P-CIP1, A Novel Protein That Interacts with the Cytosolic Domain of Peptidylglycine α-Amidating Monoxygenase, Is Associated with Endosomes*

(Received for publication, April 15, 1998, and in revised form, September 17, 1998)

Lihong Chen, Richard C. Johnson‡, and Sharon L. Milgram§

From the Department of Cell and Molecular Physiology, The University of North Carolina, Chapel Hill, North Carolina 27599 and ‡The Neuroscience Department, Johns Hopkins University, Baltimore, Maryland 21205

The cytosolic domain of the peptide processing enzyme peptidylglycine α-amidating monoxygenase (PAM) contains signals that direct its trafficking in the secretory and endosomal pathways. Using the yeast two-hybrid system, Alam et al. (Alam, M. R., Caldwell, B. D., Johnson, R. C., Darlington, D. N., Mains, R. E., and Eipper, B. A. (1996) J. Biol. Chem. 271, 28636) identified three proteins that interact with a fragment of the PAM cytosolic domain containing these targeting signals. We cloned the rat and human cDNAs encoding PAM COOH-terminal interactor protein-1 (P-CIP1). Both cDNAs contain an open reading frame that encodes a novel protein of 435 amino acids. The P-CIP1 protein is highly conserved from rat to human (85% identity) but does not display significant homology to proteins in the GenBank database. In vitro, P-CIP1 interacts with the cytosolic domain of wild type PAM-1, but does not interact with mutant PAM-1 proteins that fail to target correctly when expressed in endocrine cells. P-CIP1 contains multiple consensus serine/threonine phosphorylation sites and a region predicted to form a coiled-coil at the COOH terminus. When expressed in endocrine cells or fibroblasts, P-CIP1 is distributed in a punctate pattern in the perinuclear area but does not significantly overlap the distribution of transfected wild type PAM-1. The distribution of P-CIP1 displays significant overlap with the distribution of the secretory carrier membrane proteins, internalized Texas Red-conjugated transferrin, and Rab11. The data suggest that P-CIP1 associates with vesicles in the recycling endosomal pathway, and may play a role in regulating the trafficking of integral membrane PAM.

While all cells contain a constitutive secretory pathway to transport proteins from the trans-Golgi network (TGN)1 to the plasma membrane, neurons and endocrine cells also contain a regulated pathway (2–5). Bioactive peptides act as signaling molecules to regulate a wide range of cellular activities and are the major cargo proteins carried by regulated secretory granules. The bioactive peptides are derived from inactive precursors through a series of post-translational modifications, including endoproteolysis, exoproteolysis, and for over half of all known peptides, COOH-terminal α-amidation (6–12). Since most of these post-translational modifications occur in secretory granules, the enzymes involved must be targeted together with prohormone substrates to the regulated pathway. The mechanisms involved in this targeting are not understood. However, data from several laboratories studying the targeting of different integral membrane proteins suggests that sorting signals in the cytosolic domain of these proteins are involved (13–18).

One integral membrane protein that functions in secretory granules is peptidylglycine α-amidating monoxygenase (PAM). This bifunctional enzyme catalyzes the two sequential reactions involved in the α-amidation of bioactive peptides (10, 19). Alternative mRNA splicing produces PAM proteins that contain or lack a single transmembrane domain, providing a means to directly compare the trafficking of soluble and integral membrane secretory granule-associated proteins (19). When expressed in the neuroendocrine AtT-20 cell line, soluble PAM proteins are efficiently targeted to secretory granules, where they are stored and eventually secreted in response to secretagogues (20, 21). In contrast, the majority of the integral membrane PAM (e.g. PAM-1) is accumulated in tubuloreticular structures in the perinuclear area, although some of the expressed protein does reside in secretory granules (20–22). Integral membrane PAM is also internalized from the cell surface and transported to perinuclear vesicles; some of the internalized protein may be degraded in lysosomes or repackaged into newly forming granules (14). To identify cytosolic sorting signals responsible for targeting integral membrane PAM, we generated a series of cDNAs encoding truncation, deletion, or point mutations within the PAM cytosolic domain. We found that PAM-1 proteins truncated at amino acid residue 936 or lacking residues 928–945 of the cytosolic domain accumulate on the cell surface and are not internalized (15). We further demonstrated that a tyrosine-based signal (Glu-Tyr<sup>206</sup>-Ser-Arg-Lys) mediates the efficient internalization of PAM from the cell surface (15) and that the phosphorylation state of PAM on Ser<sup>207</sup> is one factor that governs the fate of the internalized green fluorescent protein, RT, reverse transcriptase; kb, kilobase(s); RACE, rapid amplification of cDNA ends; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; BFA, brefeldin A; PAGE, polyacrylamide gel electrophoresis; SCAMP, secretory carrier membrane proteins.

* This work was supported by National Institutes of Health Grants R29DK50744 and DK-32948, a junior faculty development award from the University of North Carolina, and a University of North Carolina University Research Council Award. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide and amino acid sequences for P-CIP1s have been deposited in the GenBank data base under GenBank accession numbers AF056208 (rat P-CIP1) and AF056209 (human P-CIP1).

§ To whom correspondence should be addressed: Cell and Molecular Physiology, The University of North Carolina at Chapel Hill, CB 7545, Chapel Hill, NC 27599. Tel.: 919-966-9792; E-mail: milg@med.unc.edu.

1 The abbreviations used are: TGN, trans-Golgi network; PAM, peptidylglycine α-amidating monoxygenase; P-CIP, PAM COOH-terminal interactor protein-1; TES, 2-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino)ethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; nt, nucleotide(s); PCR, polymerase chain reaction; GFP, enhanced green fluorescent protein; RT, reverse transcriptase; kb, kilobase(s); RACE, rapid amplification of cDNA ends; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; BFA, brefeldin A; PAGE, polyacrylamide gel electrophoresis; SCAMP, secretory carrier membrane proteins.
protein (23). Taken together our data indicate that several sorting signals are contained within the PAM cytosolic domain (15).

Since protein-protein interactions play a role in targeting proteins within the secretory pathway, Alam et al. (1) used the yeast two-hybrid system to identify three partial cDNAs encoding PAM cytosolic domain interacting proteins (P-CIPs). In two-hybrid assays, the protein encoded by each of the cDNAs interacts with the wild type PAM cytosolic domain but does not associate with mutant PAM proteins lacking the previously identified cytosolic targeting signals. P-CIP2 displays homology to known protein serine/threonine kinases (1) and may be involved in phosphorylating PAM, while P-CIP10 (Kalirin) is a member of the Db1 family of proteins (1, 24). P-CIP10 has nine putative myristylation sites (2) and associates with a variety of proteins involved in vesicular trafficking in cells.

**MATERIALS AND METHODS**

**In Vitro Binding Assays—**AtT-20 cell lines stably expressing PAM-1, PAM-1K, or PAM-1/Δ928–945 were described previously (15, 20). Wild type AtT-20 cells and the stable cell lines expressing PAM proteins were extracted in 20 mM sodium TES, pH 7.5, 10 mM mannitol, 1% Triton X-100 containing protease inhibitors (20). Fifty micrograms of each extract was immunoprecipitated with anti-PAM antibody directed against exon A (Fig. 1A, H1 629(23) using protein A-conjugated Sepharose beads (Sigma) as described previously (14). The immunoprecipitated PAM proteins were incubated with 50 nM P-CIP1 in binding buffer (10 mM Tris- HCl, pH 7.5, 150 mM potassium acetate, 1 mM MgCl2, 0.5 mM CaCl2) for 4 h at 4 °C. The beads were washed three times in 1 ml of binding buffer and the bound fractions were eluted from the beads by boiling in Laemmli sample buffer. Proteins which did not bind PAM-1 were concentrated by acetone precipitation and resuspended in Laemmli sample buffer. Bound and unbound fractions were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting using rabbit anti-PAM 

**Preparation of P-CIP1 Plasmids—**Plasmids for yeast two-hybrid systems were constructed by PCR. P-CIP1 cDNA sequence was used to construct mammalian expression vectors pEGFP.hP-CIP1 and pCIS-pCI.P-CIP1. The pCIS-pCI.P-CIP1 cDNA fragments were digested with PstI and KpnI and ligated into pEGFP-N2 (CLONTECH, Palo Alto, CA) digested with the same enzymes.

**Bacterial Expression of P-CIP1 and Anti-P-CIP1 Antiserum—**pET28.P-CIP1 or pET28.P-CIP1a plasmids were transformed into BL21(DE3), pLysS E.coli strain DH5α to express the P-CIP1 protein. Bacterial lysates were prepared in 10 mM Tris- HCl, pH 7.5, 150 mM potassium acetate, 1 mM MgCl2, 0.5 mM CaCl2, 1% Triton X-100. The expressed P-CIP1 proteins contained 6 consecutive histidine residues and the T7 epitope tag at the amino terminus. Since both of the P-CIP1 proteins were insoluble in 1% Triton X-100, bacterial lysates were boiled in binding buffer and the binding buffer was added to the supernatant and purified on Ni columns according to the manufacturer’s instructions. After elution from the Ni columns, the proteins were concentrated and the urea was removed using an UltraFree Centrifugal Filter Unit with a 10-kDa molecular mass cut-off (Millipore Inc., Bedford, MA). Full-length P-CIP1 was used for in vitro binding assays, while the P-CIP1a (amino acid residues 1–290) protein was used as immunogen. Anti-P-CIP1 (antibody 1088) was generated at Aves Labs (Portland, OR) using bacterially expressed rat P-CIP1a as immunogen. After collecting 3 eggs prior to immunization, two chickens were immunized by intramuscular injection of polyhistidine-tagged P-CIP1a. Four immunizations were performed, and 1 week following the final immunization, the IgY fraction was purified from 6 eggs collected from each chicken.

**Analysis of P-CIP1 mRNA Expression—**A rat multiple tissue Northern blot (CLONTECH, Palo Alto, CA) was hybridized with 32P-labeled rat P-CIP1 cDNA probe (nts 350–1657; 1 x 106 cpm/ml). The blot was prehybridized at 68 °C for 30 min and incubated with the probe at 68 °C for 1 h in ExpressHyb (CLONTECH, Palo Alto, CA). After incubation, the blot was washed at room temperature for 30 min in 2 x SSC + 0.05% SDS,followed by 0.1 x SSC + 0.1% SDS for 40 min at 50 °C. Blots were stripped and reprobed with 32P-labeled β-actin probe for normalization (CLONTECH). For RT-PCR analyses, total RNA from rat lung, kidney, adrenal, pituitary, ovary, and spleen was purified using RNA-STAT60 (TEL-TEST®, Inc.) and treated with DNase (Life Technologies, Inc.). cDNA was synthesized using Superscript II reverse transcriptase (Life Technologies, Inc.) and oligo(dT) primer. PCR was performed using Taq polymerase (Life Technologies, Inc.) and rat P-CIP1 gene-specific primers LC-3 (sense, 5'-AGCACCTCTCTGGTGATAGG3', nts 549–571) and LC-19 (antisense, 5'-TCTCTGGGATCTCAGTGAAT3', nts 1374–1350). The quality of each cDNA was verified using cyclophilin specific primers as described (25).

**Cloning of Full-length Rat P-CIP1 cDNA—**cDNA libraries were synthesized from 1 μg of rat adrenal gland, whole brain, testis, and kidney poly(A) mRNA using the Marathon cDNA Amplification Kit (CLONTECH). Briefly, first and second strand cDNA syntheses were performed as recommended, cDNAs were blunt-ended by incubation with T4 DNA polymerase, and adaptors were ligated to each end of the cDNA. Several gene-specific oligonucleotides were designed to amplify 1.0-kb PCR products from the cDNA libraries. These were identified by denaturing two-hybrid screen. PCR reactions were performed using adaptor-specific (AP1 or AP2) and P-CIP1 specific primers, KlenTaq DNA polymerase (CLONTECH), and the following PCR conditions: 94 °C x 30 s, 72 °C x 4 min for five cycles; 94 °C x 30 s, 70 °C x 4 min for five cycles; and 94 °C x 30 s, 68 °C x 4 min for 25 cycles. For 3′ RACE we paired sense primer LC3 and adaptor primer AP1 and amplified a distinct 1.3-kb fragment from all tissues examined. For 5′ RACE, initial PCR reactions were performed using adaptor primer AP1 and LC6 (nts 974–924; 5'-GGTTGTTCTGGTACGCTGAG-3'). Then, 5 μl of the first PCR product was diluted 1:50 in Tricine-EDTA buffer and 5 μl was used as template for a nested PCR using adaptor primer AP2 and P-CIP1 specific primer LC4 (nts 798–788; 5'-TCAATCGGGCGGGGGTTCTGAGC-3'). The resultant PCR products were cloned into the pGEM-T easy cloning vector (Promega, Madison, WI) and DNA sequencing was performed using the University of North Carolina sequencing facility.

**Transient Transfection, Immunostaining, and Confocal Microscopy—**AtT-20 and CHO cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's and F-12 media containing 10% fetal clone serum (HyClone, Logan, UT). Cells were transfected with pEGFP and pEGFP.hP-CIP1 using calcium phosphate or Superfect (Qiagen, Valencia, CA). AtT-20 cells were transfected with the same plasmids using Superfect (Qiagen, Valencia, CA). For CHO cells, the transfection efficiency was ~50% for GFP and 10% for P-CIP1 constructs while ~5% of the AtT-20 cells were transfected. In some experiments, CHO cells were co-transfected with pEGFP.hP-CIP1 and pCIS-
Results

In Vivo Interaction between PAM-1 and P-CIP1—P-CIP1 was identified by screening yeast two-hybrid library using residues 989–961 of the PAM cytosolic domain as bait (1). To confirm the yeast two-hybrid results in biochemical assays, and to test whether P-CIP1 interacted with the full-length PAM cytosolic domain (residues 899–976), we immunoprecipitated wild type full-length PAM-1 protein from transfected AtT-20 cell lysates. The immunoprecipitated PAM protein was incubated with 50 nM recombinant P-CIP1 and interactions between PAM and P-CIP1 were detected by Western blotting using P-CIP1 antisera. P-CIP1 interacted with full-length wild type PAM but did not bind to control beads lacking PAM-1 protein (wild type AtT-20 cells; Fig. 1B). Thus we conclude that P-CIP1 can interact with full-length PAM-1 in biochemical assays.

We previously used site-directed mutagenesis and expression in the neuroendocrine AtT-20 cell line to identify amino acid residues critical for the trafficking of PAM. We found that PAM proteins terminating at residue 936 of the cytosolic domain accumulated on the cell surface and were not internalized (15). Furthermore, we found that residues 928–945, including the internalization signal Gly-Tyr936-Ser-Arg-Lys, were essential for targeting integral membrane PAM. To determine whether P-CIP1 interacted with PAM through residues known to be important for PAM trafficking, we performed in vitro binding assays using PAM-1/936 and PAM-1928–945 proteins. Immunoprecipitated PAM-1/936 or PAM-1928–945 was incubated with recombinant P-CIP1 and the interaction between P-CIP1 and these mutant PAM proteins was measured. We found that in contrast to full-length PAM-1, P-CIP1 did not specifically interact with PAM-1/936 or PAM-1/928–945 (Fig. 1B). These results were not due to differences in the amount of PAM proteins in the immunoprecipitates, since immunoblots with PAM antisera revealed approximately equal amounts of each PAM protein in the assays (not shown). Thus we conclude that P-CIP1 may interact with PAM via residues within the PAM cytosolic domain that are known to be involved
in targeting the protein within the secretory and endosomal systems.

Expression of P-CIP1 mRNA and Isolation of Full-length P-CIP1 cDNAs—To begin our characterization of P-CIP1, we used Northern blot hybridization analysis and RT-PCR to examine the distribution of P-CIP1 mRNA. We found that the expression of P-CIP1 mRNA was not limited to neuroendocrine tissues. Northern blots indicated that P-CIP1 mRNA was expressed in testis, kidney, skeletal muscle, liver, lung, brain, and heart (Fig. 2A). In addition, RT-PCR indicated that P-CIP1 was expressed in pituitary gland, adrenal gland, and ovary (Fig. 2B). On Northern blots, there were two distinct P-CIP1 mRNAs, ~1.8 and 5 kb in length (Fig. 2A). These two transcripts varied in expression pattern from tissue to tissue and both were larger than the 1-kb cDNA isolated in the yeast two-hybrid library screen. Therefore we proceeded to clone the entire P-CIP1 cDNA. To clone the 5′ and 3′ ends of the P-CIP1 cDNA, we designed primers for rapid amplification of cDNA ends (RACE; Fig. 3A) and performed RACE using RNA isolated from several rat tissues including adrenal gland, brain, testis, and kidney. The resulting RACE products were subcloned and sequenced and found to contain sequences overlapping the P-CIP1 cDNA isolated in the yeast two-hybrid library screen.

The longest rat P-CIP1 cDNA isolated is 1788 nucleotides in length and contains a single long open reading frame from nucleotides 350 to 1657 (Fig. 3B). The predicted initiator methionine codon (nts 350–352) is contained within the context of a consensus translational initiation sequence defined for higher eukaryotes (27). In addition, the upstream cDNA sequence (nts 1–349) includes 5 in-frame stop codons. There are also termination codons in the alternative reading frames, and the predicted initiator methionine identified by 5′ RACE is in-frame with the protein product obtained in the yeast two-hybrid screen. Together these data provide strong evidence that this is the correct open reading frame. Although the yeast two-hybrid clone contained an in-frame translation termination codon, 3′ RACE established this to be an artifact of the library construction. There is a polyadenylation signal (AATAAA, nts 1769–1771) and a polyadenylate tail present in all the 3′ RACE products and attempts to establish a longer 3′-untranslated region using different primers were unsuccessful. To determine whether alternative mRNA splicing within the P-CIP1 coding region accounts for the mRNA size heterogeneity observed on Northern blots, we performed RT-PCR on cDNAs isolated from rat kidney, adrenal gland, brain, and testis. The oligonucleotides used in the PCR were designed to amplify the P-CIP1 coding region and overlapped the predicted initiator methionine and the termination codon. In all PCR reactions we observed a single band of the expected size amplified in each tissue (not shown), suggesting that the larger mRNA is not due to alternative mRNA splicing within the P-CIP1 coding region. The results do not rule out the possibility that alternative splicing in the 5′-untranslated region and 3′-untranslated region generates the larger P-CIP1 mRNA.

The rat P-CIP1 protein is 435 amino acids in length (Fig. 3B) with a calculated molecular mass of 49.5 kDa and pl of 5.1. The predicted protein does not contain an amino-terminal signal sequence or hydrophobic stretches typical of transmembrane domains, suggesting that P-CIP1 is a cytosolic protein. Blast searches indicate that P-CIP1 is a novel protein that does not share significant homology with known proteins. Computer-based structural analyses using PairCoil (28, 29) and Newcoil algorithms (30) indicate that amino acid residues 263–294 are predicted to form a coiled-coil with greater than 94% probability (28, 29) (Fig. 3C) and in data base searches, this region exhibits similarity to other coiled-coil proteins including myosin and intermediate filaments.

By searching EST data bases we identified two human cDNAs with high homology to the rat P-CIP1 cDNA sequence (82.2%). The cDNAs were identified in libraries prepared from human pancreatic and colon carcinomas indicating that P-CIP1 is also expressed in these tissues. We sequenced each of the human P-CIP1 ESTs to compare their sequence to each other and to the rat P-CIP1 cDNA. Human P-CIP1 cDNA contains a single long open reading frame encoding a predicted protein of 435 amino acids in length and shares 85% identity with rat P-CIP1 (Not shown).

Intracellular Localization of P-CIP1-GFP Fusion Proteins in CHO and AtT-20 Cells—We initially attempted to determine the subcellular distribution of P-CIP1 in cell lines and tissues expressing P-CIP1 but were unable to detect the endogenous protein. Therefore we transiently transfected endocrine and fibroblast cell lines with plasmids encoding P-CIP1-GFP fusion proteins. Since we consistently observed that cells expressing high levels of P-CIP1-GFP, but not GFP alone, contain large vacuoles and altered morphology (not shown), we restricted our analyses to cells expressing low to moderate P-CIP1-GFP levels.

The expression of P-CIP1-GFP chimera of the predicted size was confirmed by immunoblot analysis of membranes isolated from transfected cells (Fig. 4). We separated whole cell lysates of CHO cells expressing GFP or P-CIP1-GFP into soluble and particulate fractions, and then determined the distribution of the expressed proteins by immunoblot analysis. Most of the
P-CIP1-GFP chimera was distributed in the particulate fraction, while the majority of GFP was recovered in the soluble fraction (Fig. 4). These data suggest that the P-CIP1 protein may associate with cellular membranes and our confocal analyses confirm these biochemical results. When expressed alone, GFP was diffusely distributed throughout the cytosol and nucleus of CHO and AtT-20 cells (Fig. 5). In contrast, the P-CIP1-GFP chimera was distributed in a punctate pattern throughout the cell with a significant accumulation of the expressed protein on vesicles adjacent to the nucleus (Fig. 5). This distribution pattern was similar in fibroblasts (CHO) and endocrine (AtT-20) cells. In control experiments, we found that when nontagged P-CIP1 was expressed in cells and visualized using our P-CIP1 antisera, the expressed protein localized to the perinuclear area in a pattern indistinguishable from that observed for the P-CIP1-GFP chimera (not shown). Therefore we conclude that fusion with GFP did not alter the distribution of the P-CIP1 protein.

We previously demonstrated that integral membrane PAM proteins are concentrated in the perinuclear region of endocrine and non-endocrine cells (20, 31) in a distribution overlapping, but not identical to, the distribution of TGN38 (22). Since the distribution of P-CIP1 resembled the distribution of integral membrane PAM, we compared the localization of PAM-1 and P-CIP1 following transient transfection into CHO cells. As we observed previously in stable fibroblast cell lines expressing integral membrane PAM, the PAM-1 protein was concentrated in perinuclear vesicles. In these co-transfection experiments we observed a small, but consistent, amount of overlap between the expressed integral membrane PAM and P-CIP1 (Fig. 6A).

Characterization of P-CIP1-associated Vesicles—An identification of the nature of the perinuclear vesicles containing P-CIP1 will help with the elucidation of P-CIP1 function. Therefore we used confocal microscopy to compare the distribution of the expressed P-CIP1-GFP chimera and marker proteins concentrated in particular subcellular compartments. Since the

FIG. 3. Rat P-CIP1 cDNA sequence, structural predictions. A, the coding region is presented as a box, while the untranslated regions are indicated as lines at both ends. The partial P-CIP1 cDNA cloned from yeast two-hybrid library screen is shown below the full-length cDNA and the location of primers used in 3' or 5' RACE reactions (LC-3, LC-4, and LC-6) are indicated. B, the longest P-CIP1 cDNA isolated consists of 1788 base pairs and the coding region encodes a protein of 435 amino acids. The Kozak sequence is marked by underlining. C, the presence of the coiled-coil structure was predicted using the PairCoil algorithm (28, 29) with a window size of 28 amino acids. The probability that the second region (residues 263–294) will form a coiled-coil is 0.94. The NewCoil algorithm (30) predicts a single coiled-coil (residues 263 to 296) with the probability of 0.716 (not shown). The isoelectric point was calculated with a window size of 20 amino acids.

FIG. 4. Subcellular distribution of GFP and P-CIP1-GFP. CHO cells were transiently transfected with pEGFP or pEGFP.hP-CIP1 using Superfect. Cell extracts from wild type and transfected cells were separated into soluble (S) and particulate (P) fractions by centrifugation as described, and proteins were electrophoresed on 10% SDS-PAGE. The amount of GFP or P-CIP1-GFP protein in each fraction was determined by immunoblot analysis using rabbit anti-GFP antisera diluted 1:2000 (CLONTECH).
distribution of P-CIP1 strongly suggested a Golgi/TGN localization, we compared the distribution of P-CIP1 and TGN38, an integral membrane protein accumulated in the TGN. Although its function is still not clear, TGN38 is thought to play a role in vesicle budding in the constitutive secretory pathway (32, 33).

Cells transiently transfected with P-CIP1-GFP cDNAs were fixed, permeabilized, and stained with TGN38 antisera. Although we observed partial overlap between P-CIP1 and TGN38 in a small number of cells, in most cells the distributions of P-CIP1 and TGN38 were clearly distinct (Fig. 6). Thus we conclude that P-CIP1 is not accumulated to a large degree on TGN membranes; however, we cannot exclude the possibility that small amounts of P-CIP1 are associated with TGN.

Although we observed little overlap between P-CIP1 and TGN38 in control cells, cells treated for 60 min with the fungal metabolite brefeldin A (BFA) showed a different distribution pattern, with significant overlap between TGN38 and P-CIP1.
BFA dramatically changes the distribution and flow of membrane through secretory and endosomal systems and Lippincott-Schwartz et al. (34) demonstrated that BFA induced the mixing of the TGN and the recycling endosomal system. The significant colocalization of P-CIP1 and TGN38 following BFA treatment is consistent with the hypothesis that P-CIP1 is accumulated on endosomes.

To directly test the hypothesis that P-CIP1 associates with perinuclear endosomes, we compared the subcellular distribution of P-CIP1 and secretory carrier membrane proteins (SCAMPs), using an antibody that does not distinguish between SCAMP isoforms (26). SCAMPs are a family of integral membrane proteins that are found in cells of endocrine and nonendocrine origin and serve as a marker for organelles involved in cell surface recycling (26, 35–36). Brand et al. (35) demonstrated that SCAMPs colocalize with internalized FITC-transferrin in NRK fibroblasts. When we compared the immunocytochemical distribution of SCAMPs with the distribution of P-CIP1-GFP in CHO cells, we found that although SCAMPs display a broader distribution pattern with punctate vesicles scattered throughout the cell, SCAMPs and P-CIP1 co-localized in the perinuclear region (Fig. 7). To further characterize the P-CIP1-associated vesicles, we incubated transiently transfected cells with Texas Red-conjugated transferrin for 60 min at 37 °C and compared the distributions of P-CIP1 and Texas Red-conjugated transferrin by confocal microscopy. Vesicles containing Texas Red-conjugated transferrin were observed throughout the cells and some of the internalized protein was accumulated in perinuclear vesicles. In the periphery of the cells there was little overlap between transferrin-containing vesicles and P-CIP1; however, in the perinuclear region, we found significant overlap between the distributions of P-CIP1 and Texas Red-conjugated transferrin (Fig. 7). The co-distribution of P-CIP1 and internalized Texas Red-conjugated transferrin on perinuclear vesicles implies that P-CIP1 may associate with endosomes. To further test this hypothesis, cells were transiently transfected with P-CIP1-GFP and stained with an antibody directed against Rab11, a small GTPase that is associated with recycling endosomes (37, 38). We found significant overlap between Rab11-positive organelles and the expressed P-CIP1 protein in the perinuclear area (Fig. 7).

DISCUSSION

PAM is a post-translational processing enzyme involved in generating biologically active hormones from inactive precursors and is broadly expressed in neuroendocrine tissues. P-CIP1 was identified based on its ability to interact in the yeast two-hybrid system with a fragment of the cytosolic domain of integral membrane PAM. We now show that P-CIP1 interacts with the full-length PAM cytosolic domain and that the interaction occurs via residues that are known to be important for targeting PAM (Fig. 1). The region of PAM that interacts with P-CIP1 includes a tyrosine-based internalization motif and a phosphoserine residue known to be involved in regulating the fate of PAM proteins following internalization from the cell surface. In addition, the region of PAM that interacts with P-CIP1 may contain signals involved in controlling the fate of membrane PAM once it is removed from the immature secretory granule during the maturation process (15). Thus an interaction between PAM and P-CIP1 may play a role in the routing and steady state distribution of PAM and may be...
important for trafficking of PAM within the secretory and/or endosomal pathways. Although our data suggest that P-CIP1 may play a role in directing the trafficking of PAM in neuroendocrine cells, further co-immunoprecipitation or cross-linking experiments will be required to demonstrate that PAM and P-CIP1 are interacting partners in vivo.

P-CIP1 is a novel protein with little homology to proteins previously reported. P-CIP1 does not contain a signal sequence, hydrophobic stretches characteristic of transmembrane domains, or any known organelle targeting signals. Thus we conclude that the P-CIP1 mRNA encodes a cytosolic protein, although our membrane fractionation studies clearly show that P-CIP1 may associate with organelle membranes (Fig. 4). The 435-amino acid P-CIP1 protein contains a region predicted with high certainty to form a coiled-coil structure; this region is 31 amino acids in length and may form a helix of 4.6 nm. Coiled-coil structures mediate heteromeric and homomeric protein-protein interactions (29). The PAM cytosolic domain does not contain a coiled-coil; however, the PAM cytosolic domain does contain a basic region (residues 891–954) that may interact via charge interactions with an acidic cluster (residues 279–331) found in the P-CIP1 protein (Fig. 3). This basic region in the PAM cytosolic domain includes residues 928–945 that are known to be required for binding of P-CIP1 and PAM in two-hybrid and in vitro binding assays (Ref. 1; Fig. 1).

Rat and human P-CIP1 also contain multiple consensus serine/threonine phosphorylation sites. The fact that P-CIP1 is a substrate in vitro for protein kinases (not shown) suggests that the phosphorylation state of P-CIP1 may regulate its function; an examination of P-CIP1 phosphorylation in vivo and site-directed mutagenesis of specific serine or threonine residues will be needed to examine this possibility. It will also be interesting to determine whether phosphorylation modulates the association between P-CIP1 and PAM. However, the fact that bacterially expressed P-CIP1 binds to PAM in test tube binding assays (Fig. 1B) suggests that the dephosphorylated P-CIP1 protein is capable of interaction. In addition, PAM is phosphorylated by several kinases, phosphorylation regulates the fate of internalized PAM proteins, and the PAM cytosolic domain interacts with a serine/threonine kinase recently cloned in a two-hybrid screen (1, 23). Therefore phosphorylation or dephosphorylation of PAM may also be involved in regulating interactions between PAM and P-CIP1.

To interact within the cellular environment, P-CIP1 and PAM must be expressed in the same cells and must be co-localized (at least transiently) within the same subcellular compartment. Our Northern blot and RT-PCR analyses indicate, that like PAM, P-CIP1 is expressed in a variety of neuroendocrine tissues (Fig. 2); however, the distribution of P-CIP1 is broader than the distribution of PAM mRNA, and the expression level of PAM and P-CIP1 mRNA do not always correlate well. For example, high levels of integral membrane PAM and P-CIP1 mRNA are expressed in heart (Ref. 39; Fig. 2). In contrast, kidney and lung do not express especially high levels of PAM mRNA or protein (39), but do express P-CIP1 mRNA. Therefore, it will be interesting to compare the expression patterns of PAM and P-CIP1 using in situ hybridization and immunohistochemistry. One interpretation of these data is that P-CIP1 may associate with the cytosolic domains of other integral membrane proteins to play a broad role in regulating vesicular trafficking.

We determined the steady state distribution of P-CIP1 in CHO and AtT-20 cells following transient transfection of P-CIP1-GFP chimeras. We used transient transfection assays because we found that stable expression of P-CIP1 in cells might be toxic. We are currently generating stable cell lines overexpressing P-CIP1 under control of a inducible promoter to verify these transient transfection results and to further elucidate the function of P-CIP1. We found that in both endocrine and nonendocrine cells, the majority of the expressed P-CIP1 protein localizes in the perinuclear area (Fig. 5) but does not significantly overlap the steady state distribution of membrane PAM (Fig. 6A). These data suggest that PAM and P-CIP1 may transiently associate in cells as PAM traverses the organelles enriched in P-CIP1. A similar transient association between the endoprotease furin and the furin cytosolic domain interacting protein ABP-280 is thought to be important for the trafficking of furin (40). We also found that the perinuclear distribution of P-CIP1 does not require that PAM be expressed in the cell (Fig. 5) and that although P-CIP1 is predicted to be cytosolic, the P-CIP1-GFP chimera is recovered in the particulate fraction of cells (Fig. 4). The mechanism by which P-CIP1 associates with membranes remains to be elucidated.

Although P-CIP1 proteins in the perinuclear region may be of TGN or endosomal origin, the lack of significant overlap between P-CIP1 and TGN38 (Fig. 6B) argues that P-CIP1 does not play a major role in TGN to cell surface pathways. The fungal metabolite brefeldin A, which causes fusion of TGN and recycling endosomal system (34, 41, 42) caused a redistribution of proteins in the transfected cells leading to a significant co-localization between TGN38 and P-CIP1 (Fig. 6B). These data suggest that the P-CIP1-associated vesicles may be of endosomal origin. The fact that the distribution of P-CIP1 overlaps with the distribution of SCAMPS, proteins found in organelles of the cell surface recycling pathway, further supports our conclusion that P-CIP1 associates with endosomes. To directly test the hypothesis that P-CIP1 was associated with endosomes, we compared the distributions of P-CIP1 and internalized Texas Red-conjugated transferrin. We found that P-CIP1-associated vesicles significantly overlap with the distribution of internalized Texas Red-conjugated transferrin (Fig. 7). The overlap between P-CIP1 and internalized transferrin is only observed in the perinuclear region, not in the periphery where early sorting endosomes are found (Fig. 7). These data suggest that P-CIP1 is not accumulated on sorting endosomes but may be localized on recycling endosomes, which accumulate adjacent to the centrioles and can be loaded with transferrin-transferrin receptor complexes (43–45). Rab proteins are a large family of Ras-like small GTPases that play key regulatory roles in membrane trafficking (46, 47). Different Rab proteins are found associated with distinct membrane-bound compartments (46). Rab11 is known to associate with perinuclear recycling endosomes in both polarized (37) and non-polarized cells (38). The fact that there is a significant overlap between the subcellular distribution of P-CIP1 and Rab11 (Fig. 7) supports our conclusion that P-CIP1 is associated with recycling endosomes. In addition, the fact that BFA had little effect on the distribution of P-CIP1 (Fig. 6B) is consistent with the conclusion that P-CIP1 accumulates on recycling endosomes, since incubation of CHO cells with BFA for 60 min has little effect on the distribution of transferrin-positive perinuclear endosomes (data not shown and Ref. 34).

In neuroendocrine cells, regulated secretory granules bud from the TGN (4). During the maturation process these immature secretory granules fuse with each other and excess membrane is removed via constitutive-like vesicles (48). The soluble contents of the constitutive-like vesicles are released from cells (21, 49–51), although studies on the trafficking of integral membrane granule-associated proteins suggests that constitutive-like vesicles may fuse with recycling endosomes enroute to the plasma membrane (14, 52). It is intriguing to find that P-CIP1, which interacts with targeting signals in the cytosolic
domain of PAM, may associate with the recycling endosomal system. Further characterization of the interaction between PAM and P-CIP1 and the identification of other proteins that associate with P-CIP1 should increase our understanding of these complex trafficking pathways.

Acknowledgments—We thank Dr. David Castle (University of Virginia) for providing SCAMP antisera, Dr. James Goldenring (Medical College of Georgia) for helpful discussion, Dr. Michael Chua for help in confocal microscopy, Yang Ling for initial help with the yeast two-hybrid system, Noemi Sealock for purifying P-CIP1 protein, and Kevin Trotter for help with RT-PCR analysis of P-CIP1 expression. We also thank Drs. Richard Cheney, Kay Lund, Ann Erickson, and members of the Milgram Lab for careful reading of the manuscript and many helpful suggestions. S. L. M. thanks Drs. Dick Mains and Betty Eipper for support and encouragement throughout the last several years and for sharing advice and reagents.

REFERENCES

1. Alam, M. R., Caldwell, B. D., Johnson, R. C., Darlington, D. N., Mains, R. E., and Eipper, B. A. (1996) J. Biol. Chem. 271, 28636–28640
2. Sudhof, T. C., and Jahn, R. (1994) Neuron 6, 665–677
3. Bauerfeind, R., and Huttner, W. B. (1993) Curr. Opin. Cell Biol. 5, 628–635
4. Kelly, R. B., and Grote, E. (1993) Annu. Rev. Neurosci. 16, 95–127
5. Bennett, M. K., and Scheller, R. H. (1994) Annu. Rev. Biochem. 63, 63–100
6. Bradbury, A. F., and Smyth, D. G. (1991) Trends Biochem. Sci. 16, 112–115
7. Lindberg, I. (1991) Mol. Endocrinol. 5, 1361–1365
8. Shields, D. (1991) in Peptide Biosynthesis and Processing (Fricker, L. D., ed) pp. 37–70, CRC Press, Boston, MA
9. Steiner, D. F. (1991) in Peptide Biosynthesis and Processing (Fricker, L. D., ed) pp. 1–15, CRC Press, Boston, MA
10. Eipper, B. A., Stoffers, D. A., and Mains, R. E. (1992) Annu. Rev. Neurosci. 15, 57–95
11. Eipper, B. A., Bloomquist, B. T., Husten, E. J., Milgram, S. L., and Mains, R. E. (1993) Ann. N. Y. Acad. Sci. 680, 147–160
12. Han, K. K., and Martinage, A. (1995) Int. J. Biochem. 25, 957–970
13. Koedam, J. A., Cramer, E. M., Briand, E., Furie, B., Furie, B. C., and Wagner, D. D. (1992) J. Cell Biol. 116, 617–625
14. Milgram, S. L., Mains, R. E., and Eipper, B. A. (1993) J. Cell Biol. 121, 23–36
15. Milgram, S. L., Mains, R. E., and Eipper, B. A. (1996) J. Biol. Chem. 271, 17526–17535
16. Green, S. A., Setiahi, H., McEver, R. P., and Kelly, R. B. (1994) J. Cell Biol. 4, 435–448
17. De Bie, I., Mareckovicz, M., Malide, D., Lazere, C., Nakayama, K., Bendayan, M., and Siedah, N. G. (1996) J. Cell Biol. 135, 1261–1275
18. Dittie, A. S., Thomas, L., Thomas, G., and Touze, S. A. (1997) EMBO J. 16, 4859–4870
19. Eipper, B. A., Milgram, S. L., Husten, E. J., Yun, H.-Y., and Mains, R. E. (1993) Protein Sci. 2, 489–497
20. Milgram, S. L., Johnson, R. C., and Mains, R. E. (1992) J. Cell Biol. 117, 717–728
21. Milgram, S. L., Eipper, B. A., and Mains, R. E. (1994) J. Cell Biol. 124, 33–41
22. Milgram, S. L., Hsu, S. T., Martin, G. V., Mains, R. E., and Eipper, B. A. (1997) J. Cell Biol. 110, 695–706
23. Yun, H.-Y., Milgram, S. L., Keutmann, H. T., and Eipper, B. A. (1995) J. Biol. Chem. 270, 30075–30083
24. Alam, M. R., Caldwell, B. D., Johnsen, S. T., Darlington, D. N., Mains, R. E., and Eipper, B. A. (1997) J. Biol. Chem. 272, 12667–12675
25. Sheng, M., Cummings, J., Rolden, L. A., Jan, Y. N., and Jan, L. Y. (1994) Nature 368, 144–147
26. Brand, S. H., Laurie, S. M., Mina, O. M., and Castle, J. D. (1991) J. Biol. Chem. 266, 18949–18957
27. Kozak, M. (1992) Annu. Rev. Cell Biol. 6, 197–225
28. Lupas, A., Dyke, M. V., and Stock, J. (1991) Science 252, 1162–1164
29. Lupas, A. (1996) Trends Biochem. Sci. 21, 375–382
30. Berger, B., Wilson, D. B., Wolf, E., Teuscher, T., Mills, M., and Kim, P. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8259–8263
31. Tausk, P. A., Milgram, S. L., Mains, R. E., and Eipper, B. A. (1992) Mol. Endocrinol. 6, 2185–2196
32. Jones, S. M., Crosby, J. R., Salamero, J., and Howell, K. E. (1983) J. Cell Biol. 102, 775–788
33. Banting, G., and Poonnambalam, S. (1997) Biochim. Biophys. Acta 1355, 209–217
34. Lippincott-Schwartz, J., Yuan, L., Tipper, C., Amherdt, M., Orci, L., and Klausner, R. D. (1991) Cell 67, 601–616
35. Brand, S. H., and Castle, J. D. (1993) EMBO J. 12, 3735–3741
36. Singleton, D. B., Wu, T. T., and Castle, J. D. (1997) J. Cell Biol. 116, 2099–2107
37. Calhoun, B. C., and Goldenring, J. R. (1996) Yale J. Med. 69, 1–8
38. Ulrich, D., Reisch, S., Urbe, S., Zerial, M., Parton, R. G. (1996) J. Cell Biol. 135, 913–924
39. Braak, M. K., Stoffers, D. A., Eipper, B. A., and May, V. (1989) Mol. Endocrinol. 3, 1387–1396
40. Liu, G., Thomas, L., Warren, R. A., Enns, C. A., Cunningham, C. C., Hartwig, J. H., and Thomas, G. (1997) J. Cell Biol. 139, 1719–1733
41. Wood, S. A., Park, J. E., and Brown, W. J. (1991) Cell 67, 591–600
42. Ladowski, M. S., and Howell, K. E. (1992) Eur. J. Cell Biol. 59, 92–105
43. Touze, J., and Hollinshead, M. (1991) J. Cell Biol. 115, 635–653
44. Dunn, K. W., and Maxfield, F. R. (1992) J. Cell Biol. 117, 301–310
45. Mayer, S., Presley, J. F., and Maxfield, F. R. (1993) J. Cell Biol. 121, 1257–1269
46. Simons, K., and Zerial, M. (1993) Neuron 11, 789–799
47. Schimmoller, F., Simon, I., and Pfeffer, S. R. (1998) J. Biol. Chem. 273, 22161–22164
48. Arvan, P., and Castle, D. (1992) Trends Cell Biol. 2, 327–331
49. Arvan, P., Kuliawat, R., Prabhakaran, D., Zavacki, A. M., Elahi, D., Wang, S., and Pilkey, D. (1991) J. Biol. Chem. 266, 14171–14174
50. Kuliawat, R., and Arvan, P. (1997) J. Cell Biol. 137, 585–608

Downloaded from http://www.jbc.org/ by guest on July 25, 2018
P-CIP1, A Novel Protein That Interacts with the Cytosolic Domain of Peptidylglycine α-Amidating Monooxygenase, Is Associated with Endosomes
Lihong Chen, Richard C. Johnson and Sharon L. Milgram

J. Biol. Chem. 1998, 273:33524-33532.
doi: 10.1074/jbc.273.50.33524

Access the most updated version of this article at http://www.jbc.org/content/273/50/33524

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 50 references, 26 of which can be accessed free at http://www.jbc.org/content/273/50/33524.full.html#ref-list-1