The Prohormone Processing Enzyme PC3 Is a Lipid Raft-Associated Transmembrane Protein†

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ABSTRACT: The biosynthesis of most biologically active peptides involves the action of prohomone convertases, including PC3 (also known as PC1), that catalyze limited proteolysis of precursor proteins. Proteolysis of prohormones occurs mainly in the granules of the regulated secretory pathway. It has been proposed that the targeting of these processing enzymes to secretory granules involves their association with lipid rafts in granule membranes. We now provide evidence for the interaction of the 86 and 64 kDa forms of PC3 with secretory granule membranes. Furthermore, both forms of PC3 were resistant to extraction with TX-100, were floated to low-density fractions in sucrose gradients, and were partially extracted upon cholesterol depletion by methyl-β-cyclodextrin, indicating that they were associated with lipid rafts in the membranes. Protease protection assays, immunolabeling, and biotinylation of proteins in intact secretory granules identified an ∼115-residue cytoplasmic tail for 86 kDa PC3. Using two-dimensional gel electrophoresis and a specific antibody, a novel, raft-associated form of 64 kDa PC3 that contains a transmembrane domain consisting of residues 619−638 was identified. This form was designated as 64 kDa PC3-TM, and differs from the 64 kDa mature form of PC3. We present a model of the membrane topology of PC3, where it is anchored to lipid rafts in secretory granule membranes via the transmembrane domain. We demonstrate that the transmembrane domain of PC3 alone was sufficient to target the extracellular domain of the IL2 receptor α-subunit (Tac) to secretory granules.

Most biologically active peptide hormones and neuropeptides destined for the regulated secretory pathway of neuroendocrine cells are synthesized in the endoplasmic reticulum (ER) as larger, inactive precursors, or preprohormones, and transported to the Golgi. From the Golgi, prohormones are sorted away from lysosomal and constitutively secreted proteins and are packaged into granules of the regulated secretory pathway. The active hormones can then be secreted from the cell following specific stimuli (*1*). Endoproteolytic processing of prohormone precursors occurs predominantly in regulated secretory granules and involves the action of members of the subtilisin-like family of serine proteases. This family, currently comprising seven serine proteases, includes enzymes that play crucial roles in the biosynthesis of biologically active peptides (*2*−*4*). One of these proteases, proprotein convertase PC3 (also known as PC1), is expressed in endocrine and neuroendocrine tissues and is responsible for processing several prohormones (*5*−*13*). PC3 is originally synthesized as an inactive pre-pro precursor protein. Following removal of the signal peptide in the endoplasmic reticulum, an autocatalytic truncation reaction occurs to remove the PC3 pro sequence (*14*−*16*), resulting in the production of full-length 86 kDa PC3 (*17*, *18*). Further autocatalytic carboxyl-terminal truncation occurs in a post-*trans* Golgi network (TGN)[1] compartment(s) (*19*, *20*), yielding the 64 kDa form of PC3 that is found in neuroendocrine secretory granules (*21*).

There has been considerable interest in the interactions of proteins involved in hormone and neuropeptide biosynthesis

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[1] Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt); CT, C-terminal peptide; CYT, cytoplasmic tail peptide; EDTA, ethylenediaminetetraacetic acid; MβCD, methyl-β-cyclodextrin; PGPC, 30% DOPG/70% DOPC; tempo-PC, 1,2-dipalmitoyl-*sn*-glycero-3-phospho(tempo)choline; TGN, *trans*-Golgi network; TM, transmembrane; SUV, small unilamellar vesicles; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide HCl.

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with membrane components of the secretory pathway. Such interactions have been suggested to be important for protein targeting to the regulated secretory pathway. Several different mechanisms have been proposed for sorting proteins to the regulated secretory pathway broadly encompassed by two non-mutually exclusive models (*22*, *23*). In the "sorting-by-retention" model, the immature granule is considered to be a functional extension of the TGN into which all proteins enter by default. Proteins destined for the regulated secretory pathway are retained within immature granules through intermolecular associations, such as the formation of aggregates or binding to a sorting or retention receptor. The second model, known as "sorting for entry", proposes that protein sorting and targeting originate in the TGN. Regulated secretory proteins are proposed to bind to specific receptors located in the forming granule membrane, or aggregate with other proteins that are already bound, within the TGN. Only selected proteins are segregated into immature granules, which exclude the entry of unselected constitutive proteins. There is experimental evidence supporting membrane association as an important feature in protein sorting to the regulated secretory pathway. Membrane-associated carboxypeptidase E (CPE) has been identified as a sorting receptor capable of specifically binding the conformation-dependent sorting motif, identified at the amino terminus of POMC, proinsulin, and proenkephalin (*24*, *25*). There is also growing support for a role for protein−lipid interactions in protein targeting. The lipid raft model proposes that glycosphingolipid−cholesterol microdomains form in discrete regions of plasma cell membranes and contain selected membrane protein components. These rafts are proposed to be involved in cellular functions such as intracellular signaling (*26*) and protein sorting (*27*). Membrane-bound CPE (*28*) and PC2 (*29*) associate with detergent-resistant lipid microdomains in secretory granule membranes. CPE has also been reported to be a transmembrane protein (*30*).

In this study, we have investigated the interaction of PC3 with the secretory granule membrane. Our results indicate full-length PC3 and a carboxyl-terminally truncated 64 kDa transmembrane form of PC3 are found in lipid rafts. We predict from alignment with the C-terminal transmembrane domain of CPE that residues 619−638 of PC3 span the granule membrane and confer detergent insolubility to the protein. We examine the relationship of this predicted α-helical sequence to other predicted α-helical sequences found toward the carboxyl terminus of PC3 and propose a model of interaction of PC3 with the secretory granule membrane. Finally, we investigate the ability of the PC3 transmembrane domain to target the extracellular domain of the IL2 receptor α-subunit (Tac) to secretory granules in AtT20 cells, an endocrine cell line.

EXPERIMENTAL PROCEDURES

Materials. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DOPG), and 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(tempo)choline (tempo-PC) in chloroform were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Peptides rPC3 Y617−640 (TM peptide), rPC3 Y642−665 (cytoplasmic tail peptide, CYT peptide), and rPC3 727−751 (C-terminal peptide, CT peptide) were custom synthesized by Peptide Technologies (Gaithersburg, MD). Antibodies that were used included C-terminal CPE antibody 7-6 (*28*), PC3 N-terminal antibody 2B6, and C-terminal antibody 3B4 (gifts from I. Lindberg), PC3 fusion antibody (*21*) and PC3-TM antibody generated against rPC3 residues 617−640 (Figure 1), a polyclonal antibody against ACTH, DP4 which was generated in our laboratory (*31*). The anti-chromogranin A antibody (TK-1) was raised against purified bovine chromogranin A in our laboratory. A monoclonal antibody against the extracellular domain of Tac was purified using protein G affinity chromatography from a mouse B cell hybridoma 2A3A1H cell line (American Type Culture Collection, Manassas, VA). Recombinant 86 kDa PC3 (also known as SPC3) was prepared and purified as described previously (*20*).

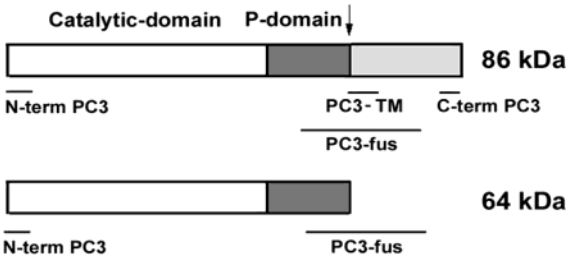

FIGURE 1: Site-specific antibodies recognize different molecular forms of PC3. The different molecular forms of PC3 were detected using four polyclonal antibodies. The N-terminal antiserum (2B6) will recognize all carboxyl-terminally truncated forms of the enzyme. The C-terminal PC3 antiserum (3B4) will recognize only full-length PC3. The PC3 fusion (PC3-fus) antibody will recognize full-length 86 kDa PC3 and some carboxyl-terminally truncated forms, including 64 kDa PC3. The PC3-TM antibody made against residues 617−640 will not recognize the mature 64 kDa PC3 thought to be generated by autocatalytic cleavage at the dibasic Arg_{617}−Arg_{618} sequence but is expected to recognize any carboxyl-terminally extended forms that include the PC3-TM epitope.

Extraction of Secretory Granule Membrane Preparations. Bovine adrenal chromaffin granules (CG) and bovine pituitary neural lobe secretory vesicles were prepared as described previously (*32*−*34*). Purified secretory vesicles were lysed by suspension in 5 mM Tris succinate (pH 5.9), followed by a freeze−thaw cycle. Membranes were pelleted by centrifugation at 20 psi in a Beckman airfuge for 5 min, washed in 5 mM Tris succinate (pH 5.9), and repelleted. Detergent extraction was carried out by solubilizing membranes in TNE buffer (50 mM Tris-HCl, 150 mM NaCl, and 2 mM EDTA) with 1× Complete Inhibitor Cocktail (Roche, Indianapolis, IN) containing either 1% (v/v) Triton X-100, Triton X-100 followed by 50 mM $NaHCO_3$ (pH 9) (*35*), or 20 or 100 mM methyl-β-cyclodextrin. Incubations were carried out for 30 min at 4 °C (Triton X-100), 20 min at 37 °C ($NaHCO_3$), or 30 min at 37 °C (MβCD). Samples were airfuged for 5 min at 22 psi to separate the soluble and particulate fractions. The protein in each soluble fraction was precipitated in 10% TCA and this pellet washed once with acetone, before equal aliquots of all samples were analyzed by Western blotting.

Sucrose Density Gradient Centrifugation. Secretory granules prepared from adrenal medulla or neural lobe (5 mg of total protein) were solubilized by incubation in 1% Triton X-100 in TNE with 1× Complete Inhibitor Cocktail at 4 °C for 30 min. Granules were also treated with 60 mM β-octyl glucoside for 30 min at 4 °C. After incubation, granules were adjusted to 1.2 M sucrose (in TNE, 0.9 mL total), loaded at

the bottom of a centrifuge tube, and overlaid with 2.6 mL of 0.8 M sucrose and 0.5 mL of 0.15 M sucrose. Centrifugation was carried out for 3 h at 240000*g* and 4 °C in a Beckman SW55Ti rotor, and 0.5 mL fractions (eight) were collected from the top of the gradient. Protein in each fraction was precipitated in 10% TCA and the pellet washed once with acetone before analysis by Western blotting.

Western Blot and Two-Dimensional (2D) Gel Analysis. Samples were separated by SDS−PAGE under reducing conditions, or by 2D gel electrophoresis (IEF pH of 4−7 followed by SDS−PAGE). 2D gel electrophoresis was performed using the IPGphor Isoelectric Focusing System with a 7 cm Immobiline DryStrip at pH 4−7 (Pharmacia Biotech), according to the manufacturer's protocol. The rehydration buffer contained 7 M urea and 2 M thiourea. For 2D gel experiments, purified total CG extracts were prepared by incubation in 5 mM HEPES, 5 mM EDTA, 0.5% Triton X-100, and 60 mM β-octyl glucoside (pH 7.4). For all remaining extractions, CG membranes were prepared and then analyzed directly or after extraction with 1% Triton X-100, or 1% Triton X-100 followed by 50 mM $NaHCO_3$ (pH 9.0). Ten microliters of the sample [in 5 mM HEPES, 5 mM EDTA, 0.5% Triton X-100, and 60 mM β-octyl glucoside (pH 7.4)] was mixed with 30 μL of rehydration buffer followed by incubation for 10 min at room temperature. Samples were then centrifuged for 10 min at 16000*g*, and samples of the supernatant (29 μL) were loaded into the first IEF dimension. Gels were electroblotted onto a nitrocellulose membrane (Schleicher & Scheull). Membranes were blocked in a solution of 3% (w/v) milk in a buffer containing 20 mM Tris-HCl, 0.5 M NaCl, and 0.05% (v/v) Tween 20 (TTBS). Primary antibodies were diluted in 1% (w/v) milk in TTBS, and immunoreactive bands were detected using the Lumi-Light Western Blotting Substrate (Roche) as described by the manufacturer.

Limited Trypsin Digestion of Chromaffin Granules. The methodology for trypsin digestion of chromaffin granules was based on a previously published procedure (*36*). The crude chromaffin granule pellet was resuspended in 0.35 M sucrose and 20 mM HEPES (pH 7.4). The protein concentration was determined using a commercial protein assay (Bio-Rad) and adjusted to 5 mg/mL. For intact chromaffin granule digestions, chromaffin granules (5 mg) were incubated with 50 μg of TPCK-treated trypsin for various times at 30 °C. Reactions were stopped by the addition of 250 μg of soybean trypsin inhibitor. Digests were then layered onto 7 mL of a 1.7 M sucrose/10 mM HEPES mixture (pH 7.4) and centrifuged in a 70.1 Ti rotor (Beckman) at 50 000 rpm for 60 min at 4 °C. Purified granules were washed and lysed in 5 mM Tris succinate (pH 5.9), and chromaffin granule membranes were pelleted by centrifugation in a Beckman airfuge (5 min at 22 psi and 4 °C). Pellets were dissolved in SDS−PAGE loading buffer for Western blot analysis. Control incubations lacking trypsin or containing trypsin preincubated with trypsin inhibitor were also performed (samples incubated for 90 min). Digests were also undertaken on lysed chromaffin granules. For these experiments, purified granules were first prepared by centrifugation through the sucrose cushion and the granules lysed prior to addition of trypsin. Samples were analyzed by Western blotting using the PC3 fusion or anti-chromogranin A antibodies.

Immunolabeling of Intact Chromaffin Granules. Intact chromaffin granules were incubated overnight at 4 °C with PC3 C-terminal or N-terminal antibodies (Figure 1) in a 320 mM sucrose/10 mM HEPES buffer/0.5% BSA/Complete Inhibitor Cocktail mixture (pH 7.4). For control absorption experiments, the PC3 C-terminal antibody was incubated with the PC3 C-terminal peptide for 30 min at 20 °C before being added to the granules. The sample was loaded on the top of a 1.6 to 2.3 M sucrose step gradient followed by ultracentrifugation for 1 h at 100000*g* in an SW55Ti rotor (Beckman). Ten fractions were collected from the top of the gradient. Intact secretory granules formed a dense band at the 1.6 M−2.3 M sucrose interface. Equal aliquots of fractions 2−5 containing intact secretory granules were subjected to SDS−PAGE and Western analysis. The protein was transferred onto a nitrocellulose membrane and probed with a secondary anti-rabbit IgG, followed by chemiluminescent detection using an enhanced chemiluminescent kit (Pierce, Rockford, IL). We also probed the same membrane with anti-chromogranin A (TK1) or PC3 fusion antibodies.

Biotinylation of Intact Chromaffin Granules. Biotinylation of granule proteins was carried out according to the manufacturer's protocol (Pierce). Briefly, 5 mg of intact granules was suspended in 1 mL of reaction buffer [0.1 M MES and 0.32 M sucrose (pH 5.5)]. EZ-Link biotin-LC-hydrazide and EDC {1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide HCl} (Pierce) were added to the granules, mixed, and incubated at room temperature for 1 h with very gentle rotation. Labeled intact granules were recovered and separated from free biotin by loading on the top of a 1.6 to 2.4 M sucrose step gradient followed by ultracentrifugation for 1 h at 100000*g* in an SW55Ti rotor (Beckman). Intact granules were collected from the interface of 2.4 and 1.6 M sucrose and centrifuged at 100000*g* for 30 min at 4 °C, and the granule pellet was solubilized with buffer A [10 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 60 mM β-octyl glucoside, 5% glycerol, and 1 mM DTT (pH 7.4)] for 30 min at 4 °C. The granule extract was spun at 12000*g* for 10 min, and the supernatant was incubated with streptavidin−Sepharose beads (Amersham Pharmacia, Uppsala, Sweden) for 1 h at 4 °C. After extensive washes with buffer A, beads were eluted in SDS−PAGE loading buffer. Samples were analyzed by Western blotting using the 2B6 (PC3 N-terminus), PC3-TM, and anti-chromogranin A antibodies.

Preparation of Model Membranes. Small unilamellar vesicles (SUV) composed of 30% DOPG and 70% DOPC (molar ratio, PGPC) or 100% DOPC were mixed and dried under a stream of nitrogen. The lipids were further dried under a vacuum for 1 h. The lipid film was resuspended in 500 μL of 1 mM Tris-HCl (pH 7.5) to bring the sample to a final concentration of 20 mM. The lipids were then sonicated in a G112SP1 Special ultrasonic cleaner (Laboratory Supplies Co., Inc., Hicksville, NY) for 10 min until they were optically clear.

Fluorescence Measurements. The tyrosine fluorescence of the peptides (CYT and CT) was measured using a Jobin Yvon-Spex FL3 spectrofluorimeter. Wavelengths were monitored using 277 nm in the direction of excitation and 307 nm in the direction of emission. The measurements were taken in 1 cm path length quartz cuvettes using 2.5 mm slits in the direction of excitation and 5.0 mm slits in the direction of emission.

Lipid Binding Experiments. Peptide binding to 30% DOPG/70% DOPC and 100% DOPC small unilamellar vesicles (SUV) was assessed by placing 16 μL of 0.5 mM peptide into 784 μL of 0.01 M sodium acetate buffer (pH 5.2) or Tris chloride buffer (pH 7.5) and 0.15 M sodium chloride (pH 5.2) to give a final peptide concentration of 10 μM. Binding was monitored using tyrosine fluorescence. Lipid was titrated into the sample from 0.01 μM to 1 mM (final concentration). Values for background samples without peptide were measured and subtracted from the value for each sample.

Measurements of the Depth of Binding of PC3 Peptides. Binding of the peptides (CYT and CT) was confirmed by measuring the quenching of tyrosine fluorescence in the presence of 30% spin-label; 30% DOPG, 30% tempo-PC, and 40% DOPC or 30% tempo-PC and 70% DOPC were mixed and dried under nitrogen. The samples were evacuated for 1 h to dispel any remaining chloroform. The samples were resuspended in 500 μL to a final concentration of 10 mM using 10 mM potassium phosphate and 150 mM sodium chloride (pH 7.5) and sonicated until they were optically clear. A final peptide concentration of 10 μM and a final lipid concentration of 200 μM were placed in a total volume of 800 μL of 0.01 mM acetate buffer (pH 5.2) or Tris buffer (pH 7.5), and the tyrosine fluorescence was measured. The percent quenching was determined by dividing the subtracted fluorescence of the peptides in the presence of tempo-PC by the fluorescence of the peptides in the absence of spin-labeled lipid.

Construction of the Tac-PC3 Membrane-Binding Domain Fusion Protein (Tac-PC3$_{616-638}$). A Tac (amino acids 1−219 of the human interleukin 2 receptor) cDNA was generated by PCR using the following primers: sense, 5′-GGCGGCG-GATCCATGGATTCATACCTGCTGATG-3′; and anti-sense, 5′-CCGCCGGAATTCATCTGTTGTTGTGACGAG-GCAGG-3′. The sense primer introduced a *Bam*HI site in the 5′ end of the PCR fragment, while the antisense primer introduced an *Eco*RI site at the 3′ end. A pCD8.1 plasmid containing the full-length Tac cDNA served as template DNA (gift of J. Bonifacino, National Institute of Child Health and Human Development, National Institutes of Health) (*37*). The fusion construct Tac-PC3$_{616-638}$ cDNA, containing amino acids 616−638 of PC3 fused to the C-terminal end of Tac, was generated by PCR with primers 5′-CCGCCGGAAT-TCGACAGGAGAGGA-3′ (sense) and 5′-CGGCGGTCTA-GAGCCATTCAGGCT-3′ (antisense) and a pcDNA 3.1 plasmid containing full-length rat PC3 cDNA as a template. The sense primer introduced an *Eco*RI site at the 5′ end of the PC3$_{616-638}$ fragment, while the antisense primer introduced an *Xba*I site at the 3′ end. After gel purification, the PCR fragment of Tac alone was inserted into the pcDNA 3.1 at *Bam*HI and *Eco*RI sites to generate the Tac/pcDNA 3.1 plasmid. The PCR fragment encoding amino acids 616−638 of PC3 was inserted into the Tac/pcDNA 3.1 plasmid at *Eco*RI and *Xba*I sites to generate the Tac-PC3$_{616-638}$/pcDNA 3.1 construct. The sequences of the plasmid cDNAs were verified by sequence analysis (Midland Certified Reagent Co., Midland, TX).

Fluorescence Immunocytochemistry. For immunocytochemical studies, AtT20 cells, a mouse pituitary tumor cell line, were grown in 12 mm circle glasses (Fisher, Pittsburgh, PA) until they were approximately 80% confluent and transfected with plasmids, containing Tac alone or Tac-PC3$_{616-638}$. Transient transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. Between 16 and 24 h after transfection, cells were rinsed with PBS, fixed in 4% paraformaldehyde, and permeabilized with 0.1% TX-100 in PBS. Primary and secondary antibodies were diluted in PBS containing 1% BSA. The following primary antibodies were used: a monoclonal anti-Tac and a polyclonal DP4 (anti-ACTH). Goat anti-mouse Alexa 488 (green) or goat anti-rabbit Alexa 568 (red) secondary antibodies (Molecular Probes, Eugene, OR) were used at a dilution of 1:1000. All fluorescent images were recorded using a Bio-Rad MRC-1000 confocal microscope and processed using Laser Sharp software and Adobe Photoshop 6.0. Image collection and quantification were carried out under double-blind conditions.

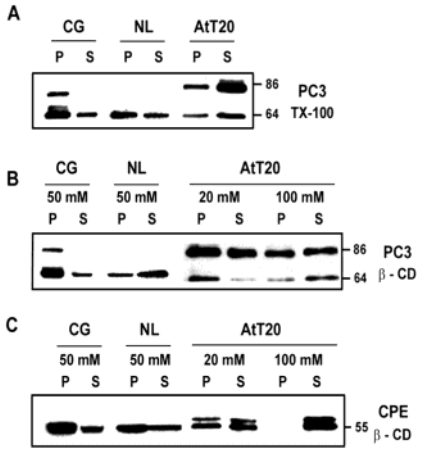

FIGURE 2: PC3 from purified secretory granules and AtT20 cells is insoluble in Triton X-100 and partially solubilized in cholesterol-depleted membranes, and a comparison with CPE. (A) Chromaffin granule membranes (CG), neural lobe secretory granule membranes (NL), and an AtT20 cell membrane preparation were extracted with 1% Triton X-100 in TNE at 4 °C for 30 min. Membrane (P, pellet) and soluble fractions (S) were separated by ultracentrifugation and samples analyzed by Western blotting using the PC3 fusion antibody. (B and C) Chromaffin granule membranes, neural lobe secretory granule membranes, and an AtT20 cell membrane preparation were extracted with the indicated concentrations of methyl-β-cyclodextrin. Membrane and soluble fractions were separated by ultracentrifugation and samples analyzed by Western blotting using the PC3 fusion antibody (B) or a carboxypeptidase E antibody (C).

RESULTS

Full-Length and Carboxyl-Terminally Truncated Forms of PC3 Associate with Lipid Rafts in AtT20 Cells, and Chromaffin Cell and Pituitary Neural Lobe Secretory Granule Membranes. The interaction between the different molecular forms of PC3 and the secretory granule membrane was examined by detergent extraction of secretory granule membranes with 1% Triton X-100 at 4 °C. The insolubility in Triton is one of the criteria by which raft proteins are identified (*38*). Insoluble and soluble fractions were analyzed by Western blot analysis and flotation on sucrose gradients. We found that the majority of full-length 86 kDa PC3 and a 64 kDa PC3 remained associated with the chromaffin granule membrane (CGM) after Triton X-100 extraction (Figure 2A). Only a small fraction of 64 kDa PC3 was solubilized. Interestingly, a small amount of intermediate 72

kDa PC3 was found in the Triton-insoluble fraction, which likely is a C-terminally truncated form of the full-length 86 kDa PC3. These experiments were repeated in dense-cored secretory vesicle membranes prepared from bovine pituitary neural lobe tissue (NLMs). Incubation of NLMs with Triton X-100 showed that two-thirds of PC3 remained insoluble (Figure 2A). Due to more efficient processing in NLMs, only the 64 kDa form of PC3 is seen. Following detergent extraction of AtT20 cells, an anterior pituitary cell line, which expresses the ACTH-endorphin hormone precursor, proopiomelanocortin, one-third of PC3 was found in the Triton-insoluble fraction. These results show that in secretory granules isolated from two tissue sources and an endocrine cell line, a significant fraction of PC3 is partitioned into the detergent-insoluble fraction after extraction with Triton X-100, a feature typical of lipid raft proteins.

The involvement of cholesterol in the membrane binding of endogenous PC3 to secretory granule membranes was also assessed. Treatment with methyl-β-cyclodextrin removes membrane cholesterol, which results in lipid raft proteins being solubilized (*39*). Extraction of CGMs with 50 mM methyl-β-cyclodextrin resulted in only a small amount of 64 kDa PC3 being solubilized, while most of the 86 kDa PC3 remained in the insoluble fraction (Figure 2B). Methyl-β-cyclodextrin treatment of neural lobe secretory vesicle membranes resulted in increased levels of soluble 64 kDa PC3. We extended these experiments to AtT20 cell membranes where we found that even at concentrations of 100 mM methyl-β-cyclodextrin, a significant fraction of immunoreactive PC3 remained membrane-associated. We compared our results with the behavior of carboxypeptidase E (CPE), a lipid raft protein found in regulated secretory granules (Figure 2C). The sensitivity of CPE to cholesterol depletion was similar to that of PC3 in chromaffin granule membranes, whereas less CPE was extracted from neural lobe membranes with 50 mM methyl-β-cyclodextrin, compared to PC3. Interestingly, in AtT20 cells, there was greater solubility of CPE than of PC3 following treatment with methyl-β-cyclodextrin.

Lipid raft association of PC3 in chromaffin and neural lobe granule membranes was further analyzed by flotation in sucrose density gradients. Following extraction of chromaffin granules with Triton X-100, and sucrose density centrifugation, a significant fraction of 86 and 64 kDa PC3 floated to low-density fractions 1 and 2 of the gradient (Figure 3A, top panel). Treatment of granules with β-octyl glucoside, which is able to solubilize lipid rafts by substitution for glycolipids, led to PC3 immunoreactivity shifting into the higher-density fractions (Figure 3A, bottom panel). As a control, a non-raft-associated soluble granule protein, chromogranin A, did not float (middle panel). Sucrose density gradient centrifugation was repeated for neural lobe secretory granules, and the results show that a proportion of ~64 kDa PC3 floated to low-density fractions 1 and 2 with Triton X-100 extraction (Figure 3B).

Limited Trypsin Proteolysis, Immunolabeling, and Biotinylation of Intact Granules Identified a Cytoplasmic Carboxyl-Terminal Domain in PC3 from Adrenal Medullary Secretory Granules. To identify sequence elements in PC3 that may be important for membrane association, the C-termini of PC3 and CPE were aligned. We found a region encompassing amino acids 619−638 that aligned with the

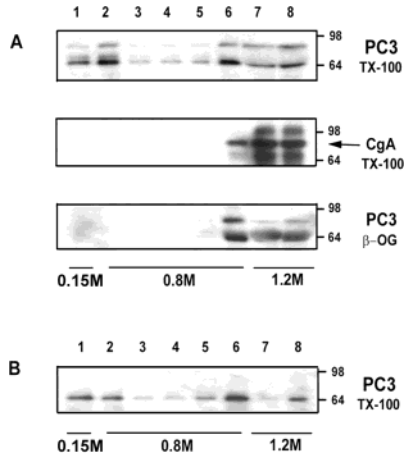

FIGURE 3: Full-length and carboxyl-terminally truncated forms of PC3 float to low-density fractions in sucrose gradients. Secretory granules were extracted with 1% Triton X-100 or 60 mM β-octyl glucoside at 4 °C and loaded at the bottom of sucrose gradients. Following centrifugation for 3 h at 240000*g*, fractions were collected from the top of the gradient and prepared for Western blot analysis. Chromaffin granules (A) and neural lobe secretory granules (B) were extracted with Triton X-100 (top panel) or β-octyl glucoside (bottom panel) and probed with the PC3 fusion antibody or chromogranin A antibody. Chromogranin A (CgA), a soluble granule protein, did not float (A, middle panel).

transmembrane domain of CPE (*30*) (Figure 4A). This led us to postulate that $PC3_{619-638}$ may be a transmembrane (TM) region and contribute to the association of full-length PC3 with the secretory granule membrane. This model would predict that ~115 amino acids at the carboxyl terminus of PC3, distal to the putative TM region, would be on the cytoplasmic side of the membrane. This carboxyl-terminal domain should be trypsin-sensitive and accessible to carboxyl-terminal-specific PC3 antibodies. To test this prediction, we incubated isolated chromaffin granules with low levels of trypsin for up to 20 min, then isolated chromaffin granule membranes, and analyzed the molecular forms of membrane-associated PC3. Chromaffin granules are unique among regulated secretory granules isolated from neuroendocrine tissues in that they contain full-length 86 kDa PC3 in addition to carboxyl-terminally truncated forms (*21*). Trypsin digestion resulted in specific and rapid loss of the full-length 86 kDa form of PC3, consistent with cleavage of cytoplasmic domains of PC3 (Figure 4B, left panel). The rapid loss of 86 kDa immunoreactivity suggests the dominant antigenic epitope for PC3 fusion contains a trypsin cleavage site. Control digests lacking trypsin or preincubated with soybean trypsin inhibitor showed no loss of 86 kDa PC3 immunoreactivity. Lysis of chromaffin granules prior to trypsin digestion resulted in the loss of all PC3 immunoreactivity (Figure 4B, middle panel). Figure 4B (right panel) shows that chromogranin A (CgA) was not cleaved by trypsin in intact granules, but was degraded in granule lysates. This experiment again demonstrates the existence of a trypsin-sensitive cytoplasmic domain in the 86 kDa form of PC3, but not CgA, a known luminal secretory granule protein. To further assess the topological orientation of PC3 in secretory granules, purified adrenal medullary chromaffin granules were labeled with amino- or carboxyl-directed PC3 antibodies (2B6 and 3B4, respectively) overnight and then fractionated on a sucrose step gradient. Gradient fractions were collected and IgG identified following SDS−PAGE and

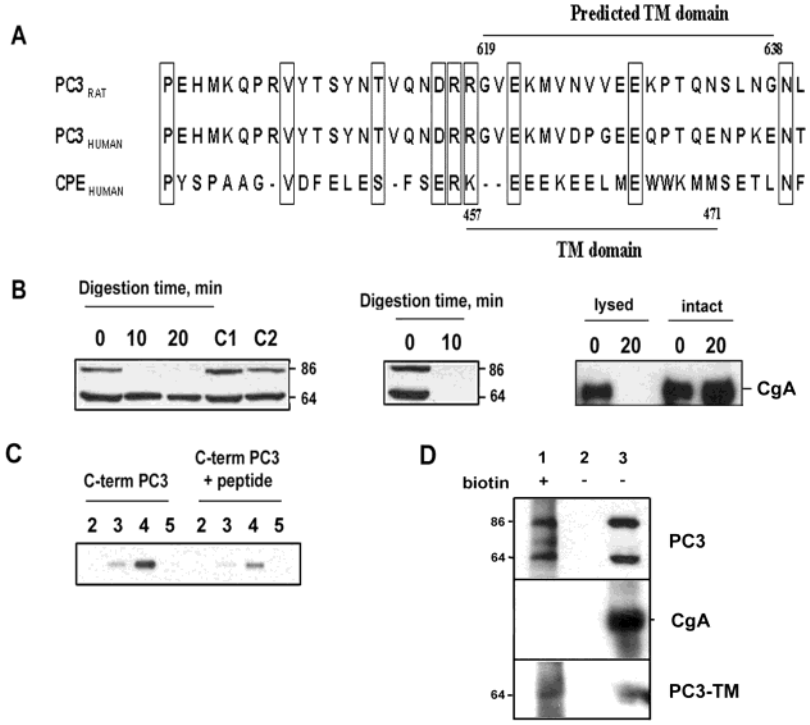

FIGURE 4: Limited trypsin proteolysis, immunolabeling, and biotinylation of intact granules identify a cytoplasmic carboxyl-terminal domain in PC3. (A) Alignment of the C-terminus of PC3 with CPE. The alignment of PC3 and CPE was performed using the CLUSTAL W multiple-sequence aligment algorithm, version 1.82, through the CLUSTAL W World Wide Web Service at the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw). The predicted transmembrane domain of PC3 corresponding to the transmembrane domain of CPE is indicated. (B, left) Intact chromaffin granules (5 mg) were incubated with trypsin (50 μg) for up to 20 min at 30 °C and lysed, and PC3 immunoreactivity was analyzed in granule membranes by Western blotting using the PC3 fusion antibody. Lane C1 was the no trypsin control. Lane C2 contained trypsin and soybean trypsin inhibitor. (B, middle) Digest of lysed chromaffin granules confirming all forms of PC3 were trypsin-sensitive. (B, right) Positive control, lysed, and intact granules were subjected to the same trypsin treatment, and digestion of chromogranin A (CgA) is shown. The presence of CgA after trypsin treatment for 20 min indicates that CgA, a luminal granule protein, was not digested by trypsin in intact granules. (C) Purified intact chromaffin granules were incubated with a carboxyl-terminal-specific PC3 antiserum or carboxyl-terminal-specific PC3 antiserum preabsorbed with the immunizing carboxyl-terminal peptide and then repurified on a sucrose step gradient. Gradient fractions 2−5 were subjected to Western blotting and bound IgG detected by enhanced chemiluminescence. (D) Purified intact chromaffin granules were incubated with biotin and EDC (lane 1) or EDC alone (lane 2), and then repurified on a sucrose step gradient. Intact granules were solubilized, and biotinylated proteins were precipitated with streptavidin−Sepharose beads. Total untreated chromaffin granule extract is shown in lane 3. Samples were analyzed by Western blotting using 2B6 (anti-PC3 N-terminal), anti-chromogranin A, and PC3-TM antibodies.

Western blotting using an anti-rabbit peroxidase-coupled antibody with enhanced chemiluminescent detection (Figure 4C). A strong IgG band was observed with the carboxyl-terminal-specific antibody in fraction 4, which contains the largest amount of the granule marker, CgA (data not shown), suggesting the carboxyl terminus of PC3 was accessible to the antibody in intact granules. The intensity of this band was markedly reduced following preabsorption of the antiserum with the carboxyl terminus peptide immunogen. Analysis of the same blots with the PC3 fusion protein antibody and chromogranin A confirmed the presence of chromaffin granules in these fractions (maximally in fraction 4) and equal granule loadings across the different experiments (data not shown).

The transmembrane orientation of PC3 was also examined by biotinylation of proteins on the outside of intact chromaffin granules (Figure 4D). Intact chromaffin granules were incubated with biotin and EDC and repurified on the sucrose gradient, and the biotinylated proteins were precipitated with strepavidin beads and analyzed by Western blotting. Figure 4D (top panel) shows that 86 and 64 kDa forms of PC3 were biotinylated. We show that the biotinylated 64 kDa form of PC3 contains the TM domain, as evidenced by the staining of this band with PC3-TM antibody (bottom panel), suggesting that this form of PC3 contains an extension on the outside of the granule. Nitrocellulose membrane was also probed with anti-chromogranin A (CgA) antibody (middle panel) to show that luminal protein CgA was not biolinylated and secretory granules were intact.

All these results taken together from three different experimental approaches clearly demonstrate that PC3 is a transmembrane protein with a cytoplasmic tail.

Identification of a Novel, Raft-Associated Carboxyl-Terminally Extended Form of PC3 Containing the Predicted Transmembrane Domain. A peptide encoding residues 617−640 encompassing the predicted transmembrane domain was synthesized and a polyclonal antiserum prepared to allow the identification of this epitope in PC3. The antiserum (named PC3-TM) was initially characterized using purified recombinant PC3 that had been incubated at 37 °C for 5 min to allow partial autocatalytic conversion to the truncated 64 kDa form (residues 1−618) (*20*). As expected, the PC3-TM antibody recognized the 86 kDa form but not the 64 kDa form of PC3 seen in lane 2 of Figure 5A. The major immunoreactive form of PC3 detected in chromaffin granule membranes by the PC3-TM antibody has a molecular mass of 64 kDa (Figure 5A, lane 4). Preabsorption of the PC3-TM antibody with the peptide immunogen blocked binding

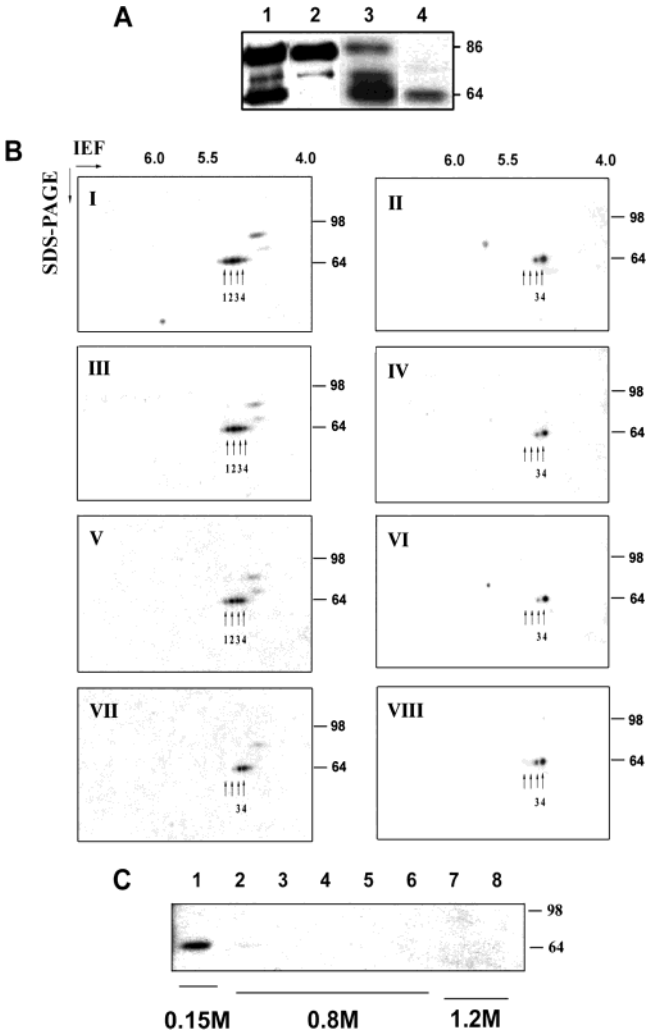

FIGURE 5: Molecular forms of PC3 containing the PC3 transmembrane domain are resistant to extraction with $NaHCO_3$ and float to low-density fractions in sucrose gradients. (A) Characterization of the molecular forms of PC3 from chromaffin granules detected by the PC3-TM antibody: lane 1, recombinant PC3 probed with the PC3 fusion antibody; lane 2, recombinant PC3 probed with the PC3-TM antibody; lane 3, purified chromaffin granule membranes probed with the PC3 fusion antibody; and lane 4, purified chromaffin granule membranes probed with the PC3-TM antibody. (B) Western blot characterization of molecular forms of PC3 fractionated by two-dimensional gel electrophoresis in total chromaffin granules (I and II) or chromaffin granule membranes extracted with TNE (III and IV), Triton X-100 (V and VI), or Triton X-100 followed by $NaHCO_3$ (VII and VIII). Membranes were incubated for 30 min in each extractant at 4 °C, and airfuged at 22 psi for 20 min. The pellets were analyzed with 2D gels, and PC3 was detected by the PC3 fusion antibody (I, III, V, and VII) or PC3-TM antibody (II, IV, VI, and VIII). (C) Chromaffin granules were extracted with 1% Triton X-100 and loaded at the bottom of a sucrose step gradient. Following centrifugation, fractions were collected from the top of the gradient and analyzed by Western blotting using the PC3-TM antibody.

(data not shown). Both the 86 and 64 kDa forms of PC3 were detected in these membranes by the PC3 fusion antibody. This suggests the PC3-TM antibody has a lower affinity for the 86 kDa form of PC3 (the amount of recombinant 86 kDa PC3 used in this experiment was significantly larger than the amount expected in chromaffin granule membranes). The failure of the PC3-TM antibody to recognize the autocatalytically generated form of 64 kDa PC3 (Figure 5A, lane 2) but detect a 64 kDa form of PC3 in chromaffin granule membranes (Figure 5A, lane 4) also suggests that there may be more than one molecular form of 64 kDa PC3.

To better characterize the 64 kDa forms of PC3 in chromaffin granules, we employed two-dimensional gels. Panels I and II of Figure 5B show that in total chromaffin granule extracts, there were four isoforms of 64 kDa PC3 detected by the PC3 fusion antibody, two of which were not recognized by the PC3-TM antibody (spots 1 and 2). We propose that spots 1 and 2 represent two mature isoforms of PC3 that are truncated at amino acid 618 (see Figure 1) with minor post-translational modifications between them and that spots 3 and 4 likely represent the isoforms of spots 1 and 2, respectively, but with the TM domain attached. Panel III of Figure 5B shows that all four forms were found in the membrane fraction after extraction of chromaffin granules with TNE buffer. All four forms were also present in the membrane pellet after Triton X-100 extraction at 4 °C (panel V of Figure 5B). However, after subsequent extraction with 50 mM $NaHCO_3$, spots 1 and 2, which do not contain the TM domain, were solubilized and absent in the remaining pellet (panel VII of Figure 5B), whereas spots 3 and 4, which contain the TM domain, were resistant to extraction by bicarbonate (panel VIII of Figure 5B). These results indicate that they are integral membrane proteins. These results also indicate that spots 1 and 2 represent 64 kDa forms of PC3 without a transmembrane domain, and instead are presumably tightly associated with other integral membrane proteins that were insoluble in Triton X-100. However, extraction with $NaHCO_3$ at pH 9 dissociated these interactions, rendering these forms of PC3 soluble.

To determine whether the carboxyl-terminally extended forms of PC3 containing the transmembrane domain were raft-associated, we repeated the sucrose density flotation experiments and probed the membranes with the PC3-TM antibody (Figure 5C). The PC3-TM antibody detected an immunoreactive band of 64 kDa PC3 in fraction 1, indicating that the 64 kDa forms of PC3 containing the transmembrane sequence are raft-associated. We name this form "64 kDa PC3-TM" to distinguish it from the 64 kDa forms that do not contain the TM domain (spots 1 and 2 on 2D gels in Figure 5B).

Interaction of PC3 Cytoplasmic Tail Peptides with Model Membranes. To determine if the cytoplasmic tail interacted with the outer leaflet of vesicle membranes, we synthesized two peptides, one of which corresponded to residues 727−751 (CT), which was predicted to bind to membranes (*40*). We also synthesized a control peptide that corresponded to residues 642−665 (CYT), which is downstream of the TM domain and predicted not to bind to membranes. We assessed the binding of these peptides to liposomes with different lipid compositions. Binding was detected via changes in the tyrosine fluorescence intensity as a function of vesicle concentration (*30*). An increase in fluorescence intensity is generally seen when a tyrosine-containing residue moves from a hydrophilic environment to a more hydrophobic environment. Figure 6 shows the change in the intensity of tyrosine fluorescence as a function of lipid concentration. There was an increase in the fluorescence intensity of the CT peptide as the concentration of PGPC vesicles increased at pH 5.2, but very little change in the fluorescence intensity of the CYT peptide was observed. Only a slight increase

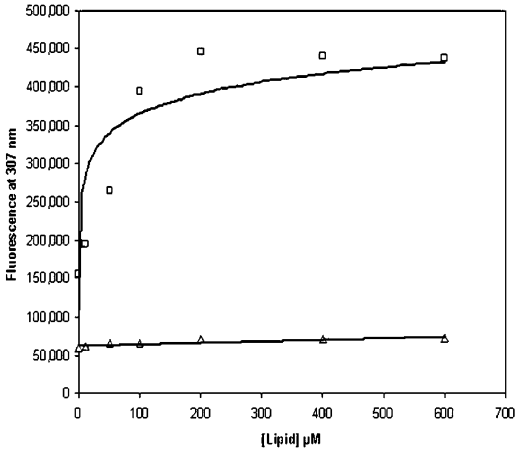

FIGURE 6: Binding of the PC peptides as a function of lipid concentration. The fluorescence of the CYT (△) and CT (□) peptides was measured in the presence of increasing amounts of 30% DOPG/70% DOPC SUV at pH 5.2. Values for background samples containing lipid but without peptide were subtracted from the value of each sample.

Table 1: Quenching of the CT Peptides in the Presence of a 30% Spin-Labeled Lipid at Neutral and Low pH[a]

	PGPC		DOPC	
	pH 7.4	pH 5.2	pH 7.4	pH 5.2
CYT peptide	0.093	0.089	0.027	0.108
CT peptide	0.339	0.448	0.003	0.093

[a] The fluorescence of the peptides was measured in the presence of SUV in the presence and in the absence of 30% tempo-PC. The percent quenching was obtained by dividing the fluorescence of the peptides in the presence of the quencher by the fluorescence in the absence of the quencher.

was observed in the fluorescence of the CT peptide at pH 7.4 (data not shown).

Binding of the CYT and CT peptides was also assessed by measuring the amount of peptide quenching in the presence of 30% nitroxide-labeled DOPC. The tyrosine fluorescence of the peptide at a concentration of 10 μM was measured in the presence of 200 μM SUV consisting of 30% PG and 70% PC (30% DOPG, 30% tempo-PC, and 40% DOPC) or 100% DOPC (30% tempo-PC and 70% DOPC). Table 1 shows the amount of quenching associated with the PC3 peptides in SUV with or without DOPG in the presence of spin-labeled DOPC at low and neutral pH. The fluorescence of the peptides in the absence of a spin-labeled lipid was used as background to determine the amount of quenching obtained in the presence of the spin-labeled lipid. The percent quenching was determined as described in Experimental Procedures. No significant quenching was observed by the CYT peptide in either DOPC or PGPC vesicles at low or neutral pH. In contrast, the CT peptide was only slightly quenched in DOPC vesicles, while in PGPC vesicles, the CT peptide was more strongly quenched. The CT peptide was quenched 33.9% at pH 7.4 and 44.8% at pH 5.2 in PGPC vesicles. These data indicate that the CT peptide strongly associated with vesicles containing negatively charged phospholipids, and only weakly associated with vesicles lacking a negative charge.

The PC3 Transmembrane Domain Is Sufficient for Targeting to Regulated Secretory Granules. A chimera (Tac-PC3$_{616-638}$) consisting of the PC3 transmembrane domain fused to Tac, the extracellular domain of the IL2 receptor α-subunit, was constructed. This construct was transfected into AtT20 cells, and the targeting of this chimeric protein to secretory granules was analyzed by fluorescence immunocytochemistry (Figure 7). Analysis of the cellular distribution of Tac alone showed staining exclusively in the cell body (Figure 7D) and no colocalization with ACTH immunoreactivity in the tips of the cell processes, where secretory granules are located (Figure 7F), indicating that Tac is not sorted to the regulated secretory pathway. In contrast, Tac-PC3$_{616-638}$ exhibited a punctate staining pattern in the cell processes that overlapped with that of ACTH (Figure 7C). To quantify these results, cells were counted and the colocalization with ACTH, the secretory granule marker in AtT20 cells, was assessed in three separate experiments (Table 2). Of 204 cells expressing Tac alone, only 13% of the cells exhibited colocalization with ACTH in cell processes. In contrast, of 200 cells expressing the Tac-PC3 fusion protein, the colocalization of Tac-PC3$_{616-638}$ with ACTH in cell processes was observed in 69% of the cells, indicating that the transmembrane domain of PC3 was sufficient for directing Tac to secretory granules.

DISCUSSION

In this paper, we show that endogenous 86 and 64 kDa forms of PC3 are associated with membranes from secretory granules purified from bovine adrenal medulla and the neural lobe of the bovine pituitary, and in membranes purified from the mouse anterior pituitary AtT20 cell line. Membrane association was partially cholesterol-dependent, although there were variations across membrane sources and molecular forms. These variations may reflect differences in the lipid composition between membranes (*28*, *41*, *42*) and differences in the mechanism(s) of interaction between the molecular forms. We also identified a membrane-spanning domain encompassing residues 619−638 of PC3, although the amino acid at the interphase between the membrane and cytoplasm has not been experimentally determined. Alignment with CPE suggests the cytoplasmic domain begins within the stretch of residues 634−638. These results support a transmembrane orientation for full-length PC3 in adrenal chromaffin granules. Two-dimensional gel analysis of chromaffin granule membranes identified two isoforms of ∼64 kDa PC3 that lacked the transmembrane sequence and were solubilized by $NaHCO_3$ but not Triton X-100, and two other isoforms of ∼64 kDa PC3 that contained this transmembrane sequence (designated 64 kDa PC3-TM), which were resistant to sodium carbonate extraction and floated in sucrose gradients. The 64 kDa PC3-TM forms behaved as integral raft-associated membrane proteins. Our results identify 64 kDa PC3-TM as a carboxyl-terminally extended intermediate presumably formed during the biosynthesis of mature 64 kDa PC3.

Several studies have reported the association of PC3 with secretory granule membranes and identified amino and carboxyl regions of PC3 as being important for association. Blazquez et al. (*43*) identified the pro region of PC3 as being important for membrane association. They suggested the carboxyl-terminal region of PC3 is not essential for membrane association since expression of a PC3 construct corresponding to proPC3, but lacking the C-terminal region (amino acids 616−752), bound to TGN/granule-enriched

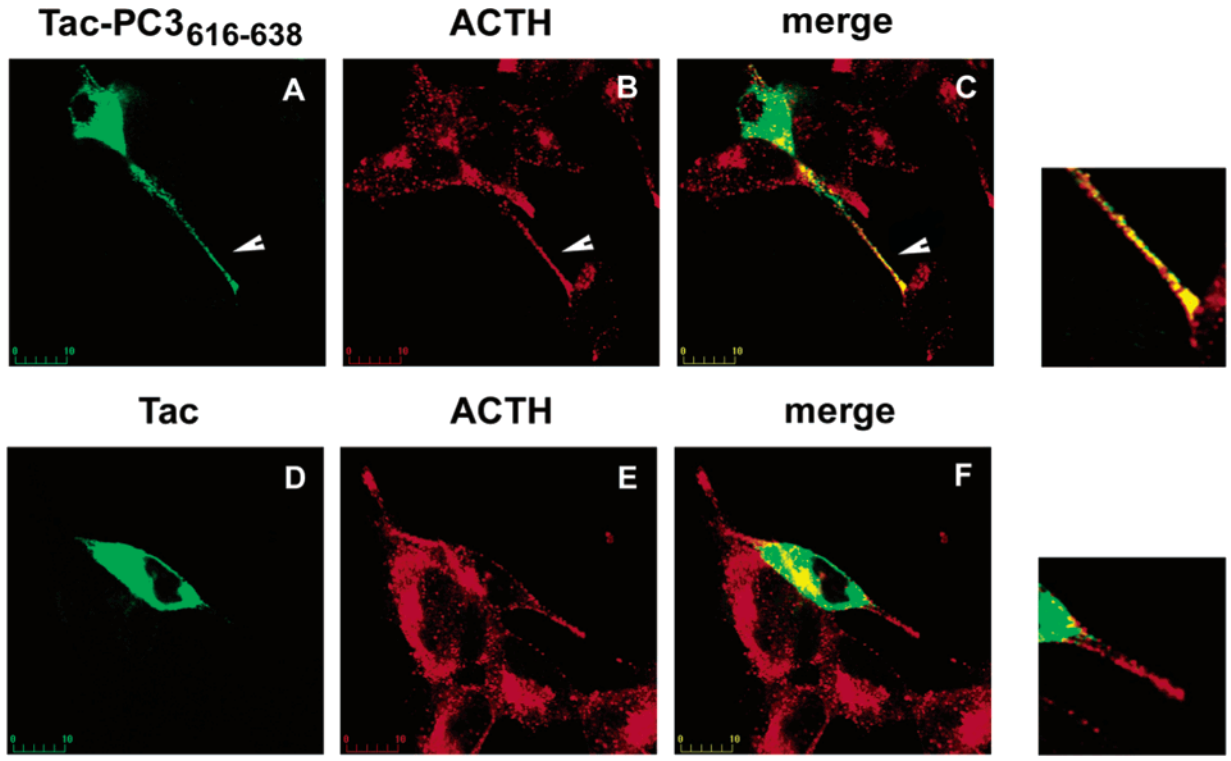

FIGURE 7: Localization of Tac-PC3$_{616-638}$ in secretory granules. AtT20 cells were transfected with Tac-PC3$_{616-638}$ (panels A−C) or Tac (panels D−F). Cells were fixed, incubated with ACTH and Tac-specific antibodies, and then incubated with appropriate secondary antibodies. Single confocal microscope sections are shown. The staining for Tac is shown in green, and that of ACTH is shown in red. Colocalization on the merged images is yellow. Arrows in panels A−C denote colocalization of ACTH and Tac-PC3$_{616-638}$ staining along and at the tips of cell processes. Panels E and F show the localization of ACTH along and at the tips of cell processes and the absence of Tac in these processes, respectively. Small panels to the right of panels C and F show images of punctate staining in cell processes from those panels, which have been enlarged to highlight the colocalization of Tac-PC3$_{616-638}$ with ACTH (top) or the staining of ACTH alone (bottom). The bars are 10 μm in length.

Table 2: Quantification of Immunocytochemical Colocalization of Tac and Tac-PC3$_{616-638}$ with a Secretory Granule Marker, ACTH[a]

	expt 1	expt 2	expt 3	mean % of cells with colocalized punctate staining
Tac	7 (5/71)	19.6 (12/61)	12.5 (9/72)	13
Tac-PC3$_{616-638}$	68.5 (48/70)	73 (46/63)	65.6 (44/67)	69

[a] AtT20 cells were transfected with Tac or Tac-PC3$_{616-638}$ and processed for immunocytochemistry as described in Experimental Procedure. Cells with processes were chosen, and the number of cells showing the presence and absence of colocalization was recorded. Cells from three separate experiments were counted. Fractions in parentheses represent the number of cells showing punctate staining of Tac or Tac-PC3$_{616-638}$ that colocalized with ACTH in cell processes over the number of cells counted. Numbers are in percent, and the means were calculated using these numbers.

membranes from AtT20 cells. However, they could not completely exclude a role for the C-terminus in membrane association as, in common with our study, they found an ~86 kDa form of PC3 containing the C-terminal region associated with chromaffin granule membranes. Interestingly, they also detected an ~66 kDa form of PC3 in chromaffin granule membrane rafts, which was identified as PC3ΔC, lacking the C-terminus. We believe that this form is actually the 64 kDa form of PC3 that contains a transmembrane domain, as identified in the study presented here. Since other properties besides the absolute molecular mass also influence the migration of proteins on SDS−PAGE, separation of mature 64 kDa PC3 from 64 kDa PC3-TM could only be achieved using 2D gels. Our work strongly supports a role

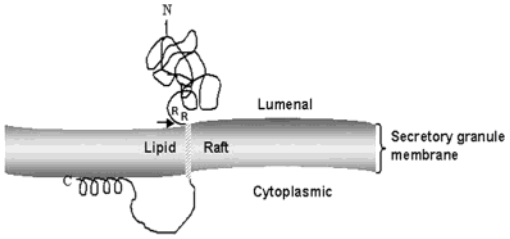

FIGURE 8: Schematic model of PC3 interaction with the secretory granule membrane. A transmembrane domain is formed by residues 619−638 which can form a partial amphipathic α-helix at acidic pH, stabilized through interactions with the lipid bilayer. The remaining ~115 amino acids would project to the cytoplasm, although a second amphipathic α-helical domain at the carboxyl terminus of PC3 (residues 727−751) may also interact with the outer granule membrane. The catalytic domain of PC3 is located within the granule lumen. The Arg$_{617}$−Arg$_{618}$ dibasic motif, which is believed to be cleaved to produce the mature form of PC3, is denoted with an arrow.

for residues 619−638 within the carboxyl terminus of PC3 in membrane association.

Our data and data from other researchers (*40*) have led us to propose a schematic model of the membrane topology of PC3 (Figure 8). In this model, the carboxyl terminus of ~115 residues of PC3 would be located on the cytoplasmic side of the membrane with the very C-terminal α-helical domain (residues 711−753) (*40*) associated with the lipid membrane on the cytoplasmic side. The protein would be anchored via the transmembrane domain (residues 619−638) to the lipid raft. The Arg$_{617}$−Arg$_{618}$ dibasic motif, which has been proposed to be cleaved to generate the mature 64 kDa form

of PC3, would be located at the luminal interface to be accessible for autocatalytic cleavage (*20*, *44*). We propose that the transmembrane orientation of PC3 is established during synthesis as the protein enters the RER cisternae. The TM domain upon interacting with the ER membrane may stop further insertion of the remainder of the C-terminal domain, leaving a cytoplasmic tail that never enters the ER cisternae. PC3 can be transported in this manner from the ER to the TGN where it becomes raft-associated through changes in the membrane composition and is then subsequently packaged into secretory granules. Truncation of the cytoplasmic tail of PC3 may occur at the TGN or after packaging into granules, as suggested previously (*19*, *45*).

Several studies have identified a role for the carboxyl terminus of PC3 for sorting to the regulated secretory pathway (*19*, *45*) and suggested that membrane association mediated by carboxyl-terminal sequences is probably important (*46*). Jutras et al. have shown that residues 667−713 or 711−753 when fused to immunoglobulin are sufficient to direct this protein to the regulated secretory pathway in vivo, suggesting that these two peptides contain sorting information. However, additional experiments which would show that they are in fact essential for sorting PC3 to the regulated pathway are still lacking. Given our model, these two sequences would reside on the cytoplasmic side of the granule membrane and may be less likely to directly participate in the sorting mechanism. Studies on CPE (*28*, *47*) and PC2 (*29*) have shown that raft association may be an important mechanism for sorting these enzymes to the regulated secretory pathway. In the case of PC3, raft association would be via the transmembrane domain (this study) or the pro region (*43*), which may then play a role in the sorting of PC3 to the regulated secretory pathway. However, since the pro region appears to be cleaved in the ER (*16*, *48*), a role for the pro domain in sorting PC3 to the regulated secretory pathway appears to be less likely. Instead, our studies demonstrate that the transmembrane domain of PC3 is sufficient to target Tac into the secretory granules in an endocrine cell line. Thus, we propose that raft association of the transmembrane domain of PC3 is the mechanism by which this enzyme is targeted into granules of the regulated secretory pathway in vivo, similar to that for CPE (*47*).

In conclusion, this study has identified two forms of PC3 (86 and 64 kDa PC3-TM) that have a transmembrane orientation and are raft-associated via the membrane-spanning domain (amino acids 619−638). This transmembrane domain has sufficient information to serve as a sorting motif for directing PC3 into secretory granules of the regulated secretory pathway.

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