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Interaction Cloning of Rabin3, a Novel Protein That Associates with the Ras-Like GTPase Rab3A

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Rab3A is a small, Ras-like GTPase expressed in neuroendocrine cells, in which it is associated with secretory vesicle membranes and regulates exocytosis. Using the yeast two-hybrid system, we have identified a rat brain cDNA encoding a novel 50-kDa protein, which we have named Rabin3, that interacts with Rab3A and Rab3D but not with other small GTPases (Rab3C, Rab2, Ran, or Ras). Several independent point mutations in the effector domain of Rab3A (F51L, V55E, and G56D) which do not alter nucleotide binding by the GTPase abolish the interaction with Rabin3, while another mutation (V52A) appears to increase the interaction. These results demonstrate that the interaction is highly specific. However, a glutathione *S*-transferase–Rabin3 fusion protein associates only weakly in vitro with recombinant Rab3A and possesses no detectable GTPase-activating protein or nucleotide exchange activity, and Rabin3 overexpressed in adrenal chromaffin cells has no observable effect on secretion. The protein possesses a sequence characteristic of coiled-coil domains and a second small region with sequence similarity to a *Saccharomyces cerevisiae* protein, Sec2p. Sec2p is essential for constitutive secretion in yeast cells and interacts with Sec4p, a close relative of the Rab3A GTPase. Rabin3 mRNA and protein are widely expressed but are particularly abundant in testes.

The complex molecular machinery that mediates vesicle fusion in the regulated secretory pathway is not yet understood, although several components have been cloned and subjected to functional analysis (3, 14). GTP is known to modulate vesicle fusion and secretion in a variety of systems, and recent evidence implicates the small, Ras-like GTPases in these processes. The Rab3A GTPase is expressed exclusively in neuroendocrine cells and is associated with secretory vesicle membranes (8, 13). Mutational analysis has recently demonstrated that Rab3A plays an important role in regulated secretion, possibly by controlling the assembly and dissolution of a inhibitory complex that prevents vesicle fusion with the plasma membrane in the absence of an appropriate stimulus (19). Rab3A interacts with a number of factors that control the guanine nucleotide-bound state of the protein (9, 21) and with a putative target, rabphilin 3, which possesses sequence similarity to synaptotagmin, an important component of the secretory machine in neurons (26). A related GTPase, Sec4p, is an essential component of the constitutive secretion pathway of the budding yeast *Saccharomyces cerevisiae*, and genetic approaches have identified several proteins in the secretory pathway with which it interacts, including proteins encoded by *SEC2*, *SEC8*, and *SEC15*, which have no known mammalian homologs (5, 24, 25).

To identify proteins that interact with Rab3A, we used the yeast two-hybrid system (10) with vectors developed by Durfee et al. (15) and a rat brain cDNA library generated in λACT. The library was screened with a GAL4 DNA-binding domain–Rab3A fusion construct as the "bait." One positive clone encoded a novel protein, which we have called Rabin3 (Rab3-interacting protein), that interacts with Rab3A but not with other small GTPases that were tested, including Rab2, H-Ras, and Ran. The protein possesses a putative coiled coil and a second small region with strong sequence similarities to the yeast protein Sec2p.

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MATERIALS AND METHODS

Interaction cloning using the yeast two-hybrid system. A cDNA library was constructed from the size-selected poly(A) RNA of a 160-g male Fischer 344 rat, using a λZAPII-cDNA kit from Stratagene. The library was ligated into the *Xho*I site of λACT, which was kindly provided by Stephen Elledge. The *Xho*I site is downstream from the activation domain of GAL4 (amino acids 768 to 881). The cDNA library was packaged with Gigapack II Gold packaging extract (Stratagene), and a total of 5×10^6 primary recombinants were obtained, with an average insert size of 1.5 kb. The λACT library was converted into a plasmid library by using the *cre-lox* site-specific recombination system. Rab3A lacking the three C-terminal amino acid residues was subcloned into pAS-CYH2 such that the open reading frame of Rab3A is in frame with the GAL4 DNA-binding domain (amino acids 1 to 147). The epitope tag, HA1, is between Rab3A and the GAL4 DNA-binding domain. *S. cerevisiae* Y190 (*MAT***a** *gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,112 URA3*::*GAL-lacZ LYS2*::*GAL-HIS3*) expressing the GAL4 DNA-binding domain–Rab3A fusion protein was transformed with the pACT library, using bacterial RNA as the carrier, and 5×10^6 primary transformants were selected for growth on medium lacking His and containing 25 mM 3-aminotriazole. Surviving colonies were screened for expression of β-galactosidase, using a 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) colony filter assay (27). Briefly, yeast colonies were transferred to nitrocellulose, permeabilized by submersion in liquid nitrogen for 2 to 3 s, and then laid onto Whatman no. 1 paper that had been presoaked in Z buffer (100 mM sodium phosphate, 10 mM potassium chloride, 1 mM magnesium sulfate, 39 mM β-mercaptoethanol) containing 0.75 mg of X-Gal per ml. The filters were then incubated at 30°C for 1 to 10 h. Blue coloration of a colony was indicative of a positive interaction. Positives were rescued from the filter, grown in selective medium, and tested against a nonspecific bait construct (SNF1–GAL4 DNA-binding domain fusion, in pAS1) (15). The cDNA from a positive clone was used as a probe to rescreen the rat brain library by colony hybridization.

Two-hybrid interaction assays. Bait vectors were constructed in pAS-CYH2. In cases where the expressed proteins would contain C-terminal Cys residues that could be prenylated, the PCR primers were designed so as to delete the final three amino acids. Independent transformants were patched onto selective medium, lifted onto nitrocellulose, and incubated with X-Gal to detect expression of β-galactosidase as described above. Transformants were also tested by a quan-

titative β-galactosidase solution assay using chlorophenol red-β-D-galactopyranoside (CPRG) as a substrate. Yeast cultures were grown to a density of approximately 7×10^6 cells per ml in dropout medium lacking leucine and tryptophan, washed in phosphate-buffered saline, resuspended in 0.5 ml of Z buffer, and lysed by vortexing with glass beads. Extracts were incubated with 8 mM CPRG in Z buffer, and the increase in A_{570} was monitored after 30 min to 2 h (16).

Northern (RNA) blot analysis. A multiple mouse-rat tissue poly(A) RNA blot (Clontech Laboratories, Inc.) was probed for expression of the Rabin3 gene, using randomly primed, ^{32}P-labeled fragments synthesized from the complete open reading frame of the Rabin3 cDNA. The blot was stripped and rescreened with an actin probe to assess the relative levels of mRNA from each tissue.

In vitro interaction of Rab3A with GST-Rabin3 fusion proteins. Rabin cDNA sequences encoding residues 101 to 460, 1 to 460, 1 to 316, and 1 to 222 were amplified by PCR using primers that incorporated *Bam*HI and *Eco*RI restriction sites at the 5′ and 3′ ends of the DNA, respectively. The amplified products were subcloned into pGEX-2T and transformed into *Escherichia coli* DH5α. The bacteria were induced with 1 mM isopropylthiogalactopyranoside (IPTG) for 5 h, and the fusion proteins were isolated by affinity chromatography on glutathione (GSH)-Sepharose (Pharmacia). Recombinant Rab3A was expressed as an unfused protein from the pET-3 vector and purified as described previously (8). Recombinant c-H-Ras was a kind gift of Alan Wolfman (Cleveland Clinic, Cleveland, Ohio). The glutathione *S*-transferase (GST) fusion proteins attached to GSH-Sepharose beads (30 ml) were incubated on ice for 20 min with approximately 0.3 mg of Rab3A in 20 mM Tris-HCl (pH 8.0)–1 mM $MgCl_2$–250 mM sucrose–0.1 mM phenylmethylsulfonyl fluoride. The beads were then rapidly washed twice with 0.7 ml of the binding buffer and denatured in Laemmli sample buffer. After separation on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and transfer to nitrocellulose (1 A, 1.5 h), the Rab3A retained by the GST fusion proteins was detected by Western blotting (immunoblotting) using an anti-Rab3A antiserum directed against a C-terminal peptide (8). Detection was by chemiluminescence (Amersham), using horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Jackson ImmunoResearch).

Expression of epitope-tagged Rabin3 in PC12 cells. A eukaryotic vector, pCH, described previously (6) was modified to incorporate two additional iterations of the HA1 tag to increase the sensitivity of tagged fusion proteins to monoclonal antibody 12CA5. Full-length Rabin3 was cloned into the *Bam*HI-*Eco*RI sites in this vector to generate a fusion protein containing the triple HA1 tag at the N terminus. This construct, or vector lacking insert, was electroporated into PC12 cells (20 mg of DNA per 10^7 cells, 960 mF, 450 V). After 3 days, the cells were harvested, swollen in hypotonic buffer (20 mM morpholinepropanesulfonic acid [MOPS; pH 7.3], 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride), and ruptured by passage through a 26-gauge needle. After centrifugation at $100{,}000 \times g$ for 60 min, the supernatant was precipitated with 10% trichloroacetic acid plus 0.1% sodium deoxycholate and dissolved in Laemmli sample buffer. The pellet was resuspended in hypotonic buffer plus 1% Nonidet P-40. The suspension was centrifuged at $10{,}000 \times g$ for 10 min, and the supernatant and pellet were dissolved in Laemmli sample buffer. Equal proportions of the fractions were separated on an SDS–10% polyacrylamide gel, transferred to nitrocellulose, and probed with monoclonal antibody 12CA5, which is specific for the HA1 epitope. Detection was by chemiluminescence, using a horseradish peroxidase-conjugated anti-mouse immunoglobulin (Jackson ImmunoResearch).

Production of anti-Rabin3 antiserum. The full-length Rabin3 open reading frame was subcloned into pGEX-2T and expressed in *E. coli* as a GST-Rabin3 fusion protein. The affinity-purified fusion protein was used as an immunogen in two rabbits to raise polyclonal antisera (Cocalico). A strong positive titer was obtained from one rabbit. The antiserum was purified by passage over a GST-GSH-Sepharose column to remove anti-GST antibodies.

Secretion assays in bovine adrenal chromaffin cells. The pCH3-Rabin3 vector was cotransfected into primary chromaffin cells in culture, together with a plasmid expressing growth hormone, and stimulated secretion was assayed as described previously (19). Transfections by calcium phosphate precipitation were performed in 35-mm-diameter wells with 3.2 mg of each plasmid. Expression of the tagged Rabin3 was determined by indirect immunofluorescence (19).

Nucleotide sequence accession number. The GenBank accession number for Rabin3 is U19181.

RESULTS

Identification of a novel Rab3A-binding protein. A yeast two-hybrid system was used to identify novel proteins that interact with the Rab3A GTPase. A Rab3A–GAL4 DNA-binding domain fusion was constructed in pAS-CYH2 (15). The final three amino acids of Rab3A were deleted in this construct, to prevent C-terminal posttranslational modification by prenylation and carboxymethylation (17). Such modifications might prevent nuclear translocation of the fusion protein, and in other experiments the full-length Rab3A-GAL4 construct was found to be highly growth inhibitory when transformed into yeast cells (not shown). Expression of the fusion protein was confirmed by Western blot analysis of yeast extracts with an anti-Rab3A antiserum (8) and with monoclonal antibody 12CA5, which recognizes the HA1 epitope incorporated into the fusion protein (15) between the GAL4 and Rab3A domains. The pAS-Rab3A(ΔCAC) construct was used to screen a rat brain cDNA library created as fusions with the GAL4 activation domain in pACT (15). From 5×10^6 primary transformants, approximately 10,000 survived initial selection on medium lacking Leu, Trp, and His and with 25 mM 3-aminotriazole added as a histidine synthesis inhibitor. Fourteen colonies were positive for β-galactosidase activity by a nitrocellulose filter assay. Of these initial positives, one transformant encoded a true positive that did not interact with a nonspecific test bait (a GAL4 DNA-binding domain–SNF1 fusion). The cDNA from this clone was used as a probe to rescreen the rat brain library, and we isolated a clone of about 2.4 kb which contains an open reading frame encoding a 460-amino-acid residue protein (Fig. 1A), the initial ATG of which obeys Kozak's rule. Multiple stop codons in all three reading frames were found upstream of this ATG, suggesting that the clone does represent the complete open reading frame. Moreover, an antiserum raised against the encoded polypeptide recognizes in various tissues a protein of the molecular mass (50 kDa) predicted from the sequenced open reading frame (see below). We have named the encoded protein Rabin3. The original clone encodes all but the first 100 amino acid residues of Rabin3. No similar protein was found in the GenBank database, but an N-terminal domain of Rabin3 is related to a large group of proteins, predominantly structural and cytoskeletal in function, that possess alpha-helical coiled-coil domains. Of these proteins, the yeast protein Sec2p (24) exhibits the closest similarity to this Rabin3 domain. Additionally, Sec2p contains another stretch of 7 amino acid residues, 100 residues C terminal to the coiled-coil domain, that is virtually identical to a similar stretch in the same relative location in Rabin3 (Fig. 1B). A second database search revealed that this sequence is unique to Sec2p and Rabin3. The complete Rabin3 open reading frame is 27.3% identical and 52.4% similar to that of Sec2p, but analysis using the randomization function of the Genetics Computer Group BESTFIT program (1) indicates that the overall similarity between the two proteins is not significant. Hydrophobicity analysis did not detect any stretches of residues of sufficient length and hydrophobicity to constitute a membrane-spanning domain. Secondary structure analyses by the methods of Chou and Fasman (11) and Garnier et al. (18) both predict a strong alpha-helical nature for the N-terminal region of Rabin3, particularly for residues 150 to 243. When displayed in a helical wheel projection, the putative Rabin3 coiled-coil domain exhibits an amphipathic character similar to that of Sec2p (24), although the predicted salt bridges and acidic repeats are less well defined (Fig. 1C). The hydrophobic face of the helix may allow protein-protein interaction with a second such helix (12), as is believed to occur in the formation of the myosin coiled coils that occur in the thick filaments of skeletal muscle (20).

Recombinant Rabin3, which has a predicted molecular mass of 50 kDa, eluted from a Superose 12 gel permeation column at an apparent size of about 100 to 150 kDa (not shown), indicating that Rabin3 either exists in solution as a homomultimer or is highly asymmetric. Transient expression of $(HA1)_3$-tagged Rabin3 in PC12 cells demonstrated that the tagged protein is predominantly cytosolic (Fig. 2A) and elutes from the Superose 12 column at an apparent molecular size of >200

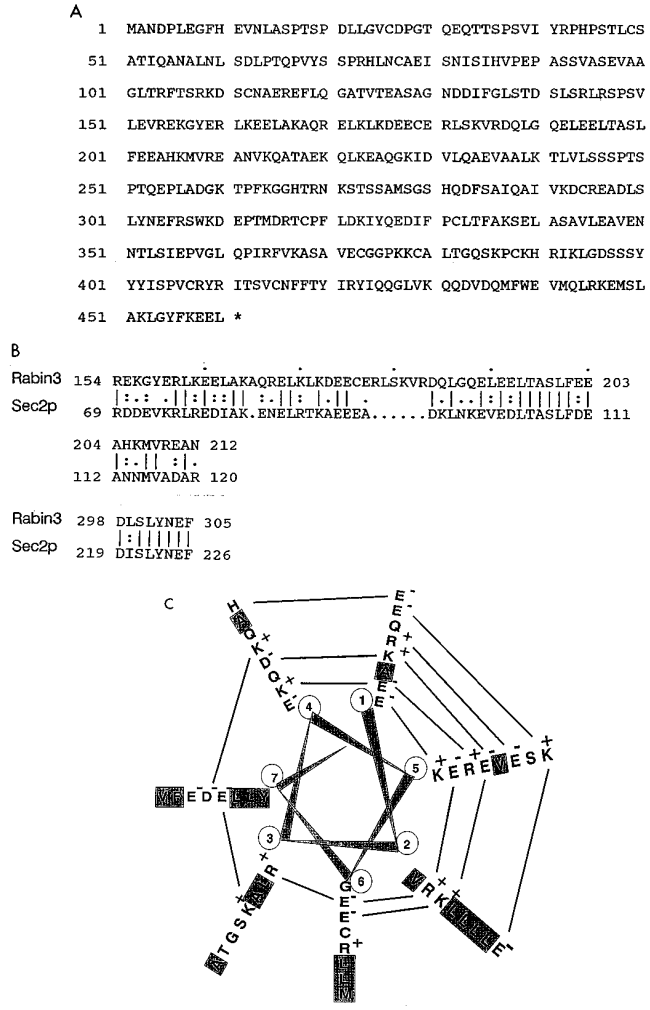

FIG. 1. Interaction cloning of Rabin3 and sequence similarity to the yeast protein Sec2p. (A) Predicted open reading frame of Rabin3, from rat brain. Clone 4B, identified by interaction with Rab3A by using the yeast two-hybrid system, encodes amino acid residues 101 to 460. (B) Regions of strong similarity to Sec2p. (C) Helical wheel projection of residues 152 to 208. Hydrophobic residues are boxed, and predicted salt bridges are shown as solid lines. The GenBank database was searched for sequences similar to that of Rabin3, using the NCBI BLAST file server (1) and the Genetics Computer Group BESTFIT program.

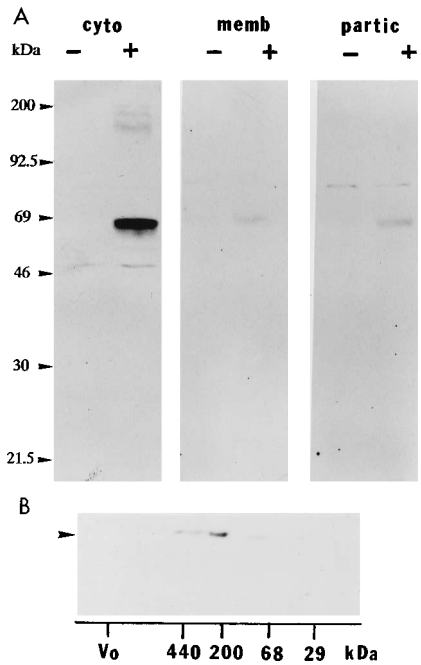

FIG. 2. Epitope-tagged Rabin3 is predominantly cytosolic in PC12 cells. (A) Subcellular distribution of $(HA1)_3$-Rabin3 in transiently transfected PC12 cells. (B) Elution of tagged Rabin3 from a Superose 12 size exclusion column. PC12 cells were electroporated with a vector, pCH3, with (+) or without (−) Rabin3 insert, which produces an epitope-tagged Rabin3 expressed from the human cytomegalovirus promoter. Transfected cells were swollen in hypotonic buffer, ruptured by passage through a 26-gauge needle, and fractionated into soluble (cyto), detergent-soluble (memb), and particulate (partic) fractions as described in Materials and Methods. After separation on an SDS–10% polyacrylamide gel and transfer to nitrocellulose, the tagged Rabin3 was detected with the anti-HA1 monoclonal antibody 12CA5. A portion of the cytosolic fraction was loaded onto a Superose 12 column equilibrated with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4)–200 mM NaCl and eluted at 0.5 ml/min. Fractions (0.5 ml) were collected, precipitated with 10% trichloracetic acid, and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. The column was calibrated by using standards of known size. Vo, void volume.

kDa (Fig. 2B), suggesting that within the cell it may exist as a complex with additional polypeptides.

Tissue distribution of Rabin3. To determine the tissue specificity of Rabin3, the cDNA encoding the open reading frame was used as a probe in a Northern blot of mRNAs from several mouse and rat tissues. Two mRNA species of approximately 1.8 and 2.5 kb were observed and were expressed ubiquitously, with particularly high levels in testes (Fig. 3). The largest insert isolated from the rat brain cDNA library corresponded closely in size to the 2.5-kb message.

To confirm the Northern blot data, an antiserum that specifically recognizes recombinant Rabin3 was used to probe rat tissue extracts (Fig. 4). A protein of the expected size (50 kDa) was detected by immunoblotting in all tissues and is expressed at the highest levels in testes. No cross-reaction was observed with preimmune serum.

While it was initially surprising to observe widespread expression of a factor that appears to interact specifically with Rab3A, a GTPase expressed only in neuroendocrine cells, we have previously detected Rab3-specific nucleotide exchange and GTPase-activating protein (GAP) activities in numerous tissues that do not express detectable levels of Rab3A (9). The Rab GDP dissociation inhibitor (GDI) is also expressed ubiquitously (21). This distribution may reflect the fact that there are a number of close homologs of Rab3A, including Rab3B, Rab3C, and Rab3D, that are widely expressed (2, 22, 28) and that probably interact with the same factors. For example, Rab3D mRNA is present only at very low levels in brain but is highly expressed in fat tissue and in lungs (2).

To test the hypothesis that Rabin3 can interact with other Rab3 isoforms, we constructed Rab3C- and Rab3D-GAL4 fusions in pAS-CYH2 and analyzed them for interaction with full-length Rabin3 in the two-hybrid assay. Interaction was assayed either after a colony lift onto nitrocellulose, using X-Gal, or by a soluble β-galactosidase assay on yeast cell extracts, using CPRG as the substrate (Fig. 5 and Table 1). Interestingly, although Rab3C produced no detectable signal, Rab3D interacted with Rabin3 at least as effectively as Rab3A (Table 1). The GTP-binding domains and effector regions of all three isoforms of Rab3 are identical, and the sequences differ only in their N-terminal and C-terminal domains. We do not know if Rab3C is expressed in a functional form as a GAL4-fusion protein.

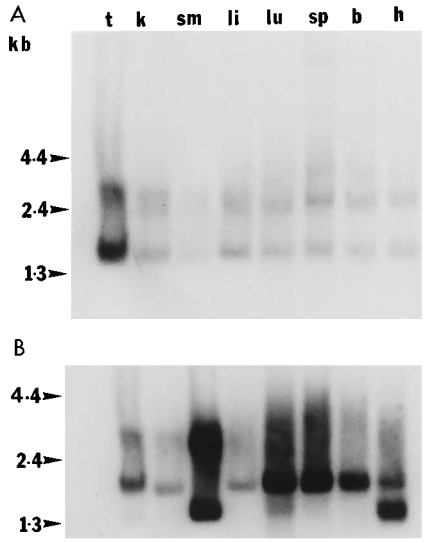

FIG. 3. Tissue distribution of Rabin3 mRNA. The open reading frame of Rabin3 was used to prepare a random-primed probe, which was hybridized to a Clontech multiple rat-mouse tissue blot. The hybridization conditions were 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7])–2% SDS at 42°C. Washing was at 50°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS followed by 0.1× SSC–0.1% SDS. The blot was exposed to X-ray film for 2 days (A). The blot was then stripped and reprobed with a β-actin probe (B). Lanes: t, testes; k, kidney; sm, smooth muscle; li, liver; lu, lung; sp, spleen; b, brain; h, heart.

Specificity of interaction between Rab3A and Rabin3. To test the specificity of the interaction of Rabin3 with Rab3 isoforms, three other cDNAs encoding small GTPases in the Ras superfamily (c-H-Ras, Rab2, and Ran1/TC4) were subcloned into pAS1 and used as bait in the two-hybrid system. Rab2 is 42% identical in amino acid sequence to Rab3A, whereas H-Ras and Ran are each approximately 30% identical. For Rab2 and c-H-Ras, the C-terminal Cys residues were deleted to prevent in vivo prenylation (Fig. 5 and Table 1).

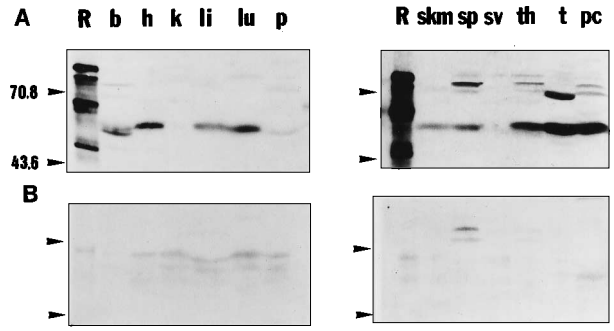

FIG. 4. Anti-Rabin3 immunoblot of mouse tissue extracts. Mouse tissues were homogenized in phosphate-buffered saline plus 1 mM phenylmethylsulfonyl fluoride and centrifuged at 2,000 × *g* for 2 min. To the supernatant were added sodium deoxycholate and Nonidet P-40 to final concentrations of 0.5% each, and the suspension was recentrifuged at 20,000 × *g* for 10 min. Supernatants were collected and analyzed for protein concentration. Equal amounts of protein (200 μg except where noted below) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose (2 h at 1 A), and immunoblotted with rabbit anti-Rabin3 antiserum diluted 1:500 (A) or with preimmune rabbit serum diluted 1:500 (B) for 3 h. The nitrocellulose was then incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (1/10,000) and detected by chemiluminescence (Amersham). As a positive control, 20 ng of GST-Rabin3 was loaded on the gel next to the tissue samples. Note that the GST-Rabin3 is partially proteolyzed during isolation. Lanes: R, GST-Rabin3; b, brain; h, heart; k, kidney; li, liver; lu, lung; p, pancreas; skm, skeletal muscle (100 μg); sp, spleen; sv, seminal vesicles; th, thymus (100 μg); t, testes; pc, PC12 cells.

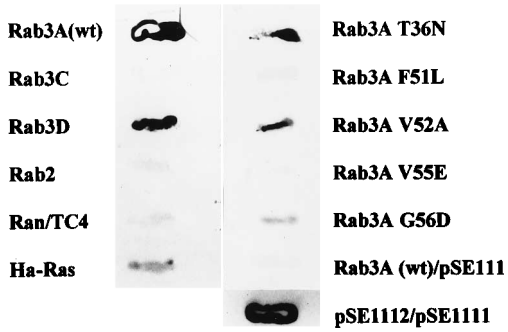

FIG. 5. Interaction of Rabin3 with the Rab3A GTPase is highly specific. In the two-hybrid interaction in situ assay, independent transformants were patched, lifted onto nitrocellulose, and incubated with X-Gal (27). Full-length Rabin3 or the original clone (residues 101 to 460) was expressed as a fusion with the DNA-binding domain of GAL4 in pAS-CYH2. Dark coloration indicates a positive signal. pSE1112 and pSE1111 served as independent positive controls for two-hybrid interaction. pSE1112 encodes SNF1 fused to the DNA-binding domain of GAL4 in pAS-CYH2, and pSE1111 encodes SNF4 fused to the activation domain of GAL4 in pACT (15). Expression of the GAL4-Rab3A fusion protein was found to partially inhibit growth of the yeast colonies. wt, wild type.

None of these other Ras-like GTPases (Ras, Ran, and Rab2) showed significant interaction in the colony lift assay, using three independent transformants, and only Ras showed any significant interaction (other than Rab3A) in the solution assay. Western blot analysis of the transformed yeast strains, using anti-HA1 tag antibody 12CA5, demonstrated that the test constructs were expressing protein at a level similar to or higher than that of Rab3A. The Ras construct expressed at least 10 to 15 times as much fusion protein as did the others, and the weak positive response to Ras may therefore represent a nonspecific interaction due to the unusually high nuclear concentration of the bait.

An intact effector domain is necessary for interaction of Rab3A with Rabin3. The effector domain of Rab3A has been

TABLE 1. β-Galactosidase solution assay using CPRG as a substrate[a]

Bait	Prey	% Activity[b]
pAS-Rab3A	pACT-Rabin3	100
pSE1112	pACT-Rabin3	10
pAS-Rab3C	pACT-Rabin3	18
pAS-Rab3D	pACT-Rabin3	167
pAS-Ran	pACT-Rabin3	0
pAS-Rab2	pACT-Rabin3	0
pAS-Ras	pACT-Rabin3	30
pAS-Rab3A(F51L)	pACT-Rabin3	0
pAS-Rab3A(V52A)	pACT-Rabin3	617
pAS-Rab3A(V55E)	pACT-Rabin3	16
pAS-Rab3A(G56D)	pACT-Rabin3	33
pSE1112	pSE1111[c]	400

[a] Transformants were grown in liquid culture and lysed, and cell extracts were tested for β-galactosidase activity (16). Western blot analysis of the expression of the fusion proteins of Rab3A, Ras, Ran, and Rab2 showed that Ras expression was 10 to 15 times greater than that of the other small GTPases (not shown).

[b] Expressed as a percentage of the interaction of Rabin3(101-460) with wild-type Rab3A (0.012 optical density unit/mg of protein/ml/min). All values are means of multiple independent assays (n = 2 to 7).

[c] pSE1112 and pSE1111 were used as a positive control for the two-hybrid assay (pSE1112 encodes SNF1 as a fusion with the GAL4 DNA-binding domain; pSE1111 encodes SNF4 as a fusion with the GAL4 activation domain).

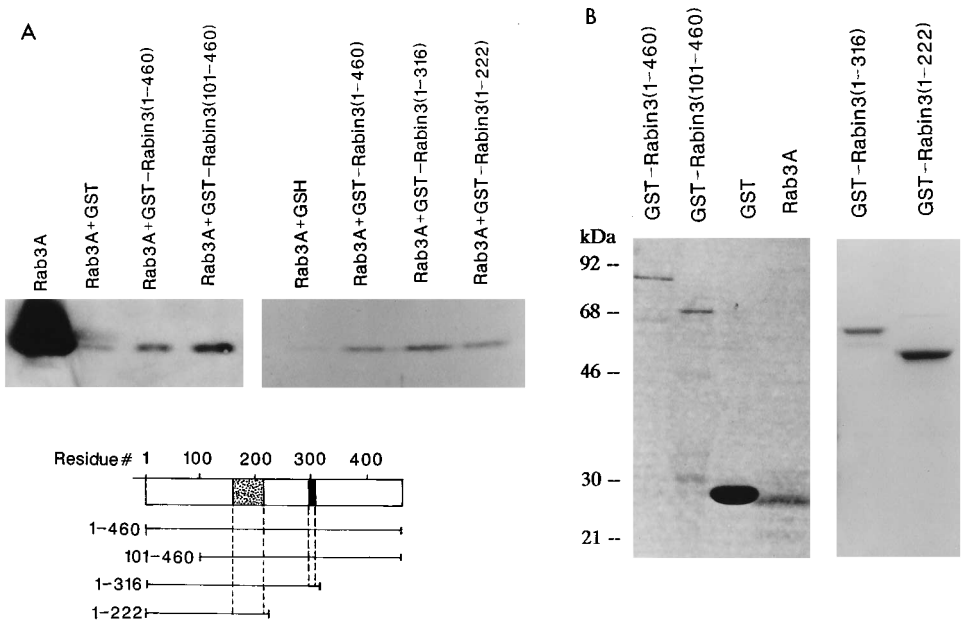

FIG. 6. Evidence for a direct interaction between Rabin3 with the Rab3A GTPase. (A) Western blot showing binding of recombinant Rab3A to GST, GST–Rabin3(101-460), GST–Rabin3(1-460), unconjugated GSH-Sepharose, GST–Rabin3(1-316), and GST–Rabin3(1-222). Lane 1 contains 10% of the total Rab3A (3 μg) added to the GSH-Sepharose beads. We estimate that about 1% of the total Rab3A is retained on the beads by GST-Rabin3. The map indicates the sizes of the Rabin3 fragments and their relationship to the location of the putative coiled-coil domain (speckled box) and the Sec2p domain (filled box). (B) Coomassie blue-stained gel showing relative levels of fusion proteins used in the binding assay. Rabin cDNA sequences encoding residues 101 to 460, 1 to 460, 1 to 316, and 1 to 222 were amplified by PCR and subcloned into pGEX-2T. Bacteria were induced with 1.5 mM IPTG for 5 h, and the fusion proteins were isolated by affinity chromatography on GSH-Sepharose. Approximately 1 μg of GST–Rabin3(1-460) was used in the binding assay. Recombinant Rab3A was expressed as an unfused protein from the pET-3 vector and purified as described previously (8).

shown to be important for functional interactions with all known regulatory factors, including Rab3-GAP, Rab3–guanine nucleotide-releasing factor (GRF), Rab-GDI, and rabphilin 3 (6, 7), and independent mutations of effector domain residues alter the interaction with each factor in a unique pattern (23). We were therefore interested in whether an intact effector domain is also essential for the interaction of Rab3A with Rabin3 and whether the phenotypic pattern matches that for other known regulatory factors. Rab3A mutants F51L, V52A, V55E, and G56D were subcloned into pAS-CYH2 and tested for interaction with Rabin3 in the two-hybrid assay. Results of the colony lift are shown in Fig. 5, and the quantitative solution assay data are presented in Table 1. The mutations in residues 51, 55, and 56 all disrupt the interaction of Rab3A with Rabin3. Surprisingly, however, the V52A mutant showed a dramatically increased response in the two-hybrid assay, suggesting that it possesses a higher affinity for Rabin3 than does the wild-type Rab3A. Western blots of yeast lysates showed that all the mutants were expressed (not shown). These data confirm the specificity of the interaction between these two proteins and suggest that the interaction may be mediated at least in part through the effector domain of Rab3A.

A second mutation, T36N, which is equivalent to the S17N dominant-inhibitory mutation of Ras and which drastically reduces the ability of Rab3A to bind GTP (7), caused no significant reduction in the interaction with Rabin3. This result indicates that Rabin3 can recognize the GDP-bound or nucleotide-free state of Rab3A.

In vitro interaction of Rab3A with GST-Rabin3 fusion proteins. A positive interaction in the two-hybrid assay may reflect direct association between bait and prey or an indirect association mediated by an endogenous bridging protein. To determine whether Rab3A associates directly with Rabin3, we attempted to reconstruct the interaction in vitro, using recombinant Rab3A and a GST fusion of Rabin3 attached to GSH-Sepharose (Fig. 6). We used Ras and unfused GST as negative controls. The proteins were mixed for 20 min on ice, rapidly washed and subjected to SDS-PAGE, transferred to nitrocellulose, and probed with anti-Rab3A antiserum (8) or an anti-Ras antibody, Y13-259. A significantly larger amount of Rab3A was detected bound to the GST-Rabin3 constructs than to GST alone. No such difference was detected when Ras instead of Rab3A was used (not shown). The association between Rab3A and Rabin3 was quite weak, however, and was not resistant to extensive washing. Various deletion mutants were constructed, expressed as GST fusion proteins, and tested for binding of Rab3A. These fragments (all of which contain the putative coiled-coil domain) all appeared to associate with Rab3A in the pull-down assay (Fig. 6).

Functional assays of Rabin3. The full-length GST-Rabin3 fusion protein exhibited no detectable guanine nucleotide exchange activity or GAP activity when [α-^{32}P]GDP- or GTP-loaded Rab3A was used as a substrate.

Bovine chromaffin cells are nondividing secretory cells that release catecholamines and soluble protein contents of their secretory granules in response to nicotinic agonist stimulation. In previous experiments using human growth hormone as a reporter for the regulated secretory pathway, we demonstrated by transient transfection that overexpression of wild-type Rab3A or Rab3A-Q81L inhibited regulated, Ca^{2+}-dependent exocytosis from the cells (19). We have used this approach to investigate the effects of the cloned Rabin3 gene and mutants on regulated exocytosis. Transfection with a plasmid encoding wild-type Rabin3, Rabin3(1-222), or Rabin3(1-316) had no effect on the secretion of coexpressed growth hormone. Transfection of Rabin3 wild-type plasmid also did not significantly

alter the almost complete inhibition of growth hormone secretion caused by overexpression of Rab3A (data not shown). Expression of the Rabin3 was confirmed by indirect immunofluorescence using the anti-HA1 tag antibody 12CA5 (19). Thus, these data do not reveal a role for Rabin3 in the late stages of the regulated secretory pathway that are probed by this assay.

DISCUSSION

The Rab3A GTPase appears to function in neuroendocrine cells as an inhibitor of regulated exocytosis (19). To understand this function at a molecular level, it is essential to identify the proteins with which Rab3A interacts. To date, these proteins include the Rab geranylgeranyltransferase (17) and GDI (21), which associate with many members of the Rab family; rabphilin 3, a brain-specific protein that may be a specific target for Rab3A (26); and two regulatory factors, GAP and GRF, which control the guanine nucleotide state of Rab3A but which have not yet been purified or cloned (9). Using the yeast two-hybrid system, we have now identified a sixth protein, which we have named Rabin3, that interacts with the Rab3A GTPase when coexpressed in yeast cells. The interaction appears to be of low affinity and may be mediated in part at least through the effector domain of Rab3A. It does not require posttranslational modification of the Rab3A C terminus. Rabin3 interacts with Rab3D in addition to Rab3A but did not interact with other small GTP-binding proteins tested, including another isoform, Rab3C, and another member of the Rab subfamily, Rab2. As further evidence for the specificity of the interaction, several point mutations in the putative effector domain of Rab3A abolished expression of β-galactosidase activity in the two-hybrid interaction assay.

Interestingly, the phenotypic pattern for the interaction of Rabin3 with the mutants that we tested in the two-hybrid assay is similar to that found previously for the exchange factor GRF. In particular, we had found previously that the V52A mutant is supersensitive to GRF (23), and in the present study we observed a similar supersensitivity of V52A to interaction with Rabin3. The other mutants examined (F51L, V55E, and G56D) are all insensitive to both GRF and Rabin3 (7). Moreover, the T36N mutant of Rab3A, which behaves biochemically like the S17N dominant-negative mutant of Ras, scores positive in the two-hybrid assay, suggesting that Rabin3 can recognize the GDP-bound state of Rab3A, another property in common with GRF (7). We have been unable to detect any Rab3A-specific guanine nucleotide exchange activity of recombinant Rabin3. Nonetheless, these results raise the intriguing possibility that Rabin3 is a component of an exchange factor for Rab3, perhaps acting to target a catalytic subunit to the GTPase.

Rab3-GRF is specific for the GDP-bound state of Rab3A. We do not yet know whether this property is also possessed by Rabin3. The in vitro interaction is of too low an affinity to provide reliable quantitative data, and we have not yet identified mutants of Rab3A that we know to be locked principally in the GTP-bound state when expressed in intact cells. We note that GRF also has an very low affinity for Rab3A, of around 20 μM (7).

Rabin3 is expressed ubiquitously, with particularly high levels of message and protein in testes. Several regulatory factors for Rab3 are also ubiquitous, including the exchange factor Rab3-GRF, Rab3-GAP, and Rab-GDI (9, 21), although rabphilin 3, a putative target for Rab3A, has so far been detected only in neuronal cells (26). This broad pattern of expression is understandable in view of the existence of other Rab3 isotypes that are found in numerous tissues. Indeed, we found that Rab3D, which is expressed at high levels in lung and adipose tissue, interacts as efficiently with Rabin3 as does Rab3A. Similarly, H-Ras, K-Ras, and N-Ras can all interact with the p120 Ras-GAP and NF1 (4).

Although the complete open reading frame of Rabin3 does not possess significant similarity to any other protein sequence in the current database, two regions show strong homology to Sec2p (24), which is a cytosolic protein from *S. cerevisiae* that has been demonstrated by genetic criteria to interact with Sec4p, a small GTPase in the Rab subfamily that is 53% identical to Rab3A in amino acid sequence. Both Sec4p and Sec2p are essential components of the constitutive secretory pathway in budding yeast cells. However, when Rabin3 was introduced into a yeast strains expressing temperature-sensitive mutants of Sec2p (NY26 and NY130, kindly provided by Peter Novick), it was unable to complement the growth defect at the nonpermissive temperature (23a). Sec2p is a component of a cytosolic complex of at least 500 kDa in size (24). Epitope-tagged Rabin3 expressed in PC12 cells elutes from a sizing column at about 200 kDa and in earlier fractions than when expressed in bacteria, suggesting that it may form a complex with other cytosolic proteins.

The challenging aspect of these data is the lack of any detectable function for Rabin3. As far as we can judge, the recombinant protein possesses no GAP or exchange factor activity and does not perturb regulated exocytosis when overexpressed in chromaffin cells. It is unlikely that the yeast two-hybrid screen is detecting a nonspecific interaction, given the gain or loss of interaction when single-point mutations are introduced into the effector domain of the Rab3A. We therefore consider this explanation unlikely. A second possibility is that the interaction is indirect and that a third component is necessary before function can be observed. For instance, the Rab-specific geranylgeranyltransferase possesses a Rab-targeting subunit that does not possess catalytic activity. The very close correspondence between the phenotypes of the effector domain mutants for GRF sensitivity and Rabin3 interaction suggests the intriguing possibility that Rabin3 is a targeting subunit for the Rab3 exchange factor. As mentioned above, size estimates of Rabin3, using epitope-tagged protein transfected into PC12 cells, do indicate that it may associate with other proteins, although we do not yet know their identity. It will clearly be of interest to determine whether the complex possesses guanine nucleotide exchange activity.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Baldini, G., T. Hohl, H. Y. Lin, and H. F. Lodish.** 1992. Cloning of a Rab3 isotype predominantly expressed in adipocytes. Proc. Natl. Acad. Sci. USA **89:**5049–5052.
3. **Bennett, M. K., and R. H. Scheller.** 1993. The molecular machinery for secretion is conserved from yeast to neurons. Proc. Natl. Acad. Sci. USA **90:**2559–2563.
4. **Bollag, G., and F. McCormick.** 1991. Differential regulation of Ras GAP and neurofibromatosis gene product activities. Nature (London) **351:**576–579.
5. **Bowser, R., H. Muller, B. Govindan, and P. J. Novick.** 1992. Sec8p and Sec15p are components of a plasma membrane-associated 19.5S particle that

may function downstream of sec4p to control exocytosis. J. Cell Biol. **118:** 1041–1056.
6. **Brondyk, W. H., C. J. McKiernan, E. S. Burstein, and I. G. Macara.** 1993. Mutants of Rab3A analogous to oncogenic Ras mutants. Sensitivity to Rab3A-GTPase activating protein and Rab3A-guanine nucleotide releasing factor. J. Biol. Chem. **268:**9410–9415.
7. **Burstein, E. S., W. H. Brondyk, and I. G. Macara.** 1992. Amino acid residues in the Ras-like GTPase Rab3A that specify sensitivity to factors that regulate the GTP/GDP cycling of Rab3A. J. Biol. Chem. **267:**22715–22718.
8. **Burstein, E. S., and I. G. Macara.** 1989. The Ras-like protein p25 is partially cytosolic and is expressed only in neural tissue. Mol. Cell. Biol. **9:**4807–4811.
9. **Burstein, E. S., and I. G. Macara.** 1992. Characterization of a guanine nucleotide-releasing factor and a GTPase-activity protein that are specific for the ras-related protein p25rab3A. Proc. Natl. Acad. Sci. USA **89:**1154–1158.
10. **Chien, C.-T., P. L. Bartel, R. Sternglanz, and S. Fields.** 1991. The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc. Natl. Acad. Sci. USA **88:**9578–9582.
11. **Chou, P. Y., and G. D. Fasman.** 1978. Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzyme Regul. **47:**145–147.
12. **Crick, F. H. C.** 1952. Is α-keratin a coiled coil? Nature (London) **170:**882–883.
13. **Darchen, F., A. Zahraoui, F. Hammel, M. P. Monteils, A. Tavitian, and D. Scherman.** 1990. Association of the GTP-binding protein Rab3A with bovine adrenal chromaffin granules. Proc. Natl. Acad. Sci. USA **87:**5692–5696.
14. **DeBello, W. M., H. Betz, and G. J. Augustine.** 1993. Synaptotagmin and neurotransmitter release. Cell **74:**947–950.
15. **Durfee, T., K. Becherer, P. L. Chen, S. H. Yeh, Y. Yang, A. E. Kilburn, W. H. Lee, and S. J. Elledge.** 1993. The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. Genes Dev. **7:**555–569.
16. **Eustice, D. C., P. A. Feldman, A. M. Colberg-Poley, R. M. Buckey, and R. H. Neubauer.** 1991. A sensitive technique for the detection of β-galactosidase in transfected mammalian cells. BioTechniques **11:**739–742.
17. **Farnsworth, C. C., M. Kawata, Y. Yoshida, Y. Takai, M. H. Gelb, and J. A. Glomset.** 1991. C terminus of the small GTP-binding protein smg p25A contains two geranylgeranylated cysteine residues and a methyl ester. Proc. Natl. Acad. Sci. USA **88:**6196–6200.
18. **Garnier, J. D., K. J. Osguthorpe, and B. Robson.** 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. **120:**97–120.
19. **Holz, R., W. H. Brondyk, R. A. Senter, L. Kuizon, and I. G. Macara.** 1994. Evidence for involvement of Rab3A in Ca^{2+}-dependent exocytosis from adrenal chromaffin cells. J. Biol. Chem. **280:**1–6.
20. **Kavinsky, C. J., P. K. Umeda, J. E. Levin, A. M. Sinha, J. M. Nigro, S. Jakovicic, and M. Rabinowitz.** 1984. Analysis of cloned mRNA sequences encoding subfragment 2 and part of subfragment 1 of α- and β-myosin heavy chains of rabbit. J. Biol. Chem. **259:**2775–2781.
21. **Matsui, Y., A. Kikuchi, S. Araki, Y. Hata, J. Kondo, Y. Teranishi, and Y. Takai.** 1990. Molecular cloning and characterization of a novel type of regulatory protein (GDI) for *smg* p25A, a *ras* p21-like GTP-binding protein. Mol. Cell. Biol. **10:**4116–4122.
22. **Matsui, Y., A. Kikuchi, J. Kondo, T. Hishida, Y. Teranishi, and Y. Takai.** 1988. Nucleotide and deduced amino acid sequences of a GTP-binding protein family with molecular weights of 25,000 from bovine brain. J. Biol. Chem. **263:**11071–11074.
23. **McKiernan, C. J., W. H. Brondyk, and I. G. Macara.** 1993. The Rab3A GTPase interacts with multiple factors through the same effector domain. J. Biol. Chem. **268:**24449–24452.

23a. **McKiernan, C. J., and I. G. Macara.** Unpublished data.

24. **Nair, J., H. Muller, M. Peterson, and P. Novick.** 1990. Sec2 protein contains a coiled-coil domain essential for vesicular transport and a dispensable carboxyl terminal domain. J. Cell Biol. **110:**1897–1909.
25. **Salminen, A., and P. J. Novick.** 1989. The Sec15 protein responds to the function of the GTP binding protein, Sec4, to control vesicular traffic in yeast. J. Cell Biol. **109:**1023–1036.
26. **Shirataki, H., K. Kaibuchi, T. Sakoda, S. Kishida, T. Yamaguchi, K. Wada, M. Miyazaki, and Y. Takai.** 1993. Rabphilin-3A, a putative target protein for *smg* p25A/*rab*3A p25 small GTP-binding protein related to synaptotagmin. Mol. Cell. Biol. **13:**2061–2068.
27. **Vajtek, A. B., S. M. Hollenberg, and J. A. Cooper.** 1993. Mammalian Ras interacts directly with the serine/threonine kinanse Raf. Cell **74:**205–214.
28. **Zahraoui, A., N. Touchot, P. Chardin, and A. Tavitian.** 1989. The human Rab genes encode a family of GTP-binding proteins related to yeast YPT1 and Sec4 products involved in secretion. J. Biol. Chem. **264:**12394–12401.