Structural Features of the GTP-binding Defective Rab5 Mutants Required for Their Inhibitory Activity on Endocytosis*

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Rab5 is a Ras-like small GTPase that regulates early events of endocytosis. Previous work indicates that two GTP-binding defective Rab5 mutants (Rab5:S34N and Rab5:N133I) are dominant inhibitors of endocytosis. In this report, we have initiated experiments to address the structural features necessary for the inhibitory activity of these two Rab5 mutants. Second-site mutations were introduced into Rab5:S34N and Rab5:N133I, respectively, and the resulting double mutants were expressed in cultured BHK-21 cells via a Sindbis virus expression vector. Endocytic activity of the cells was monitored by following the uptake of a fluid-phase endocytic marker (horseradish peroxidase). The effects of the Rab5 mutants on endosome fusion in vitro were also examined. Truncation of the C-terminal isoprenylation motif CCSN abolished the inhibitory activity of both Rab5:S34N and Rab5:N133I. The same held true when the secondary mutation was a substitution mutation (F57S) in the effector domain. Another substitution mutation in this region (I53A) had no effect on the inhibitory activity of either Rab5:S34N or Rab5:N133I. The final mutation (R81A) was created immediately downstream of the second GTP binding motif (WDTAGGER), i.e., in the loop 4 region based on the structural model of Ras. This mutation greatly decreased the isoprenylation of Rab5:N133I and its inhibitory activity on endocytosis. It is believed that Rab5 function requires protein-protein interactions with Rab5-specific regulators and effectors. Some of these interactions are disrupted by Rab5:S34N and Rab5:N133I. By analogy to Ras, both Rab5:S34N and Rab5:N133I are likely to sequester a Rab5-specific guanine nucleotide exchange factor. This interaction requires the effector domain Phe57 residue and C-terminal isoprenylation of Rab5.

The Rab family of Ras-related small GTPases regulates intracellular membrane trafficking (for a recent review, see Ref. 1). These proteins are specifically localized to the cytoplasmic surface of each intracellular compartment, such as endoplasmic reticulum (Rab1 and Rab2) (2, 3), cis-Golgi (Rab1 and Rab2) (2, 3), medial-Golgi (Rab6) (4), trans-Golgi (Rab9) (5), early endosome (Rab4 and Rab5) (2, 6), late endosome (Rab7 and Rab9) (2, 5), and plasma membrane (Rab5) (2). While most Rab proteins are ubiquitous, some, including Rab3A (7), Rab3D (8), Rab15 (9), Rab17 (10), and Rab25 (11), are found only in certain tissues, suggesting that they are involved in specialized membrane-trafficking events. The functional association of Rab proteins with intracellular membrane trafficking was first demonstrated in yeast (12) and more recently in higher eukaryotes (1). Rab5 in particular has been shown to play a role in the regulation of endocytosis (13, 14).

Overexpression of GTP-binding defective Rab5 mutants in cultured BHK-21 cells blocks both receptor-mediated and fluid-phase endocytosis (13, 14). In addition, Rab5-specific antisera and Rab5 mutants specifically inhibit endosome fusion in vitro (Ref. 15 and this report). These data strongly indicate that Rab5 regulates endocytosis at the level of endosome fusion, although the mechanism by which Rab5 exerts its function remains to be established. Like other GTPases, it is believed that Rab5 cycles between GTP-bound and GDP-bound states. The conversion between these two states is regulated by factors including GAP, GEF, and GDI. GD1 was discovered first as a 54-kDa protein that inhibits GDP dissociation from Rab3A (also called smg p25A) (16). Recently, it has been reported that this GDI also acts on other Rab proteins including Rab5 (17). GAP and GEF stimulate GTPase activity and GDP release, respectively. These activities have been detected for Rab3A (18), Ypt1 (19, 20), Ypt6 (21), and Sec4 (20). In addition to these regulators of the GTP hydrolysis cycle, it is suggested that an effector molecule interacts with the GTP-bound form of Rab to promote vesicle docking and/or the membrane fusion event. To this end, Shirakata et al. (22) have identified an 85-kDa protein that specifically interacts with the GTP-bound form of Rab3A and serves as a potential candidate for the effector molecule of Rab3A. The Rab5-specific regulators and effector(s) are largely unknown.

To facilitate the understanding of Rab5 function, we have generated a number of Rab5 mutants, some of which show altered biological activity (14). The two GTP-binding defective mutants (Rab5:S34N and Rab5:N133I) are of particular interest because they dominantly inhibit endocytosis. This inhibition may result from competition for factors essential for Rab5 function. Therefore, the mutants are valuable tools for dissection of protein-protein interactions between Rab5 and its regulatory factors. In this report, we have initiated a study of the structural features that are required for the inhibitory activities of these two mutants and shed light on the interactions between Rab5 and Rab5-specific factors.

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Rad phagmid mutagenesis system that was based on Kunke1's method (23). The cDNA sequences of Rab5;S34N and Rab5-N133I were subcloned into the unique XbaI restriction site of the PG2 plasmid (24) as previously described (14). Because of the presence of a bacteriophage M13 replication origin, PG2 could be used to produce single-stranded DNA in the presence of the helper phage M13K07. Single-stranded DNA was isolated from the PG2 of Rab5;N133I clone and used as templates for mutagenesis. Three mutagen oligonucleotides were chemically synthesized including 5'-CAAGAGAGATCCGCTGGGCGTCCTG-3', 5'GGGGCCGGTCCTGTCCTACAAA-3', and 5'-GCTTCAACAGGTACATACATAC-3'. They were responsible for the substitution mutants I53A (alanine for isoleucine at residue 53), F57S, and R81A, respectively.

Deletion of the C-terminal tetrapeptide isoprenylation motif (AC4 mutant) was generated using a polymerase chain reaction-based strategy. The DNA template was either PG2-Rab5;S34N or PG2-Rab5-N133I. The 5' primer was 5'-GCTCTAGAATGGCTAGTCAGAGGC-3', which contained an XbaI linker followed by a sequence complementary to the cDNA sequence upstream of the four 3' terminal codons of Rab5. The AC4 mutant cDNAs were amplified by 25 cycles of polymerase chain reaction in the presence of the DNA template and oligonucleotide primers described above.

The entire cDNA sequence of the Rab5 mutants was determined by DNA sequencing (25), and all of the mutations were verified.

Preparation of Recombinant Sindbis Viruses Capable of Expressing the Rab5 and Rab5 Mutants-The cDNAs of the Rab5 mutants were isolated after XbaI (Life Technologies, Inc.) digestion of either the PG2-Rab5 plasmids (for the I53A, F57S, and R81A mutants) or the polymerase chain reaction-amplified AC4 mutant cDNAs. These Rab5 mutant cDNAs were then inserted into the unique XbaI restriction site of the Sindbis virus expression vector pToto1000-3'24 (14, 26), which was previously linearized by XbaI digestion and treated with calf intestinal alkaline phosphatase.

Generation of Sindbis virus stock from the vector plasmid pToto1000-3'24 was previously described (14, 26). Briefly, the plasmid was linearized by XbaI (Life Technologies, Inc.) digestion and used as a template to produce the genome-length recombinant Sindbis virus RNA by in vitro transcription using SP6 RNA polymerase (Life Technologies, Inc.). This viral RNA was infectious and could initiate a complete cycle of virus replication upon transfection of appropriate host cells. In our case, the in vitro transcripts were used to transfect confluent BHK-21 cell monolayers by the Lipofectin-mediated transfection method (27). The cells were maintained in α-MEM containing 5% fetal calf serum (3

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In Vitro Endosome Fusion Assay—Early endosomes loaded with either dinitrophenol-derivatized β-galucuronidase or mannansylated anti-dinitrophenol IgG were prepared from the 774-E clone (a murine macrophage cell line) as previously described (30). The two sets of endosomes were mixed in the presence of an ATP regeneration system, Sephadex G-25 filtered cytosol, and recombinant Rab5 proteins wherever indicated. Endosome fusion was evidenced by the β-galucuronidase activity in the immunocomplexes (30).

In Vitro Isoprenylation Assay—Rab proteins could be used as a source of Rab prenyltansferase to prenylate Rab proteins in vitro (5). 3 μg of recombinant Rab5 proteins were prenylated in a 10-μl reaction mixture containing 774-E cytosol (3.2 mg/ml), 0.5 μM [1-3H]geranylgeranyl pyrophosphate (33,000 dpm/μmol, American Radiolabeled Chemicals, St. Louis, MO), an ATP regeneration system (1 mM ATP, 8 mM phosphocreatine, 31 units/ml creatine phosphokinase), and the reaction buffer (20 mM HEPES/KOH, pH 7.2, 0.5 mM EDTA, 50 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 250 mM sucrose, 0.2 mM GDP). The reaction was conducted at 37°C for either 1 or 4 h. The reaction was stopped by dilution (1:2) with the reaction buffer followed by affinity purification of the prenylated recombinant Rab proteins using the glutathione-Sepharose resin as described above.

RESULTS AND DISCUSSION

The Ras superfamily of small GTPases contains five subfamilies including Ras, Rho, Ran, Arf, and Rab (reviewed in Refs. 31 and 32). Among this group of more than 50 proteins, Ras is the best characterized in terms of its structure-function relationship. A large number of defined mutations in Ras have been extensively studied, and their biochemical properties and functional consequences are known. For example, the mutations in the four GTP/GDP binding motifs result in altered guanine nucleotide binding, GTP hydrolysis, and biological function. Because the GTP/GDP binding motifs are highly conserved among the Ras-related small GTPases, the corresponding mutations in other small GTPases are expected to have similar effects. This contention has proven true in many cases. Rab5 is no exception. Like the corresponding mutations in Ras, the S34N and N133I mutations completely abolished the GTP binding ability of Rab5 (14, 15). Furthermore, these two Rab5 mutants showed dominant inhibitory effect on both receptor-mediated and fluid-phase endocytosis when expressed in cultured BHK-21 cells (13, 14). This inhibition was demonstrated to be at the level of endosome fusion (Ref. 15 and this report). Of the two Rab5 mutants, it was consistently observed that the Rab5:S34N mutant was a more potent inhibitor both in vivo (14) and in vitro (see below). The dominant inhibitory effect shown by the two Rab5 mutants may be explained if the mutants sequester certain factors required for endogenous Rab5 function. In order to investigate these putative protein-protein interactions, we introduced second-site mutations in the Rab5:S34N and Rab5:N133I mutants and examined whether these second-site mutations reversed their inhibitory effects on endocytosis and endosome fusion.

Four secondary mutations were generated including I53A, F57S, R81A, and AC4. These four mutations were chosen based on previous mutational analyses of Rab5 (14) and other Rab proteins (19, 33). The AC4 mutant had the C-terminal isoprenylation motif (CCSN) deleted. As a result, this mutant was not
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Fig. 1. Expression of Rab5 and Rab5 mutants in BHK-21 cells. Shown is an autoradiogram of a 12% gel containing Rab5 and Rab5 mutants metabolically labeled with [35S]methionine in BHK-21 cells. Lane 1, a sample from vector virus-infected cells as a negative control; lane 2, wild-type (WT) Rab5; lane 2, the Rab5:S34N mutant; lane 4, the double mutant containing S34N,I53A mutations; lane 5, the double mutant containing S34N,F57S mutations; lane 6, the double mutant containing S34N,R81A mutations; lane 7, the double mutant containing S34N,A4C4 mutations; lane 8, the Rab5:N133I mutation; lane 9, the double mutant containing N133I,I53A mutations; lane 10, the double mutant containing N133I,F57S mutations; lane 11, the double mutant containing N133I,R81A mutations; lane 12, the double mutant containing N133I,A4C4 mutations. Molecular mass standards (in kilodaltons) are indicated on the left.

Fig. 2. Horseradish peroxidase uptake in BHK-21 cells expressing the Rab5 mutants. Shown is a bar graph indicating a 1-h horseradish peroxidase (HRP) accumulation in BHK-21 cells infected with either the vector virus as a control or the recombinant viruses expressing the Rab5 mutants (indicated). Each infection was done in triplicate, and the calculated standard deviation is shown. These results were reproducible in independent experiments.

The aforementioned mutations were introduced into the Rab5:S34N and Rab5:N133I mutants. The double mutants were expressed in cultured BHK-21 cells via a Sindbis virus vector, and the protein expression was analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 1, the Rab5 mutants were expressed to the same levels. At 5 h postinfection, the level of Rab5 expression was about 10-fold over endogenous Rab5 as quantified by Western blot (data not shown). Because of post-translational isoprenylation and possibly other unidentified modifications, Rab5 was resolved as a doublet by SDS-polyacrylamide gel electrophoresis (Fig. 1). It was previously shown that the faster mobility form was the isoprenylated mature form associated with membranes (35). Therefore, the relative abundance of this form was indicative of how well the protein was post-translationally processed. By this criteria, it was apparent that the N133I but not the S34N mutation resulted in decreased post-translational processing (Fig. 1, lanes 7 and 12). The faster mobility of the A4C4 mutants was due to deletion of the C-terminal tetrapeptide rather than post-translational isoprenylation. Interestingly, the R81A mutation also significantly decreased the post-translational processing of the mutants, especially the Rab5:N133I mutant (Fig. 1, lanes 6 and 11). The I53A and F57S mutations affected these events to a smaller degree. In addition, it was noted that the F57S mutation resulted in mobility shift of the proteins (Fig. 1, lanes 5 and 10). This phenomenon was probably due to alterations in charges and SDS binding.

We next examined the functional significance of these secondary mutations on Rab5:S34N and Rab5:N133I. The Rab5 mutants were expressed in cultured BHK-21 cells via a Sindbis virus vector. Endocytic activity of the cells was examined by following the uptake of a fluid-phase endocytic marker (horseradish peroxidase). In comparison with the control cells infected with the vector virus, cells that were infected with the recombinant viruses expressing either Rab5:S34N or Rab5:N133I showed decreased horseradish peroxidase uptake (Fig. 2). This inhibitory activity of Rab5:S34N and Rab5:N133I was reversed either by deletion of the C-terminal isoprenylation motif (A4C4) or by a substitution mutation in the effector domain (F57S) (Fig. 2). Another effector domain mutation (I53A) had no effect (Fig. 2). For the R81A mutation, while it only slightly reduced the inhibitory activity of Rab5:S34N, it essentially abolished that of Rab5:N133I (Fig. 2).

These results indicated that C-terminal isoprenylation is essential for Rab5:S34N and Rab5:N133I to inhibit horseradish peroxidase uptake in BHK-21 cells. We further demonstrated the importance of C-terminal isoprenylation for the inhibitory effect of Rab5:S34N and Rab5:N133I on endosome fusion in vitro. Recombinant Rab5 and Rab5 mutants were produced as glutathione S-transferase fusion proteins in E. coli and affinity purified using glutathione-Sepharose resin. The recombinant proteins were isoprenylated by incubation with the cytosol prepared from the macrophage cell line J774-A.1. Under these conditions, recombinant Rab5 and Rab5:S34N were efficiently prenylated, while the prenylation of Rab5:N133I was much less efficient (Fig. 3) (36). No prenylation was observed for the Rab5:A4C4 mutant that lacked the C-terminal tetrapeptide CCSN (Fig. 3). Upon addition to the in vitro endosome fusion assay, the prenylated Rab5:S34N and Rab5:N133I effectively blocked endosome fusion (Fig. 4A), while their nonprenylated counterparts were poor inhibitors (Fig. 4B), demonstrating that C-terminal isoprenylation is necessary for Rab5:S34N and Rab5:N133I to inhibit endosome fusion. Since Rab5:N133I was a poor substrate for isoprenylation both in vivo (Fig. 1) and in vitro (36) (Fig. 3), this could partly explain the observation that Rab5:N133I was a less potent inhibitor than Rab5:S34N. Along this line, the R81A mutation reversed the inhibitory activity of Rab5:N133I (Fig. 2) because this mutation nearly abolished its post-translational isoprenylation (Fig. 1). It should be pointed out, however, that the C terminus-truncated Rab5:S34N and Rab5:N133I mutants retain residual inhibitory activity (Fig. 2), indicating that the nonprenylated mutants can still sequester the putative factor (but very inefficiently).

Rab5:S34N and Rab5:N133I may block endocytosis and endosome fusion by competing for a Rab5-specific GEF. While this factor is yet to be identified, several lines of evidence support
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Fig. 3. In vitro isoprenylation of recombinant Rab5 and Rab5 mutants. 3 μg of affinity-purified recombinant Rab5 proteins were prenylated by incubation with J774-E cytosol (3.2 mg/ml) as described under “Experimental Procedures.” The incubation was conducted at 37 °C for either 1 or 4 h (indicated) in the presence of [3H]geranylgeranyl pyrophosphate. The [3H]-labeled (prenylated) proteins were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by fluorography and autoradiography. The results were reproducible in independent experiments. WT, wild type.

This contention. First, both Rab5 mutants require common structural features (i.e., the effector domain Ph57 residue and C-terminal isoprenylation) to inhibit endocytosis, suggesting that they sequester the same factor. Second, it has been reported that the Ras equivalents of Rab5:S34N and Rab5:N133I (Ras:S17N and Ras:N116I) inhibit endogenous Ras function by sequestering a Ras-specific GEF (37–39), and the inhibition by Ras:S17N requires C-terminal isoprenylation (37). Third, neither Rab5 mutant contains detectable GDP or GTP in cells (data not shown). It is known that nucleotide-free forms are favored by some nucleotide exchange factors (33, 40, 41). Finally, Burstein et al. (33) have shown that the effector domain phenylalanine residue of Rab3A is important for its interaction with a GEF. C-terminal processing is also important for this interaction (42), although their initial experiments suggested the opposite (33). While it is possible that C-terminal isoprenylation is directly involved in the interaction between Rab5 and its GEF, it cannot be ruled out that isoprenylation may play an indirect role in such an interaction. In other words, isoprenylation may serve to attach the Rab protein to the membrane where the protein-protein interaction actually occurs.

Although some nucleotide exchange factors have been found in cytosol, the efficiency of interaction with respective small GTPases can be greatly improved upon activation and localization to the target membranes (43), presumably by increasing the local concentration.

Two possibilities may account for the observation that the Ph57 residue in the effector domain is crucial for the inhibitory activity of Rab5:S34N and Rab5:N133I. The Ph57 residue may be directly involved in the interaction with a Rab5-specific GEF. Alternatively, the F57S mutation might cause changes in other regions of the molecule that in turn contribute to the disruption of protein-protein interactions between the Rab5 mutants and the GEF. While this latter possibility cannot be ruled out, it seems less likely for the following reasons. Based on the structural model of Ras, single substitution mutations in this region cause only limited local changes rather than alter the overall conformation of the molecule (34). Furthermore, the F57S mutation has little effect on GTP binding, and the corresponding Rab3A mutant responds to Rab3A-GAP normally (39), indicating no dramatic changes in protein conformation. Expected effects from the double mutation T52A,L53S (41) in the effector domain has little effect on the inhibitory activity of Rab5:S34N and Rab5:N133I. Taken together with our previous observation that the biological activity of Rab5 is not significantly altered by the double mutation T52A,L53S (14), these data indicate that Ile53 is not a critical residue for Rab5 function. These results are intriguing since the isoleucine residue is highly conserved among Ras-related small GTPases and is essential for the biological function of Ras and some Rab proteins. In the case of Ras, this isoleucine residue is so restricted that even isoleucine analogs cannot functionally replace it (44). This residue is essential for Ras to respond to Ras-GAP (44, 45) and for its biological function (46, 47). In the case of some Rab proteins (e.g. Ypt1 and Rab3A), the corresponding amino acid residue is also important for interaction with their specific GAPs and for their biological function (19, 33). A conservative mutation from isoleucine to methionine at this position of Ypt1 results in a lethal phenotype in yeast and abolishes its sensitivity to GAP (19). In addition, this position is essential for almost every interaction between Rab3A and its regulators and a putative effector, Rab3A-GEF (33), Rab3A-GAP (33), Rab3A-GDI (42), and Rab3A-rabphilin (48). These results suggest certain functional diversity in this conserved isoleucine residue among Ras-related small GTPases.

It is of interest to compare Rab5 and other small GTPases with the heterotrimeric GTPases involved in signal transduction, especially given the recent crystallography data on the α subunit of a heterotrimeric GTPase (Gα) (49). Although there is little homology in primary sequence, the GTP/GDP binding motifs are highly conserved, and the three-dimensional structure of the nucleotide-binding domain is remarkably similar. Furthermore, the C-terminal region is essential for interaction with a guanine nucleotide exchange factor.

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