Identification of the pH-dependent Membrane Anchor of Carboxypeptidase E (EC 3.4.17.10)*

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Carboxypeptidase E (CPE), a peptide hormone-processing enzyme, is present within secretory granules in both a soluble form and a form which is membrane-bound at pH 5.5 but soluble at neutral pH. Antisera raised against a peptide corresponding to the predicted COOH-terminus of CPE bind to the membrane-associated form of CPE but not to the soluble form. This COOH-terminal region is predicted to form an amphiphilic α-helix, containing several pairs of hydrophobic residues separated by hydrophilic residues. Synthetic COOH-terminal peptides 11–24 residues in length are able to bind to bovine pituitary membranes and can be extracted by conditions that extract the membrane-bound form of CPE. The influence of pH on the membrane binding of a 21-residue COOH-terminal peptide is similar to the membrane binding of CPE: at pH values < 6 the majority of the peptide is membrane-bound, while at pH values above 8 less than 20% is membrane-bound. Both the 21-residue COOH-terminal peptide and the purified membrane form of CPE, but not the soluble form, partition into Triton X-114 only at low pH (pH < 6). Combined polar and hydrophobic interactions of the COOH-terminal peptide appear to be responsible for the reversible, pH-dependent association of CPE with membranes.

Within secretory granules isolated from the adrenal medulla (Fricker and Snyder, 1982), pituitary (Hook and Loh, 1984), or insulinoma cells, (Docherty and Hutton, 1983), CPE is present in both soluble and membrane-associated forms. The membrane-associated form of CPE is not extracted with detergent when low pH buffers are used (pH 5–6) and is only partially extracted by high salt buffers (Supattapone et al., 1984; Hook, 1984; Fricker, 1988c). Most of the membrane-associated CPE is solubilized by a combination of high salt and detergent at pH 5–6, or by moderately high pH buffers, without detergent or salt (Supattapone et al., 1984; Fricker, 1988c; Mackin and Nee, 1987). Thus, this enzyme has a tight association with the granule membrane.

Solubilization of membrane-associated CPE leads to a 2-fold increase in Vmax and a substantial decrease in the Km value for substrate hydrolysis (Fricker, 1988c). Once solubilized and purified to apparent homogeneity, the enzymatic properties of the soluble and membrane forms of CPE are very similar (Supattapone et al., 1984; Fricker and Snyder, 1983). Most of the physical properties are also similar for the two forms, with the exception of the molecular weight. The membrane-associated form (52–53 kDa) is slightly larger than the soluble form (60 kDa) when analyzed by denaturing polyacrylamide gel electrophoresis (Supattapone et al., 1984). Both forms have the same partial amino-terminal sequence and contain many of the same tryptic fragments (Fricker et al., 1986). The isolation and sequence analysis of numerous cDNA clones encoding CPE from bovine (Fricker et al., 1986) and rat (Fricker et al., 1989; Rodriguez et al., 1989) libraries did not find any evidence that the multiple forms of CPE arise from different RNA species, and Southern analysis of genomic DNA indicates a single CPE gene. This suggests that soluble and membrane forms of CPE arise from post-translational processing of a single precursor protein.

Despite the tight association of CPE with membranes, the predicted amino acid sequence does not contain any hydrophobic regions that would be expected to span the lipid bilayer. Instead, a potential amphipathic helix exists within the COOH-terminal region of the protein (Fricker et al., 1986; Fricker, 1988b). This sequence is predicted by the Chou-Fasman algorithm to form an α-helix with 8 hydrophobic residues on one face and all of the charged groups on the other (Fig. 1). In this study, we have used antisera raised against COOH-terminal peptides to verify that the membrane-associated form, but not the soluble form of CPE, contains this region. In addition, we have found that synthetic COOH-terminal peptides containing this potential amphipathic region are able to bind to membranes and that the properties of this binding are similar to those of the membrane-associated form of CPE. These observations support the hypothesis that the COOH-terminal region of CPE is responsible for its pH-dependent membrane association.
pH-dependent Membrane Anchor of CPE

FIG. 1. Top, predicted α-helix forming potential of rat CPE, deduced from the nucleotide sequence of a cDNA clone. The Gene Pro program (Hoeffer Scientific) was used for this analysis, with a window setting of 10. Middle, amino acid sequence of the COOH-terminal region of CPE. Charged amino acids (+/-) and hydrophobic (h) are indicated for this region of CPE, amino acids 411-434, relative to the NH2 terminus of the bovine CPE (Fricker precursor, 1988b). The predicted amino acid sequences of the COOH-terminal 24 residues of bovine and rat CPE are identical (Fricker et al., 1989; Rodriguez et al., 1989). Bottom, α-helical net and axial projection of an α-helical structure of residues 419-434.

EXPERIMENTAL PROCEDURES

Peptides were synthesized with an Applied Biosystems 430A Peptide Synthesizer using standard t-butyloxycarbonyl chemistry and phenylacetamidomethyl resins (Erickson and Merrifield, 1976). Cleavage of peptides from the resins was accomplished using hydrogen fluoride. Amino acid compositions were obtained on a Beckman 6300 amino acid analyzer after hydrolysis in constant boiling HCl under reduced pressure at 110 °C for 24 h and were within 5% of the predicted values. Analysis of the peptides on an Applied Biosystems 477A sequencer revealed the presence of less than 5% deletion peptides. Peptides were coupled to keyhole limpet hemocyanin by glutaraldehyde, and injected into rabbits by injection schedules used previously (Aletta et al., 1988).

Electrophoresis in 10% polyacrylamide gels containing sodium dodecyl sulfate was performed by standard procedures (Harlow and Lane, 1988). After electrophoresis at 150 volts for 2-3 h, the gel was cut, one section was stained for protein using silver staining (Morrison, 1981), and the other section was blotted onto nitrocellulose membranes (Harlow and Lane, 1988). The blots were blocked with 3% bovine serum albumin in Tris buffer, pH 7.4, containing 150 mM NaCl (TBS) for 30 min and then probed with the indicated antisera diluted 1:1000 in the bovine serum albumin/Tris solution. After 2 h at room temperature, the blots were washed three times with TBS, TBS containing 0.01% Triton X-100, and then TBS again. The blot was then incubated for 1 h at room temperature with goat anti-rabbit antisera conjugated to horseradish peroxidase, diluted 1:1000 in the bovine serum albumin/TBS buffer. After washing three times with TBS, the blots were treated with a solution containing 0.6 mg/ml 4-chloro-2-naphthol and 0.005% H2O2 in TBS for 5-10 min at room temperature.

CPE was purified from frozen bovine pituitary glands (Pel-Freez) by a modification of the previously described procedure (Fricker and Snyder, 1983). Several pituitaries were homogenized (Polytron) in 10 volumes of 0.1 M NaAc, pH 5.5, and the centrifuged at 50,000 × g for 30 min at 4 °C. The supernatant was removed, and the pellet was suspended (Polytron) in the same NaAc buffer and then centrifuged again at 50,000 × g for 30 min. The second supernatant was combined with the first and applied to a p-aminobenzoyl-L-Arg affinity column (obtained from Dr. Thomas Plummer, Jr., New York State Dept. of Health, Albany, NY). The membrane-bound form of CPE was extracted by homogenizing the pellet in 0.1 M NaAc buffer, pH 5.5, containing 1 M NaCl and 1% Triton X-100 and then centrifuging at 50,000 × g for 30 min. This supernatant was applied directly to a similar affinity column, and both columns were treated in the same manner. The flow-through fractions were recycled several times, and then the columns were washed with 10 volumes (200 ml) of 1 M NaCl containing 1% Triton X-100, followed by 2 volumes of 10 mM NaAc, pH 5.5. The columns were then eluted with 50 mM Tris, pH 8.0, containing 100 mM NaCl, and 10-ml fractions were collected. CPE was assayed using the substrate dansyl-Phe-Ala-Arg, as described previously (Fricker and Snyder, 1983).

The peptide YF-21 was labeled with 125I using either the chloramine-T method (Harlow and Lane, 1988) or Enzymobeads (Bio-Rad). The Enzymobeads were used according to the manufacturer's instruc-
tions, and the radiolabeled peptide was purified on Sephadex G-10 as described below. For the chloramine-T iodination, 2 nmol of peptide in 25 µl of 0.5 M sodium phosphate, pH 7.4, containing 250 µCi of ¹²⁵I was combined with 25 µl of chloramine T (2 mg/ml). After 1 min at room temperature, 50 µl of sodium metabisulfite (2.4 mg/ml) was added and the mixture applied to a 1-ml column of Sephadex G-10 (Pharmacia LKB Biotechnology Inc.), equilibrated in phosphate-buffered saline. Fractions (0.3 ml) were collected, with approximately 50% of the radioactive eluting in fraction 2 and the remainder eluting predominantly in fractions 5 and 6. The purity of the labeled peptide in fraction 2 was determined by high pressure liquid chromatography analysis using a reverse-phase C18 column (300 Å, 2.1 x 10 cm) eluted with a gradient from 0 to 70% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.25 ml/min over a period of 20 min. Absorbance was monitored at 220 nm and 280 nm. One-milliliter fractions were collected. The majority (>90%) of the radiolabeled peptide eluted from the column in fraction 9.

Bovine pituitary membranes were prepared by extracting the glands twice with 0.1 M NaAc, pH 5.5, as described above for the purification of the membrane-associated form of CPE. For studies on the release of CPE from the membranes, the pellet from a single purification was resuspended in 15 ml of water, and 1 ml aliquots added to 1 ml of the appropriate buffer. To give the final concentration indicated (Table II). Following centrifugation for 30 min at 50,000 x g, the supernatant was removed and the pellet was reextracted with the same buffer and then resuspended in 10 ml of 0.1 M NaAc, pH 5.5. Aliquots (25 µl) of all fractions were assayed for CPE activity using d-fenugreek-Ala-Ala-Arg, as described (Fricker and Snyder, 1982).

Membranes for the studies on peptide reassociation were prepared from frozen bovine pituitary glands or from rat brain, liver, placenta, and heart by extracting tissue homogenates twice with 0.1 M NaAc, pH 5.5, and then centrifuged at 50,000 x g for 15 min. The pellets were resuspended (Polytron) in 0.1 M NaAc and then centrifuged at 50,000 x g for 15 min. After washing twice with 0.1 M NaAc, pH 5.5, the membranes were resuspended in 40 ml of 10 mM NaAc, pH 5.5. The protein concentration of the pituitary membranes was 35 µg/ml, determined by the Bradford method (Harlow and Lane, 1988).

A typical reassociation experiment, 25 µl of the heat-inactivated, carbonate-extracted membranes were combined with 50,000-100,000 cpm of radiolabeled YF-21 in a final volume of 250 µl of 0.1 M NaAc, pH 5.5, containing 0.01% Triton X-114. The mixture was incubated for 2 h at 4 °C on a rocking platform shaker (Thermolyne), and then centrifuged at 50,000 x g for 45 min, and then centrifuged at 50,000 x g for 15 min. The supernatant was removed, 250 µl of 0.1 M NaAc, pH 5.5, added, and the pellet resuspended by mixing on a vortex shaker. The resuspended membranes were centrifuged again at 15,000 x g for 5 min, the supernatants were removed, and the pellets resuspended in the buffers indicated (Table II; Fig. 4). The amount of radioactive in each fraction was determined in a γ counter.

The partitioning of purified CPE and radiolabeled peptide into Triton X-114 was examined using a modification of the previously described procedure (Bordier, 1981). Either radiolabeled YF-21 (approximately 30,000 cpm) or purified CPE (15 relative units) were incubated at 4 °C with 1% Triton X-114 in a final volume of 250 µl of 0.1 M NaAc, pH 5.5, containing 0.01% Triton X-100. The mixture was incubated for 2 h at 4 °C on a rocking platform shaker (Thermolyne), and then centrifuged at 15,000 x g for 5 min at 4 °C. The supernatant was removed, 250 µl of 0.1 M NaAc, pH 5.5, added, and the pellet resuspended by mixing on a vortex shaker. The resuspended membranes were centrifuged again at 15,000 x g for 5 min, the supernatants were removed, and the pellets resuspended in the buffers indicated (Table II; Fig. 4). The amount of radioactive in each fraction was determined in a γ counter.

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Sequence-specific Antibodies to CPE—Several species of CPE can be purified from soluble and membrane extracts, with apparent masses of 53 and 55 kDa for the soluble forms, and 55 and 58 kDa for the membrane-associated forms (Fig. 2A). Antisera raised against an NH₂-terminal peptide 15 residues in length recognize all of these polypeptides (Fig. 2B). Similar results were obtained with antisera raised against CPE purified from soluble extracts of bovine pituitary (data not shown). An antisem raised against a COOH-terminal peptide 11 residues in length (WF-11, Fig. 1) binds only the species of CPE purified from the membrane extracts (Fig. 2C), indicating that the soluble and membrane-associated forms differ in the length of the COOH-terminus. To investigate the extent of the difference in length, antisera were also raised against a COOH-terminal peptide 24 residues in length (SF-24, Fig. 1). These antisera recognize the membrane forms of CPE, and also shows a faint signal with the higher molecular weight form of soluble CPE (Fig. 2D), indicating that this soluble form of CPE contains some portion of the COOH-terminal 24 amino acids. Preimmune sera from each rabbit did not bind to any form of CPE (data not shown).

Binding of CPE Synthetic Peptides to Membranes—To determine whether the CPE COOH-terminal region alone could serve as a membrane anchor, a peptide corresponding to the COOH-terminal 20 residues of the predicted CPE sequence was synthesized. An NH₂-terminal tyrosine residue was added to permit radioactive labeling (YF-21, Fig. 1). After labeling with ¹²⁵I by the Enzymobead (Bio-Rad) method, the peptide

![Figs 1-4](http://www.jbc.org)
was incubated with heat-inactivated, Na₂CO₃-extracted bovine pituitary membranes for several hours and then centrifuged for 5 min at 15,000 × g. Approximately 24% of the added labeled peptide was recovered in the supernatant, and an additional 6% could be extracted with 0.1 M NaAc, pH 5.5 (Table I). The remaining 70% of the radiolabel was associated with the membranes. This distribution of the peptide was not due to nonspecific binding of the peptide to the tubes: less than 2% of the radioactivity was recovered in the wash or "pellet" fractions when membranes were not included in the incubation (Table I). Another radiolabeled peptide, corresponding to the NH₂-terminal precursor portion of CPE did not demonstrate significant binding to the membranes (Table I). The tissue specificity of the membrane binding of radiolabeled YF-21 was examined; similar results were obtained with membranes prepared from bovine pituitary, rat brain, rat liver, rat placenta, and rat heart (Table I). For these tissues, approximately 17–24% of the added ¹²⁵I-YF-21 is recovered in the supernatant, and an additional 6–9% is recovered in the wash. The remainder of the radiolabel (68–77%) is associated with the membrane fraction.

Once associated with membranes, the synthetic COOH-terminal peptide can be solubilized by the same conditions that extract the membrane-associated form of CPE (Table II). For this analysis, the radiolabeled synthetic peptide was allowed to associate with bovine pituitary membranes, the unbound peptide was removed by centrifugation, and the membranes were washed once with 0.1 M NaAc buffer, pH 5.5. Two further extractions of the washed membranes with the NaAc buffer only removes 8–10% of the total radiolabeled peptide, with 82% of the peptide remaining membrane-bound (Table II). The addition of 1% Triton X-100 to the extraction buffer does not significantly alter the amount of peptide extracted. When high salt and Triton X-100 are used together, 70% of the bound peptide can be recovered in the first extract, and 43% of the remainder (13% of the original amount) in the second extract, with only 17% of the peptide remaining associated with the membranes. Extraction with 0.1 M NaHCO₃ recovered 86% of the peptide in the first extract, and 57% of the remaining peptide (8% of the original amount) in the second extract.

This behavior of the COOH-terminal peptide is similar to that of native CPE (Table II). The membrane-associated form of CPE can be extracted from bovine pituitary membranes in high yield (77–80%) by either high pH buffer, or by 1 M NaCl, 1% Triton X-100 in NaAc, pH 5.5, buffer. Detergent alone (1% Triton X-100 in 0.1 M NaAc) is able to extract 13% of the CPE activity. Without detergent (0.1 M NaAc), only 1–2% of the CPE activity is detected in the extracts. These results are consistent with previous studies investigating the properties of the membrane association of CPE (Supattapone et al., 1984; Fricker, 1988c).

CPE COOH-terminal peptides other than the radiolabeled YF-21 also bind to bovine pituitary membranes in a pH-dependent manner (Fig. 3). For this analysis, nonlabeled SF-24, EF-20, and WF-11 (Fig. 1) were incubated with heat-inactivated, Na₂CO₃-extracted membranes for several hours and then the membranes were recovered by centrifugation. The membranes were extracted sequentially with different buffers, and the amount of peptide in each extract (and in the initial supernatant fraction) was determined by slot-blot analysis, using the antisera raised against WF-11. Very low

### Table I

| Tissue                      | Peptide     | ¹²⁵I % of total radioactivity | Supernatant | Wash | Pellet |
|-----------------------------|-------------|-----------------------------|-------------|------|--------|
| Bovine pituitary            | ¹²⁵I-YF-21  |                             | 24          | 6    | 70     |
| None                        | ¹²⁵I-YF-21  |                             | 97          | 1    | 2      |
| Bovine pituitary pr         | ¹²⁵I-YM-14  |                             | 98          | 1    | 1      |
| Rat brain                   | ¹²⁵I-YF-21  |                             | 23          | 9    | 68     |
| Rat liver                   | ¹²⁵I-YF-21  |                             | 19          | 6    | 75     |
| Rat placenta                | ¹²⁵I-YF-21  |                             | 20          | 9    | 71     |
| Rat heart                   | ¹²⁵I-YF-21  |                             | 17          | 6    | 77     |

*YF-21 is a COOH-terminal peptide (Fig. 1), and YM-14 corresponds to the precursor region of rat CPE ("pro" peptide; YAEAQEP-GAPAAGM)."
amounts of either SF-24, EF-20, or WF-11 are recovered in the initial supernatant fraction when membranes are present (Fig. 3, lane 1), indicating that most of the peptide has bound to the membranes. It is unlikely that significant amounts of peptide bind to the microfuge tubes since without added membranes, most of the peptide is present in the initial supernatant fraction. Extraction of the membranes twice with 0.1 M NaAc, pH 5.5 (Fig. 3, lanes 2 and 3), and then twice with 1 M NaCl in NaAc buffer (Fig. 3, lanes 4 and 5) does not solubilize much of the SF-24 or EF-20, and only a small amount of the WF-11. Large amounts of all three peptides are released from the membranes upon extraction with 0.1 M NaHCO₃, pH 9 (Fig. 3, lane 6). The membranes remaining after NaHCO₃ extraction could not be examined for peptides by slot-blot analysis. However, standard curves using different concentrations of SF-24 and WF-11 (Fig. 3B) indicated that the amount of peptide recovered in the various extracts was comparable to the amount added (2 nmol), and it is unlikely that a substantial amount of the peptide remained bound to the membranes.

**pH Dependence of Membrane Binding and Partitioning into Triton X-114**—The influence of pH on the release of labeled peptide and CPE activity from membranes was also investigated. Membrane-associated peptide, prepared as for the experiment in Table II, remains largely membrane-associated (>90%) when extracted with buffers of pH 5.5 or less (Fig. 4). Increasing the pH of the extraction buffer decreases the amount of radiolabeled peptide remaining with the membranes, with a maximal effect at pH 8.5 (with only 20% of the remaining membrane-associated). At pH values around 6.5-7.0, approximately 50% of the peptide is extracted. The pH dependence of the binding of CPE to membranes is similar to that of the peptide: at pH 5.5 nearly all of the membrane-associated CPE activity remains membrane-bound, whereas only 20% remains membrane-bound upon extraction with buffers of pH > 8.0 (Fig. 4). Approximately 50% of the CPE activity is released from the membranes by buffers of pH 7, similar to previous results (Fricker, 1988a).

The partitioning of the membrane-associated forms of CPE and the COOH-terminal peptide YF-21 into Triton X-114 also shows a pH dependence (Fig. 5). At pH values of 4-6, approximately 60-70% of the purified membrane-associated form of CPE partitions into the Triton X-114 (Fig. 5). Less than 20% of the membrane form of CPE partitions into the Triton X-114 phase at pH values greater than 7, and the remainder of the added CPE activity is recovered in the aqueous phase. In contrast, less than 10% of the soluble form of CPE partitions into the Triton X-114 at any pH value examined, with greater than 90% of the CPE activity recovered in the aqueous phase. These results are not due to the activation or inhibition of CPE: the total amount of CPE activity recovered in both the aqueous and the detergent phases is similar for all pH values. When 0.01% Triton X-114 is used, which is below the amount necessary to achieve the phase separation (Bordier, 1981), all of the CPE activity for either soluble or membrane forms is recovered in the supernatant at pH values of 4-9 (data not shown). The radiolabeled COOH-terminal peptide YF-21 also partitions into the Triton X-114 in a pH-dependent manner, although a maximum of 30% of the added peptide partitions into Triton X-114 at pH 4 (Fig. 5). Less than 10% of the added peptide is found in the Triton X-114 at pH values greater than 6.

**DISCUSSION**

The COOH-terminal portion of the CPE polypeptide chain seems to be responsible for the association of this protein with secretory granules membranes because 1) the soluble and membrane-associated forms of CPE differ in the COOH-terminal region, and 2) synthetic peptides corresponding to this region bind to membranes and can be released by conditions that also release CPE. Furthermore, the membrane interactions are very strong and seem to be a combined result of hydrophobic and ionic interactions, consistent with the predicted amphiphilic structure of the COOH-terminal region.

The initial hypothesis, which was based on sequence analysis of a CPE cDNA clone, proposed that the COOH-terminal region of CPE forms an amphiphilic a-helix which participates in the association of CPE with membranes (Fig. 1). Furthermore, it was noted that endoproteolytic activity at the COOH-terminal pair of basic amino acids (Arg₁⁴³ Lys₁⁴⁴) would remove this putative membrane anchor (Fricker et al., 1986). The results with the antisera raised against specific regions of CPE confirm that the soluble form of CPE does not contain the full length COOH-terminus. However, be-
cause antisera raised against a 24-residue COOH-terminal peptide (SF-24) display some binding activity for the larger molecular weight species of CPE purified from soluble extracts (Fig. 2). This soluble form could not arise by endoproteolytic activity at the pair of basic residues (Arg193-Lys194). Further studies are needed to address the exact cleavage sites which give rise to the different forms of CPE, and to examine the three-dimensional structure of the COOH-terminal region of the protein.

The observation that two molecular weight species of CPE are present in both soluble and membrane extracts is consistent with the results of two-dimensional gel analysis of bovine adrenal chromaffin granule extracts. Using antibodies to CPE, Laslap and colleagues (Laslap et al., 1986) detected two closely migrating spots of molecular weight 53,000 and 56,000 for both soluble and membrane extracts, corresponding to glycoproteins previously named "J" and "K" (Gavine et al., 1984). In contrast, when CPE was purified to apparent homogeneity, only a single band was detected on denaturing polyacrylamide gels, with a molecular weight of 50,000 for the soluble protein and 52,000 for the membrane-associated form (Fricker and Snyder, 1983; Supattapone et al., 1984). The purification scheme used at that time took several days, and it is likely that some proteolysis occurred. In the present study, a single-step purification is used to rapidly isolate the CPE. Similar results have been obtained in over a dozen different purifications, with some variability in the relative amounts of the different molecular weight forms. The inclusion of serine and sulfhydryl protease inhibitors did not substantially influence the results. The difference in molecular weight is probably not a result of N-linked carbohydrate: treatment of the purified CPE with grade II endoglycosidase F (which also contains glycopeptidase F activity) shifted all of the species of CPE to lower molecular weights, but the relative differences between the species was not altered. The possibility that O-linked glycosylation could account for the molecular weight variation observed for CPE was not addressed by these experiments.

The finding that the soluble and membrane-associated forms of CPE differ in the COOH-terminal region does not preclude the possibility that other portions of the molecule also contribute to membrane binding. However, some of the common mechanisms for the attachment of proteins to membranes can be excluded from structural considerations. Many proteins are attached to membranes through a phosphatidyl-inositol group on the COOH-terminus (Low, 1987). It is not likely that this mechanism is involved in the binding of CPE since the addition of the phosphatidylinositol group to the protein requires the presence of a hydrophobic COOH-terminus of approximately 20 residues which is subsequently removed before the phosphatidylinositol group is attached (Low, 1987). If this were the case for CPE, then the antisera raised against the predicted COOH-terminus of CPE would not react with the membrane-associated forms of CPE. Further evidence that the membrane attachment of CPE is not due to a phosphatidylinositol-specific phospholipase C (obtained from Martin Low, Department of Physiology and Cellular Biophysics, Columbia University) to release the membrane-bound form of CPE. The attachment of fatty acids such as myristate to the NH₂ terminus of CPE could also be ruled out, since CPE isolated from membranes has been found by protein sequence analysis to have a free amino terminus identical to the soluble form of CPE (Fricker et al., 1986).

Direct evidence that the COOH-terminus of CPE participates in the association of the protein with the membranes was obtained from studies with the COOH-terminal peptides. A radiolabeled peptide corresponding to the COOH-terminal 20 amino acids of CPE is able to associate with heat-inactivated, Na₂CO₃-extracted bovine pituitary membranes (Table I). A fraction of the radiolabeled peptide (20–25%) does not associate with the membranes and is recovered in the supernatant fraction. It is possible that some oxidation of the peptide occurred during the iodination process, and that one or more of these oxidized forms do not bind to membranes. Both the chloramine-T method and the Enzymobead (Bio-Rad) method were used to label the peptide. When YF-21 was labeled with the chloramine T procedure, which is expected to cause more oxidation of the peptide, the maximal amount of membrane binding was only 50–70%, compared with the 75–80% observed with enzymatically labeled peptide. This suggests that oxidation of the peptide, presumably at the Met and/or Trp residues, affects membrane binding.

The fraction of peptide bound to the membranes is not significantly influenced by longer incubation times (overnight), higher amounts of membranes (four times the typical amount), or the incubation temperature (4 versus 25 °C). The presence of high concentrations (0.1 mM) of unlabeled peptide does not interfere with binding of 125I-YF-21, suggesting that saturable binding proteins are not involved. The heat inactivation and Na₂CO₃ extraction of the membranes prior to the addition of labeled peptide are not essential for the binding. However, these procedures slightly increase the amount of peptide bound, presumably by reducing enzymatic degradation of the peptide. When unlabeled peptides are used in place of the iodinated peptide, the majority of the peptide remains bound to membranes upon extraction with low and high salt buffer at pH 5.5, but is extracted at high pH (Fig. 3). These data suggest that the pH-dependent membrane association is a result of the native peptide sequence and is not specific for the iodinated peptide. The finding that all three COOH-terminal peptides (SF-24, FF-20, and WF-11; Fig. 1) are capable of binding to membranes supports the proposal that the binding is the result of the potential amphipathic region since WF-11 lacks the highly charged region (Fig. 1).

The similar conditions for the release of membrane-bound COOH-terminal peptide and membrane-bound CPE activity (Table II; Fig. 4) is further evidence that the COOH-terminal region of the polypeptide chain is responsible for the membrane association of CPE. The slope of the release of membrane-associated CPE versus pH is steeper than the slope for the release of the peptide. This could be due to differences in the stability of the structure of this peptide when attached to the protein compared to the free peptide. Alternatively, other sequences could also be involved with the membrane binding of CPE, in addition to the COOH-terminal region. The inflection points of the pH dependence curves are approximately 6.5–7 for both the peptide and the membrane-associated form of CPE. The calculated pI for the COOH-terminal peptide is 3.85, and so it is unlikely that the pH-dependent membrane binding is a result of the net charge of the peptide. Furthermore, the shortened peptide WF-11, with a pI of 6.78, also exhibits the pH dependence, whereas the amidated-terminal "pro" peptide, with an acidic pI of 3.10, does not bind to the membranes at all. The pI for soluble and membrane-associated forms of CPE are both around 5–6 (Laslap et al., 1986; Mackin and Noe, 1987).

Partitioning of proteins into Triton X-114 has been used to assess whether the proteins are intrinsic or extrinsic mem-
brane proteins (Bordier, 1981; Seth et al., 1985). Proteins that are associated with membranes only through electrostatic interactions do not usually partition into the detergent phase (Bordier, 1981). The finding that substantial amounts of the membrane forms of CPE partition into Triton X-114 at low pH (Fig. 5) is further evidence that these forms of CPE represent integral membrane proteins. The maximum amount of CPE that was found in the detergent phase was 70% of the amount added, and the remainder was recovered in the aqueous phase. Similar results are found with amphipathic viral proteins, such as the external proteins of adenovirus, which show a maximum of 60% of the protein partitioning into Triton X-114 phase at low pH values (Seth et al., 1985). The COOH-terminal peptide YF-21, but not the soluble forms of CPE, also partitioned into the Triton X-114 in a pH-dependent manner, which is further evidence that the COOH-terminal region is responsible for the partitioning of the membrane forms of CPE.

It is interesting that the pH range of the dissociation of CPE from membranes matches that of the expected environment of CPE, or any secretory granule protein. Within the endoplasmic reticulum and most of the Golgi apparatus, the internal pH is neutral (Anderson and Pathak, 1985), and the majority of the CPE would be soluble. Upon compartmentalization of CPE into the trans Golgi network, the acidification of the budding secretory granules would cause some of the CPE to bind to the membranes. At the final pH of the mature secretory granules, which is thought to be approximately pH 5–6 (Russell, 1984; Johnson and Scarpa, 1976; Anderson and Pathak, 1985), most of the CPE with a full length COOH-terminus would be associated with the membranes. Then, when the secretory granules fuse with the cellular membrane to release the soluble contents, the neutral pH of the external environment would cause the membrane-bound form of CPE to be released. Consistent with this hypothesis is the finding that antisera specific for the membrane-associated forms of CPE are able to immunoprecipitate [35S]Met-labeled CPE secreted into the media from GHCl cells.

The predicted structure of the membrane anchor of CPE is an amphipathic helix, although there is no experimental evidence that this peptide forms an α-helix. Further studies utilizing circular dichroism and NMR are needed to address this issue. An amphipathic helix has been proposed to be involved with the binding of apolipoprotein A-I to phospholipid membranes. Interestingly, an amphipathic helical structure has been proposed to be involved in the pH-dependent hydrophobicity of influenza hemagglutinin (Harter et al., 1988). It is possible that amphipathic regions are a general mechanism for the attachment of proteins to phospholipid membranes.

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