Kalirin, a Cytosolic Protein with Spectrin-like and GDP/GTP Exchange Factor-like Domains That Interacts with Peptidylglycine \(\alpha\)-Amidating Monooxygenase, an Integral Membrane Peptide-processing Enzyme

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Although the integral membrane proteins that catalyze steps in the biosynthesis of neuroendocrine peptides are known to contain routing information in their cytosolic domains, the proteins recognizing this routing information are not known. Using the yeast two-hybrid system, we previously identified P-CIP10 as a protein interacting with the cytosolic routing determinants of peptidylglycine \(\alpha\)-amidating monooxygenase (PAM). P-CIP10 is a 217-kDa cytosolic protein with nine spectrin-like repeats and adjacent Dbl homology and pleckstrin homology domains typical of GDP/GTP exchange factors. In the adult rat, expression of P-CIP10 is most prevalent in the brain. Corticotrope tumor cells stably expressing P-CIP10 and PAM produce longer and more highly branched neuritic processes than nontransfected cells or cells expressing only PAM. The turnover of newly synthesized PAM is accelerated in cells co-expressing P-CIP10. P-CIP10 binds to selected members of the Rho subfamily of small GTP binding proteins (Rac1, but not RhoA or Cdc42). P-CIP10 (kalirin), a member of the Dbl family of proteins, may serve as part of a signal transduction system linking the catalytic domains of PAM in the lumen of the secretory pathway to cytosolic factors regulating the cytoskeleton and signal transduction pathways.

Cytosolic proteins are involved in the formation of secretory granules (1–6) and in the trafficking and localization of integral membrane proteins needed for the synthesis of bioactive peptides (7–16). We have used one of the few integral membrane proteins known to be involved in the biosynthesis of neuropeptides, peptidylglycine \(\alpha\)-amidating monooxygenase (PAM),\(^1\) to search for cytosolic proteins involved in these processes (17). PAM is a bifunctional enzyme and integral membrane forms contain an NH\(_2\)-terminal signal sequence, the two catalytic domains that catalyze the sequential reactions required for peptide amidation, a single transmembrane domain, and a short cytosolic domain (18).

PAM is involved in the production of all \(\alpha\)-amidated peptides and functions only after neuroendocrine-specific endoproteases and carboxypeptidases have exposed the COOH-terminal glycine residue that serves as the nitrogen donor for amide formation (19). Immunocytochemical evidence indicates that PAM begins to function in the trans-Golgi network (TGN), but most peptide amidation occurs in immature secretory granules (20). Using immunoelectron microscopy, integral membrane forms of PAM have been localized to the TGN, especially to distal tubuloreticular regions, and to large dense core vesicles (21).

When expressed independently in neuroendocrine cells, each luminal catalytic domain of PAM is targeted to large dense core vesicles (22). Integral membrane forms of PAM are localized to the TGN region of both neuroendocrine and nonneuroendocrine cells (7, 8). A small percentage of membrane PAM is present on the cell surface or in endosomes at steady state. Elimination of the distal 40 amino acids of the 86-amino acid cytosolic domain results in relocation of membrane PAM to the plasma membrane (8, 9). When transferred to a plasma membrane protein such as the interleukin 2 receptor \(\alpha\)-chain (Tac), the cytosolic domain of PAM directs the majority of the protein to the TGN region and confers the ability to undergo internalization from the plasma membrane (9).

Mutation of a tyrosine residue in the COOH-terminal domain of PAM greatly diminishes internalization of PAM from the cell surface without dramatically altering its TGN localization (9). The TGN localization of membrane PAM is greatly compromised upon deletion of an 18-amino acid domain that includes the tyrosine residue essential for internalization. Integral membrane PAM proteins are phosphorylated, and mutagenesis studies indicate that phosphorylation affects routing (23).

Using a rat hippocampal library and the yeast two-hybrid system, we recently identified partial cDNAs encoding two PAM COOH-terminal interactor proteins (P-CIPs) (17). The biological relevance of these interactions is supported by the fact that the interactions are eliminated when the 18-amino acid segment identified as essential for proper routing of PAM is eliminated. P-CIP2 is similar to serine/threonine dual specific

\(\alpha\), amino acids; nt, nucleotide(s); DH, Dbl homology; PH, pleckstrin homology; PAGE, polyacrylamide gel electrophoresis; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)aminomethane sulfonylic acid; Ab, antibody; mAb, monoclonal antibody; ECT, expressed sequence tag; GST, glutathione S-transferase; CD, cytosolic domain; LAR, leukocyte common antigen-related; MCS, multiple cloning site.

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\(^1\) The abbreviations used are: PAM, peptidylglycine \(\alpha\)-amidating monooxygenase; TGN, trans-Golgi network; kb, kilobase pair(s); RHP, RACE hybrid primer; PCR, polymerase chain reaction; pBS, pBlueScript II (SK–); aa, amino acids; nt, nucleotide(s); DH, Dbl homology; PH, pleckstrin homology; PAGE, polyacrylamide gel electrophoresis; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)aminomethane sulfonylic acid; Ab, antibody; mAb, monoclonal antibody; ECT, expressed sequence tag; GST, glutathione S-transferase; CD, cytosolic domain; LAR, leukocyte common antigen-related; MCS, multiple cloning site.

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ility protein kinases, while P-CIP10 contains at least five spectrin-like repeats (17). In this study we identify the full-length P-CIP10 protein as a member of the Dbl family of GDP/GTP exchange factors (24) and establish the phenotype of stably transfected AtT-20 cell lines expressing PAM-1 and P-CIP10.

**Materials and Methods**

**Cloning of Full-length P-CIP10 cDNAs**—The 2.0-kb P-CIP10 cDNA fragment identified using the yeast two-hybrid system (17) was used to screen a 1 × 10^6 plaque-forming units from a random primed rat hippocampus P-BST cloning kit (Stratagene). Seven positive clones were plaque-purified, and the two largest cDNA inserts recovered were overlapping 4.7-kb (clone 10/28) and 4.1-kb (clone 10/34) fragments. Both strands of these cDNAs were sequenced completely; only the 5′ ends of clones 10/28 (upstream sequence from nucleotide 36) and 10/34 (upstream sequence from nucleotide 419) differed. Attempts to extend these sequences by 5′-rapid amplification of cDNA ends (RACE) were unsuccessful. The five shorter cDNAs were fragments of the larger pieces. DNA manipulations were carried out according to standard protocols.

Since no in-frame stop codon was identified in clones 10/28 and 10/34, 3′ RACE was used to extend the 3′-end of the 10/28 cDNA (25). Briefly, poly(A)+ RNA from adult rat pancreas (100 μg) or olfactory bulb (140 ng) was reverse transcribed with the Promega reverse transcription system (Madison, WI) using a RACE hybrid primer (RHP) 5′-GGAATTCGAGCTCAGTAT-3′ (0.75 μM) and avian myeloblastosis virus reverse transcriptase (15 units). After the initial 35-cycle amplification using 5′-CAGGATGCTTCTTCAAGTG-3′ (nt 4375–4392 of final P-CIP10a) and RHP, an aliquot of the PCR product was used in a nested PCR with 5′-CTCTTAGACGCACCTCTGCAATT-3′ (nt 4592–4611 of P-CIP10a) and RHP. A 770-base pair fragment (674 nt of new 3′ sequence) was obtained from independent amplifications of both tissues was purified, subcloned into pBluescript II (SK−) (pBS), and sequenced. The additional 3′-sequence contained no in-frame stop codon, so 3′ RACE was repeated as above using sense primers 5′-CTCTGTTTCCCTCCTGTG-3′ (nt 5097–5116 of P-CIP10a) for the first round of amplification and 5′-GCGTATGAGCGCTAGCTGATGT-3′ (5′-nt 5144–5164 of P-CIP10a) for the second round of PCR reactions with the same primer set. The 650-base pair fragment (444 nt of new 3′ sequence) obtained in this second 3′-RACE reaction had an in-frame stop codon. Reverse transcriptase-PCR was used to verify that the novel sequence contained in the 3′-RACE products was contiguous to clone 10/28.

**Full-length P-CIP10 cDNA Construction**—P-CIP10a (10/28 cDNA at the 5′-end) and P-CIP10b (10/34 cDNA at the 5′-end) cDNAs were constructed using a PCR-generated PBS-P-CIP10-3′ (nt 4255–5724) intermediate. Full-length P-CIP10a cDNA (PBS-P-CIP10a) was created by a three-way ligation of Bsp10I/ BamHI-digested PBS, a 4.45-kb fragment obtained from clone 10/28 by digestion with Bsp10I (5′-MCS)-AspEI (nt 4401) and a 1.32-kb fragment obtained from AspEI/BamHI-cut PBS-P-CIP10-3′. To create the full-length P-CIP10a cDNA (PBS-P-CIP10b), clone 10/34 was digested with BstBI (nt 2674) and XhoI (3′-MCS), and the 95/34 sequence downstream of BstBI was replaced with the 3.4-kb BstBI fragment (nt 2291 of P-CIP10a)-XhoI (3′-MCS) fragment from PBS-P-CIP10a.

**Construction of Expression Vectors**—Construction of pGEX-CIP10, an expression vector encoding a GST fusion protein containing P-CIP10a (aa 447–1138), was described (17). To construct pET-His-DH, a bacterial expression vector encoding all of the Dbl homology (DH) domain and most of the pleckstrin homology (PH) domain of P-CIP10a (P-CIP10a (aa 1254–1537), the 857-base pair fragment from clone 10/28 was subcloned into pET28a (Novagen) in frame with the histidine tag. A mammalian expression vector encoding full-length P-CIP10a (pSCEP.P-CIP10a) was constructed by inserting the full-length cDNA piece from pBS-P-CIP10a into pSCEP (28). To construct pBS.Myc.P-CIP10, the c-Myc epitope (underlined) with a Gly5 linker (MEQKLI) was joined in-frame to Gly5 of P-CIP10a using standard methods. The full-length cDNA insert was then transferred to pSCEP to generate pSCEP.Myc.P-CIP10. All PCR-generated cDNA was confirmed by DNA sequencing.

**In Vitro Translation and Northern Blot Analysis**—Truncated forms of P-CIP10 cDNAs were used as templates for in vitro transcription and translation reactions. PBS.10a (nt 1–1142) was generated by digesting pBS-P-CIP10a with BglII (nts at nt 1142) and BamHI (cuts in 3′-MCS) and religating. PBS.10b (nt 1–1526) was generated in the same manner from PBS-P-CIP10b. Radioiodinated proteins ([35S]methionine, 10 μCi/μl) was added. After freezing and thawing three times, extracts were diluted with 3 volumes of the guanine-nucleotide depletion buffer (30) and centrifuged. For each binding reaction, about 50 μg of GST-Pase
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Tissue Distribution of P-CIP10 mRNA—P-CIP10 was identified in a hippocampal/cortical cDNA library prepared from 3-week-old rat pups that had been subjected to a single maximal electroconvulsive stimulus (17). Before trying to clone a full-length P-CIP10 cDNA, we used Northern blot analysis to determine the size of the P-CIP10 transcript and the tissues in which it was expressed. The two largest cDNA fragments isolated (10/28 and 10/34) were identical except at their 5′-ends (Fig. 2A). No in-frame stop codons were found at either end of either cDNA. The GC content of the 5′-ends of both clones was high, and attempts to extend the sequences by 5′-RACE were unsuccessful (25); sequence and in vitro translation data (see below) indicated that an initiator Met was included in each clone. By sequentially employing 3′-RACE, we extended the sequence to include an in-frame stop codon (Fig. 2A). No sequence diversity was found in the newly amplified 3′-fragments. The fragments were assembled to form two full-length P-CIP10 cDNAs, P-CIP10a (5′-end of 10/28) and P-CIP10b (5′-end of 10/34) (Fig. 2A). A single long open reading frame with a stop codon near the 3′-end was found in both P-CIP10 cDNAs. The GC-rich nature of the 5′-end of both P-CIP10a and P-CIP10b and the presence of a single Met residue in both unique regions raised the possibility that a transcriptional start site was present in each clone. The nucleotide sequence surrounding each Met agreed with the consensus translational initiation sequence defined for higher eukaryotes (Fig. 2B) (31, 32). To determine whether these potential translational initiation sites were functional, we performed coupled in vitro transcription/translation reactions. We truncated P-CIP10a and P-CIP10b at a common site less than 1200 nt from the potential translational initiation sites so that the predicted 20-amino acid difference between the translation products of P-CIP10a and P-CIP10b would be detectable (Fig. 2B). Each P-CIP10 cDNA yielded a protein of the size predicted if translation were initiated at the Met in each clone. The nucleotide sequence surrounding each Met agreed with the consensus translational initiation sequence defined for higher eukaryotes (Fig. 2B) (31, 32). To determine whether these potential translational initiation sites were functional, we performed coupled in vitro transcription/translation reactions. We truncated P-CIP10a and P-CIP10b at a common site less than 1200 nt from the potential translational initiation sites so that the predicted 20-amino acid difference between the translation products of P-CIP10a and P-CIP10b would be detectable (Fig. 2B). Each P-CIP10 cDNA yielded a protein of the size predicted if translation were initiated at the Met in each clone unique 5′-region (Fig. 2C); for both P-CIP10a and P-CIP10b, the next Met is more than 80 amino acid residues downstream. The P-CIP10a transcription/translation reaction proceeded much more efficiently than the P-CIP10b reaction, and we used the P-CIP10a cDNA for all further studies.

Structure Predicted for the P-CIP10 Protein—P-CIP10a encodes a protein of 1899 amino acids with a calculated molecular mass of 217 kDa and pI of 5.67 (Fig. 2D). P-CIP10 is largely hydrophilic, with the characteristics of a cytosolic protein. The NH2 terminus of P-CIP10 lacks a hydrophobic signal sequence, and no hydrophobic stretches typical of transmembrane domains are present. By homology search and computer-based structural analysis, P-CIP10 can be divided into five regions: a short NH2-terminal region, a region of spectrin-like repeats, a DH domain, a PH domain, and the COOH-terminal region (Fig. 2E).

The NH2-terminal 150 amino acids of P-CIP10 are homologous to Trio, a new member of the Dbl family of proteins identified by virtue of its interaction with the cytosolic domain of the leukocyte common antigen-related (LAR) transmembrane protein-tyrosine phosphatase (33). The next 1000 amino acid residues of P-CIP10 are most homologous to Trio (33), spectrin, and fodrin (34, 35). Spectrin and fodrin are cytoskeletal proteins involved in the maintenance of plasma membrane structure by cross-linking to actin and to various integral and membrane-associated proteins (36, 37). Secondary structure predictions indicate that this region of P-CIP10 is almost entirely α-helical and that the NH2-terminal part of P-CIP10 can

FIG. 1. Expression of P-CIP10 mRNA is prominent in specific brain regions. A, total RNA (10 μg) from the indicated adult rat tissues was subjected to Northern blot analysis. P-CIP10 transcripts were visualized with the P-CIP10 (nt 1365–3398) probe (17). Inset, poly(A)+ RNA (8 μg) was visualized with the same probe. P-CIP10 transcripts were visualized in a coronal section through the anterior hypothalamic area (B) or the olfactory bulb (D) using an antisense riboprobe; the sense riboprobe control for a corresponding section is shown for comparison (C). AOB, accessory olfactory bulb; IGr, internal granular layer; IPl, internal plexiform layer; Mf, mitral cell layer; EPI, external plexiform layer; GI, granule cell layer. Bar, 200 μm.

RESULTS

The beads incubated with nonlabeled cell extracts were eluted by boiling in 50 mM Tris-HCl, pH 7.5, 1% SDS, diluted, and subjected to immunoprecipitation (9) with Ab JH2000.
FIG. 2. Cloning strategy, in vitro transcription/translation, and sequence of P-CIP10. A, the partial cDNAs isolated from the yeast two-hybrid PAM CD interactor screen, I-10 and I-19, are indicated. Using I-10 (P-CIP10 (nt 1363–3398)) as probe, cDNAs 10/34 and 10/28 were isolated from a rat brain cDNA library. RACE products 1 and 2 (RACE#1 and RACE#2) were obtained through sequential use of 3'-RACE. P-CIP10a and P-CIP10b were constructed from clones 10/28 and 10/34 with RACE products 1 and 2. B, the nucleotide sequences at the putative translational initiation sites in P-CIP10a and P-CIP10b are compared with the optimal translational initiation site (Kozak) (31, 32); identities are in boldface type. C, for in vitro transcription/translation, pBS.10a (nt 1–1142) and pBS.10b (nt 1–1525) were used as templates. The SDS-PAGE analysis and apparent molecular masses of the translation products are shown; the molecular mass predicted for each translation product is shown in parenthesis. D, amino acid sequence obtained by translating the longest open reading frame of the P-CIP10a cDNA (U70373; P-CIP10b is U88156). The protein sequence reported previously (aa 447–1138) (17) is set off by arrows with double bars; the sequence marked above by a cross-hatched bar is P-CIP19 (aa 473–823). The boxed region (aa 992–1013) shows the peptide used to raise antisera. The beginning and end of each spectrin-like repeat is indicated by double-headed arrows. DH and PH domains are overlined with thick and thin lines, respectively. The triplet encoding Lys1794 was absent in approximately half of the PCR products sequenced, presumably reflecting a splicing variant. Probable human P-CIP10 clones were identified by screening dbEST and dbSTS; the regions of rat P-CIP10 to which the human ESTs correspond are indicated by dots under the sequence, and sites at which the amino acid sequence predicted for human P-CIP10 differs from that of rat P-CIP10 are indicated. a, AA028043; b, AA027938, four frame shifts inserted; c, H09889; d, AA115289, one frame shift, and AA115265. E, major domains of rat P-CIP10 are drawn to scale.
be arranged into nine spectrin-like repeats 103–138 amino acids in length (Figs. 2E and 3A).

Separated from the spectrin-like repeats by 50 amino acids is a 200-amino acid region (aa 1258–1457) with significant homology to the DH domain defined by Dbl, Dbs, and Ost (24, 38–40) (Fig. 2, D and E, and Fig. 3B). DH domains were identified first in a family of oncogenic proteins and subsequently shown to catalyze the exchange of bound GDP for bound GTP on specific members of the Rho subfamily of small GTP binding proteins (24).

FIG. 3. Analysis of P-CIP10 amino acid sequence.

A, the sequences of the nine spectrin-like repeats in P-CIP10 are aligned. The consensus features defining the A, B, and C helices of a spectrin-like unit (68) are indicated above the sequence of P-CIP10. Amino acids conforming to these rules are shown in boldface type. Hydrophobic residues are indicated by asterisks. Residues at a and d positions are generally hydrophobic. The number to the right of each sequence indicates the final residue of that repeat. B, alignment (Clustal) of DH and PH domains of P-CIP10 with related proteins, Trio (33), Dbs (40), Ost (38), Dbl (39), SCD1 (43), FGD1 (42), and Tiam (41). The only adjustments made to the alignment were in region 1 of the PH domain. DH domains start from residue 1258 for P-CIP10a, 1237 for Trio, 446 for Ost, 636 for Dbs, 499 for Dbl, 194 for SCD1, 376 for FGD1, and 1044 for Tiam. The six structural regions forming a PH domain are as defined (44, 46).

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homology to the DH domain of P-CIP10 is the first DH domain of human Trio (90% identity) (Fig. 3B). The DH domains of Dbl, Ost, and Dbs share 42–46% identity with that of P-CIP10. Additional proteins that share significant homology with the DH domain of P-CIP10 include Tiam, FGD1, and yeast SCD1 (Fig. 3B) (41–43).

The region of P-CIP10 immediately following the DH domain (Fig. 2, D and E; aa 1458–1555) constitutes a PH domain. PH domains are poorly conserved in sequence and are defined by their common three-dimensional structural motifs (44, 45). The PH domain of P-CIP10 has greatest similarity to the PH domain of Trio, followed by the PH domains of Dbl, Dbs, and Ost (Fig. 3B). Although not essential for in vitro GEF activity, the PH domain is generally essential to cellular function (46). PH domains are thought to aid in protein localization by protein-protein or protein-lipid interaction (44, 45, 47).

Except at its extreme COOH terminus, P-CIP10 exhibits homology to Trio. The COOH-terminal third of the 2861-amino acid Trio protein contains a second DH/PH domain, immunoglobulin-like repeats, and a putative serine/threonine protein kinase domain and these last two domains bear no homology to P-CIP10. Since the human ESTs identified as homologues of P-CIP10 (Fig. 2D) exhibit a greater degree of identity to rat P-CIP10 than does human Trio, we conclude that Trio and P-CIP10 are encoded by separate genes. The sequence homology exhibited by P-CIP10 and Trio suggests that these two proteins define a subfamily of the Dbl proteins.

Expression of P-CIP10 in AtT-20 Cells—To study the properties of P-CIP10 and its effects on cellular function, P-CIP10a and Myc.P-CIP10 cDNAs (Fig. 2A) were used to doubly transfet AtT-20 cells stably expressing PAM-1 (AtT-20/PAM-1 cells). AtT-20 corticotrope tumor cells do not express P-CIP10 and Myc.P-CIP10 cDNAs (Fig. 2B). Additional proteins that share significant homology with the DH domain of P-CIP10 include Tiam, FGD1, and yeast SCD1 (Fig. 3B) (41–43).

To localize P-CIP10, AtT-20/PAM-1 cells expressing Myc.P-CIP10 were biosynthetically labeled for 30 min and subjected to differential centrifugation (Fig. 4E). PAM-1 is recovered in fractions enriched in endoplasmic reticulum (P1 and P2) as well as in fractions enriched in TGN (P2 and P3) and secretory granules (P3). Each particulate fraction as well as the cytosolic fraction contained Myc.P-CIP10. The association of P-CIP10 with particulate fractions, many of which contain intact PAM-1 (23), suggests that P-CIP10 may function by interacting with membranous organelles.

Expression of P-CIP10 Changes AtT-20 Cell Morphology—Since many members of the Dbl family of proteins interact with members of the Rho family of GTPases and affect cytoskeletal organization and cell shape (48, 49), we examined the morphology of our stably transfected AtT-20 cells (Fig. 5). Many cells expressing P-CIP10 were larger than wild type AtT-20 or AtT-20/PAM-1 cells. In addition, cells expressing P-CIP10 often had very long processes, some of which were branched. Photomicrographs of randomly selected fields of each cell type were analyzed; the number of giant cells, the percentage of cells having neuritic processes, and the lengths and shapes of processes were quantified (Fig. 5E). Approximately 15% of the total population of P-CIP10-expressing cells had greatly enlarged cell bodies, 5–6 times more than for nontransfected as well as for the PAM-1-expressing cells. Twice as many of the P-CIP10-expressing cells had processes, although the average number of processes per cell for cells with processes was unaltered. The percentage of branched processes and the percentage of processes longer than 100 μm were dramatically increased for the cells expressing P-CIP10.

Expression of P-CIP10 Alters the Metabolism of PAM-1—In AtT-20 cells, the 120-kDa PAM-1 protein is cleaved by neuroendocrine-specific endoproteases after it exits the TGN (50). Cleavage yields soluble 45-kDa PHM and a 70-kDa membrane

![Image](http://www.jbc.org/)

**Fig. 4.** Expression of P-CIP10. A, extracts of AtT-20 cells expressing PAM-1 alone or PAM-1 and Myc.P-CIP10 were incubated with Myc mAb bound to Protein G-Sepharose. The bound proteins were resolved by SDS-PAGE and visualized with P-CIP10 antibody. B, AtT-20/PAM-1 cells expressing P-CIP10a (lane 1), no additional protein (lane 2), or Myc.P-CIP10 (lane 3) were incubated in medium containing [35S]Met/Cys for 15 min. P-CIP10 proteins were immunoprecipitated from cell extracts using P-CIP10 antibody. C, AtT-20/PAM-1 cells expressing P-CIP10 were incubated in medium containing [35S]Met/Cys for 15 min (P) and chased for 1, 2, or 4 h before immunoprecipitation of P-CIP10. D, data for AtT-20 cells expressing P-CIP10 and Myc.P-CIP10 were densitized and normalized to the amount of P-CIP10 present at the onset of the pulse (n = 3). E, biosynthetically labeled AtT-20 cells expressing Myc.P-CIP10 were subjected to subcellular fractionation; Myc.P-CIP10 was immunoprecipitated (Ab JH2000) from resuspended particulate fractions (1000 × g for 5 min (P1), 4000 × g for 15 min (P2), 37,000 × g for 15 min (P3), and 435,000 × g for 15 min (P4)) and the final supernatant (S); proteins were visualized by fluorography. All results were replicated three times.
protein containing the PAL, transmembrane, and COOH-terminal cytosolic domains. Active, 45-kDa PHM is stored in large dense core vesicles from which its secretion can be regulated by secretagogues. Membrane PAM proteins that are localized to the cell surface can be cleaved at a site near the transmembrane domain, leading to the accumulation of bifunctional 105-kDa PAM in the medium (8).

To determine whether expression of P-CIP10 changed the metabolism of PAM-1, we performed pulse/chase metabolic labeling experiments on AtT-20/PAM-1 cells and AtT-20/PAM-1 cells expressing P-CIP10a or Myc.P-CIP10. Quadruplicate wells of each cell type were incubated in medium containing [35S]Met/Cys and either harvested immediately (P) or incubated in the presence of unlabeled Met/Cys for 2 h prior to harvest. PAM proteins were immunoprecipitated from detergent-solubilized cell extracts (C) and medium (M) using a PHM antibody. B, PAM proteins were immunoprecipitated from medium samples obtained after a 4-h chase of cells labeled as described above. The entire analysis was replicated three times; the asterisk indicates nonspecific bands (8, 9).

FIG. 6. Expression of P-CIP10 affects the metabolism of PAM-1. A, AtT-20 cells expressing only PAM-1 (PAM-1 cells), PAM-1 with P-CIP10a (P-CIP10a cells), and PAM-1 with Myc.P-CIP10 (Myc.P-CIP10 cells) were incubated with [35S]Met/Cys for 15 min and harvested immediately (P) or incubated in the presence of unlabeled Met/Cys for 2 h prior to harvest. PAM proteins were immunoprecipitated from detergent-solubilized cell extracts (C) and medium (M) using a PHM antibody. B, PAM proteins were immunoprecipitated from medium samples obtained after a 4-h chase of cells labeled as described above. The entire analysis was replicated three times; the asterisk indicates nonspecific bands (8, 9).

DISCUSSION

Since our previous mutagenesis studies identified the CD of membrane PAM as essential in establishing its steady state localization in neuroendocrine cells, we screened a rat hippocampal cDNA library for PAM CD interactors using the yeast two-hybrid system (17). We previously identified partial cDNAs encoding a putative protein serine/threonine kinase (P-CIP2) and a protein with spectrin-like repeats (P-CIP10). Elucidation of the complete structure of P-CIP10 revealed the presence of elements common to signal transduction pathways, and expression of P-CIP10 in AtT-20 cells altered PAM processing and cell morphology. The itinerary taken by membrane PAM proteins in AtT-20 cells is complex, and it is anticipated that additional proteins capable of interacting with the CD of PAM will be identified.

The expression of P-CIP10 is highest in specific areas of rat brain. PAM is expressed in the same areas, but PAM is also expressed at high levels in many tissues lacking P-CIP10. For example, atrium, anterior, and neurointermediate pituitary express little P-CIP10 but high levels of PAM. P-CIP10 may fulfill a function unique to its sites of expression, or homologous protein(s) may be involved in the routing of PAM in tissues lacking P-CIP10. The existence of P-CIP10 transcripts with different 5′-ends and different sizes suggests that alternate splicing generates diverse forms of P-CIP10. Routing signals in the CD of PAM are recognized in both neuroendocrine and nonneuroendocrine cells (7, 8), and proteins homologous to P-CIP10, but expressed more widely, may be involved in these interactions.

The co-expression of P-CIP10 and PAM-1 in AtT-20 cells increased the rate and extent of disappearance of newly synthesized PAM-1. After traversing the Golgi stacks, newly syn-
The spectrin-based membrane skeleton restricts the mobility of to specific soluble and integral membrane proteins (36, 37, 56).

\[ \text{membrane} \] and form an extensive cytoskeletal meshwork by binding homologous to the 5-nm-long structural units that make up spectrin.

\[ \text{cretory granules identifies the TGN/immature secretory gran} \]

\[ \text{newly synthesized PAM by enzymes located in immature se} \]

\[ \text{fact that P-CIP10 expression facilitates cleavage of} \]

\[ \text{Golgi membranes in Madin-Darby canine kidney cells may play} \]

\[ \text{efficiently internalized, with mutagenesis studies suggesting} \]

\[ \text{like vesicles. Membrane PAM that reaches the cell surface is} \]

\[ \text{PAM leaves the immature granules, perhaps via constitutive-} \]

\[ \text{the regulated secretory pathway of neuroendocrine cells (52),} \]

\[ \text{and several other receptor type protein-tyrosine phosphatases} \]

\[ \text{the regulated secretory pathway of neuroendocrine cells (52),} \]

\[ \text{characterized forms of P-CIP10 lack a homologous region. Phogrin, a} \]

\[ \text{large dense core vesicle biogenesis and the signal transduction} \]

\[ \text{Rac (66). Overexpression of Tiam1 or activated Rac1 in T-} \]

\[ \text{Rac1 has been shown to interact with a variety of protein} \]

\[ \text{the COOH-terminal region of P-CIP10 is most homologous} \]

\[ \text{the COOH-terminal region of P-CIP10 is most homologous} \]

\[ \text{C-terminal domain with the cytosolic domain of the LAR} \]

\[ \text{COOH-terminal domain with the cytosolic domain of the LAR} \]

\[ \text{the 5-nm-long structural units that make up spectrin (68).} \]

\[ \text{Spectrin provides structural support to the plasma membrane} \]

\[ \text{to specific soluble and integral membrane proteins (36, 37, 56).} \]

\[ \text{The spectrin-based membrane skeleton restricts the mobility of} \]

\[ \text{an isoform of } \beta \text{-spectrin associated with Golgi} \]

\[ \text{The fact that P-CIP10 expression facilitates cleavage of} \]

\[ \text{as a subcellular site at which PAM/P-CIP10 interactions occur.} \]

\[ \text{The DH/PH domain of P-CIP10 is most homologous to the} \]

\[ \text{Many members of the Dbl family, through their DH/PH do-} \]

\[ \text{main, act as GDP/GTP exchange factors for members of the} \]

\[ \text{by its spectrin-like domains.} \]

\[ \text{Kalirin, PAM Interactor with Spectrin, DH, and PH Domains} \]

\[ \text{Acknowledgments—We thank Dr. Richard Cerione and Dr. Shubha} \]

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\[ \text{Beads: myc mAb GST-Rac1 GST-RhA GST-cde42 GST} \]

\[ \text{Extracts: PAM-1 PAM-2 PAM-1 PAM-2 PAM-1 PAM-2 IPT} \]

\[ \text{FIG. 7. P-CIP10 Binds to Rac1. A, extracts of AtT-20 cells expressing} \]

\[ \text{P-CIP10 to act as a GDP/GTP exchange factor remains to} \]

\[ \text{to be tested. The PH domain of known Dbl family members is} \]

\[ \text{and factors affecting its interaction with particulate fractions} \]

\[ \text{and cytosolic interactors with P-CIP10 and studies on the ef-} \]

\[ \text{as a GDP/GTP exchange factor remains to} \]

\[ \text{molecules (53–55).} \]

\[ \text{Perhaps most intriguing about the identification of a Dbl} \]

\[ \text{we have identified a signal transduction pathway linking the} \]

\[ \text{we propose giving P-CIP10 the name kalirin to signify its ability to} \]

\[ \text{Kali, Hindu goddess with} \]

\[ \text{perhaps most intriguing about the identification of a Dbl} \]

\[ \text{perhaps most intriguing about the identification of a Dbl} \]

\[ \text{large dense core vesicle biogenesis and the signal transduction} \]

\[ \text{These interactions suggest a relationship between large dense core vesicle biogenesis and the signal transduction} \]

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\[ \text{and Cdc42 expression vectors and Dr. Henry Keutmann (Massachusetts} \]

\[ \text{of the P-CIP10 peptide. We thank Marie Bell for helping with all aspects of this work and Kate Deanehan} \]

\[ \text{many hands) and its spectrin-like domains.} \]

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Kalirin, a Cytosolic Protein with Spectrin-like and GDP/GTP Exchange Factor-like Domains That Interacts with Peptidylglycine α-Amidating Monooxygenase, an Integral Membrane Peptide-processing Enzyme

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