An Evolutionarily Conserved Domain in a Subfamily of Rabs Is
Crucial for the Interaction with the Guanyl Nucleotide Exchange
Factor Mss4*

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Mss4 is a guanine nucleotide exchange factor that specifically binds to, and promotes GDP-GTP exchange on, a subset of the Rab GTPases (Burton, J. L., Burns, M. E., Gatti, E., Augustine, G. J., and De Camilli, P. (1994) EMBO J. 13, 5547–5558). In order to identify the domain(s) of the GTPase that is important for this interaction, protein chimerae were constructed between Rab3a, which binds Mss4, and Rab5a, which does not bind Mss4. We have identified the amino-terminal portion of Rab3a as the Mss4-binding region, with the effector domain being critically required for binding and the flanking regions further enhancing the interaction. Sequence comparisons have revealed that Mss4-binding Rabs share more homology with each other than with Rabs that do not bind Mss4. The region of highest homology between these Rabs, which defines them as members of the same evolutionary branch within the Rab subfamily, coincides with the domain shown here to be critical for Mss4 binding. A mutation in the zinc-binding domain of Mss4 (Mss4 D96H), a region that is highly conserved between Mss4 and its yeast homologue Dss4, completely abolished its property to bind to, and promote GDP-GTP exchange on, Rab3a. Thus, the preservation of the Mss4/Dss4-GTPase interaction appears to have been a critical factor in the evolution of this subset of Rab proteins.

The Rab GTPases, members of the Ras GTPase superfamily, are thought to play a central role in vesicular transport events within the cell. The molecular mechanism utilized by these proteins to control membrane traffic remains unclear. Experimental evidence suggests that their cycling between GDP- and GTP-bound conformations is critical for their function, and accessory proteins that influence the nucleotide state and their membrane-association have been identified (1–3). Such proteins include guanine nucleotide exchange factors (GEFs), which stimulate GDP-GTP exchange, GTPase-activating proteins (GAPs), which stimulate GTP-hydrolysis, and guanine dissociation inhibitors (GDIs), which stabilize the GTPase in a GDP-bound conformation and promote membrane dissociation of the GDP-bound form of the molecule (2–5).

Determining the domains of the Rab GTPases that are important for their subcellular localization and for their interaction with their accessory proteins may provide clues as to how these GTPases function in membrane trafficking. The carboxyl terminus or “hypervariable domain” of the Rabs has been shown to be important for their specific localizations within the cell (6–8). The “effector domain” (L2/b2 according to Ras nomenclature), together with the L3/b3 domain, the hypervariable domain, and the geranylgeranylated C terminus has been demonstrated to be involved in binding to Rab GDI, suggesting that GDI may shield the hypervariable domain and the lipid moiety of the Rabs, thereby preventing membrane association (9). Very little is known however, about the Rab GAPS or GEFs and the domains of the Rabs with which they interact.

The only two GEFs molecularly identified thus far for Rab GTPases are Mss4 (and its yeast counterpart, Dss4) and the recently identified p619 protein, which appears to stimulate guanine nucleotide release from both Arf1 and some Rab family members (10–12). Mss4 binds to, and stimulates GDP-GTP exchange on, a subset of the Rab GTPases that belong to the same branch of the Ras superfamily (13). To identify the Mss4 binding site on the GTPase, we constructed chimeric molecules between Rab3a, which binds Mss4, and Rab5a, which does not bind Mss4. Using this approach, we have established that the Rab domain necessary for binding to Mss4 coincides with its amino-terminal domain, which defines Mss4-binding proteins as members of the same branch within the Rab family tree. A point mutation in the zinc-binding domain of Mss4 disrupted Mss4 binding to, and GDP-releasing activity from, Rab3a, confirming the critical role of this domain in Mss4 function.

**EXPERIMENTAL PROCEDURES**

Materials—The following reagents were purchased from commercial sources: [35S]-GTP-γ-S (1300 Ci/mmol) and [35S]-protein A (9.2 μCi/μg) from DuPont NEN; [35S]sulfur labeling reagent from Amersham Corp.; restriction enzymes, Vent DNA polymerase, Amylose-Sepharose, and anti-MBP antibodies from New England Biolabs (Beverly, MA); T4 DNA ligase (5 units/μl) from Boehringer Mannheim; Sequenase 2.0 sequencing kit from U.S. Biochemical Corp.; pET15b bacterial expression vector from Novagen (Madison, WI); Ni2+ -agarose from Qiagen (Chatsworth, CA); goat anti-rabbit IgG conjugated to alkaline phosphatase from Bio-Rad; nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate tetrazolium, and imidazole from Sigma; Altered Sites II Mutagenesis system from Promega (Madison, WI); and nitrocellulose circular filters (0.45 μm) from Millipore (Bedford, MA).

DNA Constructs—All DNA manipulations were performed using standard procedures (14). Rab3aΔC1 and Rab3aΔC2 truncation mutants were made by introducing a premature stop codon in the Rab3a cDNA at aa positions 218 and 187, by site-directed mutagenesis using the pALTER-Ex1 vector of the Altered Sites II mutagenesis system and the mutagenic oligonucleotides 5’-CATCAGGATTGAGCCTGCTGA-3’.
and 5'-ATCTGTTGAGAATGTCGGAGTGCCATA-3', respectively. For chimeras between Rab3a and Rab5a (Rab3/5), the Rab5a cDNA was subcloned into the pALTER-Ex1 vector, and restriction sites were created within Rab5a by site-directed mutagenesis to allow easy insertion of various Rab3a cDNA pieces that were produced by the polymerase chain reaction (PCR) with Vent DNA polymerase. For Rab3/5 I and III constructs, a Rab5a cDNA was used in which an EcoRI restriction site was removed from the vector using the oligonucleotide 5'-GGCCGAGATTCCTGA-3', and EcoRI and SacI restriction sites were created within the Rab5a cDNA by base pair changes at nucleotide position 144 with the oligonucleotide 5'-CAATTTTCAATTCTCCAGAGAAGTCCGCCTGTC-3', respectively. Note that the resulting SacI restriction site within the Rab5a cDNA results in a conservative aa change of isoleucine to leucine at aa position 73. For Rab3/5 I, DNA encoding aa 51–73 of the Rab3a cDNA replaced the coding DNA for aa 48–72 of Rab5a by subcloning into the EcoRI and SacI restriction sites within the Rab5a cDNA. Rab3/5 III was produced by amplifying the Rab3a cDNA region encoding aa 1–73, which was used to replace the cDNA region encoding aa 1–72 of Rab5a using the NcoI and SacI restriction sites. For Rab3/5 II, IV, V, and VI constructs, a Rab5a cDNA/pALTER-Ex1 construct was made that, like the Rab5a cDNA above, had a deleted EcoRI site in the vector and a created EcoRI site in the Rab5a cDNA, but no SacI site. For Rab3/5 II, the cDNA encoding aa 51–102 of Rab5a was used to replace the region encoding aa 48–101 of Rab5a using the EcoRI and EcoRV restriction sites. Rab3/5 IV was made by PCR amplification of the Rab3a cDNA region encoding aa 1–102, which replaced the Rab5a cDNA encoding aa 1–101 using the NcoI and EcoRV restriction sites. For Rab3/5 V, aa 1–47 of Rab3a and aa 1–48 of Rab5a were replaced by aa 1–48 of Rab3a by subcloning the PCR product for this region of Rab3a into the NcoI and EcoRI restriction sites of the Rab5a cDNA. For Rab3/5 VI, aa 1–47 of Rab5a were replaced by aa 20–48 of Rab3a using the NcoI and EcoRV restriction sites. Rab3/5 VII was made by PCR amplification of Rab3/5 III using oligonucleotides that result in the removal of the DNA encoding the first 29 aa of Rab3a in the Rab3/5 III construct. Sequence for all of the chimeric constructs was confirmed by dideoxysequencing. All of the above constructs, as well as wild-type Rab3a and Rab5a cDNAs, were PCR-amplified using Vent DNA polymerase with the appropriate oligonucleotides containing NdeI and BamHI restriction sites for subcloning into the bacterial expression vector pET15b.

For the Ms4 D96H point mutation, the coding region of Ms4 was amplified by PCR and subcloned into the pALTER-Ex1 vector using the XhoI and HindIII restriction sites. The mutation was introduced using the mutagenic oligonucleotide 5'-AATCTCACAGTGCGTCGGCT-3', and positions 217, 218, and 219 with the oligonucleotide 5'-AACATGAATGGTACCTGGGATACAGCTGG-3', respectively. Note that the presence of the mutation was confirmed using dideoxysequencing. All of the above constructs, as well as wild-type Rab3a and Rab5a cDNAs, were PCR-amplified using Vent DNA polymerase with the appropriate oligonucleotides containing NdeI and BamHI restriction sites for subcloning into the bacterial expression vector pET15b.

**Recombinant Protein Production and Purification**—The above DNA constructs were transformed into BL21 (DE3) bacteria for protein expression. Cells were induced at 30 °C for 4–5 h in the presence of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside and then lysed by sonication in Buffer A (50 mM Hepes, pH 8.0, 100 mM NaCl, 5 mM MgCl2), 30 mM imidazole, and protease inhibitors. TX-100 was then added to a final concentration of 0.5%. Insoluble material was pelleted, and the supernatant was incubated overnight with Ni2+-agarose. The Ni2+-resin was washed then washed in sequence with 150 ml of Buffer A plus 0.5% TX-100, 50 ml of Buffer B (50 mM NaPO4, pH 7.2, 300 mM NaCl, 10% glycerol), pH 6.0, and 50 ml of Buffer A and then eluted with Buffer A containing 250 mM imidazole. Imidazole was removed by dialysis overnight in Buffer A plus 1 mM dithiothreitol. Proteins produced from constructs Rab3/5 IV and VII were largely insoluble. The insoluble protein fraction was therefore used in gel overlay experiments after solubilization in SDS sample buffer. For Ms4 solution binding assays, the insoluble protein present in inclusion bodies was partially solubilized in 50 mM Tris, pH 7.5, 8 μl urea. Ms4 fused to the maltose-binding protein (Ms4-MBP) was affinity-purified on amylose-Sepharose as described previously (13) and then further purified to homogeneity by ion exchange chromatography on ProteinPack8HR. Co-precipitation assays between Mss4 and Rab3a were performed as in Burton et al. (13), and the presence of Rab3a was determined by Western blotting using anti-Rab3a monoclonal antibodies (42.1) (kind gift of Dr. Reinhardt Jahn).

**RESULTS**

**Regions of Rab3a Important for Mss4 Interaction**—Ms4 has been shown previously to bind and promote GDP-GTP exchange on only a subset of Rab proteins belonging to the same branch of the Ras superfamily (13). To address the specificity of this Ms4-Rab interaction, we constructed several chimeras between a Rab that binds Ms4, Rab3a, and one that does not, Rab5a (Fig. 1). Each DNA chimera was subcloned into the bacterial expression vector pET15b, which adds a histidine tag to the amino terminus of the expressed protein. The recombinant proteins were produced in bacteria and tested for Ms4 binding by using the gel overlay technique described previously (13). The recombinant Rab proteins were either purified by affinity chromatography on Ni-NTA resin (soluble proteins left-hand panels (Fig. 2) or recovered from inclusion bodies and
solubilized in gel sample buffer (Fig. 2, right panels, Rab 3/5 IV and VII) and electrophoresed on three identical SDS-polyacrylamide gel electrophoresis gels. One gel was stained with Coomassie Brilliant Blue to visualize the amount of protein loaded. Proteins from the other two gels were transferred to nitrocellulose and overlaid with either Mss4-MBP to determine Mss4 binding activity, or 35S-GTPγS to determine GTP binding activity (Fig. 2).

We first tested the roles of the switch I region, the so called effector domain (L2/b2 according to the Ras crystal structure) and switch II (L4/a2) domains of the Rab3a GTPase in Mss4 binding. We reasoned that these switch domains of Rab3a may bind the GEF Mss4, because they are proposed to undergo a dramatic conformational change between the GDP- and GTP-bound state (17). In the Rab3/5 I and Rab3/5 II constructs, the switch I domain of Rab5 (Rab3/5 I) or both the switch I and II domains of Rab5 (Rab3/5 II) were substituted with the corresponding regions of Rab3a (Fig. 1). Both of these chimeric proteins were unable to bind Mss4, suggesting that other Rab3a sequences are necessary for the interaction (Fig. 2, middle panel, data for Rab3/5 II not shown). We therefore constructed Rab3/5 chimeras in which the amino terminus of Rab5, including the switch I (Rab3/5 III) or both the switch I and switch II domains (Rab3/5 IV) were replaced by the corresponding domains of Rab3a (Fig. 1). Both of these chimeric proteins were able to bind Mss4 (Fig. 2, middle panels), although the construct lacking the switch II domain was clearly less effective in binding (Fig. 2, middle right panel, compare Rab3/5 III with Rab3/5 IV; see also Fig. 3). The Rab3a switch I domain in combination with sequences amino-terminal to this domain are crucial for Mss4 interaction because constructs Rab3/5 V and Rab3/5 VI, which lack the switch I domain of Rab3a, do not bind Mss4 (Fig. 2).
Mss4 D96H is unable to bind Rab3a. Wild-type Mss4, but not Mss4 D96H, bound to Ni²⁺ resin is able to co-precipitate Rab3a in a nucleotide-dependent manner (top left panel). Approximately equal amounts of wild-type and D96H Mss4 proteins are present on the Ni²⁺ beads (top right panel). The histogram shows the quantity of Rab3a bound relative to the amount of Mss4 (wild-type or D96H) linked to the resin (bottom panel). Rab3a, which co-precipitated with Mss4, was detected using the monoclonal antibody, 42.1 (1:1000), a rabbit anti-mouse antibody bridge (1:1000), and ¹²⁵I-protein A (0.1 μCi/ml). Mss4 and Mss4 D96H present on the resin were determined by Western blotting using polyclonal anti-Mss4 antibodies (1:100) and ¹²⁵I-protein A (0.1 μCi/ml). Rab3a and Mss4 present on the resin were measured by densitometry of autoradiographs from the corresponding ¹²⁵I-labeled protein bands.

In a separate study aimed at identifying Mss4-binding proteins by the yeast two-hybrid system, a Rab3a clone lacking the first 29 aa from the amino terminus was isolated (data not shown). We therefore attempted to further define the Mss4-binding domain by removing these 29 aa from the Rab3/5 III chimera. The resulting chimera, designated as Rab3/5 VII (Fig. 1), which contains aa 30–73 of Rab3a and aa 72–215 of Rab5a, was found to bind Mss4 albeit at considerably lower levels than what is observed for Rab3/5 III, suggesting that aa 1–30 of Rab3a also participate in Mss4 binding (Fig. 2, middle right panel). The hypervariable domain located at the carboxyl terminus of Rab3a has also been shown to be unnecessary for binding Mss4 in this yeast two-hybrid screen (data not shown). Accordingly, two truncated forms of Rab3a, Rab3αΔC1 and Rab3αΔC2, bind to Mss4 at wild-type levels (Fig. 2).

In order to quantitate the level of interaction between Mss4 and representative Rab3/5 chimeras, a solution binding assay was performed with ³⁵S-labeled Mss4-MBP and His-tagged Rab3/5 III, Rab3/5 IV, Rab3/5 VII, Rab3α, and Rab5a bound to Ni²⁺ resin. Levels of Mss4 binding were ascertained by counting the radiolabeled Mss4 specifically associated with the Rab-bound Ni²⁺ resin. Rab3/5 IV bound Mss4 at levels approaching those of full-length Rab3a, whereas Rab3/5III and Rab3/5VII were much less efficient in interacting with Mss4 (Fig. 3). Therefore, the inclusion of the Rab3α switch II domain in Rab3/5 IV clearly increases Mss4 binding when compared with Rab3/5 III. These results are in full agreement with those found using the Mss4 overlay assay (Fig. 2). Taken together, our results implicate an 1–73 of Rab3a, which includes the effector domain, as a critical region for Mss4 interaction, with residues 74–102 of Rab3α further enhancing Mss4 binding.

Note that Rab3/5 III, IV, and VII, which bind Mss4, do not bind significant levels of GTP by gel overlay (Fig. 2, bottom right panel) or in solution (data not shown). We have previously shown that the GEF Mss4 binds tightly to the GTPase in the absence of nucleotide, suggesting that it catalyzes GDP-GTP exchange by stabilizing the nucleotide-free form of the GTPase (13). Thus, a dissociation between Mss4-binding and GTP-binding properties of the GTPase is not unexpected.

**Mss4 Point Mutation Disrupts Mss4 Binding Activity and GDP-releasing Activity on Rab3a**—To complement the above results, we wished to address which domain of Mss4 is critical for binding and stimulating guanine nucleotide exchange on Rab proteins. Sequence comparisons of Mss4 and the related yeast protein Dse4 had shown that the region of highest similarity was a small stretch in the carboxyl terminus of the molecule, which includes conserved cysteines (10). Yu and Schreiber (18) have recently determined by nmr the structure for the human Mss4 protein. They found that the conserved cysteines (Cys²⁵-X₂-Cys³⁶ and Cys⁸⁴-X₂-Cys⁹⁵) enables Mss4 to bind Zn²⁺. Using site-directed mutagenesis, they showed that substituting a histidine for an aspartic acid at position 96 (Mss4 D96H) in this zinc-binding domain reduces Mss4-mediated GDP release from Sec4 by 60%. However, the binding activity toward Sec4 was not investigated (18). We therefore produced Mss4 proteins harboring a point mutation at aa position 96 (Mss4 D96H) in this zinc-binding domain reduces Mss4-mediated GDP release from Sec4 by 60%. However, the binding activity toward Sec4 was not investigated (18). We therefore produced Mss4 proteins harboring a point mutation at aa position 96 (Mss4 D96H) or 94 (Mss4 C94S) to determine how these mutations affected Mss4 binding activity and GDP-releasing activity toward Rab3a. Due to the instability of the Mss4 C94S protein, the biochemical properties of this protein could not be addressed. In contrast, Mss4 D96H was stable and was further investigated.

His-tagged wild-type Mss4 and Mss4 D96H proteins were...
linked to Ni²⁺ resin and incubated with recombinant untagged Rab3a. The beads were pelleted, washed, and assessed for the presence of Rab3a binding by Western blotting using Rab3a antibodies (Fig. 4, top panel). As shown previously, wild-type Mss4 bound the nucleotide-free form of Rab3a, and this interaction was disrupted in the presence of 1 mM GTP (Fig. 4, upper panel, compare lanes 1 and 2; Ref. 13). In contrast, the point mutant Mss4 D96H did not bind Rab3a, irrespective of the presence of guanine nucleotide, although the amount of Mss4 protein present on the beads was similar for the Mss4 D96H and wild-type Mss4 (Fig. 4, lanes 3 and 4, upper right panel). Quantitation of Rab3a protein bound relative to Mss4 present on the beads is depicted in Fig. 4 (bottom panel). Mss4D96H was 26-fold less efficient than wild-type Mss4 in binding to Rab3a.

We next investigated the enzymatic activity of the Mss4 D96H mutant protein toward Rab3a using the GDP release filter binding assay. We found that an 8-fold molar excess of Mss4 D96H did not stimulate the GDP-release rate from Rab3a, whereas wild-type Mss4 increased this rate 5-fold (Fig. 5). These results are consistent with the lack of significant binding seen between Rab3a and the Mss4 D96H mutant in the co-precipitation studies (see above). Taken together, these results suggest that the aspartic acid at position 96 of Mss4 is involved in binding the Rab GTases.

**DISCUSSION**

In the present study, we have taken advantage of the selective binding of Mss4 to a subset of Rab GTases to identify Rab domains important for this interaction. Using chimeric Rab3/Rab5 proteins, we have demonstrated that the binding requires the amino-terminal region of Rab3a. The effector domain is crucially required for binding, while the regions amino-terminal and carboxyl-terminal to this domain further enhance binding.

**FIG. 6. Color figure illustrating Mss4-binding Rabs share high homology in the β1–α2 regions.** A pile up of small GTPases reveals that the Mss4-binding Rabs (boldface type) are more homologous with each other than with Rabs and other GTases that do not bind Mss4. In particular, there are blocks of identical or conservative aa changes (shaded regions) that are unique to the Mss4-binding GTases. Boldface, shaded residues represent residues conserved in the Rab GTases which bind Mss4. Blue indicates aa conserved in all of the Rab GTases, while red identifies residues that are conserved in all but one of the Rabs shown.

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of binding Mss4 may have been an important influence in the evolution of these proteins and strongly supports the idea that Mss4 binding is critical for the function of these Rab.

It is of interest to compare our results with those of Macara et al. (20, 21) concerning domains of Rab3a that are important for interaction with Rab3a-GRF, a partially purified cytosolic exchange factor whose primary structure is unknown. Using site-directed mutagenesis, they have found that mutations in the Rab3a switch I domain decrease the affinity of Rab3a for Rab3a-GRF and cause the protein to no longer be sensitive to Rab3a-GRF activity (20). In addition, they have found that removal of the last 34 aa of Rab3a does not affect Rab3a-GRF binding, both of these results are similar to those reported here for Mss4 (20). In spite of these similarities, several pieces of evidence suggest that Rab3a-GRF is not Mss4. First, Mss4 and Rab3a-GRF differ in molecular mass. Whereas Mss4 is a 17-kDa protein, the proposed molecular mass for Rab3a-GRF is about 295 kDa (13, 21). Second, Mss4 binds to both lipid-modified (geranylgeranylated) and -unmodified forms of Rab3a and promotes GDP-GTP exchange on both proteins (13, 22). In contrast, Rab3a-GRF prefers the lipid modified form (22, 23). Third, Rab GDI was reported to be more effective in blocking the GDP-releasing activity of Rab3a-GRF than of Mss4 when complexed with Rab3a (22). Finally, Mss4 has also been shown to stimulate GTPyS release, whereas this activity could not be detect for Rab3a-GRF (13, 23). Taken together, these studies suggest that Mss4 and Rab3a-GRF are distinct exchange factors, although they interact with similar domains on Rab3a and may thereby promote GDP release by an analogous mechanism. The Rab domains involved in the binding to p619, a recently identified GEF for Arf1 and at least some Rab proteins (12), remains to be identified.

Previous studies have investigated the domains of Ras that are important for interaction with its GEFs, mSOS and Cdc25. The last 23 aa of Ras have been shown to be dispensable for binding, in agreement with the results reported here for the Rab3a-Mss4 interaction (24, 25). However, a mutational analysis of Ras has shown that the switch II domain (which consists of L4/α2), but not the switch I domain, is important for binding to its GEFs (26–28). Since Mss4 exhibits little sequence homology with the GEFs for Ras, it is perhaps not surprising that Mss4 and these GEFs interact differently with their respective GTPases.

In this study we have further corroborated the key function of the carboxyl-terminal region of Mss4 in Rab-binding. This region, which contains a Zn²⁺-binding motif (18), is most highly conserved between Mss4 and its yeast homologue Dss4 (10). Furthermore, Dss4 was originally isolated by virtue of its ability to suppress the yeast sec4–8 strain when mutated in this region (D108G) (11). A single point mutation in this conserved domain of Mss4 (D96H) was previously shown to reduce GDP release from the nonphysiological substrate Sec4 by 60% (18). We show here that the same mutation not only abolishes Mss4-mediated GDP-release from Rab3a but also completely abolishes Mss4 binding to Rab3a.

In conclusion, we have defined domains on Rab3a and Mss4 that are important for their reciprocal interactions. Our results suggest that the Zn²⁺-binding face of Mss4 binds to the GTPase at the region that surrounds its effector domain (β1, L1, α1, L2, β2, and L3). Conservation of this Mss4-binding interface appears to have been a key constraint during the evolutionary diversification of a subset of Rabs implicated in the secretory pathway (13).

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REFERENCES
