Carboxypeptidase E, a Prohormone Sorting Receptor, Is Anchored to Secretory Granules via a C-Terminal Transmembrane Insertion†

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ABSTRACT: Carboxypeptidase E (CPE) is a sorting receptor that directs the prohormone pro-opiomelanocortin (POMC) to the regulated secretory pathway, and is also a prohormone processing enzyme in neuro/endocrine cells. It has been suggested that the 25 C-terminal amino acids are necessary for the binding of CPE to secretory granule membranes, but its orientation in the membrane is not known. In this study, we examined the structure and orientation of the membrane-binding domain at the C-terminus of CPE. In vitro experiments using model membranes demonstrated that the last 22 amino acids of CPE (CP peptide) insert in a shallow orientation into lipid bilayers at low pH. Circular dichroism analysis indicated that the CP peptide adopts a partial α-helical configuration at low pH, and helix content increases when it is bound to lipid. Protease protection experiments, immunolabeling, and immunoisolation of intact secretory granules with a C-terminal antibody revealed a cytoplasmic domain in CPE, consistent with a transmembrane orientation of this protein. We conclude that the membrane-binding domain of CPE must adopt an α-helical configuration to bind to lipids, and that CPE may require another integral membrane "chaperone" protein to insert through the lipid bilayer in a transmembrane fashion.

Endocrine cells synthesize and secrete peptide hormones in a highly regulated manner. Peptide hormones and their processing enzymes are initially synthesized as larger precursors that, upon being transported to the trans-Golgi network (TGN), are targeted to the regulated secretory pathway (RSP). During the sorting process, proteins destined for the RSP are separated from constitutive and lysosomal proteins and are packaged into budding immature secretory granules. Within this subcellular compartment, prohormones undergo endoproteolysis to yield their constituent peptide hormones. The mature secretory granule is the storage compartment of the endocrine cell, from which peptide hormones are released upon stimulation in a Ca^{2+}-dependent manner.

Carboxypeptidase E (CPE) is a processing enzyme that cleaves basic residues from the C-terminus of endoproteolytically cleaved peptide hormones. The enzyme is present exclusively in the Golgi and secretory granules of neural and endocrine cells. In a manner similar to that of prohormones and other processing enzymes, CPE is initially synthesized with a 14-amino acid N-terminal prodomain, which is removed early in the secretory pathway (*1*). Within the RSP, CPE exists in two forms: a 53 kDa soluble, enzymatically active form and a 55 kDa membrane-bound form that has decreased enzymatic activity when bound to membranes (*2*). The role of CPE in the RSP is twofold. The membrane-bound form of CPE serves as a sorting receptor that targets pro-opiomelanocortin (POMC) to the RSP, presumably operating within the TGN (*3*, *4*), and the soluble form functions as a prohormone processing enzyme within secretory granules (*5*, *6*). As a sorting receptor, CPE binds two acidic residues on the sorting signal motif of POMC at a specific binding site consisting of two basic amino acids, Arg_{255} and Lys_{260} (*7*). This interaction between CPE and the sorting signal of POMC results in the active segregation of the prohormone within the TGN, thereby targeting it to the RSP. Since POMC molecules can aggregate in the presence of Ca^{2+} and low pH (*8*), a complex of POMC aggregates may form in the TGN, bind to membrane CPE, and be carried into budding immature secretory granules. It is within this compartment that the membrane-binding domain of CPE is removed to yield its soluble, enzymatically active form. However, the proportion of soluble CPE in secretory granules appears to vary from tissue to tissue; approximately 40% of the total cellular CPE seems to remain membrane-associated within pituitary secretory granules (*1*), while 70−80% of the total CPE may be soluble within the secretory granules of pancreatic β cells (*9*).

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Carboxypeptidase E is tightly anchored to the membranes of the TGN and secretory granules. We have recently shown that CPE is associated in a cholesterol-dependent manner to lipid rafts within the membranes of the TGN and secretory granules, and that association with rafts is necessary for its function as a sorting receptor to the RSP (*10*). In the secretory granules of chromaffin cells (*11*), bovine pituitary (*12*, *13*), pancreatic β cells (*9*), and AtT-20 cells (*14*), the majority of membrane-bound CPE immunoreactivity remains associated with the membrane after extraction with detergent, and is only removed upon extraction with detergent and high salt. The membrane-binding region of CPE is thought to reside within its 24 C-terminal amino acids, comprising amino acids 411−434 of rat CPE (*12*). This study showed that a synthetic 21-residue peptide corresponding to the C-terminal sequence of CPE was able to bind to membranes in a detergent-resistant manner, indicating that this sequence appears to be sufficient and necessary for anchoring CPE to membranes of secretory granules. That this membrane association occurred only at an acidic pH may indicate that CPE is not inserted into the membrane until it reaches the acidic compartments of the regulated secretory pathway, i.e., the TGN and secretory granules. This study also demonstrated that the C-terminal sequence of CPE contains a predicted amphipathic α-helical conformation (*12*); however, its exact orientation within the lipid bilayer was not established. In this paper, we demonstrate that its 22 C-terminal residues anchor CPE to membranes of secretory granules by adopting an α-helical structure that fully spans the membrane.

MATERIALS AND METHODS

Materials. For peptide binding and acrylamide quenching experiments, dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), and 1-palmitoyl-2-(12-doxyl)stearoylphosphatidylcholine (12SLPC) were purchased from Avanti Polar Lipids (Alabaster, AL). 10-Doxylnonadecane was purchased from Aldrich Chemicals (Milwaukee, WI). Acetyl-$K_2GL_9WL_9K_2A$-amide and acetyl-$K_2CWL_9AL_9K_2A$-amide were purchased from Research Genetics (Huntsville, AL). The peptides were purified by HPLC, and their purity was assayed by mass spectrometry as described previously (*15*, *16*). They were stored at 4 °C in ethanol. The C-terminal peptide of CPE [CP peptide ($RKE_3KE_2LMEW_2KM_2SETLNF$)] was custom-synthesized by Peptide Technologies (Gaithersburg, MD), and the purity was confirmed by MALDI-TOF[1] mass spectrometry. It was dissolved in 10 mM Tris-HCl and 150 mM NaCl (pH 8.0) at a concentration of 0.3−0.8 mM and stored at 4 °C. For protease protection and immunoisolation experiments, secretory granule fractions from bovine intermediate and neural lobe pituitary were isolated and purified as previously described (*17*). Carboxypeptidase Y was purchased from Calbiochem (La Jolla, CA). For immunoisolation experiments, intact secretory granules were subsequently bound to magnetic beads using the Dynabeads M-500 Subcellular kit (Dynal, Lake Success, NY). Detection of immunoreactive CPE was by the SuperSignal Chemiluminescence System (Pierce, Rockford, IL).

Fluorescence Measurements. Fluorescence was measured at room temperature with a Spex Tau 2 Fluorolog spectrofluorometer operating in steady state mode. Unless otherwise noted, measurements were made in a semi-micro quartz cuvette (10 mm excitation path length, 4 mm emission path length). The excitation and emission slit widths were 1.5−2.5 and 5 mm, respectively. Trp emission spectra were measured at a rate of 1 nm/s using excitation at 280 nm.

Preparation of Model Membranes. Small unilamellar vesicles (SUVs) were prepared from mixtures containing 2.5 μmol of total lipid composed of DOPC or an 80% DOPC/20% DOPG mixture (molar ratio). The lipid mixtures, prepared from solutions in $CHCl_3$ or ethanol, were dried under a stream of N_2 at ∼30 °C, dissolved in a few drops of $CHCl_3$, dried again with N_2, and then further dried under high vacuum for 30 min. Typically, samples were then hydrated with 500 μL of an appropriate buffer (see below) and then sonicated in a bath sonicator (model G112SP1T, Lab Supplies Co., Hicksville, NY) for 25 min or until they were optically clear.

Assessment of Peptide Binding to Lipid Vesicles. Samples containing 2 or 10 μM peptide were prepared by mixing ∼15−35 μL of a CP peptide stock solution with one of the following buffers: 10 mM Tris-HCl and 150 mM NaCl (pH 8); Tris-acetate buffer (6.7 mM Tris-HCl, 150 mM NaCl, and 167 mM sodium acetate), adjusted to pH 4.1; or Tris-acetate buffer adjusted to pH 5.5. The final volume was 650 μL. After tryptophan fluorescence emission spectra were measured, samples were titrated with 3−10 μL aliquots of SUVs (5 mM lipid) prepared in the same buffers as described above. Vesicle plus peptide addition did not alter sample pH. Trp fluorescence emission spectra were measured after each addition. Lipid-induced shifts in peptide fluorescence reached final values immediately after each addition (not shown). Background intensities (without peptide) were found to be negligible in control experiments and were generally not subtracted before calculation of λ_{max}.

Acrylamide Quenching Experiments. For samples containing the CP peptide, SUVs composed of 20% DOPG and 80% DOPC (molar ratio) and peptide were prepared as described above. The SUVs (32−160 μL) were mixed with 12−55 μL of peptide and Tris-acetate buffer (pH 4.1) to yield final concentrations of 200 μM or 1 mM lipid and 10 μM peptide in a total volume of 800 μL. For the transmembrane peptides (acetyl-$K_2GL_9WL_9K_2A$-amide and acetyl-$K_2CWL_9AL_9K_2A$-amide), the level of acrylamide quenching was measured in 200 μM DOPC vesicles prepared by ethanol dilution into 10 mM phosphate and 150 mM NaCl (pH 7.1, total volume of 800 μL) as previously described (*16*, *18*). Aliquots of acrylamide were added from a 4 M stock solution dissolved in water. Approximately 2−3 min after each aliquot was added the Trp fluorescence was measured at an excitation wavelength of 295 nm and an emission wavelength of 340 nm. Addition of acrylamide did not affect pH. Backgrounds from samples lacking peptide were subtracted, and the resulting fluorescence values were corrected both for dilution and for the inner filter effect arising from acrylamide absorbance (*19*).

Measurement of Circular Dichroism. Circular dichroism (CD) measurements were made in 1 mm path length quartz

[1] Abbreviations: MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; λ_{max}, wavelength of maximum fluorescence emission intensity; PMSF, phenylmethanesulfonyl fluoride; DMP, dimethyl pimelimidate; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; CPD-II, domain II of carboxypeptidase D.

cuvettes at room temperature using a Jasco J-715 CD instrument. Samples without lipid contained 5 μM peptide (23 μL from the stock solution) in 0.1× Tris-acetate adjusted to pH 4.1 or 7.2 to give a final volume of 650 μL. The final pH value was 4.1 or 7.3, respectively. Samples with lipid were prepared similarly in the presence of 20% DOPG/80% DOPC (molar ratio) sonicated SUVs (final lipid concentration of 200 μM). Approximately 100−150 spectra were collected and averaged for each measurement. Background values from samples lacking peptide were subtracted, and after conversion to molar ellipticity (per peptide bond), the fractional α-helix content was calculated using SELCON3 (*20*).

Protease Protection Assay. Intact secretory granules from bovine intermediate and neural lobe pituitaries were isolated as previously described (*17*). Granules were resuspended in 1 mL of cold homogenization buffer [10 mM Tris (pH 7.4) containing 250 mM sucrose], and 200 μL aliquots were used for each digestion reaction. As a positive control, membranes from granules were prepared as previously described (*3*), and 40 μg of protein from these preparations was used in parallel reactions. Each aliquot of intact granules and membranes was incubated in the presence or absence of 40 units/mL carboxypeptidase Y for 30 min at 37 °C, as described previously (*21*). The reaction was terminated by placing the tubes on ice and adding PMSF to a final concentration of 1 mM. C-Terminal immunoreactivity of CPE was determined by Western blotting, using an antibody (CPE 7-6) raised against the five C-terminal amino acids, which was confirmed by epitope mapping. To ensure that the granules were intact during and after the digestion reaction, both intact and lysed preparations of granules were pelleted in homogenization buffer as described previously (*17*). The supernatant was removed, and the total protein was precipitated with 5 volumes of ice-cold ethanol for 16 h at −20 °C. Western blot analysis for chromogranin A, an abundant soluble protein in secretory granules, was carried out. As a negative control, blots were also probed with an N-terminal antibody to CPE, CPE 2-6. To rule out the possibility that C-terminal CPE was being degraded by carboxypeptidase Y during the Western blot process, CPY was added to aliquots of intact granules after the incubation period, immediately put on ice, and treated with PMSF as described above.

Immunoisolation of Intact Secretory Granules. Dynabeads (4×10^8) were coupled to 200 μg of either CPE 7-6 (C-terminal CPE) or CPE 2-6 (N-terminal CPE) antibodies overnight at 4 °C according to the manufacturer's instructions. Antibody−bead cross-linking was carried out through incubation with 20 mM DMP (Pierce), made in 0.2 M triethanolamine (pH 8.2), for 30 min at 20 °C. The reaction was stopped by resuspending the beads in 50 mM Tris (pH 7.5). The antibody-coated beads were blocked overnight at 4 °C with 5% nonfat dry milk in HES [20 mM Hepes, 5 mM EDTA (pH 7.4), 250 mM sucrose, 1 mM PMSF, and a complete cocktail of protease inhibitors] before the immunoisolation experiment. As a negative control, 100 μg of either C-terminal or N-terminal CPE peptide was added prior to incubation with granules to preabsorb anti-C-terminal or anti-N-terminal antibodies, respectively. For immunoisolation, purified intact secretory granules from bovine neural lobe pituitary (NL) were resuspended in HES and incubated overnight at 4 °C with 5×10^7 beads per 400 μg of granules. Granule-bound beads were magnetically separated, washed six times with HES buffer, and incubated with M-PER Mammalian Protein Extraction Reagent buffer (Pierce) containing 1% Triton X-100 and 60 mM octyl β-glucoside, for 30 min at 4 °C to elute the granules. The beads were then washed once with 0.1 M glycine (pH 2.5) and three times with HES, followed by extraction with SDS−PAGE sample buffer. Samples (isolated granules and beads) were subjected to SDS−PAGE and Western blot analysis.

Immunolabeling of Intact Secretory Granules. Intact NL granules were immunolabeled by incubation overnight at 4 °C with CPE 7-6 antibody in 80 mM metrizamide, 250 mM sucrose, and 0.5% BSA followed by density gradient centrifugation. For preabsorption experiments, the antibody was incubated with 200 μg of CPE C-terminal peptide for 30 min at 20 °C before adding it to the granule preparation. Granule preparations were loaded on top of a 0.3 to 0.15 M metrizamide/sucrose step gradient (*17*), followed by ultracentrifugation for 1 h at 100000*g* in a Beckman SW55Ti rotor. Intact secretory granules formed a dense band at the 0.23 M−0.3 M interface. Equal aliquots of fractions 2−6 (collected from the top) containing intact secretory granules associated with the CPE C-terminal antibody were subjected to SDS−PAGE and Western blot analysis. The protein was transferred onto a nitrocellulose membrane and probed with a secondary anti-rabbit IgG, followed by chemiluminescent detection using an ECL kit (Pierce).

Molecular Modeling of Mouse CPE. A homology model of mouse CPE was built using the CPD-II and CPT structures as templates and the SegMod algorithm (*22*) implemented in the program GeneMine. The CPD-II and CPE sequences are quite similar, except for two significant additions to the CPE sequence: an insertion from Leu_{152} to Leu_{183} and 24 additional C-terminal residues. Whereas CPD-II has only nine residues corresponding to the first insertion, CPT has 24 residues in this region. Therefore, CPT was used as the template for the modeling of this insertion. The CPD-II and CPT structures were superimposed by best fitting the homologous five-residue regions before (DLNRN) and after (PETxA) prior to modeling the insertion. Although CPT provides a better template for this region than CPD-II, neither is very homologous to CPE here, and the modeled loop should not be overinterpreted. The membrane-binding region of CPE (residues 411−434) was not modeled due to the absence of a homologous sequence in any other carboxypeptidase. Overall, the model of mouse CPE presented here is similar to that of human CPE (*23*).

RESULTS

Interaction of the CP Peptide with Model Membranes. To study the interaction of the C-terminal tail of CPE with the lipid bilayer, the binding of a peptide consisting of the last 22 residues of CPE (CP peptide) to model membranes was studied. Binding was detected via shifts in the λ_{max} of the Trp in the sequence of the peptide. A shift of Trp fluorescence to shorter wavelengths (blue shift) is commonly observed when a Trp-containing peptide moves from an aqueous solution to a membrane-bound state. Figure 1 shows the dependence of the Trp λ_{max} of the CP peptide on lipid concentration. There is a blue shift in the presence of DOPC SUVs at pH 4.1, which reaches a plateau at high lipid concentrations (<150 μM). In contrast, very little blue shift

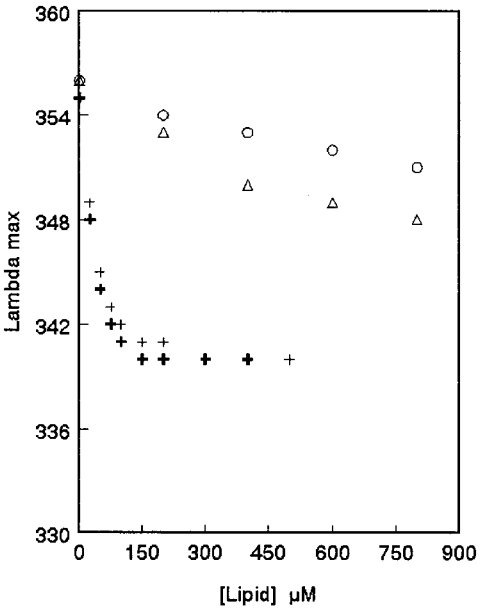

FIGURE 1: Assessment of CPE binding to lipid vesicles. Aliquots (2 μM) of the CP peptide were dissolved in Tris-acetate buffer at pH 4.1 (+), 5.5 (△), or 8.0 (○) and titrated with small unilamellar vesicles (SUVs) composed of 100% DOPC. Binding of the CP peptide at pH 4.1 was also carried out in SUVs composed of 20% DOPG and 80% DOPC (bold +). Trp fluorescence emission spectra were measured at increasing concentrations of lipid after each addition of peptide.

is seen at pH 8.0, and intermediate values were measured at pH 5.5. This behavior indicates that binding to DOPC SUVs is strongest at low pH.

Subsequent experiments were performed at pH 4.1 to obtain the strongest interaction with lipid. Binding to vesicles composed of 20% DOPG and 80% DOPC (molar ratio) was similar to binding to 100% DOPC. Somewhat higher lipid concentrations (roughly 3−4-fold) were needed to fully bind 10 μM peptide compared to those needed to fully bind 2 μM peptide (data not shown). One possible explanation for the need for a higher lipid concentration to bind a higher concentration of peptide is that the vesicles have a limited number of binding sites for peptides. The data in Figure 1 suggest an upper limit of approximately one binding site per 50 lipid molecules. This number may reflect the limited amount of hydrophobic surface that is exposed on an SUV.

Peptide binding to vesicles was also detected by the quenching of CP peptide Trp fluorescence by a nitroxide-labeled PC (12SLPC) incorporated into the lipid bilayer. Quenching of 10 μM CP peptide in SUVs consisting of either 100% PC (70% DOPC/30% 12SLPC) or 20% DOPG and 80% PC (20% DOPG/50% DOPC/30% 12SLPC) (molar ratio) exhibited a dependence upon lipid concentration similar to that of λ_{max} shifts (data not shown).

Peptide Location within the Lipid Bilayer. The location of the CP peptide within the bilayer was estimated by determining the location of the Trps in the center of the peptide sequence. A deeply inserted transmembrane conformation would require these Trps to be at the center of the bilayer. The λ_{max} of Trp fluorescence emission was used to estimate the position of Trp within the bilayer. To do this, the λ_{max} of membrane-bound CP peptide was compared to the previously reported standard curve for the dependence of Trp λ_{max} upon depth within the bilayer (*24*). The λ_{max} of

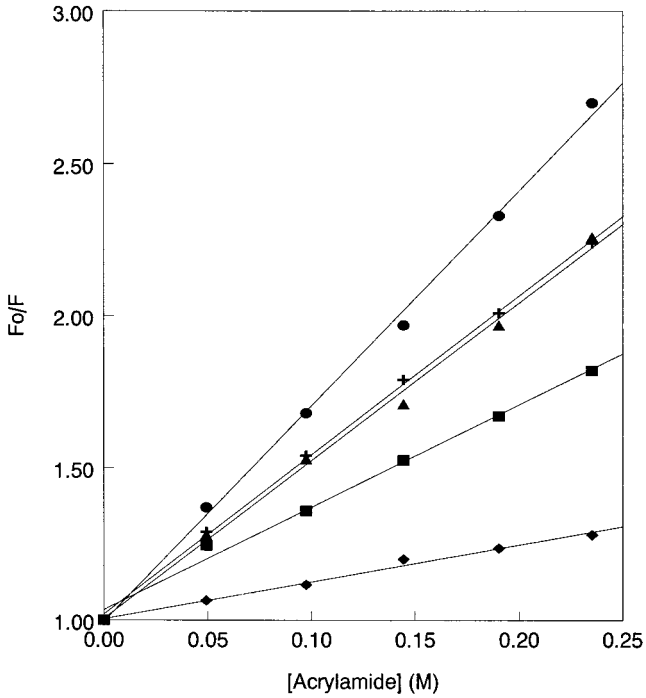

FIGURE 2: CPE is inserted in a shallow orientation in model membranes. Aliquots of acrylamide were added to samples containing 10 μM CP peptide incorporated into SUVs. F_o/F is the ratio of the fluorescence in the absence of acrylamide to that in the presence of acrylamide. The CP peptide was prepared in Tris-acetate buffer at pH 4.1 and bound to 200 μM 100% DOPC vesicles (+), 1 mM 100% DOPC vesicles (▲), or 200 μM 20% DOPG/80% DOPG vesicles (●). Acrylamide quenching curves are also shown for a control peptide with deeply located Trp, acetyl-$K_2GL_9WL_9K_2A$-amide (◆), and for a control peptide with shallowly located Trp, acetyl-$K_2CWL_9AL_9A$-amide (■). Each value represents the average of duplicate samples.

the fully lipid-bound CP peptide (340 nm, Figure 1) is even more red-shifted than that for a Trp at the polar−nonpolar boundary of the bilayer, for which λ_{max} equals 338 nm (*24*). This strongly suggests that the Trp residue, and thus the entire CP peptide, is bound at the surface of the bilayer.

Acrylamide quenching was used to confirm this conclusion (Figure 2). Fluorescence quenching of Trp by acrylamide is dependent on the degree of Trp exposure to an aqueous environment (*25*). Two transmembrane peptides with Trp residues at known positions were used to calibrate acrylamide quenching. Acrylamide quenching of the Trp fluorescence in these peptides shows that the slope of the acrylamide quenching curve is much greater when a Trp is located at the polar−nonpolar boundary of the bilayer (i.e., close to the membrane surface) than when a Trp is at the center of the bilayer (i.e., in a transmembrane orientation) (Figure 2). In experiments carried out using the CP peptide bound to 80% DOPC/20% DOPG or DOPC vesicles, the level of acrylamide quenching of the Trps is even greater than that of a Trp at the polar−nonpolar boundary of the bilayer (Figure 2), confirming a very shallow Trp location. That the quenching of the CP peptide at 200 μM lipid is not affected by a small amount of unbound peptide is shown by a similar level of quenching of the peptide at 1 mM lipid (Figure 2). The additional vesicles in the 1 mM lipid sample should bind much of any residual peptide dissolved in solution at 200 μM lipid.

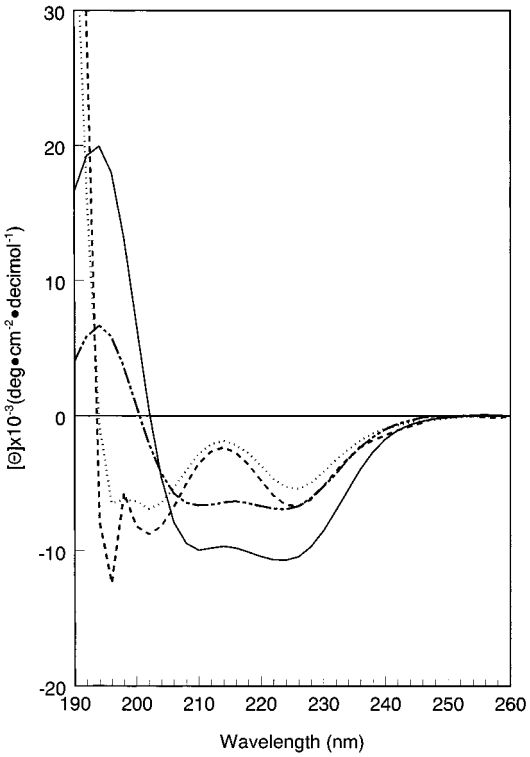

FIGURE 3: Circular dichroism spectra of CPE incorporated into model membranes. Samples contained 5 μM CP peptide in 10-fold diluted Tris-acetate buffer at pH 4.1 in the absence (-- − --) or presence (—) of 200 μM 20% DOPG/80% DOPC vesicles. The CP peptide was also prepared in Tris-acetate at pH 7.2, and CD spectra were collected in the absence (···) or presence (- - -) of lipid vesicles. Each spectrum represents the average of 100−150 measurements. The fractional α-helix content was calculated using SELCON.

We needed to ensure that the high degree of quenching by acrylamide was due to the high degree of exposure of the Trp to an aqueous solution and not to some inherent sensitivity to quenching in general. Therefore, quenching was also assessed using SUVs containing 10 mol % 10-doxylnonadecane, a quencher that deeply buries in the bilayer.[2] In these experiments, the control transmembrane peptide with a Trp close to the bilayer center exhibited strong quenching ($F_o/F = 2.64$). The control transmembrane peptide with a Trp close to the membrane surface exhibited much weaker quenching ($F_o/F = 1.55$). The CP peptide exhibited even weaker quenching by 10-doxylnonadecane ($F_o/F = 1.15$), further confirming a location of the bound peptide at the membrane surface.

Peptide Secondary Structure. The secondary structure of the CP peptide was examined using circular dichroism (Figure 3). At pH 7.2, the CP peptide exhibits an unusual circular dichroism spectrum that does not correspond to a simple mixture of helices, sheets, or random structure. This spectrum does not appear to be affected by the presence of DOPC SUVs. In contrast, at pH 4.1, the CP peptide exhibits a standard spectrum of a peptide with significant α-helical content. The amount of helix increases upon binding to DOPC vesicles, from 18% in the absence of lipid to 33% in the presence of lipid, indicating that the binding of the CP peptide to the membrane stabilizes α-helix formation.

[2] G. Caputo and E. London, unpublished results.

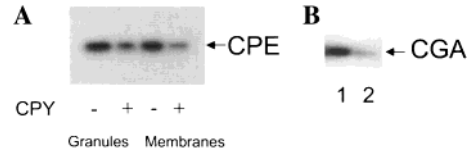

FIGURE 4: Protease protection assay. (A) Intact secretory granules from bovine intermediate lobe pituitaries were incubated with (+) or without (−) 40 units/mL carboxypeptidase Y and analyzed for C-terminal CPE immunoreactivity. As a positive control, lysed granule membranes were subjected to the same treatment. (B) Chromogranin A (CGA) immunoreactivity in supernatant after CPY treatment of lysed (lane 1) and intact (lane 2) granules. The absence of CGA in lane 2 demonstrates that the integrity of the granules is not compromised after protease digestion. Results are representative of four experiments.

Topology of CPE in Intact Secretory Granules. The biochemical studies of the CP peptide suggested that it would not by itself form a transmembrane structure. However, there is evidence that proteins that do not assume a transmembrane orientation in model membranes are able to do so in the presence of another protein (*16*). Therefore, we examined the orientation of CPE in secretory granule membranes, where it could conceivably interact with other proteins to assume a transmembrane orientation. Protease protection assays were carried out, in which intact secretory granules from bovine intermediate pituitary were treated with carboxypeptidase Y. CPY digestion markedly reduced C-terminal CPE immunoreactivity in intact granules as assessed by Western blot analysis (Figure 4A). As a positive control, lysed granules were also treated with CPY, and an equivalent decrease in CPE C-terminal immunoreactivity was observed (Figure 4A). CPY was chosen as the protease for these experiments for its selectivity in hydrolyzing peptide bonds from the carboxy-terminal end; N-terminal CPE immunoreactivity was unaffected by CPY treatment (data not shown). The absence of CGA immunoreactivity in the supernatant after CPY digestion showed that the granules were intact throughout the experiment (Figure 4B). Additionally, to rule out the possibility that C-terminal CPE was being degraded by carboxypeptidase Y during the Western blot process, CPY was added to aliquots of intact granules after the incubation period, immediately put on ice, and immediately treated with PMSF. No attenuation of CPE immunoreactivity was observed after this treatment (data not shown).

Immunoisolation and Immunolabeling of Intact Secretory Granules. To further demonstrate that membrane CPE has a cytoplasmic domain and is therefore a transmembrane protein, intact secretory granules were immunoisolated using a C-terminal antibody. Figure 5A shows that intact secretory granules were successfully isolated with magnetic beads coupled with a C-terminal CPE antibody specific for the last five residues of CPE (ETLNF), thus confirming that this epitope was exposed on the cytoplasmic face of the granule. That a significant amount of CPE immunoreactivity is eluted from beads coated with a C-terminal, but not an N-terminal, antibody indicated that secretory granules were intact and not lysed or inside-out during the purification and immunoisolation procedure. The intense band of N-terminal CPE immunoreactivity present when granules are lysed (Figure 5B), compared to the weak band observed in intact granules (Figure 5A, lane 5), further confirms granule integrity during the experiments. Additionally, preabsorbing the antibody with a CPE C-terminal peptide inhibited the binding of granules

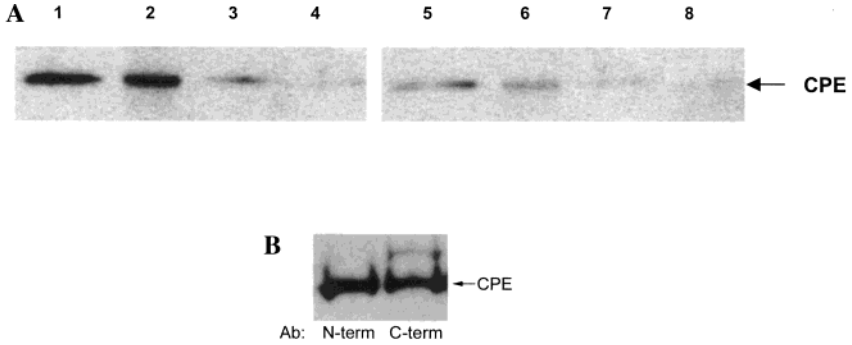

FIGURE 5: Immunoisolation of secretory granules by magnetic beads coupled to CPE antibodies. (A) Purified secretory granules from bovine neural lobe pituitary were incubated with magnetic beads coated with either anti-C-terminal CPE (lanes 1−4) or anti-N-terminal CPE (lanes 5−8) antibodies. For preabsorption controls, either C-terminal (lanes 3 and 4) or N-terminal (lanes 7 and 8) peptide was included prior to addition of granules. Western blot analysis for C-terminal CPE immunoreactivity was carried out in samples containing granules eluted from the beads (lanes 1, 3, 5, and 7) and for samples containing residual CPE immunoreactivity after elution and washing (lanes 2, 4, 6, and 8). (B) Secretory granules were lysed, and the resulting supernatant was immunoprecipitated with an anti-N terminal (N-term) or anti-C-terminal (C-term) CPE antibody. Results are representative of three experiments.

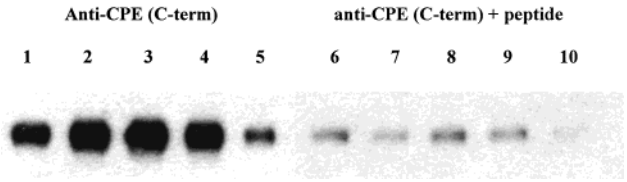

FIGURE 6: Intact secretory granules are labeled with CPE antibodies. Purified secretory granules from bovine neural lobe pituitary were incubated with an antibody against the C-terminus of CPE and subsequently centrifuged through a metrizamide/sucrose step gradient. Fractions were collected and analyzed for the presence of C-terminal CPE antibodies. Lanes 1−5 represent fractions 2−6, respectively, which contained the secretory granule fraction. Lanes 6−10 show fractions 2−6, respectively, after addition of the CPE C-terminal peptide. Results are representative of three experiments.

to the beads, showing the specificity of the reaction (Figure 5A). A significant amount of CPE immunoreactivity remained on the beads after the first elution (Figure 5A, lane 2), thus demonstrating tight specific binding of C-terminal CPE on intact granules to antibody-coated beads. To further confirm that the C-terminal epitope (ETLNF) is exposed on the cytoplasmic side of secretory granules, these organelles were labeled with the C-terminal antibody followed by isolation of intact granules using density gradient centrifugation. Specific C-terminal CPE antibodies were observed in fractions containing intact granules isolated from the 0.23 M−0.3 M interface of the gradient (Figure 6). The presence of CPE antibodies in these fractions was eliminated by addition of the C-terminal peptide during immunolabeling (Figure 6). When the results of the immunoisolation and immunolabeling experiments are taken together with the protease protection assay, they strongly indicate that this exposed epitope is part of CPE, and therefore that CPE is oriented in a transmembrane fashion in intact secretory granules.

Homology Model. A homology model of mouse CPE was built using CPD-II and CPT as templates to estimate the location of the transmembrane region relative to the enzymatic and sorting signal binding sites of CPE. Of the 410 residues modeled by homology to either CPD-II or CPT, 201 residues (49%) are identical to the nearest residue in the CPD-II structure (Figure 7). For these identical residues, the root-mean-square deviation between α-carbons is 0.61 Å. The protein core, enzymatic pockets, and inner faces of the surface helices are very well conserved (Figure 7, red residues). Bordering the modeled enzymatic pocket at its rim is an exposed loop containing two residues unique to CPE, Arg_{255} and Lys_{260}, which have been shown to be necessary for the binding of the POMC sorting signal (*7*). Two disulfide bridges are predicted to form between Cys_{28} and Cys_{90} and between Cys_{258} and Cys_{303}, and may serve to stabilize the orientation of the sorting signal binding site. Note that Cys_{90}, Cys_{258}, and Cys_{303} are conserved in CPD-II. Although there is a third pair of cysteines in mouse CPE, the model predicts that the distance between the sulfur atoms (12.5 Å) precludes the formation of a disulfide bond. As the sequences of CPD-II and CPE are highly homologous, especially in the modeled protein core and enzymatic pocket, the overall global topology of the model is presumably correct. The model cannot address the conformation and orientation of the membrane-binding region due to the absence of a homologous region in other carboxypeptidases. However, on the basis of the considerable degree of homology between CPE and CPD-II, the model strongly suggests that the N-terminal residues of the membrane-binding region are well separated from the sorting signal binding site, as well as from both the catalytic domain and the substrate-binding site of CPE (Figure 7).

DISCUSSION

The functions of CPE as both a membrane-bound sorting receptor and a soluble prohormone processing enzyme are well-documented (*3*, *26*). It is well-known that the 55 kDa form of CPE binds tightly to the membrane via its 25 C-terminal amino acids (*12*). However, the manner in which CPE associates with the membrane has not been previously examined. In this report, we have provided strong biochemical evidence showing that the C-terminus of CPE extends from the cytoplasmic leaflet of the membrane, supporting the hypothesis that CPE is a transmembrane protein.

In experiments using model membranes and a synthetic peptide representing the last 22 amino acids of CPE, we have shown that this peptide forms an amphipathic α-helix in a pH-dependent manner, and that binding to lipid increases the extent of helical formation. The finding that a CPE peptide strongly binds to model membranes only at an acidic pH is consistent with results from previous studies that

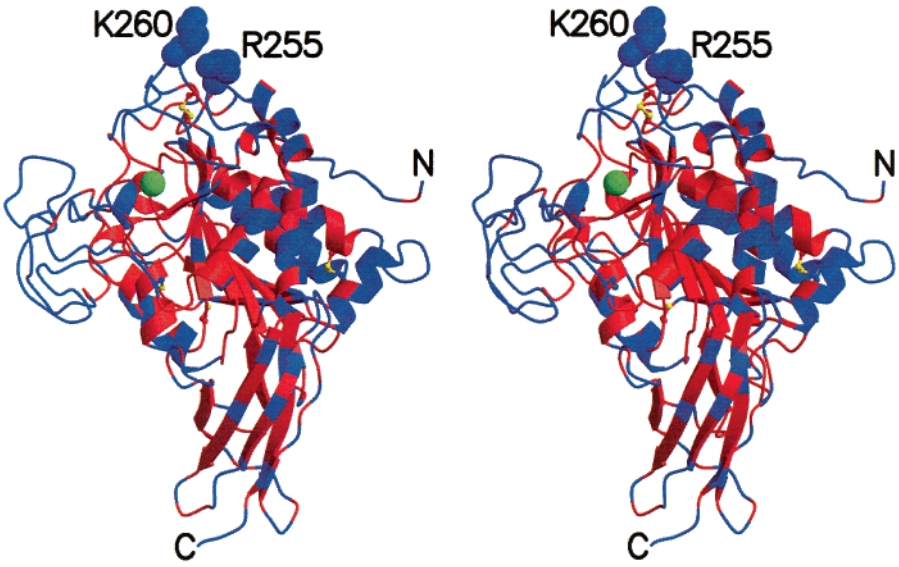

FIGURE 7: Stereoview of the CPE model, built on the basis of the homology between CPE and both CPD-II and CPT. The CPD-II, CPT, and CPE sequences were aligned initially using CLUSTAL W, version 1.74 (*37*). Manual modifications were then made to the alignment, guided by the superimposed crystal structures of CPD-II (PDB entry 1qmu) and CPT (PDB entry 1obr). The model includes residues 1−410 and is colored on the basis of identity between the CPE and CPD-II sequences (red if identical, blue if different). The N-terminal region preceding the membrane-binding domain is denoted with a C. Cysteine side chains are drawn as ball-and-stick diagrams. Note the disulfide bonds between Cys_{258} and Cys_{303} (top center) and between Cys_{28} and Cys_{90} (middle right). Arg_{255} and Lys_{260} are shown as space filling models. The zinc atom is drawn as a green sphere to mark the enzymatic pocket. This figure was prepared with the programs MolScript (*38*) and Raster3D (*39*).

showed a pH-dependent association of CPE with membranes from bovine pituitary secretory granules (*12*, *13*). Formation of the α-helical structure therefore appears to be necessary to anchor CPE to lipids. That the helical content was only 33% indicates that, at least in vitro, a partial helical configuration is sufficient for tight membrane binding. Whether this is the case in vivo is not known, since the membrane-binding domain may have an increased helical content as part of the whole protein. It is possible that, in the presence of the lumenal domain of the protein, the membrane-binding domain of CPE is able to assume a complete α-helical configuration.

We used three independent biochemical approaches to examine whether CPE is inserted in a transmembrane fashion in secretory granules. First, intact secretory granules treated with carboxypeptidase Y showed a decrease in C-terminal CPE immunoreactivity, using a C-terminal antibody specific for the last five residues of CPE (ETLNF), indicating that part of the C-terminus is exposed on the cytoplasmic side of the granule. Second, intact granules were successfully immunoisolated with the same anti-CPE antibody coupled to magnetic beads. Finally, intact secretory granules were immunolabeled with the C-terminal antibody. These experiments provide strong evidence that the extreme C-terminus of CPE is on the cytoplasmic side of the secretory granule membrane, indicating that this region of CPE spans the membrane.

The results from this study indicate that the membrane-binding region of CPE (R_{413}KEEEKEELMEWWKMM-SETLNF$_{434}$) is a transmembrane α-helix. On the basis of the experimental evidence outlined above, we propose a scheme for the transmembrane region, shown in Figure 8. Since the processing of membrane-bound CPE to the soluble form is suggested to occur at the Arg_{413}-Lys_{414} pair (*27*), these residues should lie at the lumenal interface, thus being

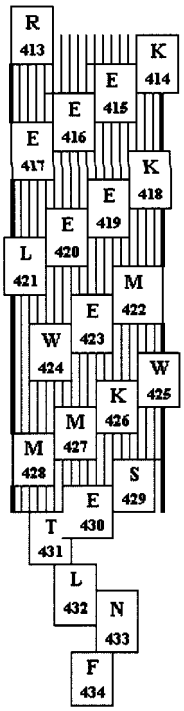

FIGURE 8: Schematic representation of residues 411−434 of mouse CPE as a transmembrane helix with lumenal and cytoplasmic leaflets at the top and bottom, respectively. This region forms a partial amphipathic α-helix at acidic pH that is stabilized through interaction with the lipid bilayer. Biochemical experiments suggest that this region spans the membrane. The Arg_{413}-Lys_{414} pair may act as an anchor at the lumenal leaflet of the membrane with as many as the last six residues (S_{429}ETLNF$_{434}$), forming a "tail" that protrudes from the cytoplasmic leaflet.

accessible to protease activity. Additionally, this pair of basic residues may serve to anchor the peptide at the surface of the lumenal leaflet of the membrane (*28*, *29*). The 22-amino acid domain of CPE is sufficiently long to penetrate the

bilayer with the last four residues exposed on the cytoplasmic side of the TGN, given that 18 amino acids is sufficient to span the cholesterol-rich membrane of the TGN (*29*, *30*). The turn of the helix at Met_{428} leaves open the possibility that as many as the last six residues may constitute a cytoplasmic tail. Protease protection, immunoisolation, and immunolabeling experiments all give strong evidence that the last four to six residues (S_{429}ETLNF_{434}) are exposed on the cytoplasmic side of the membrane. Therefore, we conclude that this region of CPE spans the membrane from residue 413 to residue 430.

The driving force for the insertion of CPE into the membrane may be protonation of the glutamate residues in the N-terminal portion of the membrane-binding domain (E_{412}RKEEEKEE_{420}). Within the acidic environment of the TGN and secretory granules (pH 6.0 and 5.5, respectively) (*31*) where CPE is located, the glutamate side chains are predicted to be partially protonated, thus enhancing the ability of the glutamate residues to form a helix. However, within the lipid bilayer, the pK_a of Glu may shift up to as high as 9 (*32*), resulting in helix stabilization due to full protonation of the glutamate residues. It has long been known that homopolymers of L-glutamic acid form very stable α-helical conformations at acidic pH that are stabilized by low ionic strengths and organic solvents due to stabilization of the protonated form of the glutamate side chain (*33*). The ability of acidic side chain neutralization to increase helix stability and the extent of membrane translocation has been described in model lytic systems (*34*, *35*) and implicated in membrane translocation of a number of natural toxin proteins (*36*). Therefore, movement of this part of the protein sequence into the hydrophobic environment of the lipid membrane would be more energetically feasible due to the increased stability of the helical structure that is formed.

Peptide binding experiments using model membranes do not support the hypothesis of a transmembrane domain in CPE, while experiments examining the insertion of endogenous CPE in secretory granule membranes argue for the existence of a transmembrane domain. Although these results appear to be contradictory, it is possible that CPE exists as a peripheral protein in model membranes, but is inserted in a transmembrane fashion in native secretory granule membranes. It is possible that the polar end of the amphipathic α-helix may need to be shielded from the hydrophobic environment of the lipid bilayer. This may be accomplished in two ways: through self-aggregation or through binding with another integral membrane protein. Self-aggregation may occur through a hydrogen bonding interaction of the protonated glutamate side chains. This mechanism has been invoked for the constitutive dimerization of a mutant form of the neu/erbB-2 receptor, which harbors a Val664Glu mutation in the transmembrane domain (*32*). Alternatively, association of CPE with another integral membrane protein within the membranes of secretory granules of endocrine cells may allow CPE to be inserted deeply into the lipid bilayer. It has previously been proposed that the polar end of the membrane-binding domain of CPE interacts with another membrane protein to bind to lipids (*26*). Interestingly, the transmembrane domain of diphtheria toxin also inserts into DOPG/DOPC SUVs in a shallow manner, like CPE. However, in the presence of molten globule-like proteins (*16*), a transmembrane insertion is achieved. Therefore, the shallow insertion of the CPE peptide in model membranes may be explained by the absence of other membrane proteins that, when present, direct CPE through the bilayer. A putative chaperone protein necessary for the transmembrane insertion of CPE has been isolated from the detergent-insoluble portion of secretory granule membranes (M. Assadi and Y. P. Loh, personal communication). Efforts to identify and characterize this protein are currently underway.

In conclusion, structural analysis of the membrane-binding C-terminus of CPE by CD demonstrated a partial α-helical configuration which was stabilized by binding to model membranes. Protease protection, immunoisolation, and immunolabeling experiments using intact secretory granules revealed a cytoplasmic domain in CPE, consistent with the insertion of the C-terminal membrane-binding region in a transmembrane fashion.

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