a marker for fluid phase endocytosis. Furthermore, the endosome fusion event was regulated by Rin1 in a concentration-dependent manner and more important, the fusion reaction mediated by Rin1 also required Rab5 [17], which is at least responsible for mediating membrane trafficking events early in the endocytic pathway [10,11].

Recent studies have suggested that EGF-stimulated endocytosis also required Rin1 and was facilitated by the constitutively active form of Ras, which in turn, potentiates Rin1's GEF activity for Rab5 [17]. Interestingly, expression of Rin1, but not the expression of a natural splice variant of Rin1 (Rin1Δ), which lacks 47 amino acids in the Vsp9 domain, induced the formation of enlarged Rab5-possitive endosomes in intact cells [17]. The addition of Rin1, but not the addition of Rin1:Δ, supported fusion between endosomes, which was further increased by the supplementation of the constitutively activated Ras: G12V mutant [17]. Consistent with these observations, it has proposed that the Vps9 domain of Rin1 plays a key role in endosomes fusion. However, the exact mechanism by which the Vps9 domain of Rin1 regulates endosome fusion is still poorly understood.

Several *in vitro* models of vesicle fusion have been used to define the behavior of endosomal compartments, and more importantly, these assays have led to an understanding of some factors that influence the fate of internalized ligands and receptors and

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[1] *Abbreviations used:* Rin1, Ras interference 1; GDI, GDP dissociation inhibitor; EEA1, early endosomal autoantigen 1; Vps9 domain, vacuolar Proteins Sorting 9; NEM, *N*-ethylmaleimide; REP-1, Rab Escort Protein-1; STAM, signal transduction adaptor molecule; HRP, Horseradish peroxidase; HBSS, Hank's balanced salt solution.

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what components are required for homo- and heterotypic fusion [18–27]. Using an *in vitro* assay that measures early endosome fusion indicates that the regulation of Rab5 function plays an essential role as a limiting factor in this process [10,28,29]. Consistent with these observations, the expression of Rab5 and/or Rab: Q79L mutant has also been shown to stimulate both EGF-receptor uptake and fluid phase endocytosis [30]. However, the expression of Rab5: S34N had an opposite effect (i.e., blocked the EGF-receptor and fluid phase endocytosis) [30]. Furthermore, other Rab5 related factors have also been required for fusion between endosomes [31–33]. In addition, Syntaxin 13 was found on early endosomes and its soluble fragment inhibited the fusion between early endosomes without affecting the fusion between lysosomes. Interestingly, a soluble fragment of Syntaxin 7 inhibited the fusion between lysosomes without affecting fusion between early endosomes [33,34]. Thus, endosome fusion is dependent on SNARE protein complexes allowing a series of intracellular membrane fusion reactions to maintain the integrity of selective compartments.

This paper describes a novel cell-free system, which utilized avidin coupled to β-galactosidase and a modified ligand of EGF with biotin to study the fusion of vesicles derived from Hela cells. We have used our newly developed endosome fusion assay on different populations of endosomes to conduct extensive mutational analysis of the structure-function relationship of the Vps9 domain of Rin1. We determined that the depletion of Rin1 inhibited the fusion reaction, and the rescue of the fusion reaction by exogenous Rin1: wild type required Rab5, EEA1, NSF and Syntaxin 13. We also observed that mutations on different conserved residues of the Vps9 domain of Rin1 altered the fusion reaction, interaction with Rab5 and association with the endosomal membrane, suggesting a possible molecular mechanism by which Rin1 regulates early endosome fusion.

Materials and methods

Cell culture and materials

Hela cells (American Type Culture Collection) were grown to confluence in Dulbecco's Modified Eagle's (DEM) medium supplemented with 5% fetal bovine serum. Iodine-125 [^{125}I] was purchased from Amersham. Kinase inhibitors were purchased from EMD Biosciences. EEA-1, Rab5, and Rab11 antibodies were from Cell Signaling Technology (Beverly, MA). Rin1 antibodies were from Novus Biologicals, and from BD Biosciences Pharmingen. Human Epidermal Growth Factor (EGF), Biotin-EGF (B-EGF), anti-EGF antibodies, Avidin β-galactodidsase (Av-Gal), and β-galactodidsase antibodies were purchased from Sigma–Aldrich. GAPDH and transferrin receptor antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). All secondary antibodies were purchased from Jackson Immuno Research Laboratories. All other chemicals were obtained from Sigma unless otherwise stated.

Endocytosis of probes and fractionation

Av-Gal and B-EGF were used as endocytic probes in our experiments. The binding and internalization of ^{125}I-B-EGF in Hela cells was measured in order to verify that the modified ligand would undergo receptor-mediated endocytosis. Both native and biotinylated EGF were iodinated by the Iodo-beads method (Pierce Chemical Co.) [30], using carrier-free ^{125}I (Amersham) according to the manufacturer's protocol. Radioactivity was measured using a Genesis γ-counter. Cells (1×10^8 cells/ml) were collected by centrifugation, washed three times in Hank's balanced salt solution (HBSS) with 15 mM HEPES and supplemented with 1 mg/ml BSA (HBSS–BSA). Endocytosis of probes was performed with suspended cells in HBSS-BSA buffer. Cells were incubated with appropriate concentrations of ligand in HBSS-BSA buffer for 120 min at 4 °C, washed with HBSS-BSA and then incubated for several time at 37 °C. After incubation, cells were washed, and the surface-bound and internalized ligands were discriminated as essentially described [35].

Briefly, for internalization studies, cells were washed with HBSS-BSA and incubated with either ^{125}I-EGF or ^{125}I-B-EGF as described above. Cells were incubated at 37 °C for the indicated time. After the incubation, the medium was collected, and then washed twice with ice-cold HBSS-BSA to remove unbound ligand. The cells were then treated 0.25 M acetic acid (pH:4.0) containing 0.5 M NaCl at 4 °C for 5 min, which remove more than 95% of the total EGF bound to the cell surface. The acid wash was combined with another wash with HBSS to determine the amount of surface-bound ^{125}I-EGF. Finally, the cells were lyzed in 0.5 N NaOH to evaluate internalized ligand. The kinetics of uptake for both ligands (EGF and B-EGF) was identical, with maximum internalization occurring within 15 min at 37 °C (Supplementary Fig. 1). The binding of B-EGF was saturated (Fig. 1, inset) and a nonspecific binding, measured in the presence of 300-fold unlabeled EGF, was less than 4% of the total counts/min in the pellet. By Scatchard analysis, we determined dissociation constants for B-EGF (Kd = 0.66 nM) and for unmodified EGF (Kd = 0.62 nM) in Hela cells and these values were comparable to values reported for unmodified EGF in the order of the nM [4,26,36,37].

For recycling and degradation studies, cells were washed with ice-cold HBSS-BSA, expose to either ^{125}I-EGF or ^{125}I-B-EGF at 4 °C for 120 min, washed three times with ice-cold HBSS-BSA, and incubated for 2 min at 37 °C. After incubation, cells were washed once with ice-cold HBSS-BSA, incubated in 0.25 M acetic acid (pH: 4.0) containing 0.5 M NaCl at 4 °C for 5 min, and washed twice with ice-cold HBSS-BSA. These cells, in which more than 95% of the radioactive EGF was intracellular, were further incubated in HBSS-BSA with excess of unlabeled EGF at 37 °C for a chase time, and the amount of degraded and intact radioactive EGF in the medium as well as cell surface and intracellular radiolabeled ligand was determined by precipitation with 10% trichloroacetic acid (TCA) (i.e., degraded ligand [TCA soluble] and nondegraded ligand [TCA insoluble]) (Supplementary Fig. 1).

The internalization of Av-Gal was carried out by incubating cells with different concentrations of ligand in HBSS-BSA for 15 min at 37 °C. After incubation, cells were washed three time with HBSS-BSA buffer, and the amount of Av-Gal activity was measured using 4-methyl umbelliferyl β-D-galactoside as described before [23]. The uptake of Av-Gal was not saturated upon increasing the amount of enzyme added to the cells (Fig. 1, inset).

For fractionation studies, cells (2×10^7/ml) were allowed to internalize either ^{125}I-Av-Gal or ^{125}I-B-EGF for 5 min at 37 °C. The cells were washed with HBSS-BSA at 800*g* following by two more washes, once with HBSS-BSA and once with homogenization (HB) buffer (0.25 M sucrose, 20 mM MES, 0.5 mM EGTA, pH 7.0). Cells were then resuspended in HB buffer (4×10^7/ml) containing phosphatase (10 mM sodium fluoride, 0.5 mM sodium orthovanadate, 2 mM sodium pyrophosphate, and 2 mM β-glycerophosphate) and protein inhibitors (5 mM AEBSF, 1.5 mM aprotinin, 0.1 mM leupeptin, and 2 mM phenylmethylsulfonyl fluoride), and passed 17 times through a stainless steel ball homogenizer. The homogenate was centrifuged at 800*g* for 10 min to generate postnuclear supernatants (PNSs). A vesicular fraction was obtained by centrifuging PNSs at 40,000*g* for 15 min, and resuspending the pellet in 1 ml of HB buffer containing phosphatase and protein inhibitors as described above. Vesicles were then applied to a 1.05 g/ml self-forming Percoll gradient (35 ml) and centrifuged at 39,000*g* for 60 min. Fractions (1.65 ml), collected from the bottom of the tube, were analyzed for the marker enzyme acid phosphatase and for radioactivity. Alternatively, early endosome fractions were