Identification of a Novel Rab11/25 Binding Domain Present in Eferin and Rip Proteins*

Rytis Prekeris‡‡§, Jason M. Davies‡, and Richard H. Scheller‡¶¶

From the ‡Howard Hughes Medical Institute, Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, California 94305-5428 and §Genentech Inc., South San Francisco, California 94080-4990

Rab11, a low molecular weight GTP-binding protein, has been shown to play a key role in a variety of cellular processes, including endosomal recycling, phagocytosis, and transport of secretory proteins from the trans-Golgi network. In this study we have described a novel Rab11 effector, EF-hands-containing Rab11-interacting protein (Eferin). In addition, we have identified a 20-amino acid domain that is present at the C terminus of Eferin and other Rab11/25-interacting proteins, such as Rip11 and nRip11. Using biochemical techniques we have demonstrated that this domain is necessary and sufficient for Rab11 binding in vitro and that it is required for localization of Rab11 effector proteins in vivo. The data suggest that various Rab effectors compete with each other for binding to Rab11/25 possibly accounting for the diversity of Rab11 functions.

Members of the Rab/Ypt GTPase family have emerged as important regulators of vesicular trafficking (1). Rab proteins have been proposed to mediate a variety of functions, including vesicle translocation and docking at specific fusion sites. Like all small GTPases, Rab cycle between active (GTP-bound) inactive (GDP-bound) conformations (2). In the GTP-bound state, Rab proteins can bind a variety of downstream effector proteins, while GDP hydrolysis leads to a conformational change in the switch region that renders the Rab GTPase unrecognizable to its effector proteins (3, 4). A key question in understanding the interactions between Rab and their effectors concerns the mechanisms by which Rab GTPases specifically bind a diverse spectrum of effectors and how this is regulated by the common structural motif used as a GTP switch. Biochemical and genetic studies have identified several hypervariable regions that might be involved in determining Rab specificity, including N and C termini, as well as the a3/β5 loop (5, 6). Indeed, the recently reported structure of Rab3a bound to a putative effector, rabphilin-3a, revealed that the Rab3a-rabphilin-3a complex interacts through two main regions (7). The first consists of conformationally sensitive switch regions of Rab3a bound to the a1 helix and the C-terminal part of rabphilin-3a. The second involves the SGAWFF domain of rabphilin-3a, which fits into a pocket formed by the three hypervariable complementary determining regions (CDRs) of Rab3a, corresponding to the N and C termini and the a3/β5 loop. Thus, it appears that the hypervariable RabCDR is involved in determining the specificity of effector binding, while the conserved switch regions impart GTP dependence and binding. It remains to be determined, however, whether this paradigm also applies to other Rab-effector complexes.

Rab11a, 11b, and 25 are closely related members of Rab GTPase family that have been implicated in regulating a variety of different post-Golgi trafficking pathways, such as protein recycling (8), phagocytosis (9), insulin-stimulated Glut4 insertion in the plasma membrane (10), and membrane trafficking from early endosomes to the trans-Golgi network (11). During the last few years several Rab11/25-interacting proteins have been identified, including Rab11BP/rabphilin-11, Rip11, nRip11, and myosin Vb (12–15). However, the mechanisms of their function, as well as molecular aspects of their interactions with Rab11, remain to be fully understood. In the present study, we report the identification of EF-hands-containing Rab11/25-interacting protein (Eferin). Furthermore, we characterized a Rab binding domain (RBD11) that is present at the C terminus of Eferin as well as other Rab11/25-binding proteins, such as Rip11 and nRip11. Using biochemical techniques, we demonstrated that RBD11 is the region that encodes the specificity for Rab11/25 but is distinct from the region interacting with the Rab switch domain, since its interactions with the Rab11/25 are not GT-dependent.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—Cell culture reagents were obtained from Life Technologies, Inc. unless otherwise specified. Miscellaneous chemicals were obtained from Sigma. Actin rhodamine conjugates were purchased from Molecular Probes (Eugene, OR). Mouse monoclonal anti-Myc antibody (9E10) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Fluorescein isothiocyanate-labeled anti-rabbit IgG and Texas Red-labeled anti-mouse IgG antibodies were obtained from Jackson Immunoresearch Laboratories (West Grove, PA).

Cell Culture and Immunofluorescence Microscopy—Madin-Darby canine kidney (MDCK II) cells were cultured, and immunofluorescence microscopy was performed as described previously (16). For immunofluorescence microscopy, cells were fixed with 4% paraformaldehyde for 15 min. Cells were then permeabilized in 0.4% saponin and nonspecific sites blocked with phosphate-buffered saline containing 0.2% BSA, 0.4% saponin, and 1% bovine serum. Following incubation with antibodies, samples were extensively washed and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Immunofluorescence localization was performed using a Molecular Dynamics laser confocal imaging system (Beckman Center Imaging Facility, Stanford University, Stanford, CA).

Received for publication, July 2, 2001

To whom correspondence should be addressed: Genentech Inc., 1 DNA Way, South San Francisco, CA 94080-4990. Tel.: 650-225-4952; Fax: 650-225-4265; E-mail: scheller@gene.com.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF395731.

† To whom correspondence should be addressed: Genentech Inc., 1 DNA Way, South San Francisco, CA 94080-4990. Tel.: 650-225-4952; Fax: 650-225-4265; E-mail: scheller@gene.com.

‡ The abbreviations used are: CDRs, complementary-determining regions; RBD, Rab binding domain; MDCK, Madin-Darby canine kidney; BSA, bovine serum albumin; GST, glutathione S-transferase; ERM, ezrin/radixin/moesin; GTP, guanosine 5'-3-O-(thio)triphosphate; GTPγS, guanosine 5'-2-O-(thio)triphosphate; GTPγS, guanosine 5'-3-O-(thio)triphosphate; GTP, green fluorescent protein; X gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.
Identification of Novel Rab11 Binding Domain

Eferin Is a Novel Rab11 and Rab25-binding Protein—Despite the accumulating evidence implicating Rab11/25 GTPases in regulating multiple membrane trafficking pathways, we still know very little about the mechanism of their function (17). Amino acid sequence analysis using PROSITE locating amino acid residues 507–653) motif indicated that the primary Rab11/25 binding domain is likely present in Rip and Eferin proteins.

Identification of a Novel Rab11 Binding Domain (RBD11) Present in Rip and Eferin Proteins—In addition to Eferin, the yeast two-hybrid screen yielded 10 other clones that specifically interacted with Rab11 and Rab25. Seven of these clones encode fragments of Rip11 (AF334812), while the other three were identified as nRip11 (AY037299), Rip11-(200–507) (13). To test whether both regions of Rip11 are involved in association with Rab11, we performed immunofluorescence studies using transiently transfected MDCK cells that showed that, similarly to Rab11, in epithelial cells Eferin-GFP is localized to the apical pole (Fig. 1E).
Identification of Novel Rab11 Binding Domain

proteins, the alignment of Rab11/25-interacting domains revealed that Eferin, Rip11, and nRip11 contain a highly conserved domain of 20 amino acids at the very C terminus of the protein (Fig. 3A). Deletion of these residues in Rip11 and Eferin resulted in loss of binding to Rab11 (Figs. 2C and 3B), suggesting that these amino acid residues might directly participate in Rab11/25 binding. To address that possibility we synthesized the peptide corresponding to Rip11-(628–653) and used it in competition assays. As previously reported (13), in non-polarized MDCK cells Rip11-GFP was distributed throughout the entire cell, whereas in polarized MDCK cells, Rip11-GFP is present predominately on the plasma membrane and shows no co-localization with Rab11a-containing endosomes. Furthermore, in polarized cells Rip11ΔRBD11-GFP has lost its polarized distribution and is present throughout the entire cell (Fig. 4, J–L). As in non-polarized cells, a large portion of Rip11ΔRBD11-GFP is present on the plasma membrane, although some Rip11ΔRBD11-GFP also shows cytosolic staining (Fig. 4J).

RBD11 Is Necessary and Sufficient to Mediate Specific Interactions between Rip11 and Rab11—The data presented above demonstrate that Rab11 is necessary for Rab11/25 binding to Eferin and Rip proteins. To determine whether RBD11 is sufficient for Rab-specific and GTP-dependent Rab11/25 binding, the peptides corresponding to both Rip11-RBD11 and Rip11-(1–17) were conjugated to BSA-coated agarose beads and used in Rab11 pull-down assays. As shown in Fig. 5A, while Rab11 did not co-sediment with BSA alone or BSA-N pept, it bound to BSA-RBD11. The binding was specific since it was observed only with Rab11a (Fig. 5B) and Rab11b (data not shown) but not Rab1a and Rab3a. Furthermore, the BSA-RBD11/Rab11a interactions could be inhibited with soluble RBD11 in a concentration-dependent manner (Fig. 5C). To test whether full-length RBD11 is necessary for Rab11/25 interactions, we expressed RBD11 as a GST fusion protein. In agreement with data reported above, Rab11a also interacted with GST-RBD11. Truncations of the RBD11 motif resulted in decreased Rab11a binding, suggesting that intact RBD11 is necessary for binding to Rab GTPases (Fig. 5D). Surprisingly, while BSA-RBD11 specifically interacted with Rab11/25 proteins, it showed no GTP dependence (Fig. 5D). Perhaps, RBD11 determines Rab specificity by interacting with RabCDR, while additional motifs interacting with Rab switch I/II domains are responsible for the GTP-dependent component.

DISCUSSION

Rab11 is a small GTPase that was implicated in regulating a variety of distinct membrane trafficking steps (8–10). How-
permeabilized with saponin and then stained for Rab11a (B) 72 h before imaging (A). Cells were plated on Transwell filters and grown for 2 days on collagen-coated glass coverslips (non-polarized) (C) to analyze subcellular localization of Rip11 and Rip11Alial cells. MDCK cells were transfected with either Rip11-GFP (D) or Rip11/H9004,2 of varying concentrations of soluble Rab11A, Rab3a, or Rab11a in the presence (E) or absence (F) of varying concentrations of soluble Rab11D (G). Bound proteins were analyzed as described above. The role of RBD11 in Rip11 subcellular localization. To determine the specificity of Rab11 binding to RBD11, varying concentrations of soluble Rab11D were incubated with Affi-Beads conjugated to BSA-RBD11 in the presence of either GTP-S (top row) or GDP-GS (bottom row). For protein pull-down assays using Affi-Bead conjugates, RBD11 peptide was attached to Affi-Beads using Imject maleimide-activated BSA (Pierce). After incubation with different Rab GTPases, beads were washed, and bound proteins analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. Start represents 20% of the recombinant protein used in the assay. A, purified recombinant Rab11A was incubated with Affi-Beads loaded with BSA alone (BSA), BSA-Rip11(1-25) (BSA-N pept), or BSA-RBD11 (BSA-RBD) in the presence of 1 mM GTP-S. B-C, to determine the specificity of Rab11 binding to RBD11, Affi-Beads-BSA (BSA) or Affi-Beads-BSA/RBD11 (BSA-RBD) were incubated either with 50 μg of Rab1a, Rab3a, or Rab11a in the absence (B) or presence of varying concentrations of soluble RBD11 (C). Bound proteins were analyzed as described above. D, to analyze the GTP dependence of Rab11 binding to RBD11, varying concentrations of Rab11a were incubated with Affi-Beads conjugated to BSA-RBD11 in the presence of either GTP-S (top row) or GDP-GS (bottom row). E, to further map the Rab11 binding domain, DNA coding for wild-type GST-RBD11 and several RBD11 truncation/deletion mutants were fused to GST. Fusion proteins were purified and used in GST pull-down assays to determine their ability to bind recombinant Rab11a. Recombinant Rab11a was bound for 1 h at 4 °C to glutathione beads coated with equal amounts of various GST-RBD11 constructs. The beads were then washed and samples eluted with 1% SDS, followed by separation on SDS-polyacrylamide gels and staining with Coomassie Blue. Gels were then scanned, and the amount of Rab1a bound was determined using Image software. The values were expressed as a percentage of Rab1a bound to wild type GST-RBD11 fusion protein.

ever, the molecular mechanisms involved in regulation and determining the specificity of Rab11 functions remain to be understood. The ever growing number of Rab11-binding proteins suggests that sequential or competitive interactions of Rab11 with different effector proteins might account for the diversity of Rab11 functions. In this study, we have identified a novel Rab11/25-interacting protein and named it Eferin. Several lines of evidence suggest that Eferin is an effector for Rab11/25 GTPases. First, Eferin binds specifically to Rab11/25 but not other Rabbs. Second, Eferin preferentially associates with the GTP-bound, thus active, form of Rab11. Third, Eferin co-localizes with Rab11 and Rab25 (data not shown). Thus, our data suggest that Eferin is an effector protein for Rab11/25 GTPases, although its role in membrane trafficking remains to be elucidated.

Immunofluorescence studies using transiently transfected MDCK cells showed that Eferin-GFP is localized exclusively to the apical plasma membrane, suggesting that Eferin might be involved in regulation of apical targeting. Interestingly, besides EF-hands, Eferin also contains a region resembling the C-terminal domain of the ezrin/radixin/moesin protein family, also known as ERM proteins, which also localize near the apical plasma membrane in actin-rich cytoskeletal structures (18–20). ERM proteins can form homo- and heterodimers via C-terminal interactions with the N-terminal FERM domain (21). The FERM/C-terminal interaction masks the binding sites for other molecules, in this way regulating ERM protein association with the cytoskeleton (22). Thus, it is tempting to speculate that the Rab11-Eferin complex might interact with the FERM domain of the ERM proteins, in this way regulating ERM protein activity and cross-linking membranes to the cytoskeleton.

The ever growing Rab11 binding protein family already includes five members. The ability to interact with several effector proteins seems to be a common feature of many Rab GTPases (23). The main challenge of future studies will be to determine the functions of all of these proteins, as well as to understand the mechanisms of their interactions with Rab proteins. Indeed, it remains unclear whether two effector proteins compete with each other for binding to Rabbs or work in a consecutive fashion. The work presented here suggests that Rips and Eferin compete for interactions with Rab11/25. Indeed, identification of a common Rab11/25 binding domain indicates that Eferin and Rips use the same binding site on Rab11/25. Interesteringly, RBD11 is not present in the other known Rab11/25 effector proteins, such as myosin Vb and
Identification of Novel Rab11 Binding Domain

Rab11BP/rabphilin-11 (12, 14, 15). It will be interesting to see whether these proteins can actually co-bind to Rab11/25 with either Eferin or Rips, perhaps forming signaling complexes that regulate transport vesicle trafficking.

While RBD11 appears to be involved in determining the specificity of interactions with Rab GTPases, its binding is independent of GTP. Perhaps, RBD11 is an equivalent of a SGAWFF motif in rabphilin-3a, which interacts with Rab3a CDRs (7). If this is the case additional motifs will be required to interact with Rab switch I/II domains to provide the GTP-dependent component of binding. Thus, it is likely that Eferin and Rip proteins interact with Rab11/25 via several motifs. At least some of the GTP-sensing motifs are encoded within the 60-amino acid domain located upstream of RBD11. Interestingly, while the putative GTP-sensing regions in Rip11 and nRip11 are very highly conserved, they have no apparent homology to Eferin. Thus Rip and Eferin proteins share the common motif involved in Rab11/25 recognition but may use different mechanisms for mediating the GTP-dependent component of Rab11/25 binding.

The functional significance of the differences in Rip and Eferin interactions with Rab11/25 remains to be determined. One possibility is that additional cellular factors can regulate the affinity of Rab11/25 binding to its effectors. Indeed, the recombinant full-length Rip11 binds poorly to Rab11a in pull-down and yeast two-hybrid assays as compared with full-length endogenous Rip11 from cellular TX-100 extracts (data not shown). Furthermore, it has been previously shown that Rip11 can also interact with γ-SNAP and cytoskeleton (13, 24). Thus, the interactions of Rips and Eferin with different factors could be used as a means of differentially regulating Rab11/25 binding. Alternatively, the Rab11/25 binding motif in Eferin and Ripl11 might be conformationally hidden and require activation before binding to Rab11/25. We have previously demonstrated that phosphorylation of Rip11 plays an important role in its trafficking (13). Thus, differential phosphorylation on Rab11/25 binding motifs could also play a role in regulating the binding of Rip11 and Eferin to Rab GTPases.

Despite the recent progress in understanding the roles of Rabs and their effectors in regulating membrane trafficking, we are only beginning to unravel the structural determinants of their function. Identification and characterization of the Rab11/25 binding regions in Rip and Eferin proteins will be of crucial importance in understanding the molecular mechanisms involved in differential regulation of the variety of Rab11-dependent trafficking pathways.

Acknowledgments—We thank Dr. Susan L. Palmieri (Stanford University, cell imaging facility) for assistance with confocal microscopy. We are grateful to Dr. T. Nagase for providing cDNA encoding KIAA0665 (Eferin). We also thank Dr. Suzie Scales for the critical reading of the manuscript.

REFERENCES
1. Gonzalez, L., Jr., and Scheller, R. H. (1999) Cell 96, 755–758
2. Bourne, H. R., Sanders, D. A., and McCormick, F. (1991) Nature 349, 117–127
3. Dumas, J. J., Zhu, Z., Connolly, J. L., and Lambright, D. G. (1999) Structure Fold Des. 7, 413–423
4. Esters, H., Alexandrov, K., Constantinescu, A. T., Goody, R. S., and Scheidig, A. J. (2000) J. Mol. Biol. 298, 111–121
5. Steinmann, H., Valenica, A., Martinez, O., Ullrich, O., Goud, B., and Zerial, M. (1994) EMBO J. 13, 575–583
6. Merihiw, E., Hatherly, S., Dumas, J. J., Lawe, D. C., Heller-Harrison, R., and Lambright, D. G. (2001) J. Biol. Chem. 276, 13982–13988
7. Ostermeier, C., and Brunger, A. T. (1999) Cell 96, 363–374
8. Ullrich, O., Reinsch, S., Urbe, S., Zerial, M., and Parton, R. G. (1996) J. Cell Biol. 135, 913–924
9. Cox, D., Lee, D. J., Dale, B. M., Calafat, J., and Greenberg, S. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 680–685
10. Kessler, A., Tomas, E., Immler, D., Meyer, H. E., Zorzano, A., and Eckel, J. (2000) Diabetologia 43, 1518–1527
11. Wileke, M., Johannes, L., Galli, T., Mayau, V., Goud, B., and Salamero, J. (2000) J. Cell Biol. 151, 1207–1220
12. Lapiere, L., Kumar, R., Hales, C. M., Navarre, J., Bhartur, S. G., Burnette, J. O., Provance, D. W., Jr., Mercer, J. A., Bahler, M., and Goldenen, J. R. (2001) Mol. Biol. Cell 12, 1843–1857
13. Prekeris, R., Klumperman, J., and Scheller, R. H. (2000) Mol. Cell 6, 1437–1448
14. Zeng, J., Ren, M., Gravotta, D., De Lemos-Chiarandini, C., Luii, M., Erdjument-Bromage, H., Tempst, P., Xu, G., Shen, T. H., Morimoto, T., Adesnik, M., and Sabatini, D. D. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2840–2845
15. Mamamoto, A., Ohtsuka, T., Hotta, I., Sasaki, T., and Takai, Y. (1999) J. Biol. Chem. 274, 25517–25524
16. Steegmaier, M., Lee, K., Prekeris, R., and Scheller, R. (2000) Traffic 1, 583–591
17. Nagase, T., Ishikawa, K., Suyama, M., Kikuno, R., Hiroswa, M., Miyajima, N., Tanaka, A., Kotani, H., Nomura, N., and Ohara, O. (1998) DNA Res. 5, 365–364
18. Sato, N., Funayama, N., Nagafuchi, A., Yonemura, S., and Tsukita, S. (1992) J. Cell Sci. 103, 131–143
19. Funayama, N., Nagafuchi, A., Sato, N., and Tsukita, S. (1991) J. Cell Biol. 115, 1039–1048
20. Bretcher, A. (1999) Curr. Opin. Cell Biol. 11, 109–116
21. Gary, R., and Bretcher, A. (1995) Mol. Biol. Cell 6, 1061–1075
22. Pearson, M. A., Rezek, D., Bretcher, A., and Karplus, P. A. (2000) Cell 101, 259–270
23. Christofofides, S., McBride, H. M., Burgoyne, R. D., and Zerial, M. (1999) Nature 397, 621–625
24. Chen, D., Xu, W., He, P., Medrano, E. O., and Whiteheart, S. W. (2001) J. Biol. Chem. 276, 13127–13135