# The C-Terminus of Prohormone Convertase 2 Is Sufficient and Necessary for Raft Association and Sorting to the Regulated Secretory Pathway

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ABSTRACT: Prohormone convertase 2 (PC2) is a member of the subtilisin family of proteases involved in prohormone maturation in the granules of the regulated secretory pathway (RSP). It has been suggested that targeting of this enzyme to the RSP is dependent on its association with lipid rafts in membranes at the trans-Golgi network. Here, we investigate the orientation of PC2 in granule membranes and the role of the C-terminus in sorting of the enzyme to the RSP. Molecular modeling and circular dichroism showed that this domain of PC2 forms an α-helix and inserts into artificial membranes. Furthermore, we show that the C-terminus of PC2 can be biotinylated at the C-terminus in intact chromaffin granules, indicating that it is a transmembrane protein. To determine if the PC2 C-terminus is necessary for raft association and sorting, we transfected a chimera of CPEΔ15 (carboxypeptidase E without the last 15 residues) and the last 25 residues of PC2 (CPEΔ15-PC2), and a truncated PC2 mutant with the last 6 residues deleted (PC2Δ6) into Neuro2a cells. Whereas CPEΔ15 was not raft-associated or sorted to the RSP, addition of the 25 residues of PC2 C-terminus to CPEΔ15 restored raft association and localization to the RSP granules, as determined by immunocytochemistry. Deletion of the last 6 residues of PC2 eliminated lipid raft association and sorting of PC2Δ6 to the RSP. These results showed that the PC2 C-terminus confers raft association and is sufficient and necessary for sorting PC2 to the RSP.

Neuroendocrine and endocrine cells synthesize and secrete neuropeptides and peptide hormones in a highly regulated manner. These peptides are synthesized as larger precursors, which may undergo several post-translational modifications during transport from the ER (endoplasmic reticulum), the site of synthesis, to the trans-Golgi network (TGN).[1] Prohormones, upon arriving at the TGN, are sorted away from constitutively secreted proteins into the regulated secretory pathway (RSP). During the sorting process, prohormones and their processing enzymes are packaged into the budding immature granules where they undergo processing. The processed peptide hormones are then stored in mature granules of the RSP until they are released in a $Ca^{2+}$-dependent manner.

Elucidating the mechanism of sorting neuropeptide and hormone precursors and their processing enzymes to the RSP is essential to understanding diseases such as hyperproinsulinemia, which presents a sorting defect of proinsulin (*1*). Thus, the mechanism of sorting proteins to the RSP is of major interest in the fields of cell biology and medicine. Two models have been proposed for sorting proteins to the RSP. The "sorting-for-entry" model proposes that regulated secretory proteins aggregate in the TGN and become associated with membranes (*2*). Subsequently, proteins are selectively packaged in immature secretory granules (ISGs), where they are processed and secreted. The "sorting-by-retention" model proposes that all proteins enter ISGs by default. Proteins of the constitutive secretory pathway and lysosomal proteins are then removed via the constitutive-like pathway (*3*). Proteins of the RSP are retained in the secretory granules due to the formation of aggregates and/or interaction with a retention receptor. Furthermore, protein−protein and protein−lipid interaction have been shown to play a major role in protein sorting to the RSP. A number of prohormones (*1*, *4*) are sorted to the RSP by protein−protein interaction. For example, pro-opiomelanocortin (POMC) is sorted to the RSP by binding to a sorting receptor, carboxypeptidase E (CPE), via a specific sorting signal on the prohormone (*5*, *6*). A number of RSP prohormone processing enzymes, such as CPE, prohormone convertase 2 (PC2), and prohormone convertase 1/3 (PC1/3), have been shown to be associated with lipid rafts, defined as glycosphingolipid- and cholesterol-rich microdomains (*7*−*10*). This

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[1] Abbreviations: POMC, pro-opiomelanocortin; DMSO, dimethyl sulfoxide; CgA, chromoganin A.; CPE, carboxypeptidase E; PC2, prohormone convertase 2; RSP, regulated secretory pathway; 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; EDTA, ethylenediaminetetraacetic acid; PGPC, 30% DOPG/70% DOPC; tempo-PC, 1,2-dipalmitoyl-*sn*-glycero-3-phospho (tempo)choline; TGN, trans-Golgi network; SUV, small unilamellar vesicles; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide HCl; tPA, tissue-type plasminogen activator.

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association has been shown to play a role in the sorting of CPE and PC2 to the RSP (*4*, *7*). The domain responsible for anchoring these processing enzymes to the raft microdomain has been identified only for CPE (*4*) and PC1 (*11*). The C-terminal 25 amino-acid-residues of CPE appears to be the domain necessary for its sorting receptor function and targeting to the RSP (*4*). In PC1/3, the C-terminal residues 619−638 have been shown to confer raft association and are sufficient for targeting of the enzyme to secretory granules (*11*). The role of the C-terminus of PC2 for raft association and sorting of the enzyme to the RSP, however, remains controversial, amidst two conflicting reports (*8*, *9*).

PC2 (*12*, *13*) is a member of the major subtilisin/kexin-like proprotein convertase family involved in the maturation of peptide hormones and neuropeptides in neural and endocrine cells (*14*). All members of this family share similarity in their structure. They contain an N-terminal signal peptide, a propeptide, a subtilisin-like catalytic domain, a conserved P-domain, and a C-terminal domain. PC2 is synthesized as an inactive pre-pro-protein. The signal peptide is removed in the ER to form the pro-protein. Two independent cleavages in the propeptide then convert the 75-kDa pro-protein to a 68-kDa mature form during transit through the secretory pathway (*15*). Maturation of PC2 has been shown to occur in the secretory granules, a late secretory compartment (*16*). The involvement of the propeptide in one study (*8*), and the C-terminal domain in another (*9*), for sorting of PC2 to the RSP has been reported. Taylor et al. (*8*) showed that the pro-region plays a significant role in aggregation and membrane association of the enzyme in AtT20 cells and therefore implicated this domain in the sorting of PC2 to the RSP. Furthermore, these investigators showed that deletion of the last 25 residues from the PC2 C-terminus did not affect sorting of this enzyme in AtT20 cells. On the other hand, Creemers et al. (*9*) showed that the luminal domain of furin was not sorted to the RSP in AtT20 cells, but when fused to the C-terminus of PC2 it entered the RSP. They suggested that the C-terminal domain was sufficient to target PC2 to the RSP. In our present study, we have examined the secondary structure of the C-terminal 25 residues of PC2 and the role of this domain for raft association, transmembrane orientation, and sorting of the enzyme to the RSP in Neuro2a cells.

## MATERIALS AND METHODS

*Materials*. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1glycerol)], 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). PC2 C-terminal peptide containing residues 613−637 with an additional tyrosine added to the N-terminus (Y613−637) was custom-synthesized by Peptide Technologies (Gaithersburg, MD). EZ-link biotin-LC-hydrazide, immobilized avidin, and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide HCl (EDC) were purchased from Pierce (Rockford, IL). Mouse full-length PC2 clone (pRC/CMV-mPC2) was a generous gift from Dr. N. Seidah (*12*). The following antibodies were used in this study: rabbit anti-PC2, generated against residues 622−638 (Affinity Bioreagents Inc., Golden, CO); sheep anti-PC2, generated against residues 489−586 (a generous gift from Dr. K. J. I. Shennan (*7*)); guinea pig anti-CPE, generated against residues 345−362 (*17*); anti-sheep-HRP (Research Diagnostic, Flanders, NJ); anti-rabbit-HRP (Amersham Pharmacia Biotech, UK); anti-guinea pig-HRP (Sigma-Aldrich, St. Louis, MO); ; and anti-sheep, anti-rabbit, and anti-guinea pig conjugated with either Alexa 488 or Alexa 568 (Molecular Probes, Eugene, OR).

*Vesicle Preparation*. Small unilamellar vesicles (SUVs) composed of 30% DOPG/70% DOPC (mol:mol, 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 $\mu$L of 0.001 M Tris, 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) until optically clear.

*Circular Dichroism Measurements*. Circular dichroism (CD) measurements were performed using a 1-mm-path-length quartz cuvette in a Jasco J-715 CD instrument at room temperature. Samples were measured in the presence and in the absence of lipid using either a 1 mM solution of Tris, pH 7.5, or a 1 mM solution of Tris−acetate, pH 5.2. Samples contained 200 $\mu$M PGPC SUVs and 5 $\mu$M peptide. Approximately 100−150 spectra were collected and averaged for each measurement. Background spectra were collected without peptide and subtracted from the samples. The spectra were then converted to molar ellipticity (per peptide bond), and the fractional $\alpha$-helix content was calculated using Selcon 3. The results obtained using Selcon 3 were compared using different reference sets and confirmed by the use of two additional programs provided with the Selcon 3 program, Continll and CDSstr.

*Fluorescence Measurements*. Tyrosine fluorescence of the PC2 peptide 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 SUVs was measured by placing 16 $\mu$L of 0.5 mM peptide into 784 $\mu$L of 0.01 M sodium acetate and 0.15 M sodium chloride, pH 5.2, to give a final peptide concentration of 10 $\mu$M. Binding was monitored using tyrosine fluorescence. Lipid was titrated into the sample from 0.01 $\mu$M to 1 mM final concentration. Background samples without peptide were measured and subtracted from each sample.

*Measurements of Depth of Binding of PC2 Peptides*. Binding of the PC2 peptide was confirmed by measuring the quenching of tyrosine fluorescence in the presence of 30% spin-label. To do so, 30% DOPG, 30% tempo-PC, and 40% DOPC or 30% tempo-PC and 70% DOPC were mixed and dried under nitrogen. Control samples contained 30% DOPG/70% DOPC or 100% DOPC. The samples were evacuated for 1 h to dispel any remaining chloroform. The samples were resuspended in 500 $\mu$L to a final concentration of 10 mM using 10 mM potassium phosphate and 150 mM sodium chloride, pH 7.5, and sonicated to optical clarity. A final concentration of 10 $\mu$M peptide and 200 $\mu$M lipid was placed in a total volume of 800 $\mu$L of 0.01 mM acetate buffer, pH 5.2, and the tyrosine fluorescence was measured. The percent quenching was determined by dividing the

subtracted fluorescence of the peptide in the presence of tempo-PC by the fluorescence of the peptide in the absence of spin-labeled lipid.

*Isolation of Intact Chromaffin Granules.* Intact bovine chromaffin granules were isolated as described previously (*18*). Briefly, fresh bovine adrenal glands were obtained. Fat and cortex were trimmed away from medulla on a ice-cold glass plate. Medulla were kept and minced by scissors in ice-cold homogenizing buffer (10 mM HEPES, 0.32 M sucrose). Using a tissuemizer (Tekmar Co., Cincinnati, OH), the tissue was homogenized for 2 min and passed through a double layer of gauze. The homogenate was centrifuged at 1000*g* for 10 min at 4 °C. The supernatant was transferred to a new tube and centrifuged at 10000*g* for 20 min at 4 °C. The supernatant was discarded, and the pellet was gently resuspended in the homogenizing buffer. The homogenate was overlaid on top of a sucrose step gradient (1 part 2.4 M, 3 parts 1.6 M sucrose in 10 mM HEPES) and centrifuged at 100000*g* for 1 h at 4 °C. Intact granules were collected from the interface between 2.4 and 1.6 M sucrose layers.

*C-Terminal Biotinylation of Intact Granules.* Biotinylation was performed according to the manufacture's protocol (Pierce). Briefly, 10 mg of intact granules was diluted in 1 mL of reaction buffer (0.1 M MES, pH 5.5, and 0.32 M sucrose). Freshly prepared EDC (12.5 $\mu$L of 100 mg/mL diluted in 0.1 M MES, pH 5.5) and biotin (25 $\mu$L of 50 mM in DMSO) were added to the granules in order and mixed. Reactions were incubated at 4 °C overnight with very gentle rotation. Labeled intact granules were separated from free biotin and a small amount of broken granules by overlaying on sucrose gradient (1 mL of 2.4 M, and 2.5 mL of 1.6 M sucrose in 10 mM HEPES) and centrifuging at 100000*g* for 1 h at 4 °C. Intact granules were collected from the interface of the 2.4 and 1.6 M sucrose. The granule layer was spun down at 100000*g* for 35 min at 4 °C. The granule pellet was rinsed 2 times with 10 mM HEPES and resuspended in the same buffer. After freezing/thawing three times, granule membranes were spun down at 100000*g* for 35 min at 4 °C. The supernatant was removed and kept (Sup1). The pellet was resuspended in 10 mM HEPES plus 3% $\beta$-octyl glucoside, incubated on ice for 30 min, and centrifuged at 100000*g* for 35 min at 4 °C. The supernatant (Sup2) was transferred to a fresh tube. Sup1 and Sup2 were incubated with 100 $\mu$L of avidin beads or beads alone for 1 h at room temperature with gentle rotation. The beads were then centrifuged, and the supernatant was discarded. After two washes with 10 volumes of PBS, PAGE-Sample buffer (Invitrogen, Carlsbad, CA) was added, and the solution was boiled for 10 min. Samples were electrophoresed and analyzed by western blot.

*Cell Culture*. Neuro2a cells were obtained from A.T.C.C. (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum (Life Technologies, Rockville, MD), 10 mM HEPES, and 0.075% sodium bicarbonate. Cells were transfected with various cDNA constructs, 5 $\mu$g/10-cm dish, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

*Sucrose Gradient Flotation Assay.* Neuro2a cells were transfected according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Cells were collected in TNE buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA), centrifuged at 1000*g* for 10 min at 4 °C. The cell pellet was resuspended in 1% Triton X-100 in TNE and centrifuged as above. The supernatant was incubated on ice for 30 min and centrifuged at 15000*g* for 20 min. The resulting pellet was resuspended in the 1% Triton X-100−TNE buffer and centrifuged as the last step. The pellet was then resuspended in 300 $\mu$L of Triton X-100−TNE buffer, mixed with 600 $\mu$L of 1.8 M sucrose in TNE, and loaded at the bottom of a centrifuge tube. This layer was overlaid with 2.6 mL of 1.2 M sucrose and 0.5 mL of 0.15 M sucrose. Following centrifugation at 100000*g* for 3 h in a Beckman SW55Ti rotor, 0.4-mL fractions were collected. Fractions were diluted in an equal volume of TNE, and proteins were precipitated in 20% (v/v) cold trichloroacetic acid. The pellet was washed once with cold acetone and analyzed by western blot. Nonsaturated X-ray films were scanned and quantitated using Image Quant 5.2 software.

*Indirect Immunofluorescence Microscopy*. Immunocytochemical studies were done as described previously (*19*). Briefly, Neuro2a cells were grown on glass coverslips until approximately 70% confluent. Cells were transfected overnight with various DNA constructs, unless otherwise stated, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were rinsed twice with PBS, fixed with 4% formaldehyde/PBS for 25 min, washed twice with PBS, and permeabilized in 0.1% TritonX-100/PBS for 20 min. After blocking with 1% BSA/PBS for 1 h at room temperature, the cells were incubated for 1 h at room temperature, or 16 h at 4 °C with primary antisera diluted in 1% BSA/PBS. The following antisera were used: rabbit anti-PC2 C-terminal, guinea pig anti-bovine chromogranin A, sheep anti-PC2, guinea pig anti-CPE, and rabbit anti-POMC (DP4). After three washes with PBS, the cells were incubated with appropriate secondary antibody conjugated with either Alexa-488 or Alexa-586 for 1 h at room temperature. Confocal images were captured, and quantification was carried out as described previously (*4*).

*Detergent Extraction.* Neuro2a cells were transfected in 10-cm dishes as described above. Cells were washed twice with PBS, collected, and centrifuged at 1000*g* for 10 min at 4 °C. The cell pellets were resuspended in 0.5 mL of TNE buffer, passed through a 26G needle 40 times, and then spun down at 1000*g* for 10 min at 4 °C to obtain a postnuclear supernatant (PNS). The PNS was collected and centrifuged at 100000*g* for 20 min at 4 °C, and the supernatant was saved as the soluble fraction. The pellet, representing total membrane fraction, was resuspended in 1% Triton X-100 in TNE, kept on ice for 30 min, and centrifuged at 100000*g* for 20 min at 4 °C. The supernatant was kept as the Triton X-100-soluble fraction. The pellet was resuspended in TNE, spun down as above, and kept as the Triton X-100-insoluble fraction. To identify integral membrane proteins, an aliquot of the Triton X-100-insoluble fraction was incubated with 50 mM $Na_2CO_3$, pH 11, for 30 min on ice and centrifuged as above. The supernatant and pellet, $Na_2CO_3$-soluble and -insoluble fractions, respectively, were separated and kept. All of the fractions were analyzed by western blotting.

*Construction of CPE$\Delta$15-PC2 and PC2 C-Terminal Deletion Proteins.* CPE$\Delta$15 was made as described previously (*4*). A three-piece ligation protocol was used for construction of CPE$\Delta$15-PC2. A cDNA fragment containing sequences encoding the signal peptide, pro-region, and N-terminal 145 amino acids of CPE (fragment A) was generated by restriction digestion of full-length mouse CPE cDNA with *Kpn*I

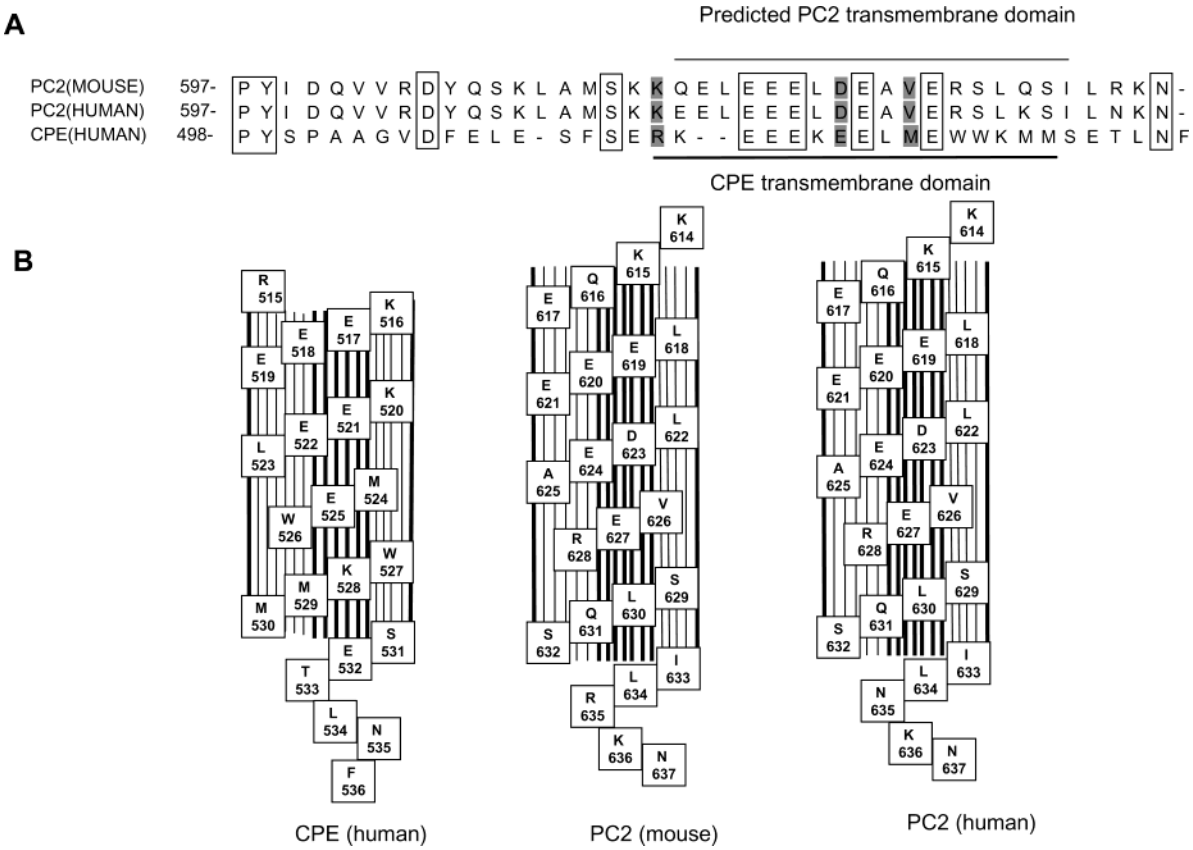


FIGURE 1: (A**)** Alignment of the C-terminus of PC2 with CPE using the "CLUSTAL W" multiple-sequence alignment algorithm. Identical residues (boxed) and chemically similar residues (gray shade) are indicated. (B**)** Schematic representation of human CPE residues (515−536), mouse PC2 residues (614−637), and human PC2 residues (614−637) as a transmembrane helix with lumenal and cytoplasmic leaflets at the top and bottom, respectively. These regions are predicted to form a partial amphipathic α-helix at acidic pH that is stabilized through interaction with the lipid bilayer. Biochemical experiments suggest that this region spans the membrane and forms a short tail that protrudes from the cytoplasmic leaflet.

and *Bgl*II. Fragment B was generated by PCR and consisted of residues 146−422 (CPEΔ15) with a *Bgl*II site at the 5′ end. PC2 C-terminal 25 residues were included in the 3′ primer with an *Eag*I site introduced at the 3′ end. Fragment A and fragment B were inserted into pcDNA3.1(+) at the *Kpn*I and *Not*I sites to generate the plasmids pcDNA-CPEΔ15-PC2. Truncated constructs of PC2 were generated by PCR, using pRC/CMV-mPC2 as a template, and consisted of residues 1−631 (PC2Δ6), 1−622 (PC2Δ15), 1−612 (PC2Δ25) with a *Hin*dIII at the 5′ end, and *Not*I at the 3′ end. The PCR product was inserted into pcDNA3.1(+) at *Hin*dIII and *Not*I sites to generate the pcDNA-PC2-Δ6, -Δ15, and -Δ25. Purified plasmid DNA was sequenced to verify the deletions. Bovine chromogranin A (CgA) construct was a generous gift from Dr. Lee Eiden. POMC cDNA was subcloned into the pcDNA3.1(+) mammalian expression vector (Invitrogen, Carlsbad, CA) at the *Nhe*1 and *Eco*RV restriction sites.

## RESULTS

*Prediction of an α-Helical Transmembrane Domain in the C-Terminus of PC2.* The alignment of CPE and PC2 (Figure 1A) shows that the last 23 amino acids of PC2 have considerable chemical homology with those of CPE. Hence, this alignment would predict that PC2 would be a transmembrane protein with a cytoplasmic tail consisting of at least the last 5 residues. It would also predict that this C-terminal domain would form an amphipathic α-helix analogous to CPE (Figure 1B) (*20*). We therefore examined the $PC2_{Y613-637}$ peptide, encompassing the predicted transmembrane domain and the cytoplasmic tail, by circular dichroism to determine if it has an α-helical structure and the ability to bind artificial membranes.

*Circular Dichroism Analysis Revealed an Amphipathic α-Helix within the C-Terminus of PC2.* The secondary structure of the $PC2_{Y613-637}$ peptide was examined by circular dichroism, and the results are shown in Figure 2. The $PC2_{Y613-637}$ peptide in the absence and in the presence of lipid contained almost no α-helix at pH 7.4. However, the amount of α-helix increased to greater than 90% at pH 5.2 in PGPC SUV, indicating that this domain forms an α-helical structure at an acidic pH within the membrane, similar to the CPE C-terminus (*20*).

*Interaction of $PC2_{Y613-637}$ Peptide with Model Membranes.* To examine the possibility of interaction of the PC2 C-terminus with granule membranes, the binding of the PC2 peptide ($PC2_{Y613-637}$) with model membranes was studied. Binding was detected via changes in the tyrosine fluorescence intensity as a function of vesicle concentration. An increase in fluorescence intensity is generally seen when a tyrosine-containing peptide moves from a hydrophilic environment to a more hydrophobic environment. Figure 3 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 PC2 peptide as the concentration of PGPC vesicles increased, at pH 5.2, but very little change

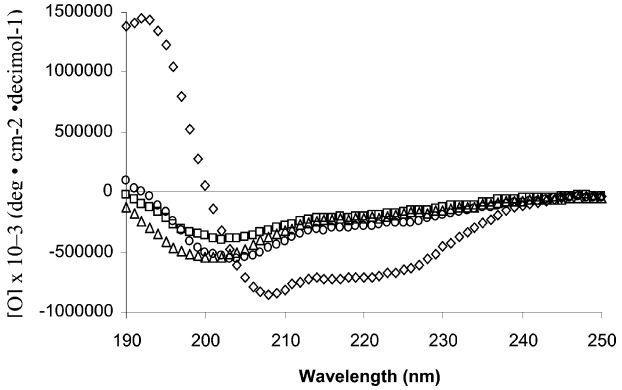


FIGURE 2: Circular dichroism spectra of PC2 incorporated into small unilamellar vesicles. Secondary structural measurements were obtained with 10 $\mu$M PC2 peptide alone at pH 5.2 (□) and pH 7.4 (△), peptide + PG/PC SUV pH 7.4 (○), or peptide + PG/PC SUV pH 5.2 (◇). Each scan represents an average of 90−125 individual readings of the peptide in 200 $\mu$M 30% DOPG/70% DOPC SUV.

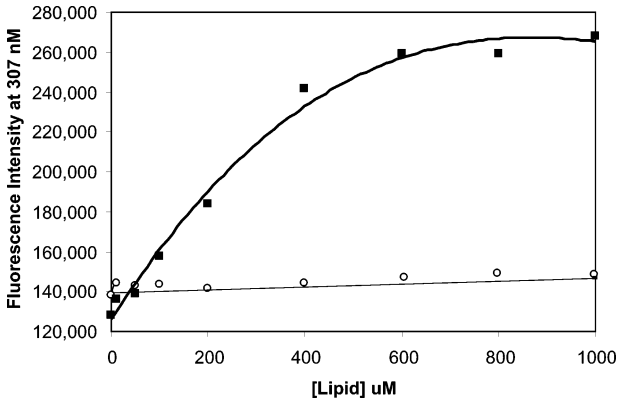


FIGURE 3: PC2 binding to small unilamellar vesicles at pH 5.2. Small unilamellar vesicles composed of either 30% DOPG/70% DOPC (■) or 100% DOPC (○) were titrated into acetate buffer, pH 5.2, containing 5 $\mu$M PC2 peptide, and the fluorescence intensity at 307 nM was measured. Background samples were subtracted from each titration point.

Table 1: Nitroxide Spin-Labeled Lipid Quenching of the PC2 Peptide at pH 7.4 and 5.2[a]

| | %Q | |
|---|---|---|
| pH | PGPC | DOPC |
| 7.4 | 20 | 4 |
| 5.2 | 31 | 20 |

[a] The PC2 peptide fluorescence intensity at 307 nm was measured at pH 5.2 in the presence of 200 $\mu$M SUV containing 30% DOPG/30% tempo-PC/40% DOPC or in SUV composed of 30% tempo-PC and 70% DOPC. The percent quenching was obtained by comparing the fluorescence intensity of the peptide in the absence of spin-labeled lipid to the fluorescence in the presence of spin-labeled lipid.

in the fluorescence intensity of the PC2 peptide was observed in the presence of 100% DOPC SUV.

Binding of the PC2 peptide was also assessed by measuring the amount of fluorescence quenching obtained by the addition of 30% nitroxide-labeled DOPC. The tyrosine fluorescence of the peptide at a concentration of 10 $\mu$M was measured in the presence of 200 $\mu$M SUV consisting of 30% PG/70% PC (30% DOPG/30% tempo-PC/40% DOPC) or 100% DOPC (30% tempo-PC, 70% DOPC). Table 1 shows the amount of quenching associated with the PC2 peptide in SUV with or without DOPG in the presence of spin-labeled DOPC at low and neutral pH. The PC2 peptide bound weakly at neutral pH to both vesicle compositions and showed decreased affinity at low pH for the DOPC vesicles. The PC2 peptide was quenched 20% in DOPC vesicles at low pH, but the amount of quenching was increased to approximately 30% in the presence of 30% DOPG at pH 5.2. These data, in addition to the circular dichroism and binding data, suggest that the PC2 peptide can associate with vesicles in the absence of negative phospholipid but becomes more strongly associated with vesicles containing negative phospholipid, where it can assume an α-helical structure.

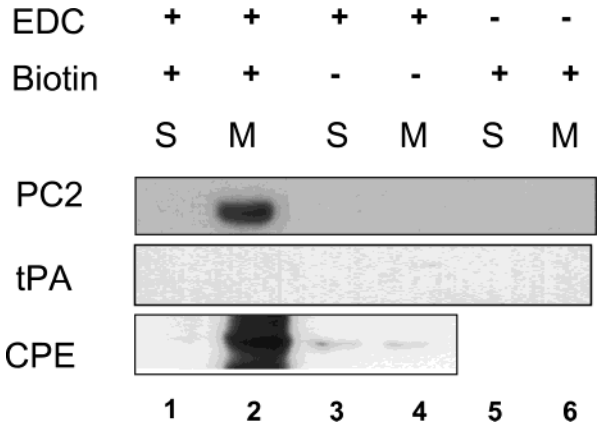


FIGURE 4: Western blot of biotinylated PC2 C-terminus in intact chromaffin granules. Peripheral and transmembrane proteins accessible on the outside of intact chromaffin granules were labeled with biotin by chemical cross-linking with EDC. The labeled intact granules were then re-isolated in a sucrose density gradient, lysed, and centrifuged at 100000*g*. The pellet containing membrane (M) and supernatant (S) fractions was analyzed. The granule membranes were solubilized with $\beta$-octyl glucoside, and the biotinylated membrane proteins were precipitated with avidin-conjugated beads. After washing, proteins were eluted from the beads and analyzed by western blot. CPE and tPA were used as positive and negative controls, respectively. Note that PC2 and CPE were pulled down by the avidin beads in the membrane fraction (lane 2), indicating their accessibility to the biotin in intact granules. The absence of labeling of tPA indicates its inaccessibility to the biotin in intact granules. No PC2 and CPE was pulled down from the soluble component of intact granules (lane 1). Additional negative controls included omission of biotin (lanes 3 and 4) or EDC (lanes 5 and 6).

*Topology of PC2 in Intact Secretory Granules.* The membrane orientation of PC2 in intact granules was examined to determine if it is a transmembrane protein. A biotinylation assay was carried out to specifically label free C-termini of proteins in intact chromaffin granules. Extracted proteins from the granule membranes were precipitated by avidin-conjugated beads and analyzed by western blot. The presence of PC2 immunoreactivity (Figure 4, lane 2) indicates that the C-terminus of PC2 was exposed on the outside of the granules, suggesting that in vivo PC2 could adopt a transmembrane orientation with its C-terminus on the cytosolic side of the granules. The absence of PC2 immunoreactivity from the granule supernatant indicates that the granules were intact during labeling (Figure 4, lane 1). Chromaffin granules were treated as above in the absence of biotin (Figure 4, lanes 3 and 4) or in the absence of cross linker (Figure 4, lanes 5 and 6) as negative controls. The absence of the PC2 in lanes 3 and 4 shows the specificity of the precipitation. As a positive control, we showed that CPE, a transmembrane protein (*20*), was labeled with biotin. As a

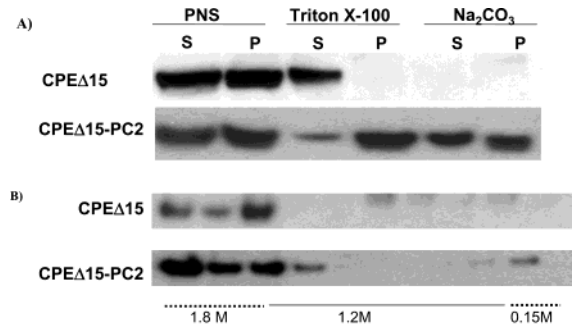


FIGURE 5: CPEΔ15-PC2 is resistant to extraction by Triton X-100 and sodium carbonate and floats to a low-density fraction in sucrose gradients. (A) Neuro2a cells were transfected with CPEΔ15 and CPEΔ15-PC2 constructs. A postnuclear supernatant of the cells was obtained and divided into three equal aliquots. The first aliquot was centrifuged to obtain membrane (PNS-P) and soluble components (PNS-S). The second aliquot was treated like the first one, and then the pellet was extracted with Triton X-100 and centrifuged to separate Triton X-100-soluble (Triton X-100-S) and -insoluble (Triton X-100-P) fractions. The third aliquot was treated like the second one, and the Triton X-100-insoluble pellet was treated with $Na_2CO_3$ to remove peripheral proteins ($Na_2CO_3$-S). Insoluble integral membranes ($Na_2CO_3$-P) were collected by centrifugation. All samples were separated and analyzed by western blot. (B) Neuro2a cells were transfected with CPEΔ15 and CPEΔ15-PC2 constructs. Postnuclear supernatant was obtained, incubated with 1% Triton X-100 for 30 min on ice, and centrifuged. The Triton X-100-insoluble pellet was resuspended in the extraction buffer, adjusted to 1.8 M sucrose, and overlaid with 1.2 and 0.15 M sucrose, respectively. After centrifugation at 100000*g* for 3 h, 10 fractions were colleted from the top and analyzed by western blotting. Guinea pig anti-CPE or rabbit anti-PC2 C-terminus antibody were used to detect CPEΔ15 or CPEΔ15-PC2 proteins, respectively.

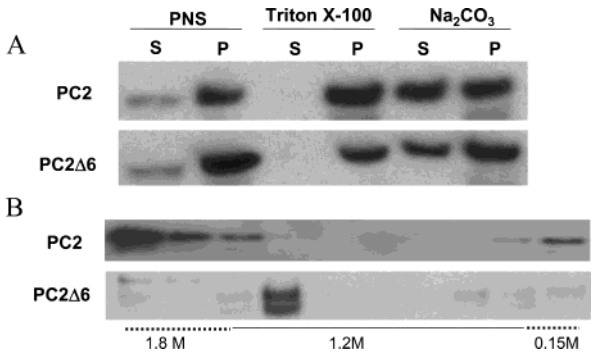


FIGURE 6: PC2 is resistant to extraction by sodium carbonate and floats to the low-density fraction in sucrose gradient. (A) Neuro2a cells were transfected with full-length or PC2Δ6 constructs. A postnuclear supernatant was obtained and divided into three equal aliquots. The first aliquot was centrifuged to obtain a membrane (PNS-P) and a soluble fraction (PNS-S). The second aliquot was treated like the first one, and the pellet was extracted with Triton X-100 and centrifuged. Triton X-100-soluble (Triton X-100-S) and -insoluble (Triton X-100-P) proteins were separated. The third aliqout was treated like the second one, and the Triton X-100-insoluble pellet was extracted with $Na_2CO_3$ and centrifuged. The soluble peripheral proteins ($Na_2CO_3$-S) and insoluble integral membrane proteins ($Na_2CO_3$-P) were separated, and all pellets and supernatants from various extractions were analyzed by western blotting. (B) Neuro2a cells were transfected with the various PC2 constructs, and a postnuclear supernatant fraction was isolated, incubated with 1% Triton X-100 for 30 min on ice, and centrifuged. The Triton X-100-insoluble pellet was resuspended in the extraction buffer, adjusted to 1.8 M sucrose, and overlaid with 1.2 and 0.15 M sucrose, respectively. After centrifugation at 100000*g* for 3 h, 10 fractions were collected from the top and analyzed by western blotting. Rabbit anti-PC2 C-terminus or sheep anti-PC2 N-terminal-region antibodies were used to detect PC2 full-length or truncated constructs, respectively.

negative control, tPA (*21*), a soluble protein found in chromaffin granules, was shown not to be labeled with biotin in intact granules.

*PC2 C-Terminus Is Sufficient for Membrane Association and Raft Localization.* PC2 has been shown previously to be a raft-associated protein (*7*). To determine if the C-terminal 25 residues of full-length PC2 is sufficient for conferring membrane association and raft localization, a chimera consisting of CPEΔ15 and the last 25 residues of PC2 (CPEΔ15-PC2) was made. The lumenal domain of CPE, CPEΔ15, has been shown to be not membrane-associated (*4*). Total membranes were isolated from the postnuclear supernatant of CPEΔ15-PC2-transfected Neuro2a cells (Figure 5A, PNS-P) and then extracted with 1% Triton X-100 or 1% Triton X-100 followed by $Na_2CO_3$. Figure 5A shows that more than 50% of CPEΔ15-PC2 was membrane-associated (Figure 5A, PNS-P) and, of that, most was Triton X-100-insoluble (Figure 5A, Triton X-100-P). More than 50% of the Triton X-100-insoluble form was also insoluble in $Na_2CO_3$, indicating localization of CPEΔ15-PC2 in membranes, primarily as an integral membrane protein.

To test whether the PC2 C-terminal domain is sufficient for raft association, sucrose density flotation assays were carried out (Figure 5B). Neuro2a cells were transfected with CPEΔ15-PC2, and the postnuclear supernatant was obtained. This fraction was extracted with 1%Triton X-100, and the insoluble proteins were subjected to sucrose density flotation. As shown in Figure 5B, a portion (7%) of the CPEΔ15-PC2 protein was floated to the lower density fraction, whereas CPEΔ15 remained in the higher density fractions. This result shows that the PC2 C-terminal 25 amino acids are sufficient to confer raft association of the protein.

*The Cytoplasmic Tail of PC2 Is Required for Membrane and Lipid Raft Association.* To determine if the cytoplasmic tail of PC2 is necessary for membrane and lipid raft association within the framework of this enzyme, we made a truncation mutation of PC2 lacking the last 6 residues (PC2Δ6). Neuro2a cells were transfected with this construct, and membrane association and raft localization of the PC2Δ6 protein were assessed. Figure 6A shows that virtually all of the PC2 is membrane-associated (PNS-P) and Triton X-100-insoluble (Triton X-100-P). Lack of solubility of a proportion of full-length PC2 in $Na_2CO_3$ indicates that there is an integral membrane component. Deletion of the last 6 residues of PC2 did not significantly affect membrane association or insolubility in Triton X-100 or $Na_2CO_3$. To determine whether the membrane-associated form of PC2Δ6 is raft-associated, flotation assays were carried out. While full-length PC2 (14%) floated to the top of the sucrose density gradient, PC2Δ6 did not (Figure 6B), indicating that the last 6 residues of PC2 is important for lipid raft association.

*PC2 C-Terminus Is Sufficient and Necessary for Sorting to the RSP.* To determine if the C-terminus of PC2 is sufficient for sorting to the RSP, we tested whether CPEΔ15-PC2 was sorted correctly in Neuro2a cells, using an immunocytochemical assay. CPEΔ15 was shown to be localized in the cell body (Figure 7A), and no colocalization with POMC, a secretory granule marker, was observed in cell processes (Figure 7C). This result indicates that CPEΔ15 was not sorted to the RSP, confirming a previous report (*4*).

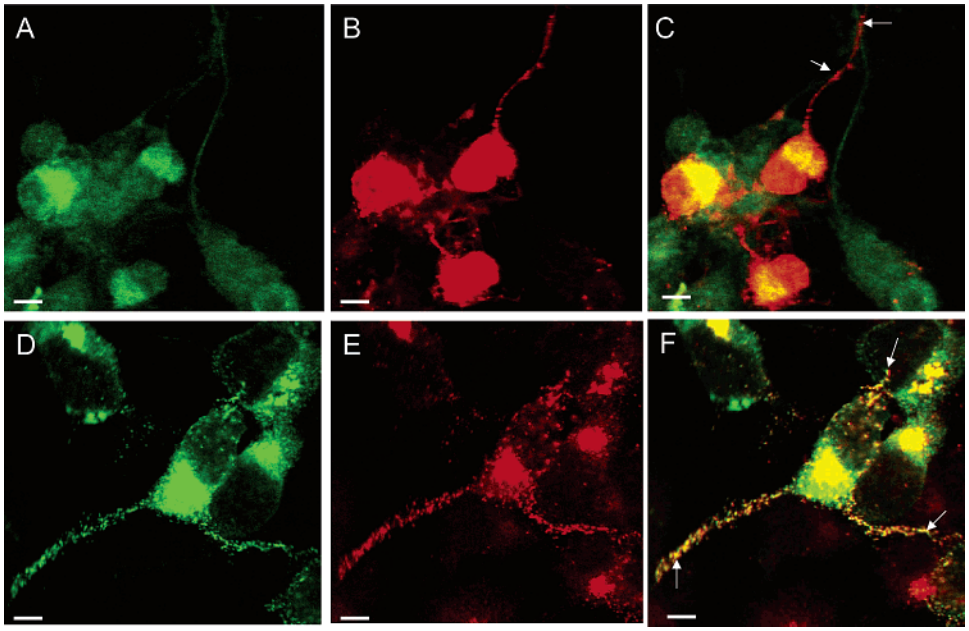


FIGURE 7: PC2 C-terminus domain is sufficient for sorting into the regulated secretory pathway. Neuro2a cells were co-transfected with POMC and CPE Δ15 (A−C) or bovine CgA and CPEΔ15-PC2 (D−F). Cells were fixed, probed with primary antibodies [rabbit anti POMC (A−C) or guinea pig anti-bovine CgA (D−F), and guinea pig anti-CPE (A−C) or rabbit anti-PC2 C-terminus (D−F)], and then incubated with appropriate secondary antibodies conjugated to Alexa 488 (green) or Alexa 568 (red). Images were captured with the use of a scanning-laser confocal microscope, and the signals were merged. Staining of POMC or bovine CgA appears red, and staining of CPEΔ15 and CPEΔ15-PC2 appears green. The arrows in panel C indicate punctate staining of POMC in cell processes. The arrows in panel F indicate colocalization of CgA and CPEΔ15-PC2. Scale bar = 10 μM.

Table 2: Quantification of Immunocytochemical Colocalization of CPEΔ15 and CPEΔ15-PC2 with Granule Markers, POMC and CgA[a]

| construct | expt 1 | expt 2 | expt 3 | mean % of cells with colocalized punctate staining |
|---|---|---|---|---|
| CPEΔ15 | 1/20 (5%) | 3/20 (15%) | 1/20 (5%) | 8.3 ± 3.3 |
| CPEΔ15-PC2 | 45/52 (86%) | 48/56 (85.7%) | 50/52 (96.2%) | 89.3 ± 2.8 |

[a] Neuro2a cells were transfected with CPEΔ15 or CPEΔ15-PC2 and granule markers, POMC or CgA, respectively. Cells were processed for immunocytochemical analysis, and images were collected as described in the Materials and Methods. Only cells with processes were chosen, and cells with colocalization of the punctate staining were counted and reported. Cells from three individual experiments were counted. Fractions (e.g., 1/20) represent the number of cells showing colocalization of staining of CPEΔ15 or CPEΔ15-PC2 with the granule markers over the total number of cells counted. Numbers in parentheses are percentages, and the means were calculated using these numbers.

In contrast, CPEΔ15-PC2 chimera showed punctate staining along the cell processes (Figure 7D) and colocalization with transfected bovine CgA, a secretory granule marker (Figure 7F). These observations were quantified by counting cells and assessing colocalization of CPEΔ15 and CPEΔ15-PC2 with the granule markers, POMC and CgA. Table 2 shows that, of 60 cells transfected with CPEΔ15, only 5 cells (8.3%) showed colocalization of CPEΔ15 with POMC in granules. In contrast, from 160 counted cells, 143 cells (89.3%) showed colocalization of CPEΔ15-PC2 and CgA. These results indicate that the PC2 C-terminal 25 amino acids are sufficient for targeting of this protein to the RSP.

To test whether the C-terminal sequence of PC2 is necessary for sorting to the RSP, Neuro2a cells were transfected with the full-length or truncated PC2 constructs (PC2Δ25 and PC2Δ6) and assayed by immunocytochemistry. Figure 8 shows that PC2 was sorted to the RSP, as evidenced by the punctate staining along and at the tips of the cell processes and colocalization with transfected bovine CgA (Figure 8C). In contrast, PC2Δ25 and PC2Δ6 showed little sorting to the RSP, as evidenced by the absence of punctate staining in the cell processes and their accumulation in prenuclear region of the cell body (Figure 8D and G, respectively). Furthermore, there was essentially no colocalization of PC2Δ25 and PC2Δ6 with the granule marker, CgA (Figure 8F and I, respectively). The immunocytochemical observations were quantified by counting cells and assessing colocalization of PC2-FL, PC2Δ25, and PC2Δ6 with the granule marker, CgA. Table 3 shows that, of 149 cells expressing PC2Δ25 counted, only 25 cells (16.7%, $n = 3$) showed colocalization of CPEΔ25 with bovine CgA. Of 159 cells expressing PC2Δ6 counted, only 27 (18.6%, $n = 3$) showed colocalization of PC2Δ6 with bovine CgA. In contrast, from 160 cells expressing pC2-FL, 156 cells (96.7%, $n = 3$) showed colocalization of PC2-FL with bovine CgA. These results indicate that the PC2 C-terminus is necessary for targeting of the protein to the RSP and, furthermore, the last 6 residues of PC2 C-terminus are the critical residues for this function in Neuro2a cells.

## DISCUSSION

Several studies have implicated the importance of the protein−lipid interaction, particularly with lipid raft microdomains, for the proper sorting and trafficking of proteins within the cell. Lipid rafts have been shown to play a role in the sorting of proteins such as influenza virus hemagglutinin (*22*) and placental alkaline phosphatase (*23*) from the TGN to the apical side of polarized epithelial cells (*23*, *24*),

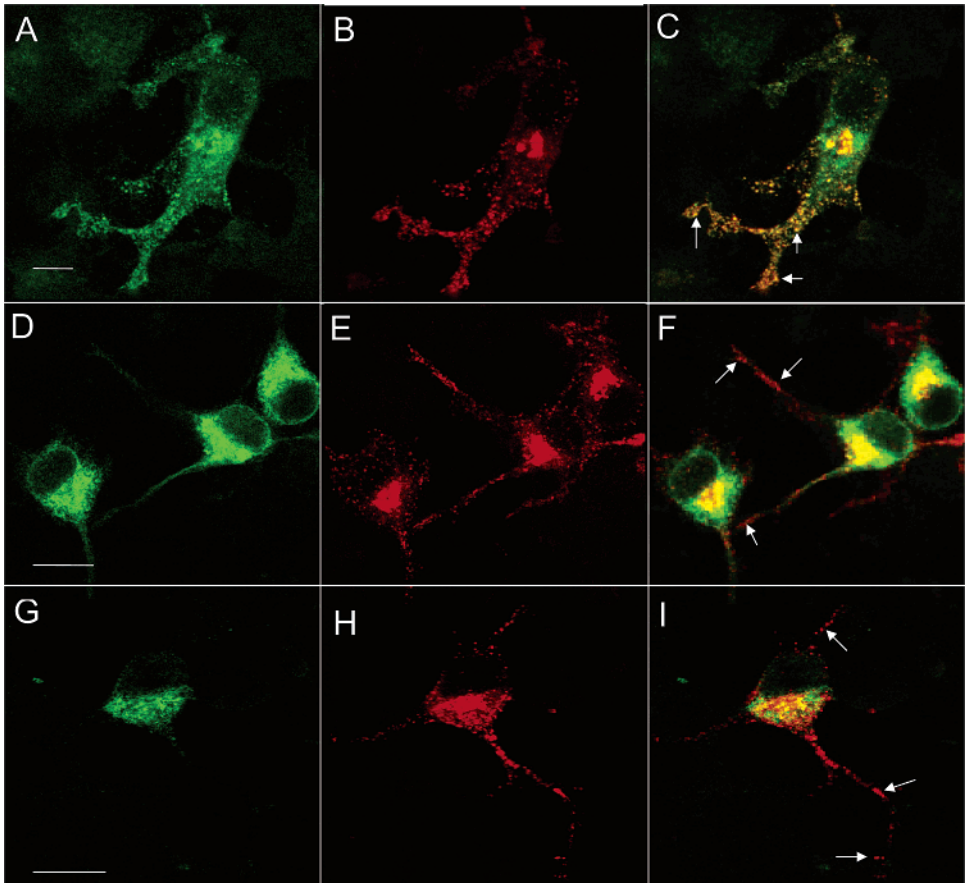


FIGURE 8: PC2 C-terminus is sufficient for sorting into the regulated secretory pathway. Neuro2a cells were co-transfected with bovine CgA and PC2-FL (A−C), PC2Δ25 (D−F), or PC2Δ6 (G−H). Cells were fixed, probed with primary antibodies [sheep anti-PC2 N-terminal region (A, D, and G), and guinea pig anti-bovine CgA (B, E, and H)], and then incubated with appropriate secondary antibodies conjugated to Alexa 488 (green) or Alexa 568 (red). Images were captured with the use of a scanning-laser confocal microscope, and the signals were merged. Staining of bovine CgA appears red, and staining of PC2 appears green. The arrows in panel C indicate colocalization of CgA and PC2. The arrows in panels F and I indicate punctate staining of bovine CgA in processes. Scale bar = 10 $\mu$m.

Table 3: Quantification of Immunocytochemical Colocalization of PC2 Constructs with Granule Marker, CgA[a]

| PC2 construct | expt 1 | expt 2 | expt 3 | mean % of cells with colocalized punctate staining |
|---|---|---|---|---|
| PC2-FL | 51/51 (100%) | 57/58 (98%) | 47/51 (92%) | 96.7% ± 2.4 |
| PC2Δ25 | 4/49 (8%) | 5/51 (10%) | 16/49 (32%) | 16.7% ± 8 |
| PC2Δ6 | 3/52 (6%) | 13/53 (24%) | 14/54 (26%) | 18.6% ± 6 |

[a] Neuro2a cells were transfected with PC2-FL, PC2Δ25, or PC2Δ6 and a granule marker, CgA. Cells were processed for immunocytochemical analysis, and images were collected as described in the Materials and Methods. Only cells with processes were chosen, and cells with colocalization of the punctate staining were counted and reported. Cells from three individual experiments were counted. Fractions (e.g., 47/52) represent the number of cells with colocalized punctate staining of PC2-FL, PC2Δ25, or PC2Δ6 with CgA over the total number of cells counted. Numbers in parentheses are percentages, and the means were calculated using these numbers.

as well as the sorting of glycosylphosphatidylinositol (GPI)-anchored proteins to the plasma membrane (*25*, *26*). Furthermore, the sorting of the prohormone processing enzymes, CPE (*27*) and PC2 (*7*), to the RSP in endocrine cells has also been reported to depend on lipid raft association. In the present study, we have identified a C-terminal α-helical transmembrane domain in PC2 that interacts with lipid rafts and shown that this interaction is necessary for sorting of the enzyme to the RSP.

The C-terminal domain of CPE forms an α-helix that can assume a transmembrane orientation and is associated with lipid rafts (*27*). However, the domain responsible for membrane association of PC2 has previously been suggested to be residues 45−84 at the N-terminal pro-region of the molecule (*7*), although the role of the C-terminus has not been investigated in this respect. Sequence alignment of the C-terminus of CPE and PC2 revealed a high degree of chemical homology, implicating a role of this domain of PC2 in raft association and sorting of the enzyme to the RSP, similar to that of CPE. Using model membranes and a synthetic PC2 peptide comprising the last 25 residues of PC2, we showed that this peptide forms an amphipathic α-helix, only at an acidic pH in the presence of lipid. The PC2 peptide showed strong binding to model membranes at pH 5.2. This indicates that formation of the α-helix appears to be necessary to anchor the C-terminus of PC2 to model membranes. Biotinylation of proteins in intact chromaffin granules demonstrated that PC2 has a cytosolic domain. This finding suggests that the C-terminus of PC2 interacts with the membrane in a transmembrane fashion, similar to CPE.

The membrane-binding region of PC2 ($K_{615}$QELEEEL-DEAVERSLQSILRKN$_{637}$) is therefore a transmembrane α-helix. This 23-amino-acid-residue domain is sufficiently long to penetrate the bilayer with the last 5−6 residues exposed on the cytoplasmic side, analogous to the membrane anchoring of the C-terminus of CPE (see Figure 1B).

The involvement of the C-terminus 25 residues of PC2 in its membrane and raft association in cells was examined by generating a CPEΔ15-PC2 chimera consisting of the lumenal domain of CPE fused to the last 25 residues of PC2. Our results show that CPEΔ15 is a peripheral membrane-associated protein, but it is not anchored to lipid rafts (Figure 5). In contrast, CPEΔ15-PC2 chimera is an integral membrane protein that is associated with membrane lipid rafts, as determined by its resistance to Triton X-100 and $Na_2CO_3$ extraction and flotation to the low-density fraction in a sucrose density gradient (Figure 5). A previous report showed that the last 4 C-terminal residues in the CPE cytoplasmic tail are required for its raft association (*4*). We therefore examined the effect of deletion of the last 6 residues of PC2 on its membrane and raft association. Deletion of the last 6 residues of PC2 C-terminus did not affect its membrane association, as evidenced by its resistance to extraction by Triton X-100 and $Na_2CO_3$ (Figure 6A). However, removing these last 6 residues significantly affected its raft association, as determined by the lack of flotation of this deletion mutant in a sucrose density gradient (Figure 6B). Our present observations and previous report suggest that these short cytoplasmic tails play a significant role in raft association of CPE and PC2.

To determine if the C-terminal transmembrane domain of PC2 is sufficient to sort the enzyme to the RSP, we tested the ability of the CPEΔ15-PC2 chimera to be correctly sorted to the RSP in Neuro2a cells. Our immunocytochemical data showed that, while CPEΔ15 was not sorted to the RSP (Figure 7A), the CPEΔ15-PC2 chimera was targeted to secretory granules (Figure 7D). Thus, the transmembrane domain of PC2 was sufficient to target a protein, CPEΔ15, which would otherwise be secreted constitutively (*4*), to the RSP. This observation confirms the work of Creemers et al. (*9*) that suggested that the C-terminus of PC2 was sufficient for sorting the enzyme to the RSP. However, we have now shown that the C-terminal transmembrane domain is not only sufficient but also necessary for sorting PC2 to the RSP in the framework of the entire molecule. This conclusion is based on our data that deletion of the C-terminal 25 residues or the 6 residues of the cytoplasmic tail resulted in lack of raft association and sorting of PC2 to the RSP, as evidenced by flotation experiments (Figure 6B) and colocalization studies by immunocytochemistry (Figure 8). However, Taylor et al. (*8*) showed that deletion of up to 50 residues from the C-terminus of PC2 did not eliminate stimulated secretion of the mutant from AtT20 cells and concluded that this domain is not necessary for sorting to the RSP. It is possible that, in AtT-20 cells, some of their PC2 deletion mutant were targeted to the RSP through heterotypic aggregation with another RSP protein, such as the prohormone POMC. The efficiency of sorting this mutant to the RSP in AtT20 cells was not analyzed in their study and may, however, have been low.

An immunocytochemical study by Blazquez et al. (*7*), showing that sphingolipid depletion with fumonisin treatment of AtT-20 cells obliterated punctate staining of PC2 in secretory granules, indicated a role of raft association in sorting of PC2 to the RSP. However, they suggested that the membrane interaction may involve the pro-region of PC2. They had no direct intracellular evidence for this proposal, but rather their suggestion came from in vitro binding studies of synthetic peptides from sequences within the pro-region to TGN/granule membranes. In fact, they reported that deletion mutations of the pro-region of PC2 when transfected into AtT-20 cells had little effect on stimulated secretion (*7*). Thus, the hypothesis that the pro-region is involved in raft association and sorting PC2 to the RSP remains tenuous at the present time.

In conclusion, our present study has shown that PC2 is a lipid raft-associated protein that is anchored to the membrane via its C-terminal domain in a transmembrane orientation. This lipid raft interaction is necessary for targeting PC2 to the RSP, similar to CPE (*4*, *20*, *27*).

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