Role of the Rab3A-Binding Domain in Targeting of Rabphilin-3A to Vesicle Membranes of PC12 Cells

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Received 18 April 1996/Returned for modification 23 May 1996/Accepted 18 June 1996

Rab3A is a small GTPase implicated in the docking of secretory vesicles in neuroendocrine cells. A putative downstream target for Rab3A, rabphilin-3A, is located exclusively on secretory vesicle membranes. It contains near its C terminus two C₆ domains that bind Ca²⁺ in a phospholipid-dependent manner and an N-terminal, Rab3A-binding domain that includes a Cys-rich region. We have determined that the Cys-rich domain binds two Zn²⁺ ions and is necessary but not sufficient for efficient binding of rabphilin to Rab3A. A minimal Rab3A-binding domain consists of residues 45 to 170 of rabphilin. HA1-tagged Rab3A and a green fluorescent protein (GFP)-rabphilin fusion were used to examine the roles of Rab3A and of rabphilin domains in the subcellular localization of these proteins. A Rab3A mutant (T54A) that does not bind rabphilin in vitro colocalized with the GFP-rabphilin fusion, indicating that Rab3A targeting is independent of its interaction with rabphilin. Deletion of the C₂ domains of rabphilin reduced membrane association of GFP-rabphilin but did not cause mistargeting of the membrane-associated fraction. However, disruption of the zinc fingers, which drastically reduced Rab3A binding, did not reduce membrane association. These results suggest that the C₂ domains are required for efficient membrane attachment of rabphilin in PC12 cells and that Rab3A binding may act to target the protein to the correct membrane.

The Rab family of small GTPases functions in vesicular traffic both between compartments within the cell and in endo- and exocytosis (for a review, see reference 16). The function of Rab proteins at the molecular level is unknown. It was initially believed that Rab proteins regulate vesicle targeting to the acceptor membrane; however, evidence against this hypothesis has been provided by a chimera of two yeast Rab proteins, Sec4p and Ypt1p, that function in post-Golgi apparatus and endoplasmic reticulum-to-Golgi apparatus transport, respectively. A chimera of the two proteins can complement a temperature-sensitive mutation in either protein without missorting of the vesicles involved in these processes (4, 14). More recent studies of Ypt1 suggest that Rab proteins facilitate the assembly of vesicle docking and/or fusion complexes (16).

Rab3A GTPase is expressed predominantly in neuronal and chromaffin cells, where it is associated with the membranes of synaptic vesicles and chromaffin granules (8, 13, 25, 31). Membrane attachment requires the posttranslational modification of the two C-terminal Cys residues of Rab3A by geranylgeranylation (15). Approximately 30% of prenylated Rab3A remains cytosolic in a complex with Rab-guanine nucleotide dissociation inhibitor (GDI) (2).

The heterologous overexpression of Rab3A in adrenal chromaffin and PC12 cells potently inhibits the initial rate of stimulated exocytosis from these cells, suggesting that the GTPase functions to control the pool of release-ready vesicles, possibly by inhibiting docking (22, 23). However, the overexpression of a putative downstream target of Rab3A, which is called rabphilin-3A, slightly potentiates secretion from transfected chromaffin cells (12). This apparent paradox can be explained if the function of Rab3A is to recruit rabphilin to the secretory vesicle membrane and if rabphilin functions to promote docking only after it has disengaged from Rab3A. Overexpression of Rab3A would reduce the probability of disengagement and inhibit docking, while overexpression of rabphilin might increase the concentration of disengaged rabphilin at the secretory vesicle. Studies of transgenic mice lacking Rab3A are consistent with this idea (20). When repetitive trains of depolarization were applied to hippocampal neurons from transgenic mice, the response was initially identical to that of wild-type neurons but showed a more rapid onset of synaptic depression, as though the transgenic neurons possessed a reduced ability to recruit vesicles to the presynaptic plasma membrane (20). Interestingly, the level of rabphilin was substantially reduced within most regions of the brains of the transgenic mice, suggesting that the rabphilin may be rapidly degraded if it is not correctly localized to the synaptic vesicle membrane by Rab3 (27).

Rabphilin-3A was first identified by its ability to interact with Rab3A preferentially in the GTP-bound form (36, 37). It is a protein with a molecular mass of 78 kDa that contains near its C terminus two C₂ domains (residues 396 to 530 and 548 to 669) which can bind calcium in a phospholipid-dependent manner (41), a central region with multiple consensus sequences for phosphorylation by protein kinases (19, 24), and a cysteine-rich region (residues 98 to 144) that resembles a double zinc finger motif (for a review, see reference 17). A proteolytic fragment consisting of the N-terminal half of rabphilin can associate with Rab3A (41). Rabphilin inhibits the GTPase activity of Rab3A promoted by Rab3-GAP (26) and also has a weak nucleotide exchange activity toward Rab3A (18). Rabphilin is present on synaptic vesicle and secretory granule membranes; however, it has no transmembrane domain and is not prenylated (30). Biochemical studies demonstrated that the binding of rabphilin to synaptic vesicle membranes is specific and independent of Rab3A (38). Protease treatment of purified synaptic vesicles abolished the ability of rabphilin to bind to vesicles; however, selective removal of Rab3A from vesicles did not alter rabphilin binding (38). These data are not

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consistent with the interpretation of the phenotype of the Rab3A-negative transgenic mice described above (27), and argue rather for the presence of a protein(s) other than Rab3A that acts as an anchor for rabphilin on secretory vesicle membranes.

To address the issue of rabphilin, we have mapped the Rab3A binding site on rabphilin. We show that the cysteine-rich region of rabphilin binds zinc and that it is necessary but not sufficient for the efficient interaction of rabphilin with Rab3A. Using a green fluorescent protein (GFP)-rabphilin fusion construct, we show that cotransfected HA1-tagged Rab3A and GFP-rabphilin colocalize in transiently transfected PC12 cells, but that an isolated Rab3-binding domain (Rab3BD) (residues 1 to 206) [rabphilin(1–206)] is not sufficient for normal membrane association of rabphilin. Rabphilin did not colocalize with synaptophysin, a marker for small clear synaptic vesicles in PC12 cells (13). Disruption of the zinc fingers, which substantially reduces Rab3 binding to rabphilin. Conversely, a Rab3A mutant that does not bind rabphilin-3A colocalized with GFP-rabphilin in PC12 cells, demonstrating that interaction between the two proteins is not necessary for the correct subcellular localization of Rab3A.

**MATERIALS AND METHODS**

**Generation of rabphilin truncations and deletion mutants.** Truncation and deletion mutants of rabphilin were made by PCR from plasmids containing full-length bovine rabphilin-3A (a gift from Ronald Holz). Primers were designed to generate -5BamHI and 3′-EcoRI restriction sites. PCR products were digested with BamHI and EcoRI and were subcloned into pGEX-2T (Pharmacia) or into pKH3 (10) and p7SGFP (11), mammalian expression vectors that contain a triple HA1 tag and a GFP (S65T) (21) tag, respectively.

To create internal deletions in rabphilin, two PCR fragments were generated, one corresponding to amino acids 1 to 94 and the other corresponding to amino acids 102 to 206, 119 to 206, or 127 to 206 to delete two, five, or seven cysteines, respectively. The fragment corresponding to amino acids 1 to 94 contained a 5′-BamHI and 3′-EcoRI site. The second fragment contained a 5′-EcoRI and 3′-EcoRI site. The PCR products were digested with the appropriate enzymes and ligated to each other. Ligation of the PCR products regenerated amino acids 95 to 96 without introducing any new amino acids. The subsequent products were ligated to the appropriate vector. The constructs were transferred to full-length rabphilin by PCR. Amino acids 1 to 206 were amplified from plasmids containing the cysteine deletions with primers containing 5′-BamHI and 3′-EcoRI restriction sites. PCRs with these fragments were subcloned in full-length rabphilin digested with BamHI and XhoI (which cuts at a site corresponding to amino acid 207). The mutations were confirmed by sequencing the constructs in pGEX-2T or pKH3 with appropriate primers.

**Expression of recombinant GST fusion proteins.** Recombinant Rab3A and rabphilin were expressed as glutathione S-transferase (GST) fusion proteins in Escherichia coli by using the pGEX-2T expression vector and were purified by affinity chromatography with glutathione-Sepharose (Pharmacia) (as described in reference 7). Uncloned proteins were eluted from the glutathione-Sepharose matrix with 10 mM glutathione (Sigma) in 50 mM Tris-HCl (pH 8.0). Glutathione was removed before binding assays by passage over a PD-10 column (Pharmacia). Alternatively, proteins were cleaved from the column with recombinant thrombin in 150 mM NaCl-50 mM Tris-HCl (pH 7.4)-2.5 mM CaCl2. Thrombin was added to full-length rabphilin by using the pGEX-2T expression vector and was purified by nickel affinity chromatography with Ni2+-charged glutathione-Sepharose slurry in 100 mM Tris-HCl (pH 8.0)-50 mM NaCl-0.5 mM MgCl2-0.5 mM CaCl2 plus 5 mM dithiothreitol and then incubated in the same buffer plus 200 mM ZnCl2 (Dupont/NEN). The blots were washed in a buffer containing 100 mM Tris-HCl (pH 6.8), 50 mM NaCl, 0.5 mM MgCl2, and 0.5 mM CaCl2 and in a similar buffer at pH 5.8. The membrane was exposed to film to visualize the zinc binding proteins and then stained with amido black (45% methanol, 10% glacial acetic acid, and 0.1% amido black) and washed (45% methanol, 10% glacial acetic acid) to confirm protein loading was detected.

**Determination of zinc binding by atomic absorption.** Rabphilin and the Zn2+-binding domain, Cys-2, of protein kinase C (PKC) (a gift from Sujoy Ghosh) were expressed as uncleaved GST fusion proteins as described above. They were diluted 20-fold to 50-fold in 0.25% nitric acid and analyzed for zinc content by atomic absorption spectrometry with a Perkin-Elmer Cetus 4100 ZL THGA atomic absorption spectrometer. Standard curves were generated by using the PKC fragment, which has been shown to bind 2 mol of zinc per mol of protein (32, 35). Protein quantitation was performed by three methods: amido black dye-binding (35), Bradford (3), and 32P-labeled PC19 assay. All three methods yielded nearly identical results for the proteins assayed. Calculated stoichiometries assumed molecular masses of 27, 36, 50, 47, and 49 kDa for GST, GST-PKC Cys-2, GST-rabphilin (1–206), GST-rabphilin (1–206)/Cys-2, and GST-rabphilin (1–206). Cys-2, respectively.

**Transfection, immunofluorescence, and partitioning of PC12 cells.** PC12 cells were electroporated with a total of 10 μg of plasmid DNA per transfection at 400 μA with a capacitance of 500 μF in a Bio-Rad Gene Pulser. The cells were plated on 60-mm dishes or glass Lab-tek chamber slides coated with poly-L-lysine (Sigma). The medium was changed after several hours, and the cells were grown for 2 days. Cells used for immunofluorescence were grown in the presence of 0.2 ng of recombinant human nerve growth factor per ml (a gift from Genentech) to induce neurite outgrowth.

For partitioning, cells were harvested into 1 ml hypotonic lysis buffer (10 mM Tris [pH 7.4], 5 mM KCl, 2 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride) and incubated at 4°C for 15 min. The cell suspension was adjusted to 0.5% SDS by adding through a 26-gauge needle. The whole-cell extract was centrifuged at 100,000 × g for 1 h. The supernatant was removed, and the pellet was rinsed twice and resuspended in 1 ml of hypotonic lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5 mM MgCl2, 0.5 mM CaCl2) to a final concentration of 10% and the samples were incubated on ice for at least 30 min and then centrifuged at 16,000 × g for 15 min. The pellets were washed in acetone and resuspended in 0.1 M NaOH-0.1% deoxycholate. After addition of Laemmli sample buffer, proteins were separated by SDS-PAGE. Expression of HA1-tagged proteins was determined by immunoblotting with anti-HA1 monoclonal antibody (diluted 1:50,000; Boehringer-Mannheim) and horseradish peroxidase-linked Fab-specific anti-mouse secondary antibody (Sigma) (1:10,000) (34). Bands were visualized by chemiluminescence (KPL).

Fluorescence microscopy was performed essentially as described elsewhere (34). Cells were fixed in paraformaldehyde and permeabilized with methanol. GFP-rabphilin-fusion proteins were visualized directly by the intrinsic fluorescence of the GFP (S65T) tag (11). HA1-tagged Rab3A was visualized with the 12CA5 anti-HA1 antibody (diluted 1:2,000) and indocarbocyanine (Cy3)-linked goat anti-mouse antibody at a dilution of 1:1,500 (Jackson ImmunoResearch). Endogenous synaptophysin was detected with an anti-synaptophysin monoclonal antibody (anti-synaptophysin-Mannheim) at a dilution of 1:50 (Sigma) and anti-mouse secondary antibody. Cells were imaged on a Bio-Rad MRC1000 confocal microscope with a 60× oil immersion lens. To determine the relative degrees of overlap of the GFP (green channel) and Cy3 (red channel) fluorescence within the transfected cells, digital images were acquired by using the multiply function in the MPL software package. This function multiplies the value for each pixel in one image by the corresponding pixel in a second image. The result is then reduced to a scale of 0 to 254. The sums of pixels of equal values in the resulting image were displayed in a histogram with a MBC software package [Bio-Rad]. Low pixel values (<20) were deleted, and the remaining pixel values were summed. To correct for differences in cell area, overlap was calculated as the pixel values in the multiplied image divided by the sum of the pixels in the separate red and green channel images. This procedure can be described by the following equation:

\[
\text{overlap} = \frac{\sum_{i=1}^{n} \min(x_i, y_i)}{\sum_{i=1}^{n} x_i + \sum_{i=1}^{n} y_i - \sum_{i=1}^{n} \min(x_i, y_i)}
\]
**RESULTS**

**Identification of the Rab3BD in rabphilin.** Although proteolytic cleavage of rabphilin has demonstrated that Rab3A binds to the N-terminal half of the protein (41), the minimal region required for this interaction has not been identified. Therefore, a variety of rabphilin truncation and internal deletion mutants were expressed as GST fusion proteins (Fig. 1A) and were tested for their ability to interact with Rab3A in vitro. The rabphilin-GST fusion proteins were attached to glutathione-Sepharose beads and incubated with \( [\gamma-^{32}P]GTP-Rab3A \). The amount of Rab3A bound was determined after rapid washing of the beads (Fig. 1B).

As expected, deletion of the C-terminal region of the protein (residues 206 to 710) did not interfere with Rab3A binding. Quantitative equilibrium binding demonstrated that the affinity of the GST-rabphilin(1–206) fusion (10 nM) was indistinguishable from that of the full-length protein (Fig. 1C) and was of similar magnitude to that measured previously for purified bovine brain rabphilin (36). Further deletions from both the N and C termini revealed that the minimal Rab3BD encompasses residues 45 to 170. This sequence includes the cysteine-rich region.

To determine the role of the cysteine-rich region in Rab3A binding, internal deletions of rabphilin were generated to remove two, five, or seven cysteines. Deletions within the cysteine-rich region of rabphilin substantially reduced its ability to bind Rab3A. However, in all three mutants, a residual level of Rab3A binding that was significantly higher than that of the nonspecific binding to GST alone was consistently observed (Fig. 1B).

**The cysteine-rich region of rabphilin binds two zinc ions.** The clustering of cysteines between amino acids 98 and 144 in rabphilin possesses sequence similarities to a variety of zinc finger protein sequences (17). Rabphilin truncations and internal deletion mutants were, therefore, assayed for their ability to bind Zn, by both a 65Zn overlay assay and atomic absorption spectrometry. For the overlay, recombinant rabphilin proteins were separated by SDS-12% PAGE with BSA as a positive control. The gel was transferred to nitrocellulose and incubated with \( ^{65}ZnCl_2 \). The membrane was then exposed to film to detect bound zinc (Fig. 2A) and stained with amido black to visualize the proteins (Fig. 2B). As shown in Fig. 2, rabphilin is capable of binding zinc, and deletion of two or five cysteine residues resulted in a decreased signal, whereas dele-

determined by scintillation counting. Binding is expressed as percent binding relative to rabphilin (1–206). Binding data are from two separate experiments performed in duplicate. SEM, standard errors of the means; nd, not determined.

(C) Equilibrium binding of rabphilin truncation mutants to Rab3A. Rabphilins were bound to glutathione-Sepharose and were incubated with 1.25 to 100 nM \( [\gamma-^{32}P]GTP-Rab3A \) at a known specific activity. The beads were washed, and the amounts of Rab3A bound were determined by scintillation counting. Nonspecific binding of Rab3A to GST attached to glutathione-Sepharose was subtracted from all points. Binding assays were performed in duplicate. Data are representative of three experiments. Values are given as mean ranges. \( \diamond \) rabphilin(1–206); \( \bigcirc \) rabphilin(1–157). The apparent \( K_d \) for rabphilin(1–206) is 10 nM.

**FIG. 1.** (A) Expression of wild-type and mutant rabphilins (Rp). Rabphilin fragments were expressed as GST fusion proteins and were purified by affinity chromatography on glutathione-Sepharose. A 5-μg amount of each protein was subjected to SDS-PAGE, and the gel was stained with Coomassie to visualize the proteins. (B) Identification of the Rab3ABD of rabphilin. Rabphilin proteins were attached to glutathione-Sepharose and incubated with \( [\gamma-^{32}P]GTP-Rab3A \). The beads were washed, and the amounts of associated Rab3A were determined by scintillation counting. Binding is expressed as percent binding relative to rabphilin (1–206). Binding data are from two separate experiments performed in duplicate. SEM, standard errors of the means; nd, not determined.

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tion of all seven cysteines reduced zinc binding almost to background.

Atomic absorption spectrometry was performed to obtain the stoichiometry of zinc binding. Rabphilin truncation and cysteine deletion mutants were expressed as uncleaved GST fusion proteins and injected into a Perkin-Elmer Cetus 4100 ZL THGA atomic absorption spectrometer. The Cys-2 zinc-binding domain of PKCy, which binds 2 mol of zinc per mol of protein, was used to generate a standard curve. The protein fragment from residues 1 to 206 bound 2 mol of zinc per mol of protein (Table 1). Deletion of an additional 45 amino acids from the N terminus of the fragment resulted in only a small decrease in zinc binding. Deletion of two or five cysteines (residues 97 to 102 or 97 to 119) decreased zinc binding to 0.6 or 0.2 mol of zinc per mol of protein, respectively (Table 1). Removal of seven cysteines abolished detectable zinc binding. These results demonstrate that rabphilin possesses a double zinc finger that is an essential component of the Rab3BD and which binds 2 mol of zinc per mol of protein.

**HA1-Rab3A and GFP-rabphilin colocalize in PC12 cells.** Wild-type, HA1-tagged Rab3A and a GFP-rabphilin fusion construct were transfected into PC12 cells by electroporation and were tested for their ability to colocalize by fluorescence microscopy (Fig. 3A). We have previously demonstrated by immunoelectron microscopy that the HA1 epitope tag does not interfere with the localization of Rab3A to dense core secretory granules in PC12 cells (13). Transfected cells were grown in the presence of nerve growth factor for 2 days to induce neurite outgrowth. The cells were fixed, permeabilized, and probed sequentially with the anti-HA1- and Cy3-linked goat anti-mouse antibodies. They were then imaged by confocal microscopy. The GFP was detected in the fluorescein channel, and the HA1 was detected in the rhodamine channel. The relative colocalizations of the two tags were quantitated by image analysis.

In cotransfected cells, HA1-Rab3A and GFP-rabphilin exhibited a punctate staining pattern and tended to colocalize in neurite outgrowths (Fig. 3A). In full-scale images, it was apparent that both fluorophores were associated with the same punctate organelles, each of which presumably represents a single dense core secretory granule. Cells transfected with untagged Rab3A and HA1-rabphilin that were probed with anti-Rab3A and 12CA5 antibodies showed similar staining patterns, demonstrating that the localization was independent of the protein tags (data not shown). A mutant of Rab3A lacking the C-terminal geranylgeranylation site (ΔCAC) was expressed diffusely throughout the cytosol but did not perturb the localization of rabphilin. Similarly, GFP alone did not colocalize with Rab3A. When cells expressing GFP-rabphilin were probed for anti-synaptophysin, no significant overlap was observed (Fig. 3B). Synaptophysin associates in PC12 cells with small, clear synaptic-like vesicles that tend to accumulate within the cell body rather than in the neurite outgrowths (13). No bleedthrough between the red and green channels was seen in cells singly transfected with either GFP-rabphilin or HA1-Rab3A.

The images were quantitated as discussed in Materials and Methods, and a mean overlap quotient was calculated for at least five cotransfected cells in each experiment. As can be seen from Table 2, the overlap quotient for GFP-rabphilin plus wild-type HA1-Rab3A is substantially higher than that for the ΔCAC Rab3A mutant or for synaptophysin (overlap quotient, 0.035 ± 0.007), or for the wild-type HA1-Rab3A plus GFP. The differences are statistically significant by a two-tailed, unpaired t test. However, when these values are taken as the extremes on a scale of degrees of overlap, it is apparent that the calculated quotient is not very sensitive and that to determine whether two proteins colocalize within the cell it is important also to compare visually the fluorescence patterns of the image sets.

### TABLE 1. Stoichiometry of rabphilin zinc binding

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mol of Zn/mol of protein (mean ± SD) (n = 3)</th>
</tr>
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<tbody>
<tr>
<td>GST</td>
<td>0.007</td>
</tr>
<tr>
<td>GST-PKCγ zinc-binding domain</td>
<td>2.0 (defined)</td>
</tr>
<tr>
<td>GST-Rab (1–206)</td>
<td>2.02 ± 0.07</td>
</tr>
<tr>
<td>GST-Rab (45–206)</td>
<td>1.5 ± 0.15</td>
</tr>
<tr>
<td>GST-Rab (Δ2Cys)</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>GST-Rab (Δ5Cys)</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>GST-Rab (Δ7Cys)</td>
<td>0</td>
</tr>
</tbody>
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*Affinity-purified recombinant GST-rabphilin (GST-Rab) fusion proteins were assayed for zinc binding with a Perkin-Elmer Cetus 4100 ZL THGA atomic absorption spectrometer. A standard curve was generated by using the Cys-2 domain of PKCy, which is known to bind 2 mol of zinc per mol of protein (32, 33). Atomic absorption measurements were performed on triplicate samples.*
fragment partitioned into the soluble fraction. A smaller fragment of HA1-rabphilin, consisting of residues 1 to 157, which cannot bind Rab3A, was entirely soluble.

The same deletion mutants were subcloned into the GFP fusion vector, p7SGFP, and were cotransfected into PC12 cells with HA1-Rab3A. As described above, full-length GFP-rabphilin shows a punctate fluorescence that colocalizes with HA1-Rab3A and is concentrated in the neurite outgrowths. The fragment from 1 to 206 also appeared to colocalize with Rab3A by calculation of the overlap quotient. However, inspection of the confocal images (Fig. 4B) reveals that the fragment from 1 to 206 was also partially expressed diffusely in the cell body, in addition to the punctate staining seen in the neurite outgrowths. In contrast, the fragment from 1 to 157 was present throughout the cytoplasm, and the overlap quotient was similar to that of free GFP (Table 2 and Fig. 4B). These results indicate that a rabphilin fragment capable of binding Rab3A but lacking the C$_2$ domains can be recruited to the correct membranes but does not associate with the membranes with high affinity. Rab3A binding alone, therefore, does not provide a sufficient signal to recruit rabphilin efficiently to the secretory granule membrane.

**Rab3A binding is not essential for membrane association of rabphilin.** To investigate further the role of Rab3A binding in the membrane targeting of rabphilin, we used mutants with deletions in the zinc finger domain which show significantly reduced binding to Rab3A in vitro. HA1-rabphilin constructs were transfected into PC12 cells and were assayed for localization by cell fractionation. As shown in Fig. 5A, the wild-type rabphilin and the Δ2Cys and Δ5Cys deletion mutants fractionated exclusively with the particulate fraction, suggesting that Rab3A binding is not required for membrane association. The
D7Cys deletion mutant could not be detected by immunoblotting. However, this mutant was clearly visible by immunofluorescence, and we suspect that the protein is particulate but is obscured by a nonspecific band present in the cell extract (Fig. 5A).

These results do not address the question of whether the HA1-rabphilin mutants are associated with the correct membrane. GFP-tagged rabphilin mutants were, therefore, transiently cotransfected into PC12 cells with HA1-Rab3A, and their subcellular distributions were examined by fluorescence microscopy. As shown in Table 2, the overlap quotients for the cysteine deletion mutants were not significantly different from that for wild-type rabphilin, suggesting that the loss of Rab3A-binding ability does not interfere with targeting to the secretory vesicle membranes. However, in Fig. 5B it can be seen that the distribution of the mutant rabphilins is increased within the cell bodies relative to that of the wild-type protein. This distribution was consistently observed in the transfected cells examined and suggests that the ability of rabphilin to bind Rab3A is not required for membrane association but may be important to increase the specificity of recruitment to the secretory vesicles.

Because neither deletion of the C terminus nor disruption of the Rab3BD led to complete mislocalization of GFP-rabphilin,
we examined the effect of double mutations. As shown in Fig. 6A, the deletion of two or five cysteines from the fragment of rabphilin from residues 1 to 206 resulted in a shift of the protein from the particulate to the soluble fraction in PC12 cells. The shift of the Δ7Cys mutant back to the particulate fraction with a concomitant decrease in mobility may be a result of the protein being ubiquitinated and targeted to the lysosome for degradation (Fig. 6A). Confocal analysis confirmed the results from the cell fractionation. The double mutants were expressed diffusely throughout the cytosol (Fig. 6B), and the overlap quotient with Rab3A was similar to that of free GFP (Table 2).

Interaction with rabphilin is not necessary for the subcellular localization of Rab3A. Because Rab3A binding is not essential for recruitment of rabphilin to membranes, we asked the converse question, i.e., whether the targeting of Rab3A to secretory vesicle membranes is dependent on rabphilin binding. As an experimental approach to this issue, we used point mutants in Rab3A that either can or cannot bind to rabphilin in vitro. Previous studies in our laboratory have demonstrated that mutations in the effector domain of Rab3A can disrupt interaction with regulatory factors without drastically changing the intrinsic biochemical properties of the GTPase (5, 6, 7, 29). The T54A mutant can interact with Rab3-GAP but not with Rab3-GRF and does not cross-link to p85, which was tentatively identified as rabphilin (29). The F51L mutant can also interact with Rab3-GAP but not with Rab3-GRF. However, it differs from T54A in showing a level of cross-linking to p85 similar to that of the wild-type Rab3A (29). These two mutants, therefore, were assayed for their ability to interact with recombinant GST-rabphilin in vitro. As shown in Fig. 7A, Rab3A (F51L) in the GTP-loaded state bound with wild-type affinity to the GST fusion, while Rab3A (T54A) did not show detectable binding above background (with GST alone as a negative control).

When these two Rab3A mutants were transiently coexpressed in PC12 cells as HA1-tagged proteins with GFP-rabphilin and examined by confocal fluorescence microscopy, we found that each showed a punctate staining concentrated in the neurite outgrowths and colocalized with GFP-rabphilin (Fig. 7B). Quantitation of the overlap quotients also indicated that both mutants colocalized with GFP-rabphilin as efficiently as the wild-type GTPase (Table 2). These results, therefore, support the conclusion that the recruitment of HA1-Rab3A to the secretory vesicle membrane is independent of its ability to associate with rabphilin. Incidentally, they also suggest that interaction with the soluble Rab3-GRF is unnecessary for membrane recruitment.

FIG. 5. (A) Partitioning of HA1-rabphilin (Rph) Cys deletions in PC12 cells. PC12 cells were transfected with 20 μg of each of the pKH3-rabphilin Cys deletion mutants and processed as described in Materials and Methods. The 87-kDa band present in all particulate (p) lanes is due to nonspecific binding of the antibody. s, soluble fraction. (B) Localization by confocal microscopy of rabphilin Cys deletion mutants in PC12 cells. PC12 cells were cotransfected with 10 μg each of pKH3-Rab3A and GFP-rabphilin Cys deletion mutants (shown in Fig. 1B) and were processed as described in Materials and Methods.
DISCUSSION

In this study, we have demonstrated that the cysteine-rich region of rabphilin (residues 98 to 144) functions as a zinc finger that binds two zinc ions and that the region is necessary but not sufficient for efficient association with Rab3A. The minimal Rab3BD consisted of residues 45 to 170. The sequence just N terminal to the zinc fingers (amino acids 45 to 98) is a putative coiled-coil domain, on the basis of analysis with the algorithm of Lupus et al. (28). When the GenBank database was searched with the Rab3BD sequence, an open reading frame was identified from the Caenorhabditis elegans genome project that possessed striking similarity to rabphilin (accession no. P41885) (40). This open reading frame, p111.7, could encode a protein with two C-terminal C2 domains, an N-terminal zinc finger, and a region with 60% identity to mammalian rabphilins that extends from residue 50 (bovine rabphilin numbering) to residue 179. This region is bounded by sequences that do not possess any significant similarities to the mammalian rabphilins. These properties suggest that the C. elegans gene p111.7 encodes a rabphilin homolog and support our mapping of the minimal Rab3BD.

When rabphilin was expressed transiently in PC12 cells as a GFP fusion protein, it colocalized with HA1-tagged Rab3A in a punctate pattern concentrated in the neurite growth cones. At high magnification, it was apparent that both proteins were associated with the same point-like structures, which we assume to be individual secretory granules. We have shown previously by immunoelectron microscopy that HA1-Rab3A associates predominantly with dense core secretory granules in PC12 cells, rather than with the small, clear, synaptic-like vesicles. Synaptophysin is a marker for these vesicles (13), and anti-synaptophysin immunostaining of PC12 cells transfected with GFP-rabphilin revealed negligible overlap. Others have reported that endogenous rabphilin associates specifically with dense core secretory vesicles in chromaffin cells (30). Therefore, we conclude that the GFP-rabphilin is being correctly targeted when expressed heterologously in PC12 cells and that we can use GFP fusions to monitor accurately the subcellular distribution of rabphilin fragments. To obtain a quantitative measure of overlap, we devised an algorithm to calculate the summed pixel values of an image produced from the multiplication of the two images produced by the red and green channels of the confocal microscope. This overlap quotient is not very sensitive but can clearly distinguish a high degree of colocalization (wild-type GFP-rabphilin and wild-type HA1-Rab3A) from no colocalization [wild-type GFP-rabphilin plus HA1-Rab3AΔCAC], which is cytosolic.

When the subcellular distributions of rabphilin mutants were analyzed, we were surprised to observe that the internal deletion of cysteines from the zinc finger domain, which sub-
stantially reduces Rab3A binding, did not prevent membrane association or the partial localization of GFP-rabphilin to secretory vesicles. An alternative explanation for the membrane association of these mutants is that they do not fold correctly and are targeted to the lysosomes for degradation. However, we could detect no evidence for proteolytic degradation of the ΔCys mutants in the context of full-length rabphilin. Moreover, the Δ2Cys(1–206) and Δ5Cys(1–206) fragments are soluble, suggesting that the membrane association of rabphilin requires an intact C terminus. Deletion of the C2 domains reduced the membrane association of the rabphilin fragment, although a fraction of the protein colocalized with the HA1-Rab3A in a punctate pattern. These results suggest that the efficient localization of rabphilin to the secretory vesicle membrane requires both Rab3A binding and a C-terminal region that includes the C2.

**FIG. 7.** (A) Equilibrium binding of Rab3A mutants to GST-rabphilin. GST-rabphilin(1–206) was bound to glutathione-Sepharose and incubated with 5 to 100 nM wild-type or mutant [γ-32P]GTP-Rab3A of a known specific activity. The beads were washed, and the amounts of Rab3A bound were determined by scintillation counting. Nonspecific binding of wild-type Rab3A to GST attached to glutathione-Sepharose was subtracted from all points. Binding assays were performed in triplicate. Data are representative of three experiments. Values ± standard deviations are given, ○, Rab3A; △, Rab3A(F51L); ●, Rab3A(T54A). (B) Partitioning of Rab3A mutants in PC12 cells. PC12 cells were transfected with 10 μg of wild-type (wt) or mutant pKH3-Rab3A and were processed as described in Materials and Methods. p and s, particulate and soluble fractions, respectively. (C) Localization by confocal microscopy of Rab3A mutants in PC12 cells. PC12 cells were cotransfected with 10 μg each of a mutant pKH3-Rab3A and GFP-rabphilin and were processed as described in Materials and Methods.
domains. A model consistent with these results is that the C2 domains provide a membrane-anchoring function, while the Rab3BD provides additional membrane-specific targeting information. This model would account for the in vitro data on rabphilin binding to vesicles that had been depleted of Rab3A (38) and can also explain the reduced level of rabphilin in Rab3A-negative transgenic mice, if mistargeted rabphilin is more rapidly degraded in neurons than is correctly targeted protein.

Finally, we have shown, in a converse experiment, that the recruitment of Rab3A to secretory vesicles is not dependent on rabphilin binding, in contrast to the model suggested by Shiri-takai et al. (38), in which it was implied that the function of rabphilin might be to recruit Rab3A to synaptic vesicle membranes. A Rab3A effector domain mutant, T54A, that does not bind rabphilin in vitro partitioned between the soluble and particulate fractions and localized within PC12 cells in a manner indistinguishable from that of wild-type HA1-Rab3A and from that of another effector domain mutant, F51L. Because both Rab3A mutants are defective in interaction with the soluble Rab3-GRF (9), we can also conclude that this exchange factor is not required for vesicle recruitment of Rab3A in PC12 cells. It is possible that another exchange factor specific for Rab3 is present on the secretory vesicle membrane or that the MSS4 exchange factor is present in these cells and can interact with the mutant Rab3 proteins.

How do the data described in the present study on rabphilin domain function relate to functional studies of rabphilin (12), in which deletion of the C2 domains produces a dominant interfering mutant that inhibits secretion from chromaffin cells? Two possible explanations come to mind. First, rabphilin might function as an adapter to connect Rab3A with another protein, and the loss of the C2 domains prevents this coupling. This hypothesis would predict that a rabphilin mutant defective in Rab3A binding would also possess a dominant interfering phenotype. It does not explain why overexpression of the wild type or a Q81L mutant of Rab3A inhibits secretion (22). Second, because the rabphilin (1–206) fragment is partially cytosolic, it could sequester a factor that is essential for vesicle docking, depleting it from its target site at the secretory vesicle membrane. This second hypothesis predicts that the N-terminal region of rabphilin binds another protein in addition to Rab3A and that this second interaction is necessary to facilitate vesicle docking. If Rab3A helps recruit rabphilin to the vesicle membrane but must then release it so as to allow it to bind to this second factor, then the overexpression of Rab3A might be expected to compete for binding and thereby reduce vesicle docking efficiency. The complete absence of Rab3A, as in transgenic mice, might also reduce docking efficiency by reducing the ability of rabphilin to be recruited to the vesicle membrane. Further studies will be necessary to distinguish these possibilities. The ability to express a GFP-rabphilin fusion protein and to detect it within living cells may provide a valuable tool in these studies.

After submission of our manuscript, Stahl et al. published similar data on the identification of the minimal Rab3A-binding domain of rabphilin and showed that this domain binds zinc ions (39). They also reported that Rab3A reversibly recruits rabphilin to the synaptosomal membrane. It is, therefore, important to consider our results in the context of these data. Stahl et al. (1996) reported a stoichiometry of one Zn2+ ion per mol of rabphilin. We believe this value to be an underestimate. In initial experiments using ZnCl2 as the standard, we frequently observed values of less than two Zn2+ ions per mol of rabphilin but also observed low values for the zinc-binding domain of PKCγ, a protein that is known to bind two Zn2+ ions per mol (32, 33). This underestimate may result from inefficient release of the Zn2+ ions from the protein. For this reason, we used PKCγ as the standard. Secondly, while our data support a model in which Rab3A recruits rabphilin to the secretory vesicle membrane, our observation that ΔCys-rabphilin mutants, which bind Rab3A poorly, remain membrane associated suggests that membrane attachment may be at least partially separable from targeting and that Rab3A is not the sole determinant of subcellular localization of rabphilin.

ACKNOWLEDGMENTS

C.J.M. and P.F.S. contributed equally to the work described in this paper.

We thank Ronald Holz for the bovine rabphilin-3A clone, Sujoy Ghosh for the Cys-2 PKCγ zinc-binding domain expression plasmid, and Genentech for the human recombinant nerve growth factor. We thank Donald Ross for assistance in performing the atomic absorption assays.

The work was supported by NIH grant CA56300 from the National Institutes of Health (DHHS) to I.G.M. C.J.M. was supported by an NIH Environmental Pathology training grant (EST3207122), and P.F.S. was supported by an NIH Cancer Biology training grant (T32CA09266).

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on May 15, 2021 by guest