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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.

Keywords

Endosome Fusion; Rab5; Rin1; Receptor; Membrane Transport; Endocytosis

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) [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].

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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 (Rin1 Δ), which lacks 47 amino acids in the Vsp9 domain, induced the formation of enlarged Rab5-positive 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 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.

Material and Methods

Cell culture and Materials

Hela cells (American Type Culture Collection) were grown to confluence in Dulbecco's modified Eagle's (DMEM) medium supplemented with 5% fetal bovine serum. Iodine-125 [¹²⁵I] 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 ImmunoResearch 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 0.5N 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 (Figure 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 ($K_d = 0.66$ nM) and for unmodified EGF ($K_d = 0.62$ 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 (Figure 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 $800 \times g$ 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 beta-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 $800 \times g$ for 10 min to generate postnuclear supernatants (PNSs). A vesicular fraction was obtained by centrifuging PNSs at $40,000 \times g$ 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 $39,000 \times g$ 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 also isolated performing a flotation-sucrose gradient fractionation method as described early [21,38].

Fusion Assay

Hela cells were incubated with either 2 mg/ml Av-Gal or 200 ng/ml B-EGF as described above. After the incubation, cells were then allowed to internalize for 5 min at 37°C to prepare the endosome fractions. The cells were washed in HBSS-BSA buffer at $800 \times g$ followed by two more washes, once in HBSS-BSA buffer and once in HB buffer. Cells were resuspended in HB buffer (4×10^8 /ml) containing protease and phosphatase inhibitors as described above, and then passed 17 times through a stainless steel ball homogenizer. The homogenate was centrifuged at $900 \times g$ for 10 min to generate a PNS. Typical homogenizations yielded $\sim 91\%$ of endocytosed marker in the PNS as determined by either radioactivity or immunoprecipitation of ligands. Homogenization never released more than $30 \pm 3\%$ of either probe from endocytic vesicles. PNSs could then be used immediately for fusion assay, or stored frozen in liquid nitrogen for later use.

To prepare endosome fractions for the fusion assay, a thawed PNS was diluted with 3 ml of HB buffer and pelleted for 1 min at $35,500 \times g$. The supernatant was centrifuged for 5 min at $50,000 \times g$. The pellet of the second centrifugation was enriched in endosomes containing ligands internalized for 5 min [22,23]. These enriched endosome fractions (further referred to as “5-min endosomes”) contained ligands internalized for 5 min at 37°C . Then, two populations of 5-min enriched endosomes (Figure 2B) containing Av-Gal and B-EGF were mixed at 4°C to a final volume of 25 ml in fusion buffer (250 mM Sucrose, 0.5 mM EGTA, 1 mM DTT, 1.5 mM MgCl_2 , 50 $\mu\text{g}/\text{ml}$ Biotin-BSA, 50 mM KCl, and 20 mM HEPES-KOH, pH 7.0). This buffer was supplemented with a regenerating system (1 mM ATP, 8 mM creatine phosphate, 40 units/ml creatine phosphokinase, [+ATP]) or with a depleting system (5 mM mannose, 25 units/ml hexokinase, [-ATP]). After incubation at 37°C , the fusion reaction was stopped by cooling to 4°C (ice) for 5 min. The vesicles were solubilized by adding 150 μl of solubilization buffer (1% Triton X-100, 0.2% methylbenzethonium chloride, 1 mM EDTA, 0.1% BSA, 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4) containing 50 $\mu\text{g}/\text{ml}$ Biotin-BSA. We have added Biotin-BSA to block any complex formation in the extravesicular medium.

To measure the formation of the immunocomplexes, the solubilized samples were then added to “wells plate” coated with anti-EGF antibodies. After a 90 min of incubation at 4°C , immunoprecipitates were washed twice with 200 μl of solubilization buffer and the β -galactosidase activity was measured by using 4-methyl-umbelliferyl β -D-galactoside substrate [23]. There was a linear relationship between the amount of immunoprecipitate added to the enzymatic assay and the amount of product formed. Fusion-dependent immunoprecipitable

enzymatic activity was expressed as a percent of the total immunoprecipitable activity in the assay. Total activity was determined by immunoprecipitating the immune complexes after mixing vesicles in the presence of detergent but in the absence of biotin-BSA. Typically, in our endosome-endosome fusion reaction the ATP-dependent fusion activity represents 27.8 ± 1.2 % of the total amount of B-EGF present in the intact endosomes.

Preparation of cytosol

To prepare cytosol, HeLa cells (10^9 cells/ml) were resuspended in HB buffer containing phosphatase and protein inhibitors, and then homogenized as described above. PNS was prepared by centrifuging at $800 \times g$. Membranes were then removed by centrifugation at $35,000 \times g$ for 15 min, followed by centrifugation at $200,000 \times g$ for 60 min. Supernatants were quickly frozen in liquid nitrogen, aliquoted in 100 μ l and stored for later use.

Immunodepletion of Cytosolic Proteins

All immunoprecipitations were performed on cytosol prepared from cells in ice-cold HB buffer containing several phosphatase and protein inhibitors as described above. After clarification, protein concentrations were measured and samples were immunoprecipitated by incubation with the appropriate antibodies followed by immobilization on Protein G-Sepharose beads (Amersham Pharmacia Biotech). The beads were then washed three times with HB buffer and the bound proteins were resuspended in sample buffer and analyzed by SDS-PAGE and Western blot with the indicated antibodies.

Plasmid constructs and protein purification

Total cellular RNA was prepared from mouse tissues using TRIZOL reagents (Invitrogen). One mg of total RNA was used in a reverse transcriptase (RT)-PCR reaction to prepare Syntaxin 13 and Syntaxin 7 constructs. Then, the Syntaxin 13 (residues 1-232) and Syntaxin 7 (residues 1-236) constructs were cloned into the pGEX vector. Rin1 mutants (i.e., Rin1: Y523F, Rin1: D537A, Rin1: P541A, Rin1: Y561F, Rin1: T572A, Rin1: E574A, Rin1: Y577F, Rin1: Y578F, Rin1: T580A) were made by site-directed mutagenesis using a Quick Change kit (Stratagene). Using pMX retrovirus system, His-tagged Rin1 constructs were expressed in NR6 cells as described early [17]. His-tagged Rin1 proteins were then purified from 500 ml cell lysates on 1 ml of Hi-trap Ni-Agarose columns (Pharmacia) by Ni-affinity chromatography according to the instruction of the manufacturer. Eluates were analyzed by SDS-PAGE and fractions containing Rin1 were pooled and dialyzed. Rin1 proteins could then be used fresh for fusion or stored frozen in liquid nitrogen for later use. Recombinant His-tagged Rab5 proteins, and His-tagged REP-1 were expressed in Sf9 cells using the baculovirus system. The purifications of Rab5 (wild type and S34N), and REP-1 from Sf9 cells were purified from cell lysates generated from 500 ml baculovirus-infected Sf9 cultures using Ni-Agarose as described above, and the Rab5/REP-1 complex was formed as previously described [39]. His-Rab GDI and GST-Syntaxin fusion proteins were expressed in BL21 (DE3) *E. coli* and purified from bacterial lysates as described above.

Binding of Rin1 mutants to endosomes

The endosomal fractions (i.e., 5-min enriched endosomes) were centrifuged at $50,000 \times g$ for 15 min, and then resuspended in HB buffer for binding experiments. For trypsinization experiments, the endosome fractions were either incubated with trypsin (T) (10 μ g/ml) at 4°C for 30 min followed by the addition of trypsin inhibitor (40 μ g/ml), or incubated with trypsin in the presence of trypsin inhibitor (TI). The treated endosomes were then diluted with HB buffer, centrifuged, and resuspended in the same buffer. Control and trypsinized endosomes (50 μ g/tube) were incubated in fusion buffer for 30 min at 37°C with different amount of purified Rin1 in the presence of Rin1-depleted cytosol. After incubation, the endosomal

membranes were washed with 3 ml of HB buffer and sedimented by centrifugation. Rin1 was detected by Western blot analysis with anti-Rin1 antibodies. The recovery of membrane in each lane on Figure 8 was monitored by Western blotting using anti-galactosidase antibodies. Alternatively, the amount of Av-Gal activity associated with endosomes in the presence of 0.5 % Trion X-100 after the binding experiments but prior Western blot analysis was measured (untreated vesicles: 125 ± 9 Units/mg protein; trypsin-treated endosomes: 123 ± 6 Units/mg protein; trypsin-treated endosomes plus trypsin inhibitor: 126 ± 6 Units/mg protein). For membrane and supernatant distribution of Rin1 proteins, the binding experiments were carried out essentially as described above, except that after incubation the samples were centrifuged to separate bound proteins (membrane, M) from unbound proteins (supernatant, S). Proteins from membrane and supernatants were separated by SDS-PAGE and transferred onto nitrocellulose membranes using standard techniques, and the presence of Rin1 was determined with anti-Rin1 antibodies as described above.

Subcellular fractionation

NR6 cell expressing Rin1: wild type and its mutants (three 100 mm dishes per Rin1 construct) were washed twice with ice-cold HBSS-BSA and one with HB buffer. After washing, cells were resuspended in 3 ml of ice-cold HB buffer supplemented with protease and phosphatase inhibitors as indicated above. Resuspended cells were passed 15 times through a stainless steel ball homogenizer at 4°C, followed by centrifugation at $900 \times g$ for 5 min at 4°C. The postnuclear supernatants were further centrifuged at $200,000 \times g$ for 60 min. After centrifugation, supernatant and pellets were separated, and then the membrane fraction was resuspended in 500 μ l of HB buffer. Both fractions were then stored at -80°C.

Electron microscopy

Gold particles (10 and 20 nm) were coated with proteins by standard techniques [40]. Binding and uptake of gold particles coated with the ligands were carried out under the same conditions described for soluble ligands. The homogenization of cells and the fusion reaction were performed using the same protocols as described above. The fusion reaction was then stopped by adding glutaraldehyde (to make 1% final concentration), which was prepared in HB buffer. After 15 min of fixation at room temperature, samples were centrifuged for 20 min at $13,000 \times g$, washed and the samples were embedded in plastic, sectioned, and analyzed by transmission electron microscopy [40].

Guanine Nucleotide Exchange Assays

Exchange kinetics were measured by monitoring the quenching of fluorescence after release of the nucleotide analog 2'-(3')-bis-O-(N-methylanthraniloyl)-guanosine 5'-diphosphate (mant-GDP; Invitrogen) [44]. The exchange reaction was carried out by incubating 1 mM of Rin1 constructs in 50 mM Tris (pH 8.0), 37 mM NaCl and 2 mM MgCl₂ in the presence of 100 pmol Rab5-mant-GDP for 30 min at 30°C. Samples were excited at 360 nm and the emission monitored at 440 nm. Data were collected using a multimode microplate spectrophotometer (Tecan). As a control, we have measured the release of mant-GDP from Rab5: wild type loaded with mant-GDP alone [Rab5 alone=100 Relative Units (RU)], or in the presence of heat inactivated Rin1 proteins [Rab5 plus heat inactivated Rin1= 99 ± 3 RU].

Statistical analysis

All experiments presented were repeated a minimum of two times in duplicate. The data represents the mean $\pm$ SD. Student's test was performed to calculate statistical significance.

Results

Internalization and intracellular localization of biotin-EGF and avidin- β -galactosidase in Hela cells

We have taken advantage of the internalization of two ligands (i.e., B-EGF and Av-Gal) to design a cell free system to detect fusion between endosomes. First, B-EGF and Av-Gal were independently internalized by receptor-mediated and fluid phase endocytosis in Hela cells, respectively. The insert in Figure 1 shows the uptake of different amounts of ^{125}I -Av-Gal and ^{125}I -B-EGF when incubated with 1.5×10^6 cells for 15 min at 37°C . We observed that the uptake of ^{125}I -B-EGF was saturated and specific, but the internalization of ^{125}I -Av-Gal was linear and not saturated in our experimental conditions.

To better comprehend the kinetics of EGF in our cells, we have also examined the percentage of ^{125}I -B-EGF that was internalized, recycled, or degraded in Hela cells after a short pulse of B-EGF. The amount of EGF recycled after a 15 min pulse is 3.7 % of the total cellular EGF, the amount degraded in 15 min is 5.8 % of the total, and the amount within the cells is 90.5 % of the total cellular EGF (Supplementary Fig. 1). As expected the uptake of ^{125}I -B-EGF was blocked with the 300-fold molar increase of unlabeled EGF. These values were comparable to values reported for unmodified EGF in the order of the nM [4, 23, 36, 37]. These data suggest that in Hela cells the kinetics we have observed are consistent with many previous observations for ligand and receptor internalization to early endosomes.

Second, the preparation of endosomes was carried out by incubating Hela cells with either ^{125}I -B-EGF or ^{125}I -Av-Gal at 37°C for 5 min. The cells were then homogenized and the subcellular localization of the probes was assessed by fractionation in Percoll gradient (1.05 mg/ml). Figure 1 shows that after 5 min of internalization, B-EGF accumulated in a light fraction. Enzyme activity of acid phosphatase, a lysosome marker, was mostly found at the bottom of the gradient, but some activity (6.6%) was present in the light fraction. We also found that 5-min endosomes containing Av-Gal accumulate in the light fraction in Percoll gradient (Figure 1).

Finally, to confirm our observations that each one of the probes is contained in early endosomes, we performed a differential subcellular centrifugation followed by a flotation-sucrose discontinuous gradient fractionation procedure. We observed that both probes localized on an endosome fraction, which contained the small GTPase Rab5, but did not contain the small GTPase Rab7 (Supplementary Fig. 2). Thus, these results indicate that both ligands are localized in endosome vesicles following internalization for 5 min at 37°C .

In vivo fusion between endosomes

Our observations indicate that both ligands can accumulate in an early endosome fraction after internalization (Figure 1). To determine whether both ligands are able to form an *in vivo* complex upon internalization, cells were incubated with Av-Gal for 1.5 min at 37°C , washed, and then chased for 1.5 min at 37°C . Then the cells were washed, incubated with B-EGF at 4°C for 2 hr, and the internalization at 37°C was started. Fusion of newly formed endosomes containing B-EGF with preexisting endosomes containing Av-Gal that have been internalized for 1.5 min and then chased for 1.5 min in intact cells were measured by determining the amount of β -galactosidase activity associated with the immunocomplex after solubilizing the cells with detergents in the presence of biotin-BSA. In Figure 2A, we observed an increase of the enzymatic activity associated with the immunoprecipitated EGF, indicating that both ligands interacted in the lumen of the endosome. Based on our internalizing conditions, endosome isolation and *in vivo* fusion reaction, we describe a *in vitro* fusion reaction between endosomes

(Figure 2B), in which two sets of vesicles containing B-EGF and Av-Gal were mixed and processed as described in Material and Methods.

In vitro endosome-endosome fusion: Characteristic and Specificity

When endocytic vesicles were mixed in the presence of ATP-regenerating system with 2 mg/ml cytosol at 37°C, a temperature dependent fusion was observed (Figure 3A). Almost no fusion was observed at a temperature below 20°C. At 4°C practically no fusion activity was detected. However, above this temperature, the fusion activity increased rapidly, reaching a maximum value at 37°C (Figure 3A). The fusion reaction was also time-dependent (Figure 3B). Optimal fusion time was determined by examining the extent of fusion after incubating the reactions for various times in the presence of 2 mg/ml cytosol at 37°C. After 15 min of incubation, fusion was ~50% of the maximum and the amount of fusion increased until 30 min of incubation. No further increase in the fusion reaction was observed after this time. Therefore, the 30 min time of incubation was chosen as the optimal fusion time for the assay (Figure 3B). The kinetics of the fusion reaction was similar to what has been reported [19, 20, 22, 43], in which the reaction shows a linear increase in fusion and then plateaus after 30 min. We have expressed the fusion reaction as a percentage of the total amount of immune complex formed after both sets of vesicles are mixed with detergent in the absence of added Biotin-BSA. The background activity (i.e., complex formation that occur after 4°C incubation in the presence of Biotin-BSA) was always approximately 1.2 % of the total activity that can be immunoprecipitated in the presence of detergent. Under our exp. conditions we observed that after 30 min of incubation the immunoprecipitable activity was 27.8 ± 1.2 % of total immunoprecipitable activity present in the system. However, the efficiency of the fusion is probably somewhat higher than the percentage of immune complex formation indicated because total immunoprecipitable activity includes the fraction of ligand present outside endocytic that was able to form immune complexes in the absence of Biotin-BSA during homogenization. Thus, the fact that a fraction of ligands present outside endocytic (i.e., leaked from the endosomes) during homogenization step in our preparation may help to explain the slower rate the *in vitro* fusion reaction compared of the fusion reaction observed in intact cells. In addition, we also observed that endosome-endosome fusion showed a significant dependence on vesicle dilution. Under our experimental conditions we observed that the undiluted vesicles (control vesicle) showed a fusion activity of 24 ± 1.2 F.U., while the 1/6 diluted vesicles showed a significantly reduced fusion activity (6.3 ± 0.6 F.U.). These observations also suggest that the concentration of vesicles in the *in vitro* assay is critical for optimal fusogenic activity, which is consistent with previous observations [20, 22, 26, 43]. The fusion dilution-dependent may be considered as other experimental factor that may contribute to the slow rate of the *in vitro* fusion reaction. Furthermore, when endocytic vesicles were incubated in fusion buffer either in the absence or in the presence of different amounts of cytosol prepared from Hela cells, an increase of ATP-dependent fusion reaction was observed (Figure 3C). The effect of cytosol was saturable at 2.0 mg/ml protein.

To determine whether the fusion activity in our cell-free assay was a consequence of a nonspecific fusion between endosomes or not, we prepared different populations of vesicles. Av-Gal was internalized by cells for 5 min and then chased for 0, 2.5, 5, 7.5, or 10 min to load the later compartments. Fusion of such vesicles with 5 min vesicles containing B-EGF was then examined. Maximal fusion activity was observed when both probes were present in 5-min endosomes (i.e., fusion between endosomes containing internalized Av-Gal for 5 min and chased for 0 min with endosomes containing internalized B-EGF for 5 min). As Av-Gal was chased longer, the ATP-dependent fusion activity decreased rapidly (Figure 3D). After a 10-min chase, fusion activity was reduced by 80% and no fusion activity was detected when Av-Gal was chased for 30 min (data not shown). The total immune complex formed did not change for each time of chase indicating that changes in fusion activity were not due to changes in

probe concentration (i.e., lysis in the absence of Biotin-BSA). Neither probes was significantly degraded under any of the conditions tested.

To characterize the cytosol ATP-dependent fusion, we tested several experimental conditions (Figure 4). First, ATP-dependent endosome fusion required KCl with an optimum concentration at 50 mM as is in the case of other *in vitro* transport systems [29,42,43]. Second, membrane-associated and cytosolic proteins were also required for the fusion reaction due to their sensitivity to trypsin treatment. Thus, trypsinization of vesicles and cytosol prior to the fusion assay resulted in strong inhibition of the fusion reaction. Third, we also observed that the addition of EGTA did not affect the fusion reaction (Figure 4A), which is consistent with previously published data [42,43].

To further characterize the cytosol ATP-dependent fusion reaction, we investigated the requirement of Rab5 in this *in vitro* assay since Rab5 has been shown to be an essential regulator of the early endosome fusion [10,28,29]. As expected, the addition of cytosol depleted of Rab5 inhibited the endosome fusion (Figure 4B). In contrast, the cytosol treated with normal IgG (control IgG) or depleted of Rab11 and Rab4 did not affect the fusion reaction. These results suggest that Rab5 is specifically required in this novel fusion reaction. The requirement of Rab5 in this *in vitro* system was further confirmed by the addition of GDI, which in turn blocked the fusion reaction (Figure 4B) by removing Rab5 from the endosomal membranes (data not shown). Because Rab5 was required in our fusion assay, we then examined whether the presence of Rin1, a Rab5- guanine exchange factor, or other effectors (i.e., EEA1) may be also required during the fusion reaction. As shown in Figure 4B, the addition of Rin1 depleted cytosol, or EEA1 to the fusion reaction showed a strong inhibitory effect, which was consistent with previous published data [25]. We also found that the addition of a soluble fragment of Syntaxin 13, but not the addition of a soluble fragment of Syntaxin 7, inhibited the fusion reaction (Figure 4B). These observations further confirmed that the fusion reaction was SNARE-dependent, as previously determined for the heterotypic fusion between Clathrin Coated Vesicles and early endosomes, and homotypic fusion between early endosomes [25]. Because PI3-kinase and NSF activities have been shown to play significant roles in the fusion reaction, we investigated the role of these two activities in the fusion reaction by incubating endosomes in the presence of NEM (a NSF inhibitor), or Wortmannin (a PI3-kinase inhibitor). As shown in Figure 4B, the addition of 1 mM NEM and 100 nM Wortmannin significantly inhibited the fusion reaction. Both inhibitory effects were time and concentration dependent (data not shown). Taken together, these results indicate that the endosome fusion reaction driven by the EGF-receptor was mediated by a specific fusion event whose characteristics are similar to those described in other endosome-endosome fusion reactions [18-27].

A similar protocol was followed to examine the endosome-endosome fusion morphologically. In these experiments, 5-min endosomes containing either 20 nm colloidal gold coated with EGF-B or 10 nm colloidal gold coated with Av-Gal were incubated under standard fusogenic conditions. Then, the vesicle pellets were processed and observed by electron microscopy as described in Material and Methods. We observed endosome fusion in the presence of cytosol and ATP-regenerating system (+ATP) at 37°C, as evidenced by the presence of more than one size of gold particles within an endosome (Figure 5A). However, we did not observe vesicle fusion when the vesicles were incubated either at 4°C in complete fusion buffer (Figure 5B) or at 37°C in complete fusion buffer in the presence of an ATP-depleting system (-ATP) (Figure 5C). As a further confirmation of these ultrastructural examinations of the morphology of endosomes during the fusion reaction, we determined the number of vesicles containing more than one size of gold particles within an endosome under several experimental conditions. After fusion reactions, we observed that the endosomes incubated in the presence of cytosol either at 4°C or in the absence of ATP did not contain two different sizes of gold particle (Figure 5D). However, we observed that ~ 25 % of the endosomes contained two different sizes of

gold particle when the endosomes were incubated in the presence of cytosol supplemented with ATP (Figure 5D). We also observed a time dependent fusion reaction with similar results to the one described in our biochemical assay in Figure 3.

Selective requirement of Rin1 during endosome-endosome fusion

Our results indicate that Rin1 regulates this novel endosome fusion since cytosol depleted of Rin1 did not fully support the fusion reaction (Figure 3). Based on these data, we then examined whether the addition of exogenous Rin1 may be able to restore fusion between endosomes in our *in vitro* system.

When these endosomes were mixed with cytosol depleted of Rin1 supplemented with different amounts of purified Rin1, we observed a significant recovery of the fusion reaction (21 ± 1.2 F.U., Figure 6A), as previously determined for the homotypic fusion between early endosomes [17, 30]. As expected, the addition of heat-inactivated Rin1 failed to support the fusion reaction (9.1 ± 1.75 F.U., Figure 6A). The results suggest that Rin1 specifically rescued the fusion between endosomes. We then investigated the characteristics of the endosome fusion rescued by Rin1. First, we found that the recovery of the fusion reaction by Rin1 required salts, energy, and temperature (data not shown). More important, we observed that the addition of purified Rin1 to the *in vitro* reaction in the absence of Rin1-depleted cytosol failed to fully support the fusion reaction (11 ± 1 F.U., Figure 6B, lane 2). These data suggest that cytosolic factor(s) may be required during the recovery of fusion reaction mediated by Rin1. As a further control in our *in vitro* system, we also carried out parallel experiments by adding purified Rin1 to the cytosol depleted of Rin1, which in turn significantly restored the fusion between endosomes (22 ± 1.1 F.U., Figure 6B, lane 3) to a point comparable to when the fusion reaction was carried out in the presence of complete cytosol (23 ± 1 F.U., Figure 6B, lane 10).

Because it has been shown that Rab5 is a key regulator in the fusion reaction [10,28,29] and is activated by Rin1 [17], we investigated whether the presence of cytosolic Rab5 might be required by Rin1 to rescue the fusion reaction. For this purpose, we removed Rab5 with anti-Rab5 antibodies from Rin1-depleted cytosol, and then endosomes were supplemented with exogenous Rin1 in the presence of cytosol depleted of Rin1 and Rab5 proteins. As shown in Figure 6, the removal of endogenous Rab5 completely blocked the rescue of the fusion reaction by the addition of exogenous Rin1 (Figure 6B, compare lanes 3 and 4). Our results suggest that Rab5 is a critical factor during the recovery of the fusion mediated by Rin1. Based on these observations, we speculate that the addition of Rab5: wild type may be required by Rin1 to support the fusion reaction. Thus, we then examined the effect of the addition of exogenous Rab5: wild type, or Rab5: S34N, a GTP-defective binding mutant, on the fusion assay. The addition of Rab5: wild type, but not Rab5: S34N mutant, further stimulated the fusion reaction (Figure 6B, compare lanes 5 and 6). These observations suggest that Rab5 is necessary for the recovery of endosome fusion mediated by Rin1. Therefore, it is reasonable to speculate that Rab5 became activated in the presence of Rin1 during the fusion reaction. The activated Rab5 will in turn allow for the recruitment of several factors, including the binding of EEA1 onto the endosomal membranes. EEA1 is a cytosolic protein that is associated with the Rab5-GTP bound form on the surface of the endosomal membranes together with other proteins [33], and therefore was required for the endosome fusion [25]. To determine whether cytosolic EEA1 may be involved in the fusion reaction rescued by Rin1, EEA1 proteins were removed from cytosol by anti-EEA1 antibodies. In Figure 6B, lane 7, we observed that the omission of EEA1 from Rin1-depleted cytosol markedly reduced the fusion between endosomes. Finally, the addition of NEM and a soluble fragment of Syntaxin 13 to this assay blocked the fusion reaction stimulated by Rin1 (Figure 6 B, lanes 8 and 9), suggesting potential interaction with SNARE proteins. Taken together, these observations establish the requirement of Rin1 during the fusion

reaction. Specifically, we demonstrated that several factors, including Rab5, EEA1, NSF and Syntaxin 13 were required for the fusion reaction mediated by Rin1.

Effect of Rin1 mutants on the fusion reaction

Based on previous data [17] and the results reported here, we then investigated the role of Rin1 in the fusion reaction by creating several Rin1 mutants (Figure 7A). We prepared several substitution mutations in the Vps9 domain of Rin1 by using information obtained from the analyses of the three-dimensional structure of Rabex-5, another Rab5-GEF containing protein, whose crystal structure has been resolved [44]. It has been reported that the five residues in Rabex5 (i.e., Asp313, Pro317, Glu351, Tyr354 and Thr357) are conserved in all Rab5-GEFs and crucial for the catalytic function on Rab5 [44]. Mutations in these residues dramatically influenced the guanine exchange activity of Rabex-5 [44]. In the sequence of Rin1 proteins, eight residues (i.e., Asp537, Pro541, Tyr561, Thr572, Glu574, Tyr577, Tyr578, Thr580) are conserved in both Rabex5 and Rin1 proteins, whereas the residue corresponding to Tyr523 is Phe299 (Supplementary Fig. 3). Corresponding mutations in other Rab5-GEFs (i.e., Rin1) are expected to show similar effects on their biochemical properties. To determine the effect of these Rin1 mutants on the fusion reaction, endosomes were incubated in fusion buffer with Rin1-depleted cytosol and supplemented with purified Rin1 mutants as indicated in Figure 7A. We observed that the addition of Rin1: wild type, Rin1: Y523F, or Rin1: T572A mutants restored the endosome fusion (Figure 7A). Moreover, the addition of Rin1: T578F mutant partially (75% of control) supported the fusion reaction. In contrast, the addition of Rin1: D537A, Rin1: P541A, Rin1: Y561F, Rin1: E574A, Rin1: Y577F and Rin1: T580A failed to restore the fusion reaction (Figure 7A) and an unexpected two of them (Rin1: D537A and Rin1: Y561F) showed a marked inhibitory effect. These data suggest a selective effect of these Rin1 mutants on endosome fusion, and subsequent regulation of the fusion reaction. They also indicate that the failure of these Rin1 mutants to support the fusion reaction may be due to their inability to interact and/or activate the Rab5 proteins.

To examine whether these Rin1 mutants were able to interact with Rab5, endosomes were incubated with different Rin1 constructs under fusogenic standard conditions (Figure 7A). After incubation, the samples were lysed, and then immunoprecipitated with anti-Rin1 antibodies. The resulting immunocomplexes were analyzed by Western blotting with anti-Rin1 and anti-Rab5 antibodies, respectively. We observed that Rin1: wild type specifically interacts with endogenous Rab5 (Figure 7B). However, four of them (i.e., P541A, E574A, Y577F, T580A) were unable to interact with Rab5, while two others (i.e., D537A, Y561F) partially interacted with Rab5. Other Rin1 mutants (i.e., Y523F, T572A, Y578F) were able to interact with Rab5 as compared with Rin1: wild type (Figure 7B). To further investigate the interaction of Rin1 with Rab5 in intact cells, NR6 cells transfected with Rin1: wild type and its mutants were lysed, and total cell lysates were immunoprecipitated with anti-Rin1 antibodies. The resulting immunocomplexes were analyzed by Western blotting with anti-Rin1 and anti-Rab5 antibodies. In cells transfected with Rin1: wild type, we observed that Rin1 co-immunoprecipitated with endogenous Rab5 (Figure 7C). Importantly, Rin1: P541A mutant was practically unable to interact with Rab5, while Rin1: D537A mutant partially interacted with Rab5. Other Rin1 mutant (i.e., Y523F) was able to interact with Rab5 as compared with Rin1: wild type (Figure 7C). As a control, we have used IgG for the immunoprecipitation reactions (Figure 7C).

To investigate whether these Rin1 mutants were able to activate Rab5, we measured the release of GDP bound to Rab5 in the presence of Rin1 mutants. Under our experimental conditions we found that the addition of Rin1: wild type to the *in vitro* exchange assay significantly released mant-GDP from Rab5 (Rin1: wild type= 44 ± 3 % of control) as compared with the release of mant-GDP from Rab5 alone (see Material and Methods). Interestingly, we did not

observe any significant release of mant-GDP from Rab5 in the presence of the following mutations: Rin1: D537A = 94 ± 3 % of control, Rin1: P541A = 97 ± 3 % of control, Rin1: Y561F = 99 ± 1 % of control, Rin1: E574A = 92 ± 6 % of control, Rin1: Y577F = 94 ± 5 % of control, and Rin1: T580A = 98 ± 5 % of control, which supports the inhibitory effect of these mutants on the fusion reaction. Other Rin1 mutants were able to stimulate the release of mant-GDP from Rab5 (Rin1: Y523F = 37 ± 4 % of control, Rin1: Y578F = 39 ± 9 % of control). Our data suggest that the deficiency of these Rin1 mutants (i.e., P541A, E574A, Y577F, T580A) to fully interact and/or activate Rab5 might explain their inability to rescue the fusion between endosomes. Taken together, these data suggest that Rin1 mutants that did not properly interact and/or activate Rab5 and did not support endosome fusion may also be defective in binding to the endosomes. Thus, to determine whether a Rin1 mutant may exert its effect by binding to endosomal membranes, we incubated 5-min enriched endosomes with increasing amounts of Rin1 proteins for 30 min at 37°C in fusion buffer. As shown in Figure 8A, we observed saturable and specific binding of Rin1: wild type to 5-min enriched endosomes. These observations suggest that a finite number of interacting sites were present on the membrane and more importantly, binding of Rin1 to membrane was specific since the trypsinization of the vesicular fractions prior to the binding reaction resulted in marked inhibition of the binding of Rin1.

To further examine whether the introduction of these mutations in the Vsp9 domain of Rin1 affected its ability to bind to the endosome fractions, we incubated 5-min enriched endosome fractions with different Rin1 mutants for 30 min at 37°C as indicated in Figure 8A. After the incubation, the samples were pelleted by centrifugation. Then, the distribution of Rin1 protein was determined (i.e., supernatant or membrane associated) by Western blot analysis. As expected, most of Rin1: wild type remained in supernatant after 30 min of incubation with trypsinized endosomes, while a significant amount of Rin1: wild type was associated to the membrane fraction after the incubation at 37°C with control membranes (Figure 8A, B). We observed that these Rin1 mutants (i.e., D537A, P541A, Y561F, E574A, Y577F, T580A) showed very little interaction with the endosome fractions; most of the proteins were found in the supernatant after the incubation (Figure 8 B, C). To further confirm our *in vitro* observations, we then investigated the subcellular distribution of Rin1: wild type and its mutants in intact cells. Significantly, we found that, whereas most of Rin1: wild type and Rin1: Y523F were membrane bound, Rin1: D537A and Rin1P541A were predominantly cytosolic (Figure 8 D). These observations were not surprising considering that these mutants did not fully interact with Rab5 (Figure 7). None of the other Rin1 mutations (i.e., Y523F, T572A, Y578F) significantly affected the binding of Rin1 to the membrane fractions as compared with the binding of Rin1: wild type to membranes (Figure 8B). Collectively these observations suggest that certain functional diversity in the residues on the Vps9 domain of Rin1 might be responsible for the binding of Rin1 to the endosomal membranes.

Discussion

We have developed a novel *in vitro* assay to investigate the structure-function relationship of Rin1 by mutational analysis. In this study, we have used two different probes (i.e., EGF and β -galactosidase), which were efficiently internalized at 37°C into endosomes in Hela cells. When two endocytic vesicles containing these ligands are brought together upon vesicle fusion (i.e., *in vitro* or in intact cells) a complex was formed. Thus, the fusion activity can be determined by measuring the enzymatic activity in the immunoprecipitated complex isolated with anti-EGF antibodies.

The results reported here show that this novel fusion reaction was time-, cytosol-, and ATP-dependent with an optimal temperature of 37°C. This *in vitro* endosome fusion was dependent on the ionic strength as well as on the presence of cytosolic and membrane factors. We observed that the fusion was sensitive to NEM and the fusion reaction was also Ca^{2+} independent since

the fusion reaction was not affected by the addition of EGTA (Figure 4). Early endosomes appear to be highly fusogenic, and selective since when some of the ligand was chased longer time (i.e., 10 min chase) we observed a dramatically decrease of the ATP-dependent fusion activity (Figure 4). These data suggest that this novel *in vitro* fusion assay is very sensitive, highly reproducible, and records fusion between early endosomes. In addition, morphological analysis of the fusion events clearly showed the formation of endosomes containing different sizes of gold particles and also the formation of large aggregated endosomes in the presence of cytosol (Figure 5A). These endosomal aggregates have been previously described and proposed as an intermediate state that assembles prior to the fusion between endosomes [42].

This novel endosome fusion reaction required Rab5 and also several Rab5-associated factors (i.e., Rin1, EEA1, GDI). The omission of Rin1 and EEA1, and addition of Rab GDI proteins had a dramatic inhibitory effect on the fusion reaction. In contrast, the addition of the Rab5/REP-1 complex (data not shown) and Rin1 increased the fusion activity between early endosomes. Furthermore, cytosol depleted of Rab4 and Rab11 did not inhibit the early endosome fusion driven by EGF-receptor, which further supported our results that this novel fusion assay recorded a Rab5-dependent fusion between endosomes. Thus, these *in vitro* results suggest that Rab5-GTP, which might be activated by Rin1: wild type (see discussion below) is a critical factor for the endosome fusion driven by EGF-receptor. These results are consistent with the observation that the addition of EGF as well as the expression of Rin1: wild type induced the formation of enlarged Rab5-positive endosomes in intact cells [17,30]. In addition, the observation that a soluble fragment of Syntaxin 13, but not a soluble fragment of Syntaxin 7, exhibited a significant inhibition of the fusion reaction further confirmed that this *in vitro* endosome fusion assay is SNARE dependent. Thus, Syntaxin 13 may function as a dominant negative inhibitor. Furthermore, we also found that this endosome fusion required NSF and PI3-kinase activities, which was evidenced by the inhibitory effect of NEM and Wortmannin inhibitors, respectively. Collectively, the requirements for this novel endosome-endosome fusion were similar to those described for other *in vitro* reconstitution cell-free assay [18-27], which has been proven to be a powerful tool for identifying key factors that mediate fusion between endosomes.

Rin1 has been suggested to be involved in the internalization and/or degradation of the EGF-receptor [17,45]. Data implicating Rin1 in EGF-receptor degradation was linked to STAM proteins via the proline rich domain of Rin1 [45]. Interestingly, the deletion of this domain of Rin1 affected the degradation of the EGF-receptor, but not the interaction and/or activation of Rin1 with Rab5 [45]. These results suggest that the proline rich domain of Rin1 might play an important role during the degradation of the EGF-receptor, while the Vps9 domain of Rin1 might be more critical in its role in endosome fusion during the endocytosis of EGF-receptor [17]. Thus, the evidence suggests that Rin1 is involved in endosome fusion because it localized with Rab5 and/or EEA1, and its expression induced the formation of enlarged Rab5-positive endosomes in intact cells [16,17].

Our observations suggest a functional role for Rin1 in this endosome fusion assay, since the depletion of cytosolic Rin1 inhibited the fusion between endosomes, and the addition of exogenous Rin1: wild type rescued the fusion reaction. Consistent with these results, the addition of Rin1: wild type supplemented with Rab5: wild type, but not with Rab5: S34N mutant, stimulated the fusion reaction, suggesting a mechanism by which Rin1 modulates endosome fusion (i.e., a positive role for Rin1 on endosome fusion is mediated by its interaction and/or activation of Rab5). This hypothesis is supported by the observation that the depletion of Rab5 from cytosol, or addition of the GTP-binding defective mutant Rab5: S34N inhibited the rescue of the fusion assay by Rin1. In addition, endosome fusion that was Rin1-dependent was blocked either by the depletion of cytosolic EEA1 or by adding a soluble peptide of Syntaxin 13 (Figure 6). NSF was also required in the fusion reaction regulated by Rin1 as

evidenced by the inhibitory effect of NEM on endosome fusion (Figure 4). In addition to the requirement of Rab5 in the regulation of the fusion by Rin1, these observations also suggest that other proteins (i.e., EEA1, Syntaxin 13 and NSF) were also required during the fusion assay mediated by Rin1. Our results also suggest that EEA1, Syntaxin 13 and NSF may function as common fusogenic factors on the fusion reaction independently of how Rab5 has been activated. Thus, it is possible to speculate that upon activation of Rab5 by Rin1, the Rab5-GTP bound form will in turn allow the recruitment of EEA1 and other associated factors required for the fusion reaction. However, there is much to be learned about these processes involving the regulation, recruitment and interactions of fusogenic factors on the surface of the endosomes.

The fact that the omission of Rab5 from the cytosol markedly blocked the fusion reaction rescued by Rin1 raises the question as to whether or not the alteration of the interaction between Rin1 and Rab5 might affect endosome fusion. To examine this hypothesis, we investigated the role of different Rin1 mutants on the fusion reaction. These Rin1 mutants (i.e., P541A, E574A, Y577F, T580A) failed to properly interact with Rab5, and more importantly did not support the fusion reaction. Noticeably, three of these Rin1 residues (i.e., Pro541, Try577 and Thr580), which we have shown to have a significant inhibitory effect on the fusion assay, are highly conserved in several Vps9 domain-containing proteins [44] (Supplementary Figure 3).

Furthermore, it is possible that other residues of Rin1 (i.e., Asp537 and Tyr561) may be involved in the interaction with Rab5 (Figure 7B) as well as in Rin1's GEF activity for Rab5 (in this paper), which may explain the strong inhibitory effect of these two mutants on the fusion reaction (Figure 7A, compare the fusion activity of cytosol depleted of Rin1 [9.1 ± 0.75 F.U.] with the fusion activity of cytosol depleted of Rin1 supplemented with Rin1: wild type [23.2 ± 1.3 F.U.], Rin1: D537A [5.2 ± 0.3 F.U.] and Rin1: Y561F [5.2 ± 0.05 F.U.], * $P < 0.01$). Thus, Rin1: D537A and Rin1: Y561F mutants may sequester Rab5, and therefore block its function. Consistent with these observations, the amino acid residues corresponding to Asp537, Pro541, Try577 and Thr580 in the Vsp9 domain of Rabex-5 have been shown to be critical for the activation of Rab5 [44]. In addition, it has been demonstrated that a natural splice variant of Rin1, lacking the N-terminal region of the Vps9 domain, did not activate Rab5, and failed to support the fusion reaction. Taken together, our data indicate that these conserved residues in the Vps9 domain of Rin1 were required to activate Rab5, and support endosome fusion.

Additionally, the binding of Rin1 to the 5-min endosomal membrane fractions was saturable, and specific. More important, we also observed that mutations on Rin1 that inhibited its interaction with Rab5 affected its association with 5-min endosomes. When Rin1: wild type was incubated with 5-min endosomes, which had been pre-treated with GDI to remove Rab5, a significant reduction (~90%) of Rin1 binding to endosomes was observed (data not shown). Therefore, these observations suggest a possible direct correlation between Rin1 and Rab5 interaction and binding of Rin1 to 5-min endosomes. Consistent with this observation, we found that Rin1 mutants (P541A, E574A, Y577F, T580A) that failed to interact with Rab5, poorly bound endosomes. However, the binding to the endosomal membranes of Rin1: D37A and Rin1: Y561F mutants, which weakly interacted with Rab5, was also significantly diminished as compared with the binding of Rin1: wild type. Furthermore, our subcellular distribution of Rin1 in intact cells revealed that, whereas most of Rin1: wild type and Rin1: Y523F mutant were membrane bound, Rin1: D537A and Rin1: P541A mutants were predominantly cytosolic (Figure 8D). Based on these findings, our data also suggest that the Vps9 domain of Rin1 was necessary, but not sufficient, for binding to the endosomal membranes since some of the mutations in Rin1 (as discussed above) did not completely inhibit their binding to the endosomal membranes. The exact domain(s) and/or additional factor(s) by which Rin1 interacts with the membrane fraction will require further investigation. In this regard, Ras binding and SH2 domains of Rin1 may be considered as a membrane switch factors

during activation of Rab5. Thus, in addition to the Vps9 domain, the activated EGF-receptor (via the SH2 domain) may direct Rin1 to the correct membrane localization and subsequent activation of Rab5, while activated Ras may potentiate Rin1 GEF activity. Alternatively, the targeting of Rin1 to membranes could be mediated by a combination of both Vps9 and Ras associated domains. Additionally, the proline rich domain of Rin1 could also promote Rin1 binding to membranes for the activation of Rab5. However, this possibility is unlikely since a Rin1 mutant, lacking the proline rich domain, fully supported the activation of Rab5 [45].

In conclusion, we have used a newly developed fusion reaction to investigate the molecular mechanism by which Rin1 regulates fusion between endosomes. Specifically, we demonstrated that the fusion reaction regulated by Rin1 required several factors, including Rab5, EEA1, NSF and Syntaxin 13. More importantly, we have identified key residues in the Vps9 domain of Rin1 critical for its biological function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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The abbreviations used are

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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Figure 1. Uptake and subcellular compartmentalization of biotin-EGF and avidin- β -galactosidase in HeLa cells

HeLa cells (6×10^7) were incubated for 5 min at 37°C with ^{125}I -biotin EGF (B-EGF, ■) or ^{125}I -avidin- β -galactosidase (Av-Gal, ●) at $0.1 \mu\text{g/ml}$ and 2 mg/ml final concentration, respectively. The cells were then washed and fractionated. A membrane fraction was mixed with 25 ml of Percoll (density 1.05 g/l) and centrifuged at $39,000 \times g$ for 60 min . The gradients were collected from the bottom and analyzed for radioactivity and acid phosphatase activity (▲). *Inset:* HeLa cells (1.5×10^6) were incubated for 15 min at 37°C with different concentrations of ^{125}I -Av-Gal (◆) or ^{125}I -B-EGF (◇) as indicated in Material and Methods. The specific uptake is shown for each ligand concentration. Values are means $\pm$ SD, $n=4$.

Figure 2. Fusion between endosomes in intact cells

(A) Cells were incubated with Av-Gal for 1.5 min uptake, washed and chased for 1.5 min at 37°C. Cells were then washed, and then incubated at 4°C for 2 h with B-EGF. Endocytosis of EGF was started by incubation at 37°C and the amount of B-EGF associated with Av-Gal was quantitated at each time by solubilizing the cells in the presence of Biotin-BSA. Results are expressed as fluorescence units, which indicates the percentage of the immunocomplex formed after solubilization of vesicles in the absence of Biotin-BSA. Total was 68 ± 1.2 fluorescence units and did not change significantly with the time of internalization. Values are means $\pm$ SD, $n=4$. (B) Outline of the method to determine an endosome-endosome fusion event in a cell-free system. Endosome fractions containing appropriate probes are co-incubated under fusogenic conditions (i.e., support vesicles fusion). These events (i.e., vesicle fusion) are evaluated by the Av-Gal::B-EGF complex formation, which may be assayed in wells coated with anti-EGF antibodies and processed as described in Material and Methods.

Figure 3. Effect of temperature, time, cytosol, and energy on the fusion reaction

(A) Five-minute endosomes isolated from Hela cells containing B-EGF and Av-Gal were mixed in fusion buffer containing 2 mg/ml cytosol, incubated for 30 min at different temperatures in the presence of an ATP-regenerating (●, +ATP) or ATP-depleting (■, -ATP) systems and processed as described under Materials and Methods. Values are means $\pm$ SD, $n=3$. (B) Five-minute endosomes generated as described above were mixed in fusion buffer in the presence of 2 mg/ml cytosol containing an ATP-regenerating (●, +ATP) or ATP-depleting (■, -ATP) systems for the indicated time at 37°C, and processed as described above. Values are means $\pm$ SD, $n=3$. (C) Five-minute endosomes generated as described above were incubated for 30 min at 37°C with different amounts of cytosol in the presence of an ATP regenerating (●, +ATP) or ATP depleting (■, -ATP) systems and processed as described above. Values are means $\pm$ SD, $n=4$. (D) Endosomes were obtained from cells that internalized Av-Gal for 5 min at 37°C followed by 0, 2.5, 5.0, 7.5 and 10 min chase at 37°C. These endosomes were then mixed with endosomes obtained from cells that endocytosed B-EGF for 5 min at 37°C. Samples were incubated in fusion buffer in the presence of 2 mg/ml cytosol containing an ATP-regenerating (■) or an ATP-depleting (□) systems for 30 min at 37°C and processed as described above. Values are means $\pm$ SD, $n=4$.

Figure 4. Characteristics of fusion between endosomes

(A) *In vitro* fusion amount endosomes were assessed by the use of buffers of different compositions in the presence of 1.5 mg/ml cytosol. The following conditions were examined: -KCl: the salt was not included in the buffer; Trypsin-treated samples: vesicles and cytosol were incubated separately with trypsin prior to mixing. For trypsinization experiments, vesicles and cytosol were incubated with 18 μ g/ml trypsin for 45 min at 4°C followed by soybean trypsin inhibitor (40 μ g/ml); as a control, trypsinization was carried out in the presence of inhibitor. EGTA/ Ca^{2+} : vesicle fusion was carried out in the absence (+ 0.5 mM EGTA) or presence of 40 μ M Ca^{2+} ; Values are means $\pm$ SD, n=5. (B) Fusion between endosomes was performed as described in Figure 3 either in the presence of 1.5 mg/ml Rab- (i.e., Rab4, Rab5, and Rab11)

or EEA1-, or Rin1-depleted cytosols, or IgG-treated cytosol (Control IgG). Proteins: vesicle fusion was carried out in the presence of 1.5 μ M GDI, 2 μ M Syntaxin 7, and 2 μ M Syntaxin 13. Values are means $\pm$ SD, n=5. NEM treatment: Cytosol and vesicles were pretreated with 1 mM NEM at 4°C for 45 min and excess of NEM was inactivated by 3 mM DTT. Kinase inhibitor: Control (DMSO alone), and Wortmannin (WN, 100 nM) were used in the fusion reaction. Nonspecific background fluorescence was measured by incubation at 4°C and subtracted from all values. Values are means $\pm$ SD, n=4.

Figure 5. Electron micrographs of fusion between endosomes

Endosome fractions containing colloidal of 20 nm B-EGF coated gold particles and 10 nm Av-Gal coated gold particles were loaded in 5-min endosomes, and then the two sets of endosomes were incubated with 1.5 mg/ml of cytosol either in the presence (A) or absence (B) of ATP for 30 min at 37°C, or in the presence of ATP at 4°C (C). After fusion, the samples were fixed in suspension, sedimented, and analyzed for transmission electron microscopy. Bars=100 nm.

(D) For each time of the fusion reaction, the presence of either 10 or 20 nm gold particles was assessed for at least 2,000 endosomes under three different experimental conditions (●, +ATP/37°C; ○, -ATP/37°C; □, +ATP/4°C). Results are expressed as a percentage of the total endosomes that contained 10 nm and 20 nm gold. Values are means ± SD, n=3.

Figure 6. Effect of Rin1 on the fusion reaction between endosomes

(A) Fusion assays were performed in the presence of 1.5 mg/ml Rin1-depleted cytosol supplemented with different amounts of purified Rin1: wild type (WT) (●) or purified heat-inactivated Rin1: wild type (○). The asterisk (*) indicates the fusion activity using complete cytosol (□). Values are means $\pm$ SD, $n=4$. (B) Endosomes were incubated with (Lanes 1, 3-9) or without (Lanes 2 and 10) 1.5 mg/ml of Rin1-depleted cytosol and subsequently allowed to fuse as indicated in the graph. Lanes 1 to 10 show the fusion reaction either in the absence (Lanes 1 and 10), or in the presence (Lanes 2 to 9) of purified Rin1: wild type (1.2 μ M). Lanes 4 and 7 show the fusion reaction in the presence of cytosol depleted of Rin1/Rab5 and Rin1/EEA1, respectively. Lanes 5 and 6 show the fusion reaction in the presence of Rab5 proteins. Fusion reactions were supplemented with equimolecular amount of Rin1 and Rab5 proteins (i.e., 1.2 μ M), mixed to form a complex, and then added directly to the fusion reaction. Lanes 8 and 9 show the fusion reaction in the presence of 2 μ M Syntaxin 13 and 1 mM NEM, respectively. Lane 10 shows fusion activity using complete cytosol treated with control IgG. Values are means $\pm$ SD, $n=2$.

Figure 7. Effect of Rin1 mutants on the fusion reaction between endosomes

(A) Fusion assays were performed under standard conditions either in the presence of 1.5 mg/ml Rin1-depleted cytosol alone, or supplemented with 1.7 μ M of purified Rin1: wild type, Rin1: Y523F, Rin1: D537A, Rin1: P541A, Rin1: Y561F, Rin1: T572A, Rin1: E574A, Rin1: Y577F, Rin1: DY578F, or Rin1: T580A. Values are means $\pm$ SD, $n=3$. (* $P < 0.01$) (B) Endosomes were incubated either in the presence of 1.5 mg/ml Rin1-depleted cytosol alone, or supplemented with 1.7 μ M of different purified Rin1 proteins under fusogenic standard conditions. After incubation, the samples were lysed, and the whole endosome lysates (WEL) were prepared and immunoprecipitated (IP) with anti-Rin1 antibodies. The resulting immunocomplexes and WEL were subjected to Western Blot (WB) analysis with anti-Rin1 and anti-Rab5 antibodies. Inset: A representative Western blot showing the relative amount of Rab5 bound to Rin1: wild type and its mutants after an immunoprecipitation with anti-Rin1 antibodies from an *in vitro* binding assay. Values are means $\pm$ SD, $n=3$. (C) Stable NR6 cell lines expressing Rin1: wild type, Rin1: Y523F, Rin1: D537A, and Rin1: P541A mutants were lysed as described in Material and Methods. The cell lysates were subject to immunoprecipitation (IP) with rabbit anti-Rin1 antibodies followed by SDS-PAGE and Western blotting (WB) with mouse anti-Rin1 and anti-Rab5 antibodies. We have used IgG as a control for the immunoprecipitation reaction. The presence of Rab5 in the immunoprecipitate was visualized with anti-Rab5 antibodies. Inset: A representative Western blot showing the

relative amount of Rab5 bound to Rin1: wild type and its mutants after an immunoprecipitation with anti-Rin1 antibodies from whole cell lysates (WCL). Values are means $\pm$ SD, n=3.

Figure 8. Binding of Rin1 mutants to endosomal membranes

(A) Endosomal membranes (control and trypsinized) and Rin1-depleted cytosol (50 µg/tube) were incubated with different amounts of Rin1: wild type in fusion buffer for 30 min at 37°C to allow for the binding of Rin1 proteins to membranes. After incubation, the endosomal membranes were washed with HB buffer and sedimented by centrifugation. The membrane-bound Rin1 proteins were separated by SDS-PAGE, and transferred onto nitrocellulose membranes. Whole endosomal lysates (WEL) were prepared to determine the total number of vesicles added to the system by determining the amount of Av-Gal associated with the endosomes. The presence of Rin1 and Av-Gal in the samples was examined with anti-Rin1 antibodies and anti-galactosidase antibodies, respectively. Data represent one of three

independent experiments. (B-C) Endosomal membranes and Rin1-depleted cytosol (50 µg/tube) were supplemented with 1.7 µM of Rin1 mutants in fusion buffer for 30 min at 37°C to allow the binding of Rin1 mutants to membranes. After incubation, the endosomal membranes were washed with HB buffer and sedimented by centrifugation. The membrane-bound and unbound Rin1 proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The presence of Rin1 in both supernatant (S) and membranes (M) were examined with anti-Rin1 antibodies. Whole endosomal lysates (WEL) were prepared to measure the total amount of Rin1 added to the system. Data represent one of three independent experiments. (D) NR6 cells stable transfected with Rin1: wild type, Rin1: Y523F, Rin1: D537A, and Rin1: P541A mutants were homogenized using a stainless steel ball homogenizer. The homogenates were then centrifuged at $800 \times g$ for 10 min. The resulting supernatants were then centrifuged at $200,000 \times g$ for 60 min to yield cytosol (S) and membrane (M) fractions that were analysis by SDS-PAGE and Western blotting (WB). The presence of Rin1 in both supernatant and membrane fractions were examined with mouse anti-Rin1 antibodies. GAPDH and transferrin receptor (Tf) were used as a marker of the cytosol and membrane fractions, respectively. Data represent one of two independent experiments.