PRA Isoforms Are Targeted to Distinct Membrane Compartments*

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Rab GTPases are a family of 20–29-kDa Ras-like GTPases localized to unique intracellular organelles (1–7). They cycle through an active membrane-bound GTP- and inactive cytosolic GDP-bound states. They contain a characteristic Cys-containing motif at the extreme carboxyl terminus that is posttranslationally modified by one or more prenyl residues (8–13). This modification is essential for membrane localization and function. The GDP-bound Rab is removed from the membrane by a cytosolic carrier protein known as GDI or GDP dissociation inhibitor, which also inhibits the dissociation of GDP and maintains Rab in the inactive state. Guanine nucleotide exchange occurs at the membrane and is catalyzed by a guanine nucleotide exchange factor of which a Rab3-specific (14) and the yeast Sec4p-specific form have been identified (15, 16). The low GTPase activity required for Rab cycles (31–33). We describe here the isolation of a second PRA isoform that shares many similarities to PRA1 including overall physical properties, tissue distribution, and broad binding specificity toward the Rab GTPases. However, it differs from PRA1 in its subcellular localization. Whereas PRA1 was localized predominantly in the Golgi complex, PRA2 was found in the ER compartment. Moreover, we showed that the localization signal resides in the COOH-terminal region of the proteins.

EXPERIMENTAL PROCEDURES

Isolation of PRA2—A EST data base search using the conserved domain of the rat PRA1 resulted in a number of similar clones, most notably that with accession numbers AW519501 and AA051031. The rat PRA2 was subcloned into a vector containing the hemagglutinin (HA) tag by ligation between the XbaI and StuI sites. PRA2 was PCR-amplified from a rat brain cDNA library using the following oligonucleotides: 5′-TAAGGCCTATGGACGTGAACCTCG-3′ and 5′-GCTCTAGATGTTCCCTCGGCTTTGCTGA-3′. The resulting PCR fragment was sequenced.

Northern Blot Analysis—A 32P-labeled full-length PRA2 probe was prepared using random hexamer labeling. Hybridization solution (5 × SSPE, 10 × Denhardt’s solution, 0.1% SDS) at 42 °C overnight.
Plasmid Construction—PRA2 was subcloned into the yeast two-hybrid prey vector, pGAD424X using PRA2.E 
9 5-GCAAGATCCATGGGACGATGAGCAATGGC-3' and PRA2.X2, 5'-CGAGAATTCTTACTCCCTGGC-3'
and PRA2.X2, 5'-CGAGAATTCTTACTCCCTGGC-3'. The PCR products were digested with
9 9 CCTGGCTTTGCTGAT-3' and PRA2.E, 5'-CGAGAATTCTTACTC-
9 9 CCTGGCTTTGCTGAT-3' and PRA2.E, 5'-CGAGAATTCTTACTC-
CCTGGCTTTGCTGAT-3'. The sequences were com-
pared using BestFit alignment program with identical residues indicated by a
bar and similar residues with a colon. The predicted hydrophobic domains are
underlined.

FIG. 1. PRA2 sequence and comparison to PRA1. The sequences were com-
pared using BestFit alignment program with identical residues indicated by a
bar and similar residues with a colon. The predicted hydrophobic domains are
underlined.
located at residues 35 to 47, immediately amino-terminal to the first hydrophobic segment of the two proteins. One notable difference between the two proteins was the length of the amino- and COOH-terminal domains flanking the two hydrophobic segments. PRA2 has a shorter amino-terminal domain of 46 residues compared with 77 in PRA1, but a longer COOH-terminal domain of 53 residues compared with 21 in PRA1. The PRA2 COOH-terminal domain has a cluster of basic residues in contrast to PRA1 which has a number of acidic residues. Moreover, this COOH-terminal domain of PRA2 has a weak coiled-coil formation, which might play a role in protein-protein interaction. A search of the data base revealed that PRA2 is identical to the rat JWA (accession number AAP60354) and highly similar to the human JM4 (accession number NP 009144) clones.

Northern blot analysis showed that PRA2 was encoded by a single 1.5–1.8-kilobase transcript and broadly expressed in most tissues (Fig. 2). The message was most abundant in the heart and brain. This expression pattern was similar to that of PRA1 (29) with one exception: there was a lower level of expression of PRA2 in the testis whereas PRA1 was highly expressed. This broad expression pattern suggests that PRA2 might also be involved in membrane trafficking events in all tissues. In both PRA1 and PRA2, the testis transcript appeared larger than that from other tissues. The reason for this remained unknown.

PRA2 Binds Multiple Rab Isoforms in a Guanine Nucleotide-independent Manner—Since PRA1 can bind both GDP- and GTP-bound Rab, we examined the interaction of PRA2 with the Rab GTPases. In the yeast two-hybrid system, PRA2 showed a positive β-galactosidase reaction when tested against the wild-type and GTPase mutant Rab1A and Rab3A (data not shown). As with PRA1, the interaction was abolished when the double Cys prenylation motif of Rab was deleted, suggesting that prenylation is required for interaction. To confirm this interaction, we performed an in vitro binding assay using recombinant Rab1A and Rab3A purified as a 6×His-tagged fusion protein from the yeast, *Saccharomyces cerevisiae*, and covalently linked to CNBr-activated Sepharose. The proteins were preloaded with GDP, GTP, or maintained in the nucleotide-free state (in the presence of EDTA). Increasing amounts of recombinant HA-tagged PRA1 or PRA2 was added to the beads at 4 °C with the bound proteins recovered and analyzed by Western immunoblot. As shown in Fig. 3A, PRA2 was recovered with the immobilized Rab1A and Rab3A but not with the control Sepharose beads. There was a slight increase in the amount of PRA2 recovered with immobilized Rab3A compared with Rab1A. PRA2 showed a slightly higher affinity for GTP-bound Rab but was clearly recovered with the GDP-bound as well as guanine nucleotide-free state of both Rab GTPases. Under the same conditions, PRA1 also showed a slight preference for Rab3A over Rab1A (Fig. 3B). There was also a slightly higher affinity for the GTP-bound Rab followed by guanine nucleotide-free and GDP-bound forms. Thus, we conclude that both PRA1 and PRA2 can interact with, at least, Rab1A and Rab3A in the guanine nucleotide-bound and free states.

PRA2 Is Localized to the ER Compartment—To determine the cellular distribution, we subcloned the HA-tagged PRA2 into the bicistronic expression vector pIRESpuro, transfected it into CHO cells, and performed a subcellular fractionation analysis. As with PRA1, the protein fractionated with both the high speed supernatant and membranes (Fig. 4). When the membrane fraction was extracted with alkaline carbonate buffer, some of the membrane-bound protein appeared in the soluble fraction similar to the behavior of PRA1 and suggested peripheral association with the membrane. However, the membrane-bound PRA2 partitioned exclusively with the detergent phase when subjected to Triton X-114 extraction and phase separation. In contrast, a significant portion of the membrane-bound PRA1 remained with the aqueous phase in the Triton X-114 extraction. Thus, both PRA1 and PRA2 are highly hydrophobic proteins tightly associated with the membrane but may also appear in the cytosol.

When transfected into CHO cells, PRA2 has a striking reticular staining pattern reminiscent of the ER (Fig. 5A). Indeed, there was extensive co-localization with calnexin, a known ER marker. We observed little, if any, co-localization with mannosidase II, a Golgi membrane marker. In contrast, PRA1 was found exclusively associated with the Golgi complex with extensive co-localization with mannosidase II (Fig. 5, C and D). Thus, the two PRA isoforms are localized to distinct intracellular compartments with PRA2 restricted to the ER and PRA1 confined to the Golgi complex (30). This distinct intracellular localization implies that PRA1 and PRA2 may contain targeting signals that direct the protein to the appropriate intracellular compartment. It also implies that each PRA might only interact with a subset of Rab in the cell even though both are capable of binding multiple Rab isoforms in either the GDP- or GTP-bound state.

The Localization Signal Resides in the Carboxyl-terminal Domain of PRA—To define the organelle-specific targeting signal, we constructed amino-terminal HA-tagged chimeras of PRA1 and PRA2, and determined their cellular localization in transfected CHO cells. Based on the structural features, we divided the two proteins into three separate domains: amino-terminal domain (Domain A), the two hydrophobic segments plus the intervening hydrophilic loop (Domain B), and the charged COOH-terminal domain (Domain C). Hybrid oligonucleotides spanning these domains were used to generate the chimeras by PCR using either HA-tagged PRA1 or PRA2 as the template. As shown in Fig. 6, chimera 1A/2BC, for example, contained Domain A of PRA1 fused to Domains B and C of PRA2. Likewise, chimera 2A/1B/2C contained Domain A of PRA2 fused to Domain B of PRA1 followed by Domain C of PRA2, and so on. The various chimeras were subcloned into the pIRESpuro vector and sequenced to confirm the expected mutations. Two days after transfection, the CHO cells were stained with anti-HA and costained with either anti-mannosidase II or anti-calnexin to identify the Golgi and ER, respectively. As shown in Fig. 7, the COOH-terminal domain of PRA2 targeted the chimera to the ER whether it contained only the
B and PRA2 (A) were fractionated by centrifugation at bound PRA.

Homogenates from CHO transfected with HA-tagged PRA1 was incubated with 20 pmol of PRA2, B, the same conditions were used as in panel A except recombinant HA-tagged PRA1 was added to the immobilized Rab. PRA1 and PRA2 bound to the beads were detected with anti-HA antibodies.

FIG. 3. Binding of recombinant PRA1 and PRA2 to Rab1A and Rab3A. A, 40 pmol of immobilized Rab1A (top panel) or Rab3A (bottom panel) was incubated with 10, 15, or 20 pmol of HA-tagged PRA2. The Rab GT-Pases were preincubated with GTP, GDP, or EDTA, as indicated. Lane C represents the control Sepharose beads incubated with 20 pmol of PRA2. B, the same conditions were used as in panel A except recombinant HA-tagged PRA1 was added to the immobilized Rab. PRA1 and PRA2 bound to the beads were detected with anti-HA antibodies.

FIG. 4. Subcellular fractionation and extraction of membrane-bound PRA. Homogenates from CHO transfected with HA-tagged PRA1 (A) and PRA2 (B) were fractionated by centrifugation at 100,000 x g for 1 h. The resulting high speed membrane fractions were further extracted with 0.1 M sodium carbonate, pH 11.5, or with 2% Triton X-114. Aq and Det refer to the supernatant and pellet fractions, respectively. Aq and Det refer to the aqueous and Triton X-114 detergent phases.

The carboxyl-terminal Domain C of PRA2-(132-188) was able to partially target GFP to intracellular membranes where a distinctive punctate staining pattern throughout the cell was clearly evident (Fig. 8D). However, membrane targeting by this domain was inefficient as a significant amount of florescent signal remained in the cytosol. Since this domain of PRA2 contained 57 amino acids, we performed a limited deletion to further define the boundaries of the localization signal. Further deletion of 17 residues in GFP-PRA2-(148-188) or 33 residues in GFP-PRA2-(164-188) completely abrogated this punctate staining pattern (Fig. 8E). Thus, the minimal localization signal must either be contained within the first 17 amino acids immediately COOH-terminal to the second hydrophobic segment or required domains in addition to that contained within Domain C. In contrast to PRA2, GFP fusion of Domain C of PRA1-(162-185), which contained only 24 amino acids, cannot target GFP to intracellular membranes (Fig. 8E). Neither can a further addition of 5 hydrophobic residues in GFP-PRA1-(157-185). When combined with the previous observation, these results suggest that the COOH-terminal domain of PRA2 can partially target proteins to the membrane and that transport of PRA1 to the Golgi complex required additional domains such as ones that can functionally interact with Rab or VAMP2.

Charged Residues at the Carboxy Terminus Direct Organelle-specific Targeting of PRA—One striking feature of the COOH-terminal domain of the PRA isoforms is their overall charge. PRA1 contained a number of acidic residues whereas the first 15 amino acids of Domain C in PRA2 contained numerous basic residues. The carboxyl terminus of PRA1 contained an additional glutamate immediately after the consensus DXE motif (residues 176-178) involved in the exit of membrane proteins from the ER (35, 36). To test whether this DXE motif might constitute the sorting signal of PRA1, we mutated these residues to alanine or to basic residues and examined their effect on cellular localization. Targeting of PRA1 to the Golgi complex was lost when either one of the acidic residues (Asp176, Glu178, or Glu179), both glutamate residues (Glu178 and Glu179), and all three acidic residues within the DXE motif were changed to alanine (Fig. 9A). It is noteworthy that mutation of the Glu179 residue alone while leaving an intact DXE motif also abolished localization to the Golgi complex. Mutation of all three acidic residues to lysine also caused mislocalization to the ER. These results indicate that the DXE motif is essential for effective Golgi localization of PRA1, and the additional glutamate residue is indispensable for exit from the ER compartment.

Since the COOH-terminal domain of PRA2 can partially direct GFP to intracellular membranes and contained a number of basic residues, we decided to perform a mutagenic analysis of this region. The presence of basic amino acids immediately following a transmembrane domain on the cytoplasmic...
side of cytochrome P-450 has been shown to exclude the protein from ER transport vesicles (37). However, we observed no alteration in the ER localization of PRA2 when the basic residues within the first 15 amino acids (Arg140, Arg142, Lys145, and Lys147) were changed to alanine (AAAA in Fig. 9B). Changing the basic residues Arg140, Arg142, Lys145, Lys147, and Lys151 to glutamate (acidic in Fig. 9B) resulted in partial localization to the Golgi complex. This might be due to introduction of a di-acidic motif that mimicked a DXE motif. If so, this suggests that the protein may exit the ER but is subsequently retrieved. Retrieval of membrane proteins has been shown to depend on a di-basic KKXX motif located at the COOH-terminal end (38, 39). Since PRA2 does not contain this KKXX motif, we explored the possibility that the basic residues located at the COOH-terminal end, KARE, might mimic this ER retrieval signal. Mutation of Lys185 or Arg187 to alanine resulted in partial localization to the Golgi complex. Similarly, introduction of either K185A or R187A in the context of a PRA2 with an acidic COOH-terminal domain resulted in predominantly Golgi localization (acidic/K185A and acidic/R187A in Fig. 9B). Thus, our results indicate that PRA2 can be transported to the Golgi complex despite the lack of a di-acidic motif for exit from the ER. But the protein is efficiently retrieved from the pre-Golgi compartment through a process that partially depended on a KXXR motif at the extreme carboxyl terminus.

**DISCUSSION**

Proteins involved in vesicle transport are conserved throughout evolution with specific members of each protein family mediating similar functions at each step of the secretory pat-
way. We have isolated a second PRA isoform from a rat brain cDNA library based on sequence similarity to a conserved domain in PRA1. Northern analysis showed that PRA2 is ubiquitously expressed in all tissues, suggesting that it might also participate in general membrane trafficking events. Although the overall identity of the two proteins is relatively low, we concluded that the two proteins belong to the same family based on their overall structural similarity and ability to bind to Rab. PRA2 also contains two extensive hydrophobic domains (36 and 35 residues). Subcellular fractionation analysis indicates that the two proteins share properties of both cytosolic and membrane proteins. A significant portion of the two proteins fractionated with the high speed supernatant. The membrane-bound PRA2 behaved as an integral membrane protein in that it partitioned with the Triton X-114 detergent phase. However, a fraction of the membrane-bound PRA2 can be extracted with alkaline carbonate buffer. In the case of PRA1, a portion of the membrane-bound protein was extracted with alkaline carbonate buffer and partitioned with the aqueous phase when extracted with Triton X-114. These properties suggest that PRA might be partially embedded in the lipid bilayer.

A more compelling argument that PRA2 is an isoform of the PRA family is its ability to bind, at least, Rab1A and Rab3A in an in vitro binding assay. The binding property was very similar to PRA1 in that both proteins can bind Rab1A and Rab3A in GDP-, GTP-, and guanine nucleotide-free states. This is similar to that of Mss4, a cytosolic protein that can bind to the transient guanine nucleotide-free state of Rab (40). However, the interaction differs with respect to the prenylation state of Rab. Binding of Rab to PRA1 and PRA2 is highly dependent on prenylation as deletion of the double cysteine-motif completely abrogated protein-protein interaction in the yeast two-hybrid system (not shown). In contrast, Mss4 can interact with the lipid-unmodified form of Rab (41). Since PRA1 can bind specifically to VAMP2, we also tested whether PRA2 could interact with some of the VAMP isoforms. We were unable to detect any interaction between PRA2 and VAMP1, VAMP2, or VAMP3 (cellubrevin) in the yeast two-hybrid system and in vitro binding assays (not shown). Thus, we can exclude VAMP1–3 as possible PRA2-interacting proteins.

Most studies on protein localization within the secretory pathway focused on sorting and transport of cargo or integral membrane proteins. These proteins enter the secretory pathway by insertion into or translocation across the ER membrane. The selection thereafter for transport, retention, or retrieval depends on specific interaction of localization signals present in the protein with the transport machinery. Similarly, proteins localized entirely on the cytoplasmic side of the membrane contain signals mediating specific targeting to an organelle. For example, a FYVE finger domain is essential in mediating specific localization of EEA1 to endosomes (42), a GRIP domain in targeting to the Golgi complex (43) or a GRASP65-binding site for Golgi localization of GM130 (26). The two PRA isoforms do not contain any of these domains, but
Fig. 8. GFP chimeras of PRA1 and PRA2. The native GFP showed an even cytosolic pattern in transfected CHO (panel A) while a GFP fusion with the full-length PRA2 (panel B) and PRA1 (panel C) targeted the protein to the ER and Golgi, respectively. Representative of the GFP-PRA1 chimeras, GFP-PRA1-(162–185) and GFP-PRA1-(157–185) were shown in panels E and G, respectively. Representative of GFP-PRA2-(132–188) was shown in panel D, GFP-PRA2-(148–188) in panel F, and GFP-PRA2-(164–188) in panel H.
have very distinct intracellular localization. We have determined that the sorting signal lies within the carboxyl terminus following the second hydrophobic domain. This domain clearly determines the intracellular localization such that replacing the PRA1 COOH-terminal domain with that from PRA2 targeted the chimera to the ER, the normal cellular localization of PRA2. Likewise, replacing the PRA2 COOH-terminal domain with that from PRA1 resulted in targeting of the chimera to the Golgi complex, the normal localization of PRA1. The importance of this domain was also evident in our previous observation where its deletion resulted in insertion of PRA1 into the ER membrane (30). For PRA2, this COOH-terminal domain was able to partially target the cytosolic GFP to the membrane, suggesting that it is capable of interacting with ER-specific transport machinery. However, efficient targeting may require an additional domain or perhaps functional interaction with Rab. This may also apply to PRA1 since the COOH-terminal domain alone or together with five upstream hydrophobic amino acids was not able to direct membrane association of GFP. However, when fused to upstream PRA2 domains, it could target the protein to the Golgi membrane.

Since PRA shared properties of integral membrane proteins, we examined whether sorting signals for membrane proteins might be responsible for PRA-specific targeting. Efficient export of vesicular stomatitis virus G glycoprotein from the ER requires a di-acidic DXXE motif (35, 36) found on the COOH-terminal side of a YXXφ motif (where φ is a hydrophobic residue) at the cytoplasmic side of the membrane (44). The carboxyl terminus of PRA1 is overall acidic in nature but contains a DXXE motif (residues 176 to 179). However, this COOH-terminal domain alone is insufficient in targeting the cytosolic GFP reporter protein to the Golgi or any intracellular membrane. In the context of a full-length PRA1, the DXXE motif is essential in Golgi localization and mutation of Asp176 (37) or Glu178 resulted in retention in the ER. Interestingly, mutation of Glu178, which contains an intact DXXE motif, also resulted in loss of Golgi localization. Thus, efficient Golgi localization of PRA1 required an additional glutamate beside the DXXE motif, and our results suggest that PRA1 might be associated with the transport machinery utilized by membrane proteins to exit from the ER.

Localization of membrane proteins in the ER may result from exclusion from ER transport vesicle or retrieval from the pre-Golgi compartment. In certain cases, a transmembrane domain with basic amino acids immediately following it on the cytoplasmic side may serve to exclude the protein from ER transport vesicles (37). We explored the possibility that the basic residues following the second hydrophobic domain might mimic this motif thereby allowing PRA2 to remain in the ER. However, mutagenesis of these basic residues to alanine showed no effect on PRA2 localization. Retention of membrane proteins in the ER can also result from protein retrieval from the pre-Golgi compartment, a process that requires a di-basic KKXX motif at the cytoplasmic tail (38, 39, 45). PRA2 does not contain a KKXX motif at the carboxyl terminus, but ends with KARE (residues 185 to 188) that might mimic this retrieval signal. We found that mutation of either one of the basic residues resulted in partial localization to the Golgi complex. This suggests that the protein normally transits from the ER to the Golgi complex, and that mutation of the terminal basic residues resulted in reduced retrieval from the Golgi complex. It is interesting to note that PRA2 does not contain a DXXE motif at the COOH-terminal domain, yet is capable of transport to the Golgi complex. Moreover, transport to the Golgi complex can be enhanced by the creation of a di-acidic motif at the COOH-terminal domain.

Our data suggest that PRA1 enters the secretory pathway at the ER compartment and is efficiently transported to the Golgi complex through its DXXE motif at the carboxyl terminus. It can progress beyond the Golgi complex since PRA1 was detectable on synaptic vesicle membrane (46). PRA2 also enters the secretory pathway at the ER compartment and is transported to the Golgi complex independent of a DXXE motif. It is then retrieved, possibly through its KKXX motif and returned to the ER compartment. Thus, intracellular localization of PRA is likely due to its interaction with the same machinery that sorts proteins for export out of the ER and retrieval from the pre-Golgi compartment. This might serve to recruit or retain Rab on the transport vesicles. Despite the broad specificity for Rab, the distinct cellular localization of PRA implies that each isoform may be restricted in its interaction to a subset of Rab.

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