Carboxypeptidase E, a Peripheral Membrane Protein Implicated in the Targeting of Hormones to Secretory Granules, Co-aggregates with Granule Content Proteins at Acidic pH*

(Received for publication, March 18, 1998, and in revised form, September 21, 1998)

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Carboxypeptidase E (CPE) is a prohormone-processing enzyme and peripheral membrane protein of endocrine/neuroendocrine secretory granules. CPE has been shown to bind to an amino-terminal peptide of pro-opiomelanocortin (N-POMC) at pH 5.5 and hypothesized to be critically involved in the targeting of hormones such as POMC to the regulated secretory pathway [Cool, D. R., Normant, E., Shen, F., Chen, H. C., Pannell, L., Zhang, Y., and Loh, Y. P. (1997) Cell 88, 73–83]. To further explore the possibility that CPE serves to mediate the association of content proteins with the membrane during granule biogenesis, the binding of CPE to granule content proteins was investigated using an in vitro aggregation assay in which the selective precipitation of granule content proteins is induced by titration of the pH to <6.0. CPE was observed to co-aggregate efficiently with pituitary and chromaffin granule content proteins at concentrations well below those that promote its self-aggregation. In addition, CPE co-precipitated at pH 5.8 with purified prolactin and with insulin, which homophilically self-aggregate yet are structurally distinct from N-POMC. N-POMC when added to the assays did not inhibit the aggregation of CPE with prolactin or insulin, indicating that these interactions do not involve a binding site for N-POMC. The data show that CPE interacts at acidic pH with a variety of different content proteins, resembling in this regard other granule membrane proteins. The results support the idea that co-aggregation of abundant membrane proteins with content proteins is an important general mechanism for the sorting and retention of secretory granule proteins during granule maturation.

Aggregation of secretory granule proteins occurs as immature secretory granules (ISGs) are formed from the trans-Golgi network (TGN) and continue to mature and acidify (1, 2). Aggregation can also be achieved by lowering the pH of isolated granule contents, including those derived from chromaffin and anterior pituitary granules (3) as well as pancreatic zymogen granules (4, 5). A number of purified granule content proteins, including the chromogranins A and B (in the presence of calcium) (6, 7), prolactin (3), and insulin (8), aggregate in vitro as the pH is lowered to < 6.5 as well, reinforcing the view that changes in the ionic milieu of the TGN and ISG are responsible for content protein condensation (9, 10). Constitutively secreted proteins such as albumin and IgG, when added to content proteins, do not precipitate, suggesting that the formation of aggregates itself may directly regulate the process of segregating constitutively secreted proteins from granule content proteins (3).

We have previously demonstrated that the luminal domains of several abundant granule membrane proteins can co-aggregate with content proteins at pH 5.5–6 (3), which mimics the conditions existing in the ISG, a site where sorting of proteins destined for storage from those constitutively secreted is known to occur (11–13). This would be consistent with the idea that membrane and content proteins use similar routes of segregation during granule maturation and that the binding of content proteins to the granule membranes is mediated by the major membrane proteins themselves. Recently, Cool et al. (14) showed that pro-opiomelanocortin (POMC) as well as a peptide consisting of its amino-terminal 26 amino acids (N-POMC) bind to pituitary secretory granule membranes with a pH optimum of 5.5–6. This peptide was chosen because the amino-terminal portion of POMC can confer granule targeting on heterologous proteins in transfected cells (15). The binding of N-POMC to membranes was inhibited not only by POMC itself but by proinsulin and proenkephalin, suggesting that common sites of association are used by several different hormones. N-POMC could also be cross-linked at pH 5.5 to carboxypeptidase E (CPE), an abundant granule membrane protein (14), suggesting that POMC binding to membranes was mediated by CPE. Moreover, in cells depleted of CPE, either from the pituitary of fat/fat mice, which bear a mutation in CPE that prevents its exit from the endoplasmic reticulum (16, 17), or in cells transfected with antisense DNA constructs, POMC was observed to be secreted largely by the constitutive pathway (14). These data were interpreted to mean that CPE serves as the sorting receptor for POMC and other related prohormones, although the hypothesis remains controversial as proinsulin incorporation into secretory granules was not greatly affected in either isolated islets or an insulin-secreting cell line derived from fat/fat mice (18, 19).

CPE is a processing enzyme found in all endocrine and neuroendocrine cells (20). It associates with membranes at low pH by virtue of an amphipathic helix in its carboxyl terminus (21), resembling in this regard other processing enzymes such as prohormone convertase 2, which has a membrane binding domain in its amino terminus (22). CPE undergoes homophilic self-association at pH 5.5 when present at high concentrations...
Fig. 1. CPE co-aggregates with pituitary granule content proteins at pH 5.8. Left panels, pituitary granule content (1 mg/ml) was incubated either alone (lanes 1–4) or with purified CPE (1.3 μg/ml) added (Pit + CPE, lanes 5–8) using the standard assay conditions (see “Experimental Procedures”). CPE was incubated in the absence of granule content as a control (lanes 9–12). The pH was reduced as indicated. The entire pellet (P, odd numbered lanes) and 20% of the remaining supernatant (S) after centrifugation (even numbered lanes) were then subjected to SDS-PAGE and transfer to nitrocellulose paper. CPE (top panels) was identified by immunoblots, whereas the major pituitary content proteins Prl and GH were visualized by staining with Ponceau S (bottom panels). Right panel, the assay was performed as described above, but in this case, equal aliquots (50%) of the supernatant and pellet samples were used for SDS-PAGE and immunoblotting. CPE in the pituitary pellet (•) or incubated alone (○) is depicted along with Prl and GH bands quantitated after staining the bottom portion of the gels with Coomassie Blue (□). Each data point represents the mean and S.E. of three to six samples from three separate experiments.

RESULTS

CPE Aggregates Together with Other Pituitary and Adrenal Chromaffin Granule Proteins—CPE is an endogenous protein of the pituitary gland (20) and was readily detectable in granule content prepared from bovine pituitary (Fig. 1). To assess centrifugation at 100,000 × g as described (23). A portion of the supernatant and the entire pellet fractions were then subjected to immunoblotting (see below). As a control, samples were subjected to pH titration but not centrifuged. Under the conditions used, the amounts of protein remaining in the tubes after removal of the assay mixture were inconsequential (data not shown). Triton X-100 at 0.016% had no effect on overall aggregation of pituitary or adrenal extracts but did prevent binding of CPE itself to the tubes. To measure insulin aggregation, pellets and supernatants from triplicate samples were subjected to a Micro-BCA assay ( Pierce ), as described previously (3).

To assess the effect of N-POMC on CPE aggregation with Prl or insulin, a modification of the assay protocol was used. The final sample volume was reduced to 25 μl, and a buffer of 5 mM HEPES, 20 mM 4-morpholineethanesulfonic acid, 130 mM KCl, and 0.016% Triton X-100 was used. After the prespin, N-POMC (120 μg/ml) was added, and after a 15-min incubation the pH was reduced to 5.8. After an additional 30 min at 37 °C, centrifugation was conducted at 100,000 × g for 30 min. This protocol allowed titration of the amount of hormone necessary to induce reproducible CPE co-aggregation to 40 μg/ml for insulin and 80 μg/ml for Prl. The aggregation of Prl and insulin was essentially identical at 23 and 37 °C (data not shown).

Immunoblotting—Immunoblotting was performed after SDS-PAGE, transfer of the proteins to nitrocellulose paper, and staining with Ponceau S to visualize the transferred proteins. In experiments in which IgG was used, SDS-PAGE was performed without a reducing agent. Antibodies directed against the amino terminus of CPE were used in the experiments shown, but carboxyl-terminal antibodies were used in initial experiments with the identical results. All antibodies were generated as described above at a 1:500–1:2000 dilution. The enhanced chemiluminescence procedure was used with preflashed film to visualize the reactive species (Amersham Pharmacia Biotech). The exposed radiographs and the images of gels stained with Ponceau S were digitized (LACIE Ltd., Beaverton, CA) and imported into Quark Express (Denver, CO) for computerized labeling and printing of figures. Quantitation of aggregation on the scanned images was conducted using NIH Image (version 1.55) on exposures that were not oversaturated. All of the data presented are representative experiments that were repeated two to four times with similar results.

Measurement of CPE Activity—The assays of CPE activity were performed using the method of Fricker and Devi (24). In brief, purified CPE was diluted (1:500) in 0.1 M NaOAc, pH 5.5, 0.01% Triton X-100, 10 μg/ml bovine serum albumin, and 1 mM CoCl2, and incubated at 4 °C, for 20 min in the presence or absence of 0.5 μM GEMSA. Dansyl-FAR (20 μM) was then added, and the reaction was allowed to continue for 7 min at 37 °C and 5 min at 4 °C before addition of HCl (0.15 M final concentration) and chloroform. After centrifugation, samples were read in a Perkin-Elmer 650-10S fluorescence spectrophotometer using excitation and emission wavelengths of 355 and 500 nm, respectively. All assays were conducted in triplicate.

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(23), another property shared by prohormone convertase 2 (22) and by the luminal segment of peptidyl glycine α-amidating mono-oxygenase (3). In the current study, we have evaluated the interaction of CPE with granule content proteins using low pH-induced aggregation as an assay. The results indicate that CPE has the capacity to interact with granule content proteins other than prohormones as pH is titrated to the range of the TGN and ISG. Thus, in its ability to interact with content proteins, CPE resembles other membrane-associated proteins including peptidyl glycine α-amidating mono-oxygenase, dopamine β-hydroxylase, and pancreatic glycoprotein 2 (3, 5), suggesting that these interactions are important in the segregation of granule membrane and content proteins during granule formation and maturation.

EXPERIMENTAL PROCEDURES

Antibodies and Purified Granule Content Proteins—Purified bovine CPE (mixture of membrane forms) and rabbit antibodies specific for the amino- and carboxy-terminal peptides were generously provided by Dr. L. Fricker (Albert Einstein College of Medicine, Bronx, NY) and were prepared as reported previously (23). Rabbit antibodies to chromogranin B (CgB) were a gift from Dr. R. Angeletti (Albert Einstein College of Medicine). Rabbit IgG, bovine insulin, and ovine prolactin (Prl) were purchased from Sigma. Human growth hormone (GH) was obtained from the National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Pituitary Program, whereas rabbit antibody was purchased from Accurate Chemical (Westbury, NY). N-POMC, synthesized by Phoenix Pharmaceuticals (Mountain View, CA), was a gift from Y. P. Loh (National Institutes of Health, Bethesda, MD).

Preparation of Granule Content Proteins—Bovine adrenal chromaffin and pituitary granules were prepared as described previously (3). Granules were lysed by freeze-thawing and sonication in 100 mM KCl, 25 mM HEPES, pH 7.5, in the presence of protease inhibitors. The released content proteins were recovered from the supernatant after centrifugation and desalted on Bio-Gel P-6 DG columns (Bio-Rad).

In Vitro Aggregation Assay—The assay was conducted as described previously (3). In brief, 50-μl samples containing granule content or the proteins of interest in 5 mM HEPES, 10 mM 4-morpholineethanesulfonic acid, pH 7.5, and 0.016% Triton X-100, with KCl or CaCl2 where indicated, were centrifuged for 30 min, and the pellet was discarded. The supernatants were then diluted slowly to the indicated pH values by adding 0.125 N HCl while vortexing. The pH was measured with a microelectrode (Microelectrodes Inc., Bedford, NH). CPE was generally used at a concentration of 1.3 μg/ml, which is well below the threshold necessary to induce self-aggregation at this pH (23). GEMSA (Calbiochem) was added in some experiments after the prespin and 20 min before pH titration. After incubation for 30 min at 23 °C, the reaction mixture was centrifuged and separated into the supernatant and pellets. Centrifugation in most cases was for 30 min at 15,000 × g in an Eppendorf centrifuge. In experiments in which adrenal chromaffin extracts were incubated in the presence of calcium, centrifugation was conducted at 56,000 × g in a TL100 ultracentrifuge, which is required for efficient recovery of CgA (3). CPE aggregation itself could be induced at concentrations of 40 μg/ml, with pellets recovered by...
whether CPE could interact with pituitary content proteins, an in vitro aggregation assay was used (3). This assay allows the measurement of weak but specific protein-protein interactions at mildly acidic pH that are difficult to detect by more direct procedures. The pH of pituitary extracts was slowly acidified, and CPE was detected by immunoblotting. As shown in Fig. 1, CPE aggregated effectively (>90%) at pH 5.8 but remained almost entirely in the unaggregated supernatant at pH 7.5. Similarly, as we have previously demonstrated (3), other pituitary content proteins, including Prl and GH, aggregate as the pH is reduced. This observation was not a consequence of the homophilic self-aggregation of CPE, which is known to occur at pH 5.5 and high concentrations of protein (23). CPE alone at levels (1.5 μg/ml) comparable with those present endogenously (1.5 μg/ml estimated from densitometric scanning) in the pituitary samples (Fig. 1, compare lanes 2 and 10), did not aggregate either at pH 7.5 or pH 5.8 (<1% in this experiment, never >6% in any experiment). However, addition of exogenous CPE (membrane form) to the pituitary extract led to the precipitation (>90%) of both endogenous and exogenous CPE (Fig. 1, lanes 7 and 8). Quantitative assessment of CPE aggregation in the pH range 5.4–7.5 is shown in Fig. 1 (right panel). CPE aggregation was more efficient than that of Prl and GH with a half-maximal value ~6.0. Although our gel system did not completely separate the different forms, much of the endogenous CPE (membrane form) was recovered almost exclusively in the supernatant at pH 7.5. Prl self-aggregation as the pH is titrated from 74% without salt to 65% with KCl (Fig. 4) or from 63%; range, 56–69% for GEMSA; n = 2). The addition of KCl did lower the efficiency of aggregation somewhat, but the effects were small, with the amount of CPE in the pellets decreasing from 74% without salt to 65% with KCl (Fig. 4) or from 42 to 32% in another experiment (data not shown).

CPE Co-precipitates with Prolactin and Insulin—Prolactin is the major component of the pituitary granule extracts. We have previously shown that Prl self-aggregates as the pH is titrated to <6.5 (3). It was therefore of interest to determine whether CPE and Prl could interact directly. CPE was incubated with Prl as the pH was titrated to 5.8. As shown in Fig. 5, in the absence of Prl, CPE did not aggregate appreciably (<4%) at either pH (lanes 1 and 3), whereas the presence of Prl, CPE was recovered almost exclusively in the supernatant at pH 7.5 (99%) and in the pellet at pH 5.8 (mean, 95%; range, 94–97%; n = 2) together with Prl (mean, 47%; range, 42–52; n = 2). This
interaction also was observed in the presence of 140 mM KCl (data not shown), indicating that the interaction is relatively strong.

The interaction of CPE with insulin, another hormone that undergoes self-aggregation at acidic pH (8), was also assessed. Insulin aggregates effectively as pH is titrated to <6.5. In fact, >99% of insulin is recovered in the pellet fraction at pH 5.8 under our standard assay conditions (data not shown). It proved necessary, however, to include 140 mM KCl in the assay mixtures to effectively prevent IgG, a marker of the constitutive secretory pathway, from co-aggregating to some extent. Fig. 6 shows the results of an experiment in which CPE and IgG were mixed with insulin. In the absence of insulin, CPE did not aggregate significantly at either pH (Fig. 6, lanes 1 and 3), whereas in the presence of insulin, CPE was recovered exclusively in the supernatant at pH 7.5 (Fig. 6, lane 5) and at pH 5.8 (Fig. 6, lane 7), primarily in the pellet (81 ± 6%; n = 3). IgG remained almost entirely in the supernatant even in the presence of insulin (Fig. 6, lanes 5 and 7, bottom panels). The amount of insulin aggregation could not be directly quantitated using SDS-PAGE. However, in experiments conducted in the absence of added CPE and measuring total protein recoveries, 64 ± 3% (n = 3) of insulin was found in the pellet fraction under these assay conditions.

N-POMC Does Not Inhibit the Aggregation of CPE with Prolactin or Insulin—As noted in the Introduction, CPE has been shown to bind to a peptide corresponding to the amino-terminal 26 amino acids of POMC at pH 5.5 (14). The question therefore arises of whether CPE has a single binding site capable of interacting with a number of different hormones, including Prl and insulin. To test this hypothesis, N-POMC was included in the assays together with CPE before pH titration. To maximize the ratio of the peptide to the precipitating hormones, much lower amounts of insulin (40 µg/ml) and Prl (80 µg/ml) were used, and the assay protocol was modified to recover a higher proportion of the aggregates (see “Experimental Procedures”). Fig. 7 shows that under these conditions CPE when incubated alone remained in the supernatant fraction (≤6%) in the presence or absence of N-POMC (top panel). In the presence of insulin (Fig. 7, middle panel), CPE aggregated well in the absence or presence of the peptide (88 versus 80%, respectively, in this experiment; 95 versus 97% in a second experiment). Similarly, in the presence of Prl (Fig. 7, bottom panel) the amount of CPE recovered in the pellet was not affected by N-POMC (56 without versus 59% with peptide in this experiment; 86 versus 93% in a second experiment). It should be pointed out that N-POMC was present in ~5-fold molar excess in the case of insulin, ~11-fold in the case of Prl, and in >1500-fold excess of CPE itself. Hence we conclude that the sites of interaction of CPE with Prl and insulin are distinct from any involved in its binding to N-POMC.

![Fig. 3. Low pH induced aggregation of CPE with the pituitary granule content proteins is not dependent on its enzymatic activity and occurs at physiological salt concentrations. A, 0.5 µM GEMSA, an inhibitor of CPE activity, was added to sample mixtures 20 min before titration to pH 5.8 (lanes 3 and 4). B, the aggregation assay was performed at pH 5.8 without added KCl (lanes 1 and 2) or in the presence of 140 mM KCl (lanes 3 and 4). The assays were performed and the samples were analyzed as described in Fig. 1. Top panels, CPE alone; middle panels, aggregation of CPE; bottom panels, aggregation of Prl and GH under the same conditions. P, pellet; S, supernatant.](image)

![Fig. 4. Low pH induced aggregation of CPE with the chromaffin granule content proteins is not dependent on its enzymatic activity and occurs at physiological salt concentrations. Aggregation assays in the presence of 0.5 µM GEMSA (A) or 140 mM KCl (B) were also performed using adrenal chromaffin granule content with exogenous CPE (1.3 µg/ml) added (lanes 3 and 4). In each case, parallel assays were conducted under the standard conditions (lanes 1 and 2). The aggregation assays were performed exactly as described in Fig. 3, i.e. in the absence of added CaCl₂ and with centrifugation at 15,000 × g. Top panels, purified CPE alone; middle panels, CPE aggregation in the adrenal extract; bottom panels, aggregation of the chromaffin granule content proteins. P, pellet; S, supernatant.](image)

![Fig. 5. CPE co-precipitates with prolactin. CPE was incubated in the aggregation assay in the presence (bottom panels) or absence (top panels) of Prl, which homophillically self-aggregates at mildly acidic pH. The pH was titrated to 5.8 where indicated (lanes 3 and 4). Pellet (P, odd numbered lanes) and supernatant (S, even numbered lanes) samples were collected and analyzed as described in Fig. 1, with CPE identified by immunoblotting and Prl by Ponceau S staining (bottom).](image)

![Fig. 6. CPE co-precipitates with insulin. CPE (top panels) was incubated in the aggregation assay (with 140 mM KCl added) in the absence (lanes 1–4) or presence (lanes 5–8) of bovine insulin (1 mg/ml). As a control for nonspecific binding, rabbit IgG (1.5 µg/ml), a constitutively secreted protein that should not interact with insulin, was incubated in the absence (lanes 1–4) or presence (lanes 5–8) of insulin in another series of tubes (bottom panels). The pH was titrated to 5.8 where indicated (lanes 3, 4, 7, and 8), which induces of insulin to precipitate. Pellet (P, odd numbered lanes) and supernatant (S, even numbered lanes) samples were collected and analyzed as described in Fig. 1, with CPE and IgG identified by immunoblotting.](image)
CPE (1.3 μg/ml) was incubated either alone (top panels) or with 40 μg/ml insulin (middle panels) or 80 μg/ml PRL (bottom panels). N-POMC (120 μg/ml) was included in one set of samples (lanes 3 and 4), whereas in the other set an equal volume of buffer was included (lanes 1 and 2). The pH was reduced in all samples to 5.8, and precipitates were recovered by centrifugation at 100,000 × g for 30 min. The entire pellet (P) and in this case 50% of the supernatant (S) fractions were then subjected to SDS-PAGE and immunoblotting as described in Fig. 1.

**DISCUSSION**

Acidification is thought to play a critical role in secretory granule formation. Incubation of secretory cells with high concentrations of lysosomotropic amines (2, 25–28) or with bafilomycin A1 (29), an inhibitor of vacular ATPases, leads to inefficient packaging of granule content proteins as well as inefficient prohormone processing and ISG maturation. The aggregation of CPE with granule proteins from pituitary and adrenal gland was a measure of its ability to bind to content proteins and is consistent with a role for acidic pH in the interaction of content proteins with the membranes during granule formation. The pH dependence in vitro is consistent with initiation of aggregation in the environment of the TGN (5.9–6.2) (30, 31), with the process continuing even more vigorously in ISGs (5.5–6) (2). Given the fact that the concentrations of content proteins are much higher (>100 mg/ml) in secretory granules than those used in our assay, it is reasonable to propose that stronger and more significant interactions between CPE and granule content proteins would take place in vivo in the TGN, where protein concentration would also be expected to be high.

An unexpected observation was that CPE aggregated well in the chromaffin granule extracts in the absence of added calcium at pH 5.8. CgB and higher molecular weight components were also present (16, 19). The pH was reduced in all samples to 5.8, and precipitates were recovered by centrifugation at 100,000 × g for 30 min. The entire pellet (P) and in this case 50% of the supernatant (S) fractions were then subjected to SDS-PAGE and immunoblotting as described in Fig. 1.

# Acknowledgments

I thank G. Kicska, M. Jackson, and Y. Jin for expert technical assistance, Dr. D. Sabatini for continued encouragement and support, Dr. Y.-P. Loh for the gift of N-POMC, and Drs. L. Fricker and L. Devi for generously providing reagents and advice.

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