Isolation and Characterization of a Dual Prenylated Rab and VAMP2 Receptor*

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Rab GTPases have been implicated in intracellular vesicle trafficking. Using the yeast two-hybrid screen, we have isolated a rat clone that interacts with Rab3A as well as with Rab1. The gene encodes a 20.6-kDa protein with two extensive hydrophobic domains and is broadly expressed in all tissues. This protein binds to prenylated Rab GTPases but not to other small Ras-like GTPases such as the Rho/Rac family. This prenylated Rab acceptor (PRA1) also binds specifically to the synaptic vesicle protein VAMP2 (or synaptobrevin II) but shows no affinity for VAMP1 or cellubrevin in both the yeast two-hybrid system and in vitro binding assays. This specificity resides, in part, in the proline-rich domain of VAMP2 as a chimera containing this domain of VAMP2 fused to VAMP1 is able to bind to PRA1. The transmembrane domain of VAMP2 is also essential as its deletion abolished binding to PRA1. Replacement of the deleted VAMP2 transmembrane domain by a CAAX prenylation signal can not restore binding to PRA1. This interaction is therefore distinct from that required for VAMP2 binding to either syntaxin or both syntaxin and SNAP-25. Deletion analysis on PRA1 indicates that the critical Rab- and VAMP2-interacting residues reside in two regions: the amino-terminal residues 30–54 and the extreme carboxy-terminal domain. This dual Rab and VAMP2 binding characteristic suggests that PRA1 may serve to link these two protein families in the control of vesicle docking and fusion.

The Rab family of GTPases plays a regulatory role in the intracellular transport of vesicles by cycling through GDP- and GDP-bound states (1, 2). The inactive GDP-bound form of Rab is recruited from the cytosol to the correct membrane compartment by a process involving dissociation of GDP-bound Rab from its cytosolic carrier, GDP dissociation inhibitor (GDI), followed by GDP-GTP exchange catalyzed by an exchange factor (3–5). There are currently two proteins promoting GDP-GTP exchange, Mss4 (6) and Rab3 GDP/GTP exchange protein or GEP (7). Mss4 promotes guanine nucleotide exchange in a subset of Rab proteins (8), whereas Rab3 GEP is specific to the Rab3 subfamily (7). Moreover, the guanine nucleotide exchange activity of Mss4 does not appear to be influenced by Rab prenylation (8, 9) in contrast to the Rab3 GEP. The specificity of these guanine nucleotide exchange proteins raises the possibility that each Rab or Rab subfamily may possess a unique set of proteins catalyzing guanine nucleotide exchange and membrane localization. The GTP-bound Rab associated with the membrane is thought to ensure proper docking and fusion of transport vesicles. The precise mechanism by which the Rab GTPase mediates vesicle fusion remains unclear, but it appears to be required for the assembly of the SNARE fusion complexes (10).

The Rab GTPases are post-translationally modified by the addition of a prenoid moiety to the characteristic cysteine-containing motif at the carboxyl terminus (11–13). This modification is thought to promote membrane localization and is essential for biological function. Prenylation involves the addition of either farnesyl (15-carbon) or geranylgeranyl (20-carbon) moiety catalyzed by three distinct prenyl transferases exhibiting characteristic prenoid and protein substrate specificity (14–18). Farnesyl transferase and geranylgeranyl transferase I are responsible for the prenylation of GTPases containing the characteristic CAAX motif at the carboxyl terminus. Most members of the Rab family, however, are modified by the addition of two GG moieties by geranylgeranyl transferase II. This modification requires presentation of newly synthesized Rab by a Rab escort protein (REP), a protein that is defective in the retinal degenerative disease choroideremia (19, 20).

The Rab GTPases are localized to distinct intracellular compartments. Rab3 is a member of the Rab subfamily associated with regulated secretory vesicles undergoing fusion with the plasma membrane. There are currently four Rab3 isoforms (A, B, C, and D). Rab3A, -B, and -C are found predominantly in the plasma membrane. There are currently four Rab3 isoforms (A, B, C, and D). Rab3A, -B, and -C are found predominantly in the plasma membrane. There are currently four Rab3 isoforms (A, B, C, and D). Rab3A, -B, and -C are found predominantly in the plasma membrane. There are currently four Rab3 isoforms (A, B, C, and D). Rab3A, -B, and -C are found predominantly in the plasma membrane."
PC12 cells, Rab3A appears to be an inhibitor of secretion (27), while Rab3B plays a stimulatory role (28).

To determine the mechanism by which Rab GTPases mediate synaptic vesicle trafficking, we have initiated a yeast two-hybrid screen (29) for proteins interacting with Rab3A (22). In addition to REP and GDI, other proteins are known to interact with Rab3 or other Rab proteins. Rabphilin-3A, a protein with two C2 domains, binds the GTP-bound form of Rab3 (30) and its interaction with α-actinin may serve to link Rab3 function to actin bundling (31). Rabin-3 interacts with a subset of Rab3 GTPases through the putative effector domain (32). Recently, a GTPase-activating protein has been described for Rab3A (33). We describe here a Rab-interacting protein isolated from rat brain that is homologous to a partial mouse Rab5/6-interacting GTPase-activating protein has been described for Rab3A (33).

Plasmids—The Rab3A sequences were cloned into the bait plasmid for PRA1(1–164). PRA1(1–110) contains the amino-terminal fragment spanning the first transmembrane domain fused to the Gal4 activation domain for PRA1(30–185) and PRA1(54–185), respectively. The following forward primer for VAMP2(N49A). Subset of the above VAMP2 primers were used to generate deleted transmembrane domain (TMD) variants of the two mutant VAMP2. To substitute the prolinc-rich domain of VAMP1 with that of VAMP2, the following oligonucleotides were used to introduce a Pci site used to PCR amplify the VAMP1 sequence corresponding to amino acid residues 34–118: 5'-AGG ACT GCA GAA ACC GCA GC-3'. The resulting PCR product was then ligated to a NcoI for Pci fragment containing the NH2-terminal 1–31 amino acids of VAMP2. The resulting chimera containing VAMP2(1–31) ligated to VAMP1(34–118) was then subcloned into pGAD vector.

Yeast Two-hybrid Screen—The Y190 yeast strain was co-transformed with the Rab3A bait plasmid and cDNA library as described (38) and plated on selective plates supplemented with 25 μM 3-amino-1,2,4-triazole. His+ colonies were screened for β-galactosidase activity 7–10 days later by filter assay (39).

Northern Blot Analysis—Northern blot containing poly(A)+-selected mRNA (CLONTECH) was probed with the entire EcoRI and XhoI fragment labeled by random hexamer primer. Hybridization containing 1–10×10⁶ cpm/ml was performed in 5×SSPE containing 50% formamide at 42°C for 18 h and washed with 2×SSPE containing 50% formamide at 50°C.

Tissue Western Immunoblot Analysis—A PCR fragment encoding amino acids 20–75 was subcloned into pQE40 (Qiagen) using the following primers: 5'-GAA GTG CAT GGC CCC CCT TTA-3 and 5'-TCG GAG GAT TG C TGG GCC TGG-3'. The His-tagged fusion protein was purified using Ni-NTA beads (Invitrogen) and was used to immunize rabbits.

Rab tissues were homogenized with a Potter-Elvehjem assembly in 10 volumes of phosphate-buffered saline containing 2% Triton X-100, and a protease inhibitor mixture containing 2 mM bromo-4-chloro-3-indolyl β-n-galactopyranoside (39).

Northern Blot Analysis—Northern blot containing poly(A)+-selected mRNA (CLONTECH) was probed with the entire EcoRI and XhoI fragment labeled by random hexamer primer. Hybridization containing 1–10×10⁶ cpm/ml was performed in 5×SSPE containing 50% formamide at 42°C for 18 h and washed with 2×SSPE containing 50% formamide at 50°C.

EXPERIMENTAL PROCEDURES

Strains and Media—The host Saccharomyces cerevisiae strain Y190 (MATa ura3-52 his3-200 ade2–101 trpl–1 901 leu2–3, 112 gal4Δalgal80 URA3::GAL-lacZ yh1-1 LYS2::GAL-3His6) was used in the yeast two-hybrid screen. Growth conditions and media were as described (36).

Purification of 6His-Tagged and GST Fusion Proteins—The influence hemagglutinin (HA) tag was added to the amino terminus of the PRA1 clone by subcloning the PCR amplified product into a HA tag containing BlueScript plasmid. The following primer pairs were used to PCR amplify the full-length PRA1(1–185): 5'-CAT GCC ATG GGC CCC CAG AAG G-3' and 5'-AGG TCC ATG GAC ACT TTA CAC-3'. The PCR fragments were blunt-end subcloned into the EcoRV site of a HA tag containing BlueScript plasmid. The XhoI to XbaI fragment was then excised and blunt-end subcloned into the SalI site of the 6His tag plQE11 (Qiagen).

Expression and purification on Ni-NTA resin (Qiagen) were as described by the manufacturer. Briefly, bacteria from 1 liter of culture were sonicated in 50 mM phosphate buffer, pH 8.0, 300 mM NaCl, 1% Triton X-100. The cleared lysate was bound to Ni-NTA resin and washed with 20–40 volumes of 50 mM phosphate, pH 6.0, 300 mM NaCl, 10% glycerol, 1% Triton X-100. The fusion proteins were eluted from the resin with 0.25 or 0.3 M imidazole in 50 mM phosphate, pH 6.0, 300 mM NaCl, 10% glycerol containing 0.05% Triton. The 6His-HA-tagged fusion proteins were detected by Western immunoblot analysis using 12CA5 anti-HA monoclonal antibodies.

The full-length and transmembrane-deleted clones of VAMP2 expressed as GST fusion proteins were generously provided by Dr. W. S. Trimble, University of Toronto. Cultures grown to mid-log were induced with 1 mM isopropyl-1-thio-β-n-galactopyranoside and incubated for another 5–8 h. Cells were resuspended in Lysis Buffer (25 mM HEPES-KOH, pH 7.5, 100 mM KCl, 2 mM EDTA, 0.4 mM PMSF, 2 mM DTT,
0.3 mM sucrose, 20% glycerol). Lysozyme was added to 10 mg/ml, and the suspension was incubated at 4 °C for 1 h. Cells were lysed by sonication in the presence of 1% Triton X-100. Insoluble material was cleared by centrifugation at 10,000 × g for 20 min. The GST fusion proteins were purified by affinity chromatography using glutathione-agarose beads (Sigma). The beads were washed with 20 volumes of Wash Buffer (25 mM HEPES-KOH, pH 7.5, 100 mM KCl, 2 mM EDTA, 0.4 mM PMSF, 2 mM DTT, 0.1% Triton X-100). The recombinant proteins were eluted with Elution Buffer (10 mM reduced glutathione, 25 mM HEPES-KOH, pH 7.5, 100 mM KCl, 2 mM EDTA, 0.4 mM PMSF, 2 mM DTT, 0.05% Triton X-100). All recombinant proteins were quantified by densitometric analysis of the immunoblots subjected to varying length of exposure to x-ray films.

In Vitro Protein Binding Assay and Co-precipitation—Unless otherwise stated, 3 pmol of the GST fusion proteins and 6 pmol of the HA-tagged PRA1 constructs were used for the in vitro affinity chromatography assays. The GST fusion proteins (GST, GST-VAMP2, GST-VAMP2-ATMD, GST-VAMP1, and GST-cellubrevin) were pre-bound for 1 h at 4 °C to 40 μl of 50% glutathione-agarose slurry in 1 ml of Binding Buffer (25 mM HEPES-KOH, pH 7.5, 140 mM KAc, 2 mM DTT, 0.1% gelatin, 10% glycerol, 0.05% Triton). The beads were washed twice in Binding Buffer before the addition of recombinant PRA1(1–185) and PRA1(1–164) in 1 ml of Binding Buffer. The bead slurry was incubated at 4 °C for 16 h and washed five times in Binding Buffer. Proteins bound to the beads were eluted with SDS Loading Buffer at 65 °C for 10 min and subjected to Western immunoblot analysis. Bound PRA1 was detected using anti-HA monoclonal antibodies and GST fusion proteins by polyclonal antibodies against VAMP2 or VAMP1. The bound antibodies were detected by horseradish peroxidase-coupled secondary antibodies and ECL. The amount of PRA1 bound to GST-VAMP2 was estimated by densitometric analysis of the immunoblots subjected to varying length of exposure to x-ray films.

Co-precipitation with recombinant PRA1 was performed on detergent solubilized rat brain extract. Frozen rat brains were thawed and homogenized with a Polytron homogenizer in 10 ml of Homogenization Buffer (10 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM PMFS) per brain. The cell debris was removed by centrifugation at 5000 × g for 10 min. The remaining supernatant was extracted with 1% Triton X-100 at 4 °C for 60 min, and insoluble membranes were removed by centrifugation at 150,000 × g for 60 min. The supernatant was diluted 20-fold with Lysis Buffer and incubated with recombinant PRA1 together with monoclonal anti-HA. Monoclonal anti-rat transferrin receptor antibodies were used as control. Guanine nucleotides (GTPγS or GTPβS) and MgCl2 were added to a final concentration of 0.2 mM and 1.2 mM, respectively. After incubation at 4 °C for 18 h, 25 μl of Protein G-Sepharose was used to recover the monomeric antibodies. The resin was washed six times with 10 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 0.01% Triton X-100. Proteins were analyzed by Western immunoblot using antibodies against Rab3A and VAMP2, as described above.

RESULTS

Structural Features of the Rab3A-interacting Clone—We used the wild type and GTPase-deficient mutant form (Q81L) of Rab3A to identify Rab-interacting proteins in a directional rat brain cDNA library we have constructed. A screen of over 3 × 108 yeast transformants produced 38 positive clones that interacted with both forms of Rab3A. Sequence and hybridization analyses indicate that eight were rabin-3 (32) and the rest cross-hybridized to each other. The longest clone (861 base pairs) represents the full-length rat cDNA based on Northern analysis and screening of a rat brain A(Zap cDNA library. The clone contains an open reading frame of 185 amino acids. Sequence analysis indicates that this clone is homologous to a partial sequence of the Rab6/5 partner C described in mouse (34). However, three discrepancies corresponding to nucleotide positions 101, 196, and 204 in the published sequence were detected. The clones were sequenced in both directions, and sequence spanning residues 77–70. Based on the binding characteristics (see below), we decided to designate the protein encoded by this clone as PRA1 for prenylated Rab acceptor. PRA1 has a predicted molecular mass of 20.6 kDa, and no signal peptide was apparent at the amino terminus. It contains two extensive hydrophobic domains at positions 78–112 and 140–164 (Fig. 1A). The first hydrophobic domain is 35 amino acids in length, and both hydrophobic domains are predicted to form β sheets. The protein contains one or more potential phosphorylation sites clustered around residues 35–50.

BLAST search of GenBank revealed that PRA1 is similar to at least two rat EST clones (accession numbers H31184 and H35218) and to a number of human EST clones. Sequence analysis and secondary structure prediction revealed that PRA1 also shows similarity to a yeast gene product, Yip3p (Fig. 1B). The similarities are localized to a region proximal to the first hydrophobic domain (residues 40–104) and to a portion of the second hydrophobic domain (residue 134–171) with an overall similarity of 40% and 32%, respectively. The two proteins contain a divergent amino-terminal 39 amino acids and showed no sequence similarity in the region between the two hydrophobic domains. It is notable that the overall structure of the mammalian and yeast protein is conserved. Both proteins contain two extensive hydrophobic domains, and the carboxyl terminus each clone contains an open reading frame of 185 amino acids.

PRA1 Is Broadly Expressed in All Tissues—Northern blot analysis indicates that PRA1 is encoded by a 950-base pair transcript expressed in all tissues with the exception of the testis, whereby a 1000-base pair transcript was detected (Fig. 2). Although PRA1 was isolated from a rat brain cDNA library by virtue of its interaction with the neuronal specific Rab3A, its broad tissue distribution suggests that PRA1 may be involved in membrane trafficking events in all tissues.

Western immunoblot analysis of detergent solubilized extracts from different tissues is consistent with the broad tissue distribution of the PRA1 message. An apparent 24-kDa protein was detected in crude Triton X-100 solubilized cellular extracts from brain, liver, kidney, skeletal, and cardiac muscles (Fig. 3) using an anti-PRA1 polyclonal antiserum. This immunoreactive protein appears larger than the predicted size of the protein and occasionally appears in a form consistent of a dimer.

PRA1 Binds to Farnesylated and Geranylgeranylated Rab—Our initial analysis from the yeast two-hybrid screen indicates that PRA1 interacts with both Rab3A and Rab1. A number of mutations were subsequently introduced to further determine the structural features within the Rab GTPase responsible for binding to PRA1. These mutations were designed to test whether interaction is dependent on conformational changes on Rab induced by the guanine nucleotide, and whether PRA1 exhibits any preference toward the prenyl group. Deletion of the carboxyl-terminal CXC prenylation signal in Rab3A (Rab3A(ΔCXC)), and Rab3A(Q81L,ΔCXC)), and the last two cysteines in Rab1 (Rab1(ΔC)) completely abrogated PRA1 interaction (Fig. 4). This indicates that PRA1 interaction requires modification of the Rab protein with a prenoid group. To address whether PRA1 is capable of distinguishing di-GG from mono-GG or farnesylated proteins, the carboxyl-terminal CXC of Rab3A was mutated to CVL and CVLS. The former was expected to be geranylgeranylated by geranylgeranyl transferase I and the latter farnesylated by farnesyl transferase. As shown in Fig. 4A, both Rab3A(CVL) and Rab3A(CVLS) interacted equally well with PRA1. This indicates that PRA1 is capable of recognizing Rab with a mono-GG group and that interaction is not limited to GG-modified Rab but also includes farnesylated Rab. A mutation affecting GTPase activity, Rab3A(Q81L), decreased but did not abolish interaction. However, this may be due to decreased efficiency in GG modification of the GTP-bound Rab, as REP is able to discriminate between the two nucleotide-bound forms of Rab (40). Recognition of Rab GTPases by PRA1 is not limited to the prenyl group. It would appear that PRA1 also recognizes domain(s) common to the Rab family of GTPases, since other prenylated Ras-like
GTPases such as RhoA(G14V) and Rac1 failed to interact with PRA1.

PRA1 Specifically Interacts with VAMP2—We next explored the possibility that PRA1 might be involved in recruiting Rab to the target membrane or in transmitting the Rab signal to downstream effector(s). Since components of the v-SNARE are possible downstream targets for a membrane bound Rab (10), we decided to examine whether PRA1 might interact with components of the SNARE complexes. VAMP is an obvious choice since studies in yeast have shown that lethality due to loss of YPT1, a Rab GTPase involved in the transport of vesicles from the ER to the Golgi complex, can be suppressed by VAMP-
like proteins (41–43). To test whether PRA1 might be a protein that can link the Rab and VAMP protein families, we examined the ability of mammalian VAMP-like proteins to interact with PRA1. Fig. 5A shows that PRA1 interacted with VAMP2 but not with closely related VAMP1 or the ubiquitous cellubrevin. Primary sequence comparisons revealed that VAMP1 and VAMP2 exhibit 90% identity with the differences residing mainly at the amino-terminal proline-rich domain and the carboxyl-terminal TMD. Although there is little similarity between the two TMD at the primary amino acid level, the overall hydrophobicity is maintained. To determine whether the proline-rich domain contributes to the specificity observed with VAMP2, we exchanged the proline-rich domains of VAMP1 and VAMP2. A VAMP2-VAMP1 chimera was constructed with the amino-terminal 31 amino acids of VAMP2 fused to the remainder of VAMP1 (amino acids 34–118). This VAMP2-VAMP1 chimera showed a positive β-galactosidase reaction when tested against PRA1. Thus, the amino-terminal proline-rich domain of VAMP2 may be partially responsible for the specificity in the interaction between the two proteins.

Interaction with PRA1 requires the carboxyl-terminal TMD of VAMP2, as its deletion (VAMP2(ΔTMD)) completely abolished interaction. We next examined whether replacing the deleted TMD of VAMP2 with a CAAX prenylation signal, CAIL or CAIS, can restore PRA1 interaction. Based on the properties of the CAAX prenyl transferases, VAMP2(ΔTMD, CAIL) and VAMP2(ΔTMD, CAIS) are expected to be modified by a GG and farnesyl group, respectively. Neither VAMP2(ΔTMD, CAIL) nor VAMP2(ΔTMD, CAIS) interacted with PRA1. Therefore, modification of VAMP2 by a prenoid group is not sufficient for PRA1 interaction and suggests that the TMD may impart structural conformation necessary for PRA1 binding.

Since targeting or recycling of VAMP2 to synaptic vesicle is affected by mutations at residues 46 and 49 (44), we examined the effects of these mutations on PRA1 interaction. Neither VAMP2(M46A) nor VAMP2(N49A) showed any significant effect on PRA1 interaction (Fig. 5A). As with the wild type VAMP2, deletion of the TMD in these two mutants, VAMP2(M46A,ΔTMD) and VAMP2(N49A,ΔTMD), abolished interaction with PRA1. To verify that the VAMP hybrids are functional, they were tested against the full-length syntaxin 1A (Syn1A), a protein known to interact with VAMP (45). Both VAMP1 and VAMP2 interacted strongly with Syn1A, whereas a weaker interaction was detected with cellubrevin (Fig. 5B). As expected, deletion of the TMD in VAMP2 had no effect on Syn1A interaction. Therefore, we conclude that PRA1 specifically interacts with VAMP2 and that this interaction requires an intact hydrophobic TMD.

FIG. 4. Yeast two-hybrid interaction of variants of Rab3A to PRA1. Interaction was observed with the wild type Rab3A, GTPase defective Rab3A(Q81L), mono-geranylgeranylated Rab3A(CVLL), farnesylated Rab3A(CVLS), and wild type Rab1. Interaction was abolished when the prenylation signal was deleted in Rab3A(ΔCX2), Rab3A(Q81L,ΔCX2), and Rab1(ΔCC). No interaction was observed with Rac1 or RhoA(G14V) family of Ras-like GTPases.

The Amino- and Carboxyl-terminal Amino Acids in PRA1 Are Essential for Rab and VAMP2 Binding—To determine the domains within PRA1 responsible for binding to prenylated Rab GTPase and to VAMP2, deletion analysis was performed on PRA1. Deletion of the carboxyl-terminal 21 amino acids (PRA1(1–185)) and the second hydrophobic domain (PRA1(1–110)) abolished interaction to both Rab3A and VAMP2 (Fig. 6). Moreover, frameshift substitutions introduced into the carboxyl-terminal 10 amino acids of PRA1(1–175) abolished binding to Rab3A and VAMP2. This indicates that the carboxyl-terminal domain of PRA1 is essential for prenylated Rab3A and VAMP2 interaction or for protein conformation. Amino-terminal deletion of 8 (PRA1(9–185)) and 29 amino acids (PRA1(30–185)) showed no effect on interaction to either Rab3A or VAMP2. However, deletion of 53 amino acids (PRA1(54–185)) abolished binding to Rab3A and significantly diminished binding to VAMP2. Thus, the critical Rab GTPase- and VAMP2-interacting domains reside in the carboxyl-terminal domain and the amino-terminal domain spanning amino acids 30–54. The results also raise the possibility that Rab and VAMP2 may bind to the same PRA1 sites.

PRA1 Binds Directly to Prenylated Rab3A and Full-length VAMP2 in Vitro—To biochemically confirm the binding to PRA1, in vitro interactions were performed using recombinant proteins. Full-length PRA1(1–185) and carboxyl-terminal truncated PRA1(1–164) were expressed with a 6xHis tag followed by the HA epitope at the amino terminus. As seen with the endogenous protein, a small fraction of the bacterially expressed proteins is often detected as a dimer on SDS gel (Fig. 7A). When added to detergent-solubilized extracts of rat brain, Rab3A was recovered with the recombinant full-length PRA1(1–185) independent of exogenously added guanine nucleotides (Fig. 7B).

Interaction between PRA1 and VAMP was examined using various VAMP isoforms expressed as GST fusion proteins. Both full-length GST-VAMP2 and GST-VAMP1 are expressed at
lower levels compared with the truncated GST-VAMP2 (ΔTMD). In all cases, smaller GST-containing proteolytic products co-purify with the full-length protein (Fig. 7C), but they do not appear to have any effect on protein binding. In vitro affinity chromatography assays indicate that the full-length PRA1(1–185) can bind to the immobilized full-length VAMP2 but not to GST, the TMD-deleted VAMP2(ΔTMD), or full-length VAMP1 (Fig. 7C). The full-length PRA1(1–185) also failed to bind to immobilized GST-cellubrevin (data not shown). Binding between PRA1(1–185) and GST-VAMP2 is sensitive to detergent such that a Triton X-100 concentration of 0.1% or higher significantly decreased the binding. In contrast to the full-length PRA1(1–185), the carboxyl-terminal deleted PRA1(1–164) failed to bind to any of the GST fusion proteins (Fig. 7C). However, longer exposure of the immunoblot did reveal that a small amount of the truncated PRA1(1–164) was bound to the full-length VAMP2. To further characterize the effect of deletion of PRA1 on its interaction with VAMP2, we performed affinity chromatography assays using full-length PRA1(1–185) and truncated PRA1(1–164) on increasing amounts of full-length VAMP2. As shown in Fig. 8A, the full-length PRA1(1–185) bound to GST-VAMP2 to a much greater extent than the truncated PRA1(1–164). We estimated a 20-fold difference in the binding of the full-length PRA1(1–185) to immobilized GST-VAMP2 when compared with the truncated PRA1(1–164) (Fig. 8B). These results are consistent with the yeast two-hybrid interaction, indicating that deletion or mutation of the carboxyl-terminal residues of PRA1 severely affected its interaction with VAMP2.

**DISCUSSION**

The PRA1 clone was isolated as a Rab3A-interacting clone in a yeast two-hybrid screen of a rat brain cDNA library. The deduced amino acid sequence predicts that PRA1 contains at least two extensive hydrophobic domains. These may form a membrane-spanning domain or the inner hydrophobic core of the protein. Sequence comparison revealed that PRA1 is homologous to the yeast gene YIP3 with sequence similarities localized to regions adjacent to the two hydrophobic domains. The yeast Yip3p also contains two extended hydrophobic domains and an overall acidic carboxyl-terminal end. This similarity raises the possibility that PRA1 and Yip3p may share a common function in mediating vesicular transport. Moreover, it is consistent with the observation that proteins involved in the secretory pathway are often conserved from yeast to mammals (46, 47).

PRA1 appears to interact with a number of prenylated Rab GTPases. It has been shown previously to interact with Rab5 and Rab6 (34), and we showed here that it also interacts with Rab3A and Rab1. Binding requires modification of the Rab proteins.

**FIG. 6.** Deletion analysis of PRA1 interaction with Rab3A and VAMP2. Schematic representations of the structures of the various PRA1 deletions are shown on the left with the boxed area representing the predicted hydrophobic domains. The full-length PRA1 interacted with both Rab3A and VAMP2. Deletion of the second hydrophobic domain (PRA1(1–110)), carboxyl-terminal domain (PRA1(1–164)), and replacement of the carboxyl-terminal 10 amino acids (PRA1(1–175)) all abolished interaction to both Rab3A and VAMP2. Amino-terminal deletion of 8 (PRA1(9–185)) and 29 amino acids (PRA1(30–185)) showed no effect on interaction to both Rab3A and VAMP2. However, deletion of 53 amino acids (PRA1(54–185)) abolished interaction to both Rab3A and VAMP2.

**FIG. 7.** Affinity chromatography assay of the interaction between PRA1 and the GST-VAMP family. A. Western immunoblot of bacterially expressed 6xHis and HA-tagged full-length PRA1(1–185) in lane 1, and carboxyl-terminal truncated PRA1(1–164) in lane 2 detected with anti-HA monoclonal antibodies. A small fraction of the recombinant proteins often appeared as SDS-resistant dimer. B. Rab3A from detergent solubilized rat brain extract co-precipitates with recombinant PRA1(1–185) in the presence of GTPyS or GDPyS. The recombinant PRA1 was recovered with mouse monoclonal anti-HA antibodies and Rab3A was detected with rabbit anti-Rab3A antibodies. Monoclonal anti-rat transferrin receptor was added in place of anti-HA in the control lane. C. In vitro binding of recombinant HA-PRA1 (6 nM) to immobilized GST fusion proteins (3 nM). Bound PRA1 were recovered and detected by Western immunoblot using anti-HA antibodies (top panel). GST-VAMP fusion proteins recovered from the beads were detected with anti-VAMP2 (middle panel) or anti-VAMP1 antibodies (lower panel). The carboxyl-terminal transmembrane domain was deleted in GST-VAMP2ΔTMD. All recombinant GST-VAMPs contain a smaller, proteolytically cleaved product co-purifying with the full-length fusion protein.
VAMP2 interacts with PRA1 in the yeast two-hybrid system equally well to both VAMP1 and VAMP2 (45) whereas only that of VAMP and Syn1 in many ways. First, Syn1 binding the TMD in Syn1 binding have been localized to the conserved cytoplasmic domain toward VAMP2 raises the possible existence of isoforms of the Rab GTPase to the SNARE complexes. It is more plausible that PRA1 is involved in mediating a protein interaction. The extensive overlap in the PRA1 domain spans the last 10 amino acids. It is interesting to note that similarity to the yeast Yip3p extends over residues 36–102 of PRA1, and suggests a possible conservation of structure and function. A notable feature of this domain is the abundance of arginine residues. In contrast to the basic nature of the minimal amino-terminal domain, the carboxyl-terminal domain of PRA1 is rich in glutamate. Deletion or substitution of this carboxyl-terminal domain also abolished binding to both Rab3a and VAMP2 in the two-hybrid system, and to VAMP2 in vitro binding assays. It is unlikely that the interaction between PRA1 and VAMP2 is due to nonspecific binding between the hydrophobic domains of the two proteins. Both VAMP1 and cellubrevin contain a similar hydrophobic domain at the carboxyl terminus, yet failed to bind to the full-length or truncated PRA1. Furthermore, deletion of the carboxyl-terminal domain of PRA1 leaves the two hydrophobic domains intact, yet binding to VAMP2 was significantly diminished. When combined with the results from the yeast two-hybrid system, it would appear that the amino- and carboxyl-terminal segments flanking the two hydrophobic domains are essential for protein-protein interaction. The extensive overlap in the PRA1 domains involved in Rab3a and VAMP2 binding raises the possibility that these two proteins may compete for the same binding site in PRA1, and is consistent with the observation that proteins in the secretory pathway often interact with multiple partners. One would predict that binding of PRA1 to the Rab GTPase will likely influence VAMP2 binding and vice versa.

How might a protein showing little or no specificity toward the different Rab proteins but highly specific for VAMP2 be
involved in regulating vesicular transport? We envision that PRA1 may play a role in regulating the interaction between VAMP2 and the t-SNARE proteins. One possibility is that PRA1 binding to VAMP2 may prevent premature interaction with the t-SNARE proteins. Recruitment of the Rab GTPase to the correct membrane compartment, and its binding to PRA1 may serve to disrupt PRA1 and VAMP2 interaction. This consequently frees VAMP2 to interact with components of the t-SNARE complex leading to vesicle docking and fusion. Hydrolysis of the bound GTP in the Rab GTPase and its subsequent extraction from the membrane by GDI may serve to trigger binding of PRA1 to VAMP2. Based on this scenario, the membrane cycling of Rab as governed by the bound guanine nucleotide may serve to regulate the availability of VAMP2 to interact with and mediate formation of the SNARE complexes through its binding to PRA1. Further studies are required to understand the role of the Rab GTPases and PRA1 in the formation of the SNARE complexes.

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