The steady state distribution of membrane forms of peptidylglycine α-amidating monoxygenase (PAM) in the secretory pathway of neurons and endocrine cells depends on signals in its cytosolic COOH-terminal domain (CD). Mutagenesis studies yielded catalytically active PAM proteins that are not properly localized or internalized. Employing the yeast two-hybrid system, we isolated two distinct cDNAs whose protein products showed a strong interaction with the CD of PAM. The interaction of these novel PAM COOH-terminal interactor proteins (P-CIPs) did not occur with a misrouted CD mutant as bait in the yeast system. Both proteins, P-CIP2 and P-CIP10, were expressed as fusion proteins that interacted in vitro with solubilized integral membrane PAM. P-CIP2 was homologous to several serine/threonine and dual specificity protein kinases, while P-CIP10 contained spectrin-like repeats. Endogenous P-CIP2 was localized to the Golgi region of AtT-20 corticotrope tumor cells, and expression of integral membrane PAM disrupted the distribution of endogenous P-CIP2. Both P-CIP2 and P-CIP10 mRNAs were found to be expressed in rat brain neurons also expressing PAM proteins. P-CIP2 and P-CIP10 may be members of a family of cytosolic proteins involved in the routing of membrane proteins that function in the regulated secretory pathway.

In neurons and endocrine cells, biologically active peptides are stored in large dense core vesicles (LDCVs), which undergo regulated release (1–3). After exocytosis, the membrane PAM are stored in large dense core vesicles (LDCVs), which undergo regulated release (1–3). After exocytosis, the membrane proteins that function in LDCVs. Peptidylglycine α-amidating monoxygenase (PAM; EC 1.14.17.3), which catalyzes the two reactions involved in the COOH-terminal α-amidation of bioactive peptides (4–6), occurs in soluble and integral membrane forms (Fig. 1) and serves as a tool to compare the routing of soluble and membrane proteins to LDCVs (4–6). Although the luminal domains of PAM catalyze the amidation reaction, the facts that the COOH-terminal domains (CD) of mammalian and Xenopus laevis PAM are highly conserved and that the nervous system expresses almost exclusively the integral membrane forms of PAM indicate that tethering of PAM to the membrane is functionally important (4–7).

In AtT-20 corticotrope tumor cells, integral membrane forms of PAM have access to immature secretory granules and accumulate in tubuloreticular structures in the distal part of the TGN (8–11). Soluble PAM proteins accumulate in LDCVs in the peripheral processes of AtT-20 cells. At steady state, only a small percentage of the membrane PAM is on the surface or in endosomes and can be detected by surface enzyme assays and binding and internalization of ectodomain antibodies (8–11) (Fig. 1).

The PAM CD contains multiple routing signals recognized at different subcellular locations. Truncation of half of the CD (Fig. 1) leads to diminished storage of PAM in LDCVs, plasma membrane localization of active enzyme, and failure of internalization (9–12). Transfer of the cytosolic and transmembrane domains of PAM to the luminal domain of the interleukin 2 receptor α chain relocated this protein from the plasma membrane to the TGN and supported internalization of bound antibody (10). Further mutagenesis studies identified residues 928–945 in the PAM CD as critically important and eliminated a role for amino acids distal to residue 957 (10). Mutation of Tyr936 to Ala (Y936A) disrupted internalization of membrane PAM without greatly affecting cleavage in LDCVs (10). The CD of membrane PAM is phosphorylated on Ser/Thr residues in cells; mutation of a protein kinase C site at Ser937 to Ala (S937A) resulted in mistargeting of the internalized PAM-PAM antibody complex (11). The importance of the cytosolic domains of many other membrane proteins in their retention and/or targeting to the appropriate subcellular compartment is well established (13–21).

MATERIALS AND METHODS

DNA Techniques—The methods of Sambrook et al. (25) were used; sequence analysis employed the Sequenase kit (U.S. Biochemical Corp.) or was performed by the Johns Hopkins Genetics Core. The polymerase chain reaction was used to introduce restriction sites for SalI and EcoRI at the 5′ and 3′ ends of amplified cDNA fragments for various versions of PAM CD, and the inserts generated by SalI/EcoRI digestion were cloned into pPC97 (22, 23). This produced plasmids pPC97-CDT (CD truncated), which encodes PAM-1 (891–961), and pPC97-dCDT (encoding PAM-1 (891–961) with residues 928–945 deleted) (Fig. 2A). For construction of the expression vectors pGEX-CIP2 and pGEX-CIP10, P-CIP2 and P-CIP10 cDNAs were excised from the isolated pPC86 library vectors by SalI/NcoI digestion and ligated to pGEX5X.2 (Phar- macia Biotech Inc.) digested with the same restriction enzymes.

Yeast Two-hybrid Screening—The yeast reporter strain HF7c, (MATa ura3–52, his3–200, ade2–101, lys2–801, trp1–901, leu2–3, 112, gal4–542, gal80–538, LYS2::GAL1::HIS3, URA3:: GAL117:: (UAS3-CyCI1::TATA, lacZ) was transformed first with pPC97-CDT, encoding the GAL4 DNA-binding CDT (GAL4(DB)-CDT) fusion protein...
Transformants were checked for production of GAL4(DB)-CDT fusion protein by Western blot analysis and for failure to grow in histidine-deficient medium. One transformant expressing the GAL4(DB)-CDT fusion protein was transformed with a cDNA library prepared from the hippocampus/cortex of 3-week-old rat pups subjected to a single maximal electroconvulsive shock-induced seizure (gift of Dr. A. Lanahan) (23). The library was constructed in pPC86 by directional cloning of random-primed cDNAs downstream of the trans-activator domain of GAL4 and a nuclear localization signal (NLS-GAL4/TA). Double transformants were allowed to grow on solid media containing histidine and leucine, or histidine and tryptophan, or histidine, leucine, and tryptophan, respectively.

To identify cytosolic factor(s) involved in the routing of membrane PAM, we used the two-hybrid system, a yeast-based genetic assay that uses the modular nature of transcriptional activators to detect protein-protein interactions (22–24). The PAM CD (CDT) was used as bait for the two-hybrid screen of a rat hippocampal cDNA library (Fig. 2A). Truncation at amino acid residue 961 eliminated the highly charged PEST segment located near the COOH terminus of PAM-1, which might cause nonspecific ionic interactions or rapid degradation (10, 11, 27). A screen of $10^5$ double transformants on histidine-deficient medium yielded 20 primary clones; four displayed strong $\beta$-galactosidase activity when tested by the yeast colony filter assay (22). Plasmids from positive colonies were rescued and named P-CIP1, -2, -10, and -19. None of the four interactor clones supported growth of yeast in deficient medium in the absence of the CDT plasmid or in the presence of the nonfunctional, internally deleted dCDT plasmid (Fig. 2B). Thus, the pattern of growth of the double transformants suggests that the interaction between the protein products of the isolated cDNAs and the PAM CD is similar to the physiological interactions that determine PAM routing (Fig. 2B).

**Predicted Structures of Interactor Proteins P-CIP2 and P-CIP10**—The sequences of the proteins encoded by P-CIP2 and P-CIP10 are shown in Fig. 3, A and B, respectively. The

**RESULTS**

**Identification of Interactor Proteins in the Yeast Two-hybrid System**—To identify cytosolic factor(s) involved in the routing of membrane PAM, we used the two-hybrid system, a yeast-based genetic assay that uses the modular nature of transcriptional activators to detect protein-protein interactions (22–24). The PAM CD (CDT) was used as bait for the two-hybrid screen of a rat hippocampal cDNA library (Fig. 2A). Truncation at amino acid residue 961 eliminated the highly charged PEST segment located near the COOH terminus of PAM-1, which might cause nonspecific ionic interactions or rapid degradation (10, 11, 27). A screen of $10^5$ double transformants on histidine-deficient medium yielded 20 primary clones; four displayed strong $\beta$-galactosidase activity. Plasmids were rescued from each of these yeast colonies and named P-CIP1, -2, -10, and -19. None of the four interactor clones supported growth of yeast in deficient medium in the absence of the CDT plasmid or in the presence of the nonfunctional, internally deleted dCDT plasmid (Fig. 2B). Thus, the pattern of growth of the double transformants suggests that the interaction between the protein products of the isolated cDNAs and the PAM CD is similar to the physiological interactions that determine PAM routing (Fig. 2B).

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sequence of P-CIP19 was contained within P-CIP10 (Fig. 3B). When P-CIP1, P-CIP2, and P-CIP10 were searched for homology to the Non-redundant Protein Data base maintained at the National Center for Biotechnology Information using the BLASTP algorithm, they were found to encode novel proteins. The 333-amino acid sequence predicted for P-CIP1 did not exhibit significant homology to any sequences in the data bases, and studies on it will be reported separately.

The predicted P-CIP2 protein that follows the GAL4 transcriptional domain contains 392 amino acids (Mₐ = 43,567) followed by an in-frame stop codon and a 264-nucleotide 3’-untranslated region. According to BLASTP analysis, the P-CIP2 sequence overlapping that of mouse KIS is underlined with a solid line; amino acid substitutions are indicated by carets. The region exhibiting homology to U2AF⁵⁵ is underlined with a dashed line. The peptide used to generate polyclonal antisera to P-CIP2 is boxed. B. P-CIP10 protein. The beginning and end of each spectra-like repeat are indicated by arrows (32–35); the repeats at both ends are incomplete. The region included in P-CIP19 is underlined. The BLASTP searches are in the XREFdb Report of June 4, 1996.

sequence of P-CIP19 is 677 amino acids long (Mₐ = 78,435) and lacks an in-frame stop codon, suggesting that full-length P-CIP10 is extended in both the COOH-terminal and NH₂-terminal directions (Fig. 3B). Secondary structure predictions for P-CIP10 indicate that it is a serine/threonine-specific protein kinase. The catalytic core domains of protein kinases typically begin with a nucleotide binding loop (GXGXXGXXV) followed by an invariant Lys, also involved in nucleotide binding (28). The region N is often an indicator of protein kinase subdomain thought to fold into a three-helix bundle (31–35). Spectrin and fodrin are involved in cross-linking actin filaments to form the meshwork underlying the plasma membrane (3, 31–33). P-CIP10 includes five full spectrin-like repeats varying in length from 105 to 131 amino acids with partial spectrin-like domains at the NH₂ and COOH-terminal ends. P-CIP19 includes two complete spectrin-like domains (Fig. 3B); secondary structure predictions suggest that the B and C helices of the second complete spectrin-like repeat in P-CIP19 are interrupted by a longer than average nonhelical loop.

Intaractor Proteins P-CIP2 and P-CIP10 Bind to the CD of Integral Membrane PAM—The interaction between membrane PAM proteins and P-CIP2 and P-CIP10 proteins was confirmed in vitro using GST fusion proteins. From the SDS-polyacrylamide gel electrophoresis patterns of the expressed GST fusion proteins (not shown), the P-CIP2 and P-CIP10 portions of the fusion proteins have apparent molecular masses of 44 and 84 kDa, respectively, in agreement with the sizes predicted from the amino acid sequence data in Fig. 3. Glutathione-Sepharose beads with immobilized GST, GST-P-CIP2, and GST-P-CIP10 were prepared, and integral membrane PAM-2 was applied to each resin. As demonstrated in Fig. 4, the 105-kDa PAM-2 protein bound to the GST-P-CIP2 and GST-P-CIP10 resins but not to the GST resin.

Integral Membrane PAM Interacts with P-CIP2 in Cultured Cells—In order to determine whether integral membrane PAM and P-CIP2 interact in a cellular environment, we generated an antiserum to a peptide from the COOH-terminal region of P-CIP2 (Fig. 3A). This antiserum visualized a single major band of 47 kDa when used for Western blot analysis of AtT-20 corticotrope tumor cell extracts (data not shown). Nontransfected AtT-20 cells were fixed and immunostained simultaneously with polyclonal antisera specific for P-CIP2 and monoclonal antibodies directed against PAM. As demonstrated in Fig. 5A, the 105-kDa PAM-2 protein bound to the GST-P-CIP2 and GST-P-CIP10 resins but not to the GST resin.
membrane PAM-1 was concentrated primarily in the perinuclear TGN region with staining at the tips of the cellular membrane. PAM-1 was concentrated primarily in the perinuclear Golgi/TGN region (A). Nontransfected AtT-20 cells showed little or no staining for PAM (A), which is expressed at very low levels in AtT-20 cells. P-CIP2 was readily visualized and was localized primarily to the perinuclear Golgi/TGN region (B). AtT-20 cells stably expressing PAM-1 showed strong staining for PAM in the Golgi/TGN region (C). Expression of PAM-1 resulted in the redistribution of P-CIP2 away from the Golgi/TGN region and a diffuse cytosolic staining pattern (D).

Interactor Proteins P-CIP2 and P-CIP10 Are Found in Neurons That Express PAM—A meaningful interaction between PAM and either P-CIP2 or P-CIP10 can occur only if the proteins are expressed in the same cells, so the localizations of PAM protein and transcripts encoding P-CIP2 and P-CIP10 in rat brain were compared using dual in situ hybridization and immunocytochemistry. Sections of brain were first analyzed by in situ hybridization using radiolabeled sense and antisense RNA probes synthesized from fragments of the P-CIP2 and P-CIP10 cDNAs. The sections were then probed with polyclonal PAM antibodies. As shown in Fig. 6, neurons that showed high levels of hybridization with P-CIP2 and P-CIP10 riboprobes could also be immunostained for PAM protein; the control sense riboprobes for P-CIP2 and P-CIP10 yielded no signal (not shown). Many nearby neurons that did not contain PAM protein also lacked the transcripts encoding P-CIP2 and P-CIP10. PAM protein is found in neurons expressing P-CIP2 and P-CIP10 transcripts, making the postulated interactions possible.

DISCUSSION
The yeast two-hybrid system was used to identify proteins that interact with the cytosolic domain of PAM and thus might be involved in the trafficking of membrane PAM in neurons and endocrine cells. Four clones exhibited the proper selection characteristics, suggesting that they were capable of physiologically relevant interactions with the cytosolic CD of membrane PAM.

P-CIP2 is a putative serine/threonine or dual specificity protein kinase containing all of the conserved residues characteristic of the catalytic core of a serine/threonine-protein kinase (Fig. 3A) (28, 29). The mass of the AtT-20 protein identified on Western blots by antisera to P-CIP2 (~47 kDa) is only a few kilodaltons greater than the mass predicted based on the partial CDNA identified in the yeast two-hybrid screen (~44 kDa). A short NH2-terminal extension in full-length P-CIP2 presumably accounts for the observed size difference. The COOH-terminal region of P-CIP2 is highly homologous to the third ribonucleoprotein consensus sequence domain of pre-mRNA splicing factor U2AF65 (36). Ribonucleoprotein consensus domains are involved in RNA recognition and binding; their significance to the function of P-CIP2 is unknown. P-CIP2 is the rat homologue of a mouse protein designated KIS, differing at only 2 of the 201 amino acid residues predicted for KIS (30). KIS was identified in a yeast two-hybrid screen for proteins that interact with stathmin, a ubiquitously expressed 19-kDa cytosolic protein that is phosphorylated in response to a variety of secretagogues (30). Although KIS was recognized as a putative serine/threonine-protein kinase, the partial sequence reported lacks the NH2-terminal part of the protein kinase catalytic core, and no direct interaction of KIS with stathmin was demonstrated (28–30).

The interaction of a protein kinase with the CD of membrane PAM could play an important role in the routing and steady state distribution of the protein. In cultured cells, the CD of membrane PAM is phosphorylated on Ser and Thr residues by an unknown protein kinase (11). Recombinant PAM CD is phosphorylated on Ser932 and Ser937 by protein kinase C (11). Expression of mutant PAM-1/S937A in AtT-20 cells resulted in its misrouting following internalization from the plasma mem-

![Fig. 4. Interaction of integral membrane PAM-2 with immobilized GST, GSTP-CIP2, and GSTP-CIP10. PAM-2 was solubilized from AtT-20 cell extracts and tested for binding to glutathione resins bearing GST, GSTP-CIP2, and GSTP-CIP10. Equal proportions of starting material (SM), the bound fraction (B), and the unbound fraction (U) were subjected to SDS-polyacrylamide gel electrophoresis and analyzed by Western blot using a monoclonal antibody (6E6) specific for the PAM CD (8, 10).](image)

![Fig. 5. Expression of integral membrane PAM alters the steady state distribution of P-CIP2 in AtT-20 cells. Nontransfected cells showed little or no staining for PAM (A), which is expressed at very low levels in AtT-20 cells. P-CIP2 was readily visualized and was localized primarily to the perinuclear Golgi/TGN region (B). AtT-20 cells stably expressing PAM-1 showed strong staining for PAM in the Golgi/TGN region (C). Expression of PAM-1 resulted in the redistribution of P-CIP2 away from the Golgi/TGN region and a diffuse cytosolic staining pattern (D).](image)

![Fig. 6. Transcripts encoding P-CIP2 and P-CIP10 occur in neuronal perikarya along with PAM proteins. P-CIP2 mRNA was found in trigeminal nucleus neurons (mesencephalic division) by in situ hybridization (silver grains) along with PAM protein (immunocytochemistry; dark stain). P-CIP10 mRNA was found in cortical neurons that also express PAM protein. Arrows indicate neurons stained for PAM protein and covered by silver grains; bar, 50 μm.](image)
brane; PAM-1/S937A appeared to be routed to lysosomes instead of returning to the TGN (11). Thus, phosphorylation of the CD plays a role in the trafficking of membrane PAM, and P-CIP2 may be directly or indirectly involved in this process.

P-CIP10 is most homologous to human α-fodrin and chick α-spectrin, and the amino acid sequence of P-CIP10 can all be arranged into spectrin-like repeats (Fig. 3A) (32, 34, 35). More distantly related members of this family include β-spectrin, Dbs (a protein with a guanine nucleotide exchange factor domain and a pleckstrin homology domain), and dystrophin. PAM is clearly expressed in neurons lacking P-CIP10, and P-CIP10 may represent only one of a family of related proteins that interact with the PAM CD. In erythrocytes and nonerythroid cells, spectrin and fodrin provide structural support to the plasma membrane, and spectrin binds to specific soluble and integral membrane proteins to form an extensive cytoskeletal meshwork that is tightly associated with the plasma membrane (32, 33, 35). An isoform of β-spectrin is associated with Golgi membranes in Madin-Darby canine kidney cells and disarranged into spectrin-like repeats (Fig. 3d) (11). Thus, phosphorylation of membrane PAM proteins and, thus, will shed light on the biogenesis of LDCVs in neurons and endocrine cells.

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