Evidence for a Symmetrical Requirement for Rab5-GTP in \textit{in Vitro} Endosome-Endosome Fusion\textsuperscript{*}

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Early endosome fusion, which has been extensively characterized using an \textit{in vitro} reconstitution assay, is Rab5-dependent. To examine the requirement for Rab5 on both fusion partners, we prepared cytosol and endosomes depleted of Rab5. Unlike control cytosol, Rab5-depleted cytosol was only marginally active in the \textit{in vitro} endosome fusion. However, fusion could be restored by the addition of wild-type Rab5 or Rab5 D136N, a mutant whose nucleotide specificity favors xanthine over guanine. The addition of Rab5 D136N restored fusion only in the presence of XTP. In the absence of XTP or in the presence of XDP, Rab5 D136N failed to restore fusion. When fusion was carried out with endosomal vesicles depleted of Rab GTPases (by preincubation with GDP dissociation inhibitor), together with cytosol immunodepleted of Rab5, fusion was virtually absent. We then used immunodepleted cytosol and GDP dissociation inhibitor-treated vesicles to determine whether Rab5 is required by both fusion partners. Using separate sets of endosomal vesicles, we found that priming both sets of Rab5-depleted vesicles with Rab5 Q79L, a GTPase-defective mutant, substantially stimulated endosome fusion. Priming one set of vesicles with Rab5 Q79L and a second set of vesicles with Rab5 S34N failed to activate fusion. When both sets of Rab5-depleted vesicles were primed with Rab5 D136N supplemented with XTP, endosome fusion was stimulated, similar to that observed with Rab5 Q79L. However, when one set of vesicles was preincubated with Rab5 D136N plus XTP and the second set with Rab5 D136N and XDP, no stimulation of fusion was observed. We conclude that Rab5-GTP is required on both fusion partners for docking and fusion of early endosomes. To confirm the fusion of Rab5-GTP-positive vesicles \textit{in vivo}, we expressed GFP-Rab5 Q79L in fibroblasts and observed fusion of Rab5-positive vesicles. We failed to record fusion of Rab5-negative vesicles with Rab5-negative vesicles. We conclude that Rab5-GTP is required on both sets of endosomes for fusion \textit{in vitro} and in living cells.

Individual Rab proteins have been localized to distinct compartments of both the endocytic and exocytic pathways (1, 2). Endocytosis requires a series of vesicle fusion events to deliver internalized solute and membrane to the endosomal sorting compartment and beyond (3). Multiple Rab GTPases are required for endocytosis, but Rab5 is a key early-acting GTPase that is rate-limiting (4–6). Rab5 also plays an essential role in phagosome-endosome fusion (7).

Like other small GTPases, Rab proteins are thought to act as regulatory molecules that recognize target proteins through a nucleotide-dependent conformational change. Several candidates for downstream targets of Rab5 have been identified including rabaptin 5 and rabphilin 3A, which are molecules that bind specifically to the GTP-bound form of Rab5 and Rab3A, respectively (8, 9). More than one effector may be required for Rab5 function (10). Association of Rab proteins with membranes depends on C-terminal isoprenylation, which is also essential for their function (11). Membrane association is regulated by Rab-GDI and REP (Rab escort protein) and is accompanied by exchange of bound GDP with GTP (12, 13). Guanine nucleotide status of Rab5 is also regulated by phosphatidylinositol 3-kinase activity (14). Conversely, the switch from the GTP- to the GDP-bound state occurs through hydrolysis of GTP, a process catalyzed by GTPase-activating proteins (15).

Complex intracellular trafficking pathways are thought to be composed of overlapping relays involving multiple GTPases often acting sequentially. However, fusion per se does not require GTP hydrolysis by Rab5. For example, the GTPase-defective mutant of Rab5, Rab5 Q79L, is fully active in promoting endocytosis and enhancing \textit{in vitro} endosome fusion (6, 16–18).

Earlier work demonstrated that GTPyS stimulates \textit{in vitro} endosome fusion under conditions where cytosolic proteins added to the assay are in limiting supply (19). Given that both GTPyS and Rab5 Q79L stimulate fusion, the question arose as to whether the GTP form of Rab5 was required on both fusion partners. To explore this question, we have made use of a mutation that has previously been shown to change the nucleotide binding specificity of a number of GTPases from GTP to XTP (20–22), including Rab5 (23–25). The Rab5 D136N mutant allowed us to selectively load one set of endosomes with the active form of Rab5 and a second set with an inactive form of Rab5. Our results indicate that the GTP-bound form of Rab5 is required on both fusion partners during an \textit{in vitro} endosome fusion assay.

EXPERIMENTAL PROCEDURES

Materials—Dinitrophenol-derivatized $\beta$-glucuronidase and aggregated anti-dinitrophenol IgG were prepared as reported (26). Anti-Rab5 monoclonal antibody 4F11 was a generous gift of Angela Wandinger-Ness (Northwestern University, Chicago, IL). Preparation and Prenylation of Wild-type Rab5 and Rab5 D136N Mutant—The preparation and purification of wild-type Rab5 and the

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\textsuperscript{*} The abbreviations used are: GDI, GDP dissociation inhibitor; GTPyS, guanosine 5′-O-(3-thiotriphosphate); ATPyS, adenosine 5′-O-(thiotriphosphate); GGPP, geranylgeranyl pyrophosphate.
Rab5 D136N mutant was carried out as described (23). Rab5 D136N (10 μM) was incubated with the semipurified REP-1/Rab geranylgeranyltransferase in 50 μl of 50 mM Hepes/KOH, pH 7.2, 5 mM MgCl₂, 0.5 mM Nonidet P-40, and 1 mM dithiothreitol containing 20 μM geranylgeranyl pyrophosphate (GGPP) for different times at 37 °C as described (17). Alternatively, 1 μM GGPP was used to label Rab5 protein as described (17). Following prenylation, Rab5 was directly used as indicated in each figure legend.

In Vitro Endosome Fusion Assay—Early endosomes were prepared from J774 E-cun macrophages as described (19). After ligand uptake, cells were washed and homogenized in 250 mM sucrose, 20 mM Hepes/KOH, pH 7.4, 1 mM EDTA, 50 mM KCl, and 1 mM GDP and incubated at 25 °C for 10 min. After incubation, 1 μl of 10 mM MgCl₂ was added to stabilize the Rab-GDP complexes. GDI (25 μl, 500 μg) was then added to the samples, which were incubated at 25 °C for 20 min. GDI-treated endosomes (vesicles: Va− and Vb−) were diluted with 50 volumes of buffer, followed by centrifugation. The pellets were resuspended in fusion buffer (26), and the samples were used for endosome fusion. As a control, the endosome fraction was treated as described above, except that GDI was omitted (vesicles: Va+ and Vb+).

Immunodepletion of Cytoplasmic Rab—Macrophage cytosol was subjected to immunoprecipitation with anti-Rab monoclonal antibody 4F11 bound to protein A-Sepharose (Amersham Pharmacia Biotech) for 60 min at 4 °C in immunoprecipitation buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 1 mg/ml bovine serum albumin, 1 mM sodium vanadate, 10 μg/ml aprotinin, 4 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride as described (7). Briefly, the supernatant was removed and used in in vitro fusion assays. The beads were washed three times with wash buffer (0.1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, and 1 mg/ml bovine serum albumin) and the bound Rab5 was then visualized by Western blotting. Rab5-immunodepleted cytosol (Cy−) and control cytosol (Cy+) were used as described in each experiment.

Construction of GFP-Rab5 Q79L Fusion Protein and Confocal Microscopy—The GFP-Rab5 Q79L cDNA used in these studies was prenylated, the purified protein was incubated with the semipurified REP-1/Rab geranylgeranyltransferase and GGPP for different times at 37 °C as described under “Experimental Procedures.” The prenylation of the Rab5 D136N mutant was nucleotide-dependent (Fig. 1B) (17, 23). The inability to completely suppress fusion activity using cytosol immunodepleted of Rab5 suggested that the remaining endosome fusion activity was due to membrane-associated Rab5. To explore this point, endosome membrane fractions were depleted of Rab5 by the addition of GDI. The release of Rab5 from the endosomes was carried out in the presence of GDP and an excess of GDI as described under “Experimental Procedures.” In agreement with previous results (29), GDI (100–120 μM) completely removed Rab5 from endosomal membranes in a dose-dependent manner (Fig. 1D). To examine the role of membrane-bound Rab5 in one or both vesicle populations, two sets of vesicles were prepared. One set of vesicles (Va) contained one of the probes necessary for in vitro endosome fusion (e.g. anti-dinitrophenol IgG), and the second set of vesicles (Vb) contained the second probe (e.g. dinitrophenol-derivatized β-glucuronidase).

Control vesicles are denoted as Va− or Vb−, whereas GDI-depleted vesicles are denoted as Va+ or Vb+. To control the influence of Rab5 present in cytosol, normal cytosol (denoted as Cy+) or immunodepleted cytosol (denoted as Cy−) was used. In Fig. 1C, maximal endosome fusion was observed when both untreated cytosol and vesicles were used (Va+/Vb+/Cy+). In contrast, virtually no fusion was observed when Rab5 was removed from both cytosolic and membrane fractions (Va−/Vb+/Cy+). Low levels of fusion activity (~16%) were recorded when one population of control vesicles was used (Va−/Vb+/Cy−) with immunodepleted cytosol. Only minimal recovery of endosome fusion was obtained when Rab5 was present in one set of vesicles in the presence of normal cytosol (e.g. Va−/Vb+/Cy+) or in neither set of vesicles, but in the presence of cytosol (Va−/Vb−/Cy−). These results clearly show that the presence of Rab5 is required in both sets of vesicles and that the Rab5 activity associated with normal cytosol was insufficient to recover fusion. The removal of Rab5 seems not to be specific to either set of endosomes since Rab removal from either set produced inhibition. Removal of Rab5 from the membranes appears to be the rate-limiting factor since the addition of Rab5 D136N to GDI-treated vesicles in the presence of XTP (but not in the presence of XDP) substantially restored fusion (Fig. 2A). The restorative activity was temperature-, ATP-, and concentration-dependent (Fig. 2A). The addition of Rab7 was unable to restore endosome fusion (data not shown). Moreover, incubation of GDI-depleted membranes with [3H]Rab5 D136N resulted in the accumulation of radiolabeled Rab5 on the membranes (Fig. 2B). Rab5 D136N binding was temperature- and ATP-dependent (Fig. 2B). Interestingly, following incubation of membranes with Rab5 D136N, Rab5 D136N/XTP was substantially more enriched on endosomal membranes than Rab5 D136N/XDP. In contrast, the addition of ATP-S blocked the

RESULTS

Removal of Rab5 from Membranes and Cytosol Is Required for Maximal Inhibition of in Vitro Endosome Fusion—As shown in Fig. 1A, in vitro fusion carried out with cytosol immunodepleted of Rab5 was substantially reduced, with ~70% reduction in normal fusion activity (Fig. 1A, column 2). To selectively activate Rab5 in the presence of other GTPases, we constructed, purified, and characterized the Rab5 D136N mutant. Rab5 D136N avidly bound [3H]XTP, and an excess of unlabeled XTP selectively blocked [3H]XTP binding. Moreover, the presence of other nucleotides (GTP, GDP, and GTP·S) did not show significant competition in the [3H]XTP binding assay (data not shown) (23, 24). The addition of Rab5 D136N to the in vitro endosome fusion assay containing Rab5-depleted cytosol and an excess of XDP (Fig. 1A, column 2) failed to stimulate in vitro fusion, as did the addition of the mutant protein alone (data not shown). The addition of Rab5 D136N restored endosome fusion using Rab5-depleted cytosol only when Rab5 D136N was supplemented with XTP (Fig. 1A, column 4). The concentrations of wild-type Rab5 (Fig. 1A, column 5) and Rab5 D136N plus XTP required for maximal recovery of fusion were similar, indicating that they are equally active in the fusion assay. To demonstrate that the Rab5 D136N mutant was prenylated, the purified protein was incubated with the semipurified REP-1/Rab geranylgeranyltransferase and GGPP for different times at 37 °C as described under “Experimental Procedures.” The prenylation of the Rab5 D136N mutant was nucleotide-dependent (Fig. 1B) (17, 23). Rab5 D136N plus XTP required for maximal recovery of fusion were similar, indicating that they are equally active in the fusion assay. To demonstrate that the Rab5 D136N mutant was prenylated, the purified protein was incubated with the semipurified REP-1/Rab geranylgeranyltransferase and GGPP for different times at 37 °C as described under “Experimental Procedures.” The prenylation of the Rab5 D136N mutant was nucleotide-dependent (Fig. 1B) (17, 23). Rab5 D136N plus XTP required for maximal recovery of fusion were similar, indicating that they are equally active in the fusion assay. To demonstrate that the Rab5 D136N mutant was prenylated, the purified protein was incubated with the semipurified REP-1/Rab geranylgeranyltransferase and GGPP for different times at 37 °C as described under “Experimental Procedures.” The prenylation of the Rab5 D136N mutant was nucleotide-dependent (Fig. 1B) (17, 23). Rab5 D136N plus XTP required for maximal recovery of fusion were similar, indicating that they are equally active in the fusion assay. To demonstrate that the Rab5 D136N mutant was prenylated, the purified protein was incubated with the semipurified REP-1/Rab geranylgeranyltransferase and GGPP for different times at 37 °C as described under “Experimental Procedures.” The prenylation of the Rab5 D136N mutant was nucleotide-dependent (Fig. 1B) (17, 23). Rab5 D136N plus XTP required for maximal recovery of fusion were similar, indicating that they are equally active in the fusion assay.
binding of the Rab5 D136N mutant in the presence of either XTP or XDP (Fig. 2B).

**GDI Depletion of Membrane Rab Inhibits Priming of Endosomes: Recovery Requires Activated Rab5 in Both Sets of Vesi-
cles**—We have previously shown that GTPγS induces the binding of cytosolic factors in the presence of low levels of cytosol (referred to as priming) and that GTPγS is a potent stimulator of in vitro endosome fusion under these conditions (19). Be-

**Fig. 1.** Effects of depletion of Rab5 from cytosol and endosomal membranes on in vitro endosome fusion. A, fusion with Rab5-depleted cytosol is restored by Rab5 in the active form. Endosome fusion was carried out with untreated cytosol (column 1) or with cytosol immunodepleted of Rab5 (column 2) in the presence of 0.6 μM Rab D136N plus 1 mM XDP (column 3) or plus 1 mM XTP (column 4). As a control, 0.6 μM wild-type Rab5 was added to the fusion assay (column 5). Untreated cytosol or cytosol treated with irrelevant IgG presented similar fusion values. The data are representative of three independent experiments. B, prenylation of the Rab5 D136N mutant. The Rab5 D136N mutant was prenylated in the absence or presence of nucleotides as described under "Experimental Procedures." Following incubation, the experiment was repeated three times with similar results. C, membrane-bound Rab5 is required for maximal endosome fusion. Endosome fusion was assessed with GDI-treated endosomes (Vα- and Vβ-) or with GDI-untreated endosomes (Vβ+ and Vα+) and with Rab5-immunodepleted cytosol (Cy-) and control cytosol (Cy+). Vesicles were mixed and then incubated for 45 min at 37 °C. Fusion was measured as described under "Experimental Procedures." D, release of Rab5 from membranes by GDI. Endosomes were incubated with different amounts of GDI at 25 °C for 20 min as described under "Experimental Procedures." After incubation, the membranes were separated by centrifugation, and membrane-associated Rab5 was analyzed by Western blotting. The experiment was repeated four times with similar results.

**Fig. 2.** Restoration of fusion with Rab5 D136N/XTP, but not Rab5 D136N/XDP. A, vesicles (Vα- and Vβ-), cytosol (Cy-), and different amount of Rab5 D136N plus 1 mM XTP were incubated in fusion buffer for 45 min at 37 and 4 °C and in the absence of ATP (a depleting system (DS) was used as described under "Experimental Procedures"). B, vesicles (Vα- and Vβ-), cytosol (Cy-), and 400 nM [3H]GGPP-labeled Rab5 D136N plus 0.5 mM XTP or 0.5 mM XDP were incubated in fusion buffer for 45 min at 37 °C in the presence of 200 μM ATP or 200 μM ATPγS or in the absence of ATP (DS). After incubation, the membranes were separated by centrifugation, and membrane-associated [3H]GGPP-labeled Rab5 D136N was analyzed by autoradiography. The data are representative of four independent experiments.
cause of these observations and the fact that the GTPγS stimulation of endosome fusion is Rab5-dependent, we investigated whether priming vesicles with Rab5 is symmetric. To test whether GDI removal of Rab GTPases from endosomal membranes interfered with the priming reaction, control vesicles (Va" and Vb") and GDI-treated vesicles (Va" and Vb") were incubated (i.e. primed) without KCl for 5 min at 37 °C in 0.05 mg/ml control cytosol (Cy") and Rab5-depleted cytosol (Cy"), respectively. As ions are absolutely required for optimal fusion activity, KCl was omitted during this step to avoid fusion during the priming step. Cytosol was removed by diluting the endosomes in 100 volumes of homogenization buffer. The vesicles were then collected by centrifugation. The endosomal pellets were resuspended in fusion buffer containing 50 mM KCl in the absence of cytosol. Fusion was measured by incubation at 37 °C as described under “Experimental Procedures.” Fig. 3A (first and second columns) shows that priming control vesicles with as little as 0.05 mg/ml cytosol stimulates endosome fusion by nearly 3-fold compared with GDI-treated endosomes. We then used the priming assay to investigate whether Rab5 Q79L or Rab5 S34N, when added to separate sets of GDI-treated vesicles, stimulates fusion. The addition of Rab5 Q79L to both sets of vesicles (e.g. Va"/Vb"/Cy") (third column) during the priming reaction greatly stimulated fusion, whereas the addition of Rab5 S34N to both sets of vesicles (fourth column) prevented priming and fusion. When Rab5 Q79L was added to one set of vesicles and Rab5 S34N to a second set of GDI-treated vesicles, fusion was prevented (fifth column). These results suggest that the GTP form of Rab5 is required in both sets of vesicles for docking and fusion to occur. Rab5 Q79L and Rab5 S34N are locked in the GTP and GDP forms, respectively. To determine whether selective activation of Rab5 (i.e. with respect to nucleotide binding and hydrolysis) displays a symmetrical requirement, we took advantage of the Rab5 D136N mutant. GDI-treated endosomes (Va"/Vb"/Cy") were preincubated separately with Rab5-immuno-depleted cytosol (0.05 mg/ml) in the presence of either Rab5 D136N/XTP or Rab5 D136N/XDP. Control vesicles were incubated without Rab5 addition. KCl was removed from the incubation buffer to prevent fusion from proceeding. After priming, the samples were centrifuged, as described above, to remove the excess of Rab5 D136N and cytosol. The vesicles were then resuspended in complete fusion buffer, and fusion was assessed (19). Fig. 3B shows that the addition of Rab5 D136N/XTP to both sets of vesicles (second column) substantially stimulated fusion compared with the Rab5-deficient control (first column). In contrast, a pronounced inhibition of priming and fusion was observed when one or both sets of GD1-treated vesicles were preincubated in the presence of Rab5 D136N/XDP, even if the other set of vesicles was treated with Rab5 D136N/XTP (fourth column). These results indicate that Rab5-GTP is required in both endosomal vesicles for priming and for subsequent endosome fusion.

Overexpression of GFP-Rab5 Q79L Results in Enlarged Fluorescent Endosomes That Fuse Selectively with Each Other—To visualize the fusion of endosomes in living cells, we took advantage of GFP constructs of Rab5 in which both fusion partners were labeled with GFP-Rab5 Q79L. Following transient expression of GFP-Rab5 Q79L in living cells, enlarged endosomes develop, which allows one to monitor endosome fusion directly by confocal microscopy. Cells expressing GFP-Rab5 Q79L show green fluorescence over the entire surface (Fig. 4). Docking and fusion between two or more green endosomes were observed in all the preparations tested. To test whether Rab5-negative vesicles were able to fuse with Rab5-positive vesicles,

**FIG. 3.** Symmetrical requirement for activated Rab5 for endosome fusion. A, restoration with Rab5 Q79L. GDI-treated endosomes (Va" and Vb") and GDI-un-treated endosomes (Va" and Vb") were primed separately for 5 min at 37 °C in KCl-free fusion buffer containing 0.05 mg/ml untreated (Cy") or treated cytosol (Cy") alone or in the presence of Rab5 Q79L or Rab5 S34N, respectively. KCl was not included during the preincubation to avoid fusion (20). After preincubation, the vesicles were diluted in homogenization buffer, and the samples were centrifuged 50,000 × g for 10 min to remove cytosol and Rab5 proteins. Samples were resuspended in complete fusion buffer and then incubated for 45 min at 37 °C as follows: primed vesicles (Va" and Vb") with Rab5-depleted cytosol (Cy") alone (first column), primed vesicles (Va" and Vb") with normal cytosol (Cy") alone (second column), Rab5 Q79L-primed vesicles with Rab5-depleted cytosol (Cy") (third column), Rab5 S34N-primed vesicles with Rab5-depleted cytosol (Cy") (fourth column), and Rab5 S34N- and Rab5 Q79L-primed vesicles with cytosol (Cy") (fifth column). Last, GDI-treated endosomes (Va" and Vb") were primed with normal cytosol (Cy") (sixth column). B, to restoration with Rab5 D136N/XTP. GDI-treated endosomes (Va" and Vb") were primed separately for 5 min at 37 °C in KCl-free fusion buffer in the presence of 0.05 mg/ml treated cytosol (Cy") alone or with Rab5 D136N/XTP or Rab5 D136N/XDP, respectively. After the priming reaction, the primed vesicles alone, the Rab5 D136N/XTP-primed vesicles, and the Rab5 D136N/XDP-primed vesicles were mixed as follows: primed vesicles with cytosol alone (first column), Rab5 D136N/XTP-primed vesicles (second column), Rab5 D136N/XDP-primed vesicles (third column), and Rab5 D136N/XTP- and Rab5 D136N/XDP-primed vesicles (fourth column). The data are representative of four independent experiments.

**DISCUSSION**

Rab5 is a rate-limiting factor required for endocytosis and endosome-endosome fusion. Work from several laboratories

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has shown that endosome fusion does not require GTP hydrolysis by Rab5 since GTPase-defective mutants of Rab5 allow endocytosis to proceed and fully support in vitro endosome fusion (6, 16, 17). In vitro endosome fusion is Rab5-dependent, and dominant-negative mutants of Rab5 inhibit fusion (4–6, 16). Vesicle docking, which precedes endosome fusion, is also Rab5-dependent. We set out to examine whether Rab5 is required on both fusion partners during endosome docking and fusion. First, we prepared and characterized Rab5 D136N, a mutation that changes the nucleotide binding specificity of Rab5 from GTP to XTP (23–25). Rab5 D136N allowed us to activate Rab5 selectively in the presence of other GTPases. We showed that Rab5 D136N (i) stimulates endosome-endosome fusion, which was cytosol-, XTP-, and Rab5 D136N concentration-dependent, and (ii) restores endosome fusion using both cytosol and/or membranes that were depleted of Rab5. We also showed that Rab5 D136N binding to membranes is temperature- and ATP-dependent. Earlier work by Ulrich et al. suggested that Rab5 binding is ATP-independent; however, these studies were carried out in a semipermeabilized cell preparation (13). To examine the question of whether both cytosolic and membrane-bound Rab5 proteins are required for docking and fusion, we immunodepleted cytosol of Rab5 with an anti-Rab5 monoclonal antibody. Immunodepletion of Rab5 from cytosol reduced its ability to support fusion by 70%. Complete recovery of fusion with vesicles and immunodepleted cytosol was achieved by the addition of Rab5 D136N plus XTP, but not XDP. Moreover, Rab5 D136N binding to membranes corresponded with the recovery of fusion and was temperature- and ATP-dependent. Interestingly, ATP hydrolysis was also required for the binding of Rab5 D136N/XTP or Rab5 D136N/XDP. To remove Rab5 fully from the in vitro system, we depleted Rab5 from endosomal vesicles by treatment with GDI in the presence of GDP. Removal of Rab5 from both cytosol and membranes completely inhibited fusion.

To explore the symmetrical requirement of Rab5 to restore fusion, two sets of endosomal vesicles were prepared, and the vesicles were primed (i.e. preincubated) either with normal cytosol or with cytosol that had been depleted of Rab5 with GDI treatment. Following a brief priming incubation in the presence of limiting levels of cytosol, vesicles were tested for fusion in the absence of cytosol. Removal of Rab5 from one or both sets of endosomes inhibited fusion. Priming both sets of vesicles with Rab5 Q79L restored endosome fusion. However, priming both sets of vesicles with Rab5 S34N had little effect on fusion. Interestingly, priming one set of vesicles with Rab5 Q79L and the other set of vesicles with Rab5 S34N resulted in no restoration of fusion. These findings suggest that Rab5 in the GTP form is required in both sets of vesicles.

To carry this analysis a step further, we examined priming of endosomal vesicles under conditions where only Rab5 was activated using the Rab5 D136N mutant. Rab5 D136N is functional only in the presence of XTP. To determine the role of Rab5 in priming, we incubated vesicles in Rab5-depleted cytosol along with Rab5 D136N in the presence or absence of XTP. We found that only in the presence of Rab5 D136N and XTP in both sets of vesicles were we able to recover fusion. To extend these observations to living cells, we expressed GFP-Rab5 Q79L in cells using the Sindbis virus expression system.2 GFP-Rab5 Q79L is functional in living cells when transiently expressed or in stable cell lines. GFP-Rab5 appears to localize to endocytic compartments as evidenced by co-localization studies using transferrin as a marker. By observing living cells expressing GFP-Rab5 Q79L, we recorded multiple endosome fusion events by video microscopy. As shown in Fig. 3, endosomes labeled with GFP-Rab5 Q79L preferentially fused with each other and failed to fuse with adjacent acid compartments that were not labeled with GFP-Rab5 Q79L.

The binding and subsequent activation of Rab5 induce the binding of cytosolic factors (i.e. rabaptin 5 and others (8, 30)) on the surface of endosomes (priming reaction), which, in turn, is required for vesicle docking and subsequent fusion. Haas et al. (31) and Mayer and Wickner (32) have shown that Ypt7 was required in both sets of yeast late endosomal vesicles for the docking step to occur. Thus, the yeast homotypic fusion event seems quite similar to macrophage endosome fusion described here. Clearly, more work needs to be done to characterize the Rab5-membrane requirement for both sets of endosome and to understand how Rab5 is linked to other proteins (i.e. rabaptin 5 (8) and EEA1 (early endosome antigen (33)) and to SNARES (soluble N-ethylmaleimide-sensitive fusion attachment receptors) to promote the docking and subsequent homotypic fusion events (34). Homotypic fusion may utilize only target SNARES, as has been suggested for endoplasmic reticulum vesicle fusion (35). Rab GTPases do appear to be required for the proper assembly of SNARES into active units (36), although Rab
GTPases are not necessary for SNARE-driven membrane fusion (27). Recent work demonstrating the fusion of lipid vesicles with SNARE molecules alone (27) suggests that the priming step, carried out by Rab5, mediates the regulation and fidelity of vesicle fusion.

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