Sorting of carboxypeptidase E to the regulated secretory pathway requires interaction of its transmembrane domain with lipid rafts

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Carboxypeptidase E (CPE) functions as a regulated secretory pathway sorting receptor for several prohormones, including pro-opiomelanocortin (POMC), proenkephalin and proinsulin. The association of CPE with lipid rafts in the *trans*-Golgi network and secretory granule membranes is necessary for its sorting receptor function. We now provide evidence that a domain within the C-terminal 25 residues of CPE functions as a signal for both raft association and the sorting of CPE to the regulated secretory pathway. A fusion protein containing the extracellular domain of the human interleukin-2 receptor Tac (N-Tac) and the C-terminal 25 amino acids of CPE was transfected into Neuro2A cells. This fusion protein floated in sucrose density gradients, indicating raft association, and co-localized with chromogranin A (CGA), a secretory granule marker. To define further a minimum sequence required for raft association and sorting, deletion mutants of CPE that lacked the C-terminal four or 15 residues (CPE-Δ4 and CPE-Δ15 respectively) were transfected into a clone of CPE-deficient Neuro2A cells. In contrast with full-length CPE, neither CPE-Δ4 nor CPE-Δ15 floated in sucrose density gradients. The sorting of both CPE-Δ4 and CPE-Δ15 to the regulated secretory pathway was impaired, as indicated by significantly increased basal secretion and a lack of response to stimulation. Additionally, there was a significant decrease in the co-localization of mutant CPE immunofluorescence with CGA when compared with full-length CPE. Finally, the sorting of the prohormone POMC to the regulated pathway was impaired in cells transfected with either CPE-Δ4 or CPE-Δ15. We conclude that the sorting of CPE to the regulated secretory pathway in endocrine cells is mediated by lipid rafts, and that the C-terminal four residues of CPE, i.e. Thr^{431}-Leu-Asn-Phe^{434}, are required for raft association and sorting.

Key words: carboxypeptidase E, intracellular protein sorting, lipid rafts, pro-opiomelanocortin, regulated secretory pathway, sorting receptor.

INTRODUCTION

Endocrine cells synthesize and secrete peptide hormones in a highly regulated manner. Peptide hormones and their processing enzymes are synthesized initially as prohormones that are targeted to the regulated secretory pathway (RSP) via the *trans*-Golgi network (TGN). During the sorting process, proteins destined for the RSP are separated from constitutive and lysosomal proteins and are packaged into budding immature secretory granules. A small subset of constitutive proteins also enters immature secretory granules, and is removed through a process termed constitutive-like secretion [1]. Prohormones then undergo endoproteolysis to yield their constituent peptide hormones, which are then stored in mature secretory granules until secretion is triggered in a Ca^{2+}-dependent manner.

The process of sorting of prohormones and other regulated secretory proteins to the RSP may involve multiple mechanisms. Most regulated secretory proteins, such as chromogranin A (CGA) and chromogranin B [2] and the prohormones pro-opiomelanocortin (POMC), prolactin and proinsulin [3] aggregate in a Ca^{2+}-dependent manner. The mechanism of sorting may involve the interaction of protein aggregates with sorting receptors, such as carboxypeptidase E (CPE) [4]. Aggregates of prohormones may also bind directly to membrane lipids in the TGN. It has been proposed that lipid rafts form a sorting platform at the TGN, at which prohormones aggregate and immature secretory granules form [5]. We have shown that the sorting of POMC and CPE requires the association of CPE with cholesterol-rich lipid rafts at the TGN [6]. Similarly, the processing enzyme prohormone convertase 2 (PC2), which aggregates in a pH- and Ca^{2+}-dependent manner [7], associates with sphingolipid components of rafts via proPC2-(45–84) [8]. Therefore lipid rafts may play a role in the aggregation- or receptor-mediated sorting of proteins destined for the RSP, although the mechanism is not known.

In the TGN, CPE exists primarily as a 55 kDa lipid-raft-associated membrane-bound protein [6]. This form is sorted to the RSP and converted into a soluble prohormone processing enzyme within secretory granules by removal of its C-terminal 25 amino acids [9]. This C-terminal region anchors CPE to membranes [10] by spanning the membrane [11], and some regions within this domain may also sort CPE to the RSP [12]. Thus the mechanism of sorting of CPE is hypothesized to include its membrane-binding region and association with lipid rafts.

In the present study, we show that a fusion construct containing the final 25 residues of CPE was raft-associated and targeted to the RSP in Neuro2A cells, demonstrating that this region of CPE can function as a domain for both raft association and

Abbreviations used: CGA, chromogranin A; CPE, carboxypeptidase E; CPE-FL, full-length CPE; CPE-Δ4 and CPE-Δ15, deletion mutants of CPE lacking the C-terminal four and 15 residues respectively; NTac, extracellular domain of Tac (the human interleukin-2 receptor); NTac–CPE25, NTac fused to the C-terminal 25 amino acids of CPE; PC1, PC2 and PC5/6, prohormone convertases 1, 2 and 5/6 respectively; POMC, pro-opiomelanocortin; RFP, red fluorescent protein; RSP, regulated secretory pathway; TGN, *trans*-Golgi network.

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sorting. We also identify a small, four-residue sequence in the C-terminal region of CPE that is necessary to target CPE to lipid rafts, and this correlates with the sorting of CPE to the RSP. Consequently, removal of this sequence results in the loss of function of CPE as a sorting receptor for POMC. We conclude that raft association is a necessary prerequisite for the sorting of CPE to the RSP, and that both events are mediated by the C-terminus of CPE.

MATERIALS AND METHODS

Construction of the N-Tac–(CPE membrane-binding domain) fusion protein (N-Tac–CPE25)

An N-Tac (amino acids 1–219 of the human interleukin-2 receptor, comprising the extracellular domain) cDNA was generated by PCR using the following primers: sense, 5′-GG-CGGCGGATCCATGGATTCATACCTGCTGATG-3′; antisense, 5′ - CCGCCGGAATTCATCTGTTGTTGTGACGAG-GCAGG-3′. The sense primer introduced a *Bam*HI site at the 5′ end and the antisense primer introduced an *Eco*RI site at the 3′ end of the PCR fragment. A pCD8.1 plasmid containing full-length Tac cDNA served as template DNA (a gift from Juan Bonifacino, NICHD/NIH, Bethesda, MD, U.S.A.) [13]. The fusion construct N-Tac–CPE25 cDNA, containing amino acids 453–477 of CPE fused to the C-terminal end of N-Tac, was generated by PCR with the following primers: sense, 5′-GCCGCC-GAATTCTTCTCTGAAAGGAAG-3′; antisense, 5′-CGGCG-GTCTAGATTAAAAATTCAAAGTTTCTG-3′. A pcDNA3.1 plasmid containing full-length mouse CPE cDNA served as template. The sense primer introduced an *Eco*RI site at the 5′ end, while the antisense primer introduced an *Xba*I site at the 3′ end. After gel purification, the PCR fragment of N-Tac alone was inserted into pcDNA3.1 at *Bam*HI and *Eco*RI sites to generate the N-Tac/pcDNA3.1 plasmid. The PCR fragment encoding the C-terminal 25 amino acids of CPE (amino acids 453–477) was inserted into the N-Tac/pcDNA3.1 plasmid at *Eco*RI and *Xba*I sites to generate the N-Tac–CPE25/pcDNA3.1 construct. The sequences of the plasmid cDNAs were verified by sequence analysis (Midland Certified Reagent Co., Midland, TX, U.S.A.).

Construction of CPE C-terminal deletion proteins

A three-piece ligation protocol was used for construction of C-terminally truncated CPE mutants. A cDNA fragment containing sequences encoding the signal peptide, pro-region and N-terminal 145 amino acids of CPE (fragment A) was generated by restriction digestion of full-length mouse CPE cDNA with *Kpn*I and *Bgl*II. Fragment B was generated by PCR and consisted of residues 146–437 [full-length CPE (CPE-FL)], residues 146–433 (CPE-Δ4) or residues 146–425 (CPE-Δ15) of CPE, all with a *Bgl*II site at the 5′ end and an *Eag*I site introduced at the 3′ end. Fragment A and fragment B (CPE-FL, -Δ4 or -Δ15) were inserted into pcDNA3.0 at the *Kpn*I and *Not*I sites to generate the plasmids pcDNA-CPE-FL, pcDNA-CPE-Δ4 and pcDNA-CPE-Δ15 respectively. Purified cDNA was sequenced to verify the deletions.

Construction of fusion protein comprising POMC and red fluorescent protein (RFP)

Human POMC cDNA was generated by PCR with *Nhe*I and *Bam*HI restriction sites introduced at the 5′ and 3′ ends respectively. This fragment was inserted in-frame into the PdsRed1-N1 plasmid (Clontech, Palo Alto, CA, U.S.A.), which codes for a RFP. The resulting plasmid, POMC–RFP, was sequenced through the junction of POMC and RFP to verify the in-frame insertion of POMC.

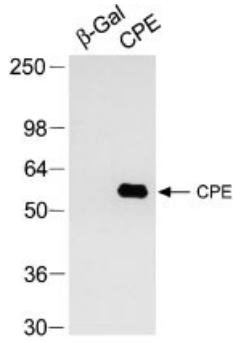

Figure 1 Clonal variant of wild-type Neuro2A cells that does not express CPE

Neuro2A cells were transfected with β-galactosidase (left lane) or full-length human CPE (right lane) and assessed for CPE expression by Western blot using an antibody directed against the N-terminal region of CPE. A 4 μg sample of protein was loaded in each lane, and equal loading was verified by Ponceau S staining of the membrane after transfer.

Cells and secretion studies

Neuro2A cells were obtained from A.T.C.C. (Manassas, VA, U.S.A.) and maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum (Life Technologies, Rockville, MD, U.S.A.). One clonal variant was found to be deficient in CPE expression by Western blot analysis of cell lysates (see Figure 1), and was therefore used in the present study. Cells were transfected with cDNA constructs using LIPOFECTAMINE 2000™ (Invitrogen, Carlsbad, CA, U.S.A.). For secretion studies, cells were plated in six-well dishes in triplicate. Following transfection overnight, media were removed and cells were rinsed twice with warm PBS. Cells were then preincubated for 2 h in basal release medium (10 mM Hepes, 129 mM NaCl, 5 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 2.8 mM glucose, pH 7.4). This medium was removed and replaced with either basal or stimulation medium (10 mM Hepes, 79 mM NaCl, 5 mM $NaHCO_3$, 50 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 2 mM $BaCl_2$, 2.8 mM glucose, pH 7.4) in parallel sets of wells, and cells were incubated for 30 min. Both cells and media were assessed for CPE immunoreactivity by Western blot analysis using the N-terminal antibody CPE 2-6. Blots were quantified using ImageQuant and NIH Image software. For analysis of stimulated secretion, untreated and treated cells showed the same levels of intracellular CPE as assessed by Western blot; therefore the extent of stimulated secretion was calculated based on secreted CPE only. Basal CPE secretion was calculated as a percentage of the total CPE in both media and cell lysates. Statistical significance was calculated using Student's *t* test.

Sucrose gradient floatation

Cells transfected with CPE deletion constructs or with the N-Tac–CPE25 fusion construct were extracted in cold 1% Triton X-100 in TNE (50 mM Tris/HCl, 150 mM NaCl, 2 mM EDTA, pH 7.4) and incubated on ice for 30 min. For treatment with β-octyl glucoside, cells were extracted in 60 mM β-octyl glucoside and incubated for 30 min at 4 °C. The lysate was made up to a volume of 1.2 ml of 1.2 M sucrose (in TNE), loaded in the bottom of a centrifuge tube and overlaid with 1.6 ml of

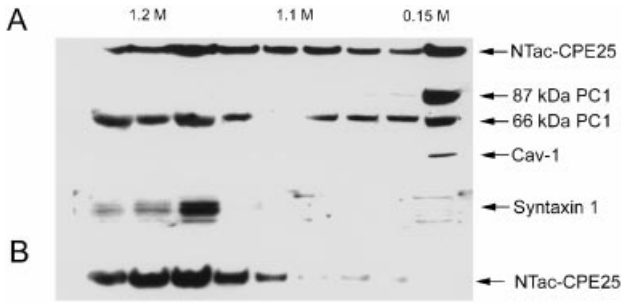

Figure 2 The membrane-binding domain of CPE is sufficient for raft association

(**A**) Neuro2A cells were transfected with N-Tac–CPE25. Cells were extracted with 1% Triton X-100 for 30 min at 4 °C. Lysates were adjusted to 1.2 M sucrose, overlaid with 1.1 M and 0.15 M sucrose, and centrifuged as described in the Materials and methods section. Fractions (0.4 ml) were collected from the bottom and analysed for (from top to bottom) CPE C-terminal region, PC1, caveolin-1 (Cav-1) and syntaxin-1 immunoreactivity by Western blot. Results are representative of those from three experiments. (**B**) Neuro2A cells transfected with N-Tac–CPE25 were extracted with 100 mM β-octyl glucoside, and CPE immunoreactivity in sucrose density gradient fractions is shown.

1.1 M sucrose and 0.8 ml of 0.15 M sucrose. Following centrifugation at 39000 rev./min in a Beckman SW 50.1 rotor for 15 h, 0.4 ml fractions were collected by bottom puncture. Samples were diluted 2-fold with TNE and protein was precipitated in 20% (v/v) trichloroacetic acid. The pellet was washed once with acetone before Western blot analysis. An antibody directed against the N-terminal region of CPE (CPE 2-5) was used for detection of CPE mutants, and an antibody directed against the C-terminal region (CPE 7-8) was used for detection of the N-Tac–CPE25 fusion protein. In order to verify raft and non-raft fractions, blots were also probed with antibodies against the raft proteins PC1 [14] and caveolin-1 [15] and the non-raft protein syntaxin-1 [16] (Santa Cruz Biotechnology).

Fluorescence immunocytochemistry

For immunocytochemical studies, Neuro2A cells were grown in two-well plastic chamber slides (Lab-Tek) until approx. 80% confluent. After overnight co-transfection with the CPE deletion mutants, N-Tac or N-Tac–CPE25 and POMC–RFP or bovine CGA, media were replaced and cells were incubated for an additional 24 h before fixation and immunostaining. The following primary antibodies were used: CPE 2-6 for N-terminal CPE immunoreactivity; CPE 7-8 for C-terminal immunoreactivity; anti-CGA, which recognizes bovine CGA [17]; anti-p115, against the Golgi protein (Transduction Laboratories, Lexington, KY, U.S.A.); a monoclonal antibody against N-Tac purified from a mouse B cell hybridoma (A.T.C.C.); and a monoclonal antibody against RFP (Clontech). For detection, the Alexa 488 (green) or Alexa 528 (red) secondary antibodies (Molecular Probes, Eugene, OR, U.S.A.) were used at a dilution of 1:1000. Fluorescent cells were viewed using a BioRad MRC-1000 confocal microscope, and images were captured and processed using LaserSharp software and Adobe Photoshop 6.0. Image collection and quantification were carried out under double-blind conditions.

RESULTS

The C-terminal 25 residues of CPE are sufficient for raft localization and sorting to the RSP

A chimaera consisting of the entire 25-residue transmembrane and cytoplasmic domains of CPE [11] fused to N-Tac (N-Tac–

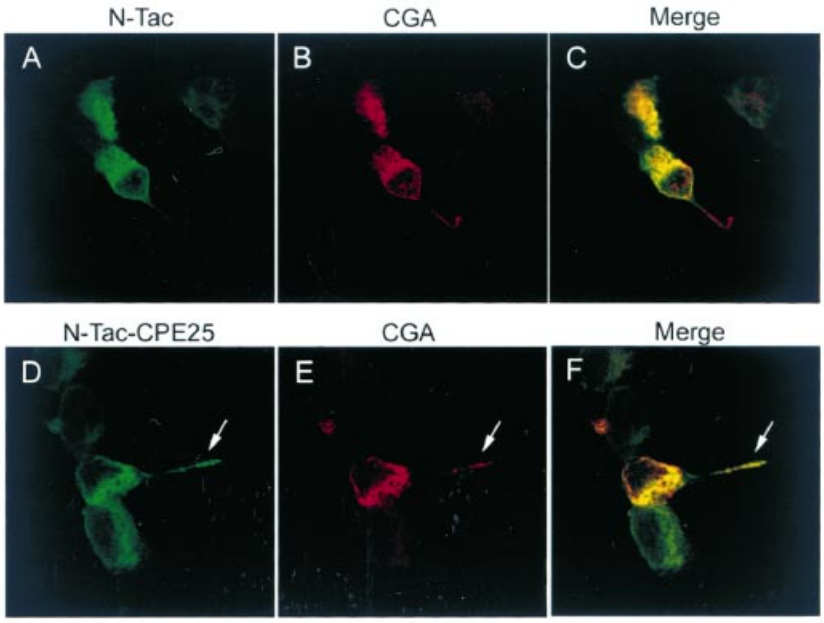

Figure 3 Localization of N-Tac–CPE25 in secretory granules

Neuro2A cells were transfected with N-Tac (**A**–**C**) or N-Tac–CPE25 (**D**–**F**). Cells were fixed, incubated with CGA- and N-Tac-specific antibodies, and then incubated with appropriate secondary antibodies conjugated to Alexa 488 (green) or Alexa 568 (red). Images were captured with the use of a scanning-laser confocal microscope and the signals were merged. The staining for N-Tac appears green, and that of CGA appears red. The arrows in (**B**) and (**C**) indicate the localization of CGA and the absence of N-Tac at the tips of cell processes. Arrows in (**D**)–(**F**) indicate co-localization of CGA and N-Tac–CPE25 staining at the tips of cell processes.

Table 1 Quantification of immunocytochemical co-localization of various forms of CPE with a secretory granule marker, CGA

Cells transfected with N-Tac or N-Tac–CPE25 were processed for the immunocytochemical localization of Tac and CGA, and images were collected as described in the Materials and methods section. Cells with processes were chosen, and the number of cells showing co-localization of punctate staining was recorded. Cells transfected with CPE-FL, CPE-Δ4 or CPE-Δ15 were processed for the immunocytochemical localization of CPE and CGA. Cells from three separate preparations were counted. Fractions (e.g. 6/44) represent the number of cells showing punctate staining of CPE that co-localized with CGA in cell processes over the total number of cells counted. Numbers in parentheses are percentages, and the means were calculated using these numbers.

CPE construct	Expt 1	Expt 2	Expt 3	Cells with co-localized punctate staining (%)
N-Tac	6/44 (13.6%)	5/49 (10.2%)	ND	11.9
N-Tac–CPE25	6/15 (40%)	3/20 (15%)	8/12 (66.7%)	40.6
CPE-FL	12/16 (75%)	15/17 (88.2%)	18/22 (81.8%)	81.7
CPE-Δ4	1/5 (20%)	3/13 (23%)	4/16 (25%)	22.7
CPE-Δ15	1/13 (7.7%)	3/8 (37.5%)	2/17 (11.8%)	19

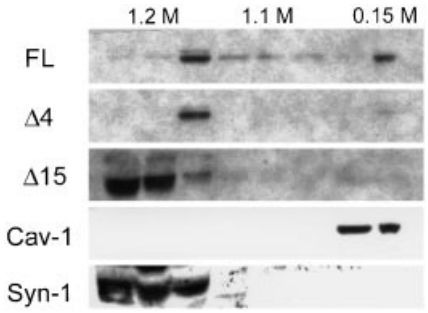

Figure 4 C-terminally truncated mutants of CPE are not raft-associated

Neuro2A cells were transfected with CPE-FL, CPE-Δ4 or CPE-Δ15. Triton X-100 lysates were adjusted to 1.2 M sucrose, overlaid with 1.1 M and 0.15 M sucrose and centrifuged as described in the Materials and methods section. Fractions (0.4 ml) were collected from the bottom and analysed for CPE N-terminal region, caveolin-1 (Cav-1) and syntaxin-1 (Syn-1) immunoreactivity by Western blot. Results are representative of those from two experiments.

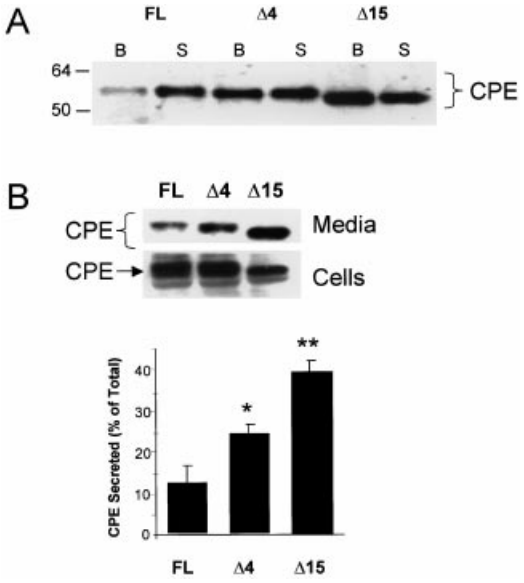

Figure 5 CPE deletion mutants are mis-sorted to the constitutive secretory pathway

Neuro2A cells were transfected with CPE deletion mutants and assayed for basal secretion and the secretory response to stimulation by K^+/Ba^{2+} by quantitative Western blot. (**A**) Representative Western blot of secreted CPE immunoreactivity after a 30 min incubation in basal (B) medium, followed by a 30 min stimulation (S) in medium containing 50 mM KCl and 2 mM $BaCl_2$. Cells in both treatment groups showed the same transfection efficiencies for each construct. (**B**) The upper panels show a representative Western blot of secreted (Media) and cellular (Cells) CPE immunoreactivity after a 2 h incubation in basal medium. The lower panel shows the densitometric quantification of both media and cells in three experiments, with secreted CPE given as a percentage of total (media + cells) CPE. Values are means ± S.E.M. ($n = 3$); $^*P < 0.01$; $^{**}P < 0.001$ compared with CPE-FL.

CPE25) was constructed. This construct was transfected into a clonal variant of the cell line Neuro2A that lacked CPE, as assessed by Western blot analysis (Figure 1), and raft association was determined by subjecting detergent-extracted cell lysates to sucrose density centrifugation. Collected fractions were assessed for C-terminal CPE immunoreactivity by Western blot. As a control, transfected cells were extracted with 60 mM β-octyl glucoside, a detergent that solubilizes raft proteins [18], including CPE [6]. Figure 2(A) shows that N-Tac–CPE25 was present throughout the gradient, with a significant portion in the low-density fractions. That the top fraction represented the raft-enriched fraction was verified by the presence of caveolin-1 in this fraction (Figure 2). Additionally, the detection of both the 66 and 87 kDa forms of PC1 in low-density fractions verified the presence of rafts in the TGN and secretory granules. In contrast, the non-raft protein, syntaxin-1, remained in high-density fractions (Figure 2), as reported by Lang et al. [16] using the same extraction buffer as in our present study. However, another study [19], using a different extraction buffer at a more acidic pH followed by homogenization of the extract, found that a small amount of syntaxin-1 floated. This could have been due to the aggregation of some syntaxin-1 with another raft-associated protein under the extraction conditions used. Solubilization with β-octyl glucoside resulted in a shift of CPE immunoreactivity to high-density fractions (Figure 2B). Therefore the C-terminal 25 residues of CPE are sufficient to target CPE to rafts. We were unable to detect N-Tac immunoreactivity by Western blot with our antibody; however, it is well documented that N-Tac does not recruit proteins to raft microdomains [20].

Fluorescence immunocytochemistry showed an exclusively perinuclear distribution of N-Tac (Figure 3A). There was no co-localization of N-Tac with CGA in the tips of the cell processes, where secretory granules are located (Figure 3C), indicating that N-Tac is not sorted to the RSP. In contrast, N-Tac–CPE25 showed a punctate staining pattern in the cell processes that overlapped with that of CGA (Figure 3F).

In order to quantify these results, cells were counted and co-localization with CGA, a secretory granule marker, was assessed. Table 1 shows that, of 93 cells transfected with N-Tac alone, very few (12 %; $n = 2$) showed co-localization of N-Tac with CGA in cell processes. In contrast, the co-localization of N-Tac–CPE25 with CGA in cell processes was observed in more cells (41 %; $n = 3$), indicating that the C-terminal 25 residues of CPE were sufficient to direct N-Tac to secretory granules.

A minimum sequence within the C-terminal region is necessary for raft-mediated sorting of CPE

In order to map the minimum sequence required for raft association of CPE, CPE-deficient Neuro2A cells were transfected with C-terminal deletion mutants of CPE. These cells were

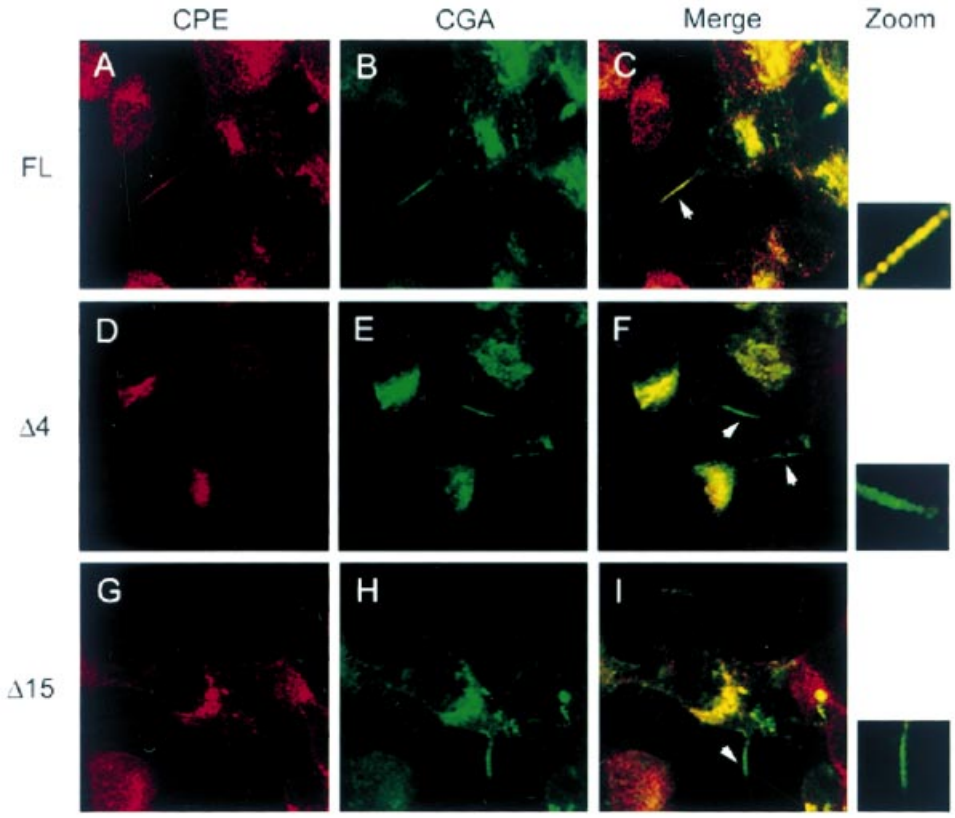

Figure 6 CPE truncation mutants are not localized in secretory granules of the RSP

Neuro2A cells were co-transfected with bovine CGA and CPE-FL (**A–C**), CPE-Δ4 (**D–F**) or CPE-Δ15 (**G–I**). Cells were fixed, incubated with antibodies against CGA and the N-terminal region of CPE, and then incubated with appropriate secondary antibodies conjugated to Alexa 488 (green) or Alexa 568 (red). Images were captured with the use of a scanning-laser confocal microscope and the signals were merged. The staining of CGA appears green, and that of CPE appears red. The arrows in (**C**) indicate co-localization of CGA and CPE staining at the tips of cell processes. Arrowheads in (**F**) and (**I**) indicate the punctate staining pattern of CGA and the absence of CPE-Δ4 and CPE-Δ15 respectively. Small panels on the right show areas of punctate staining in cell processes that have been enlarged to highlight the co-localization of CGA and CPE-FL (top) or the staining of CGA alone (middle and bottom).

extracted with cold Triton X-100 and subjected to sucrose gradient floatation. Raft association was determined for CPE mutants in which the last four (CPE-Δ4) and last 15 (CPE-Δ15) residues were deleted. As expected, a significant portion of CPE-FL floated to the top of a sucrose step gradient (density of 0.15 M sucrose), as did caveolin-1, a raft protein (Figure 4). However, both CPE-Δ4 and CPE-Δ15 mutants did not float to the top, but were found in high-density fractions containing syntaxin-1 in the same sucrose step gradient (Figure 4). These results suggest that disruption of the C-terminal end of CPE corresponding to its cytoplasmic domain interferes with its raft association.

To determine whether the same domains that conferred raft association were also necessary for the targeting of CPE to the RSP, secretion experiments and immunocytochemistry were carried out in Neuro2A cells transfected with full-length or mutant CPE constructs. The extent of stimulated secretion was calculated based on Western blot analysis of media alone (Figure 5A), since transfection efficiencies were the same for each construct (see the Materials and methods section). The secretion of CPE-FL was significantly ($P < 0.01$) stimulated, by 2.5 ± 0.01-fold ($n = 3$), in response to 50 mM KCl and 2 mM $BaCl_2$. In contrast, there was no stimulated secretion observed for either CPE-Δ4 (1.08 ± 0.03-fold, $n = 3$) or CPE-Δ15 (0.82 ± 0.03-fold, $n = 3$). For both deletion mutants, basal secretion was significantly elevated, by 1.6 ± 0.2-fold ($P < 0.01$) for CPE-Δ4 and by 2.6 ± 0.2-fold ($P < 0.001$) for CPE-Δ15 (Figure 5B), indicating that removal of as few as the final four residues was sufficient to cause mis-sorting and constitutive secretion of CPE.

Immunocytochemistry showed that CPE-FL had a punctate staining pattern in the cell processes through to the tips that co-localized with CGA, a secretory granule protein (Figures 6A–6C), indicating that CPE-FL is packaged into secretory granules. This co-localization was evident in most of the cells examined (Table 1). In contrast, both CPE-Δ4 (Figures 6D–6F) and CPE-Δ15 (Figures 6G–6I) had a largely perinuclear distribution that did not co-localize with the punctate staining pattern of CGA in the cell processes in most of the cells examined (Table 1). Only cells with processes were counted, since cells without processes all showed perinuclear staining.

CPE requires its raft association/sorting domain to sort POMC to the RSP

We have shown previously, by *in vivo* cholesterol depletion by lovastatin treatment [6], that CPE requires association with cholesterol-rich lipid rafts in order to sort its prohormone cargo, POMC, to the RSP. Since lovastatin can potentially affect organelle biogenesis [21], we have in the present study examined

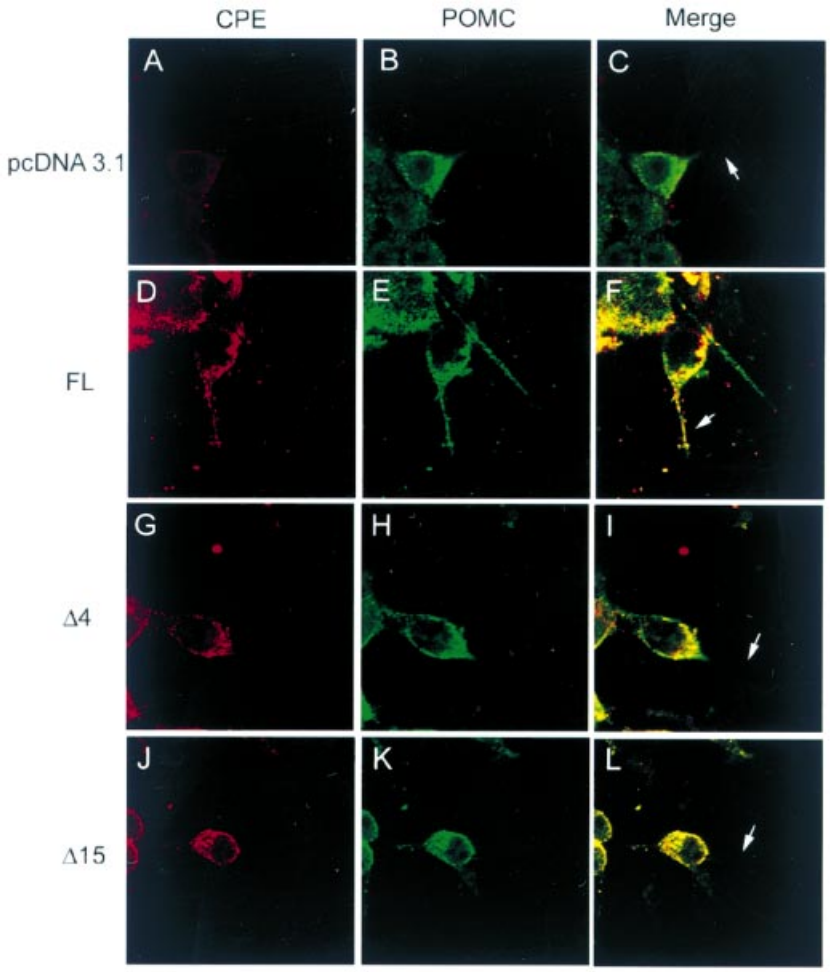

Figure 7 Sorting of POMC to the RSP requires raft-associated CPE

Neuro2A cells were transfected with POMC–RFP alone (**A**–**C**), or were co-transfected with POMC–RFP plus CPE-FL (**D**–**F**), CPE-Δ4 (**G**–**I**) or CPE-Δ15 (**J**–**L**). Cells were fixed, incubated with antibodies against RFP and the N-terminal region of CPE, and then incubated with appropriate secondary antibodies conjugated to Alexa 488 (green) or Alexa 568 (red). POMC–RFP fluorescence appears green, and that of CPE appears red. The arrows indicate the presence of cell processes. Punctate staining of POMC–RFP in the cell processes is seen only in cells transfected with CPE-FL (**D**–**F**). Each panel is representative of 30–90 images.

Table 2 Quantification of POMC–RFP localization in secretory granules in the presence of CPE constructs

Cells transfected with POMC–RFP and the indicated CPE constructs were processed for immunocytochemical localization of POMC and CPE, and images were collected as described in the Materials and methods section. Cells with processes were chosen, and the number of cells showing punctate staining of POMC in the cell processes was recorded. Cells from three separate preparations were counted. Fractions (e.g. 8/27) represent the number of cells showing punctate staining of POMC–RFP in cell processes over the total number of cells counted. Numbers in parentheses are percentages, and the means were calculated using these numbers.

CPE construct	Expt 1	Expt 2	Expt 3	Cells showing punctate staining of POMC (%)
No CPE	8/27 (29.6%)	4/27 (14.8%)	4/15 (26.7%)	23.7
CPE-FL	10/13 (76.9%)	13/20 (65.0%)	14/19 (73.7%)	71.9
CPE-Δ4	6/15 (40.0%)	3/9 (33.3%)	2/10 (20.0%)	31.1
CPE-Δ15	2/13 (15.4%)	2/15 (13.3%)	1/13 (7.7%)	9.8

the ability of CPE deletion mutants, which are not raft-associated, to sort POMC. Neuro2A cells were transfected with POMC–RFP alone, or co-transfected with POMC–RFP plus CPE-FL, CPE-Δ4 or CPE-Δ15. The targeting of POMC–RFP to the RSP was determined by immunocytochemistry. Neuro2A cells transfected with POMC–RFP alone showed a largely perinuclear distribution of POMC–RFP fluorescence in the absence of CPE (Figures 7A–7C). In contrast, in Neuro2A cells co-transfected with CPE-FL, there was a punctate staining pattern of POMC–RFP fluorescence along and at the tips of the cell processes that co-localized with that of CPE, indicating localization in secretory granules (Figures 7D–7F). In cells transfected with CPE-Δ4 (Figures 7G–7I) or CPE-Δ15 (Figures 7J–7L), POMC–RFP had a largely perinuclear localization: $\sim 69\%$ of cells expressing CPE-Δ4 and $\sim 90\%$ of cells expressing CPE-Δ15 showed no punctate staining of POMC–RFP, indicating a lack of sorting to the RSP in the majority of these cells (Table 2).

DISCUSSION

The association of membrane proteins with lipid raft microdomains has been implicated in their proper trafficking and compartmentalization. Lipid rafts were originally proposed to

play a role in the sorting of proteins from the TGN to the apical side of polarized epithelial cells [18,22]. Rafts may also be important in the sorting of glycosylphosphatidylinositol-anchored proteins to, and their retention in, the recycling endocytic compartment [23,24].

The sorting of membrane proteins to the RSP in endocrine and neuroendocrine cells may also be dependent on interaction with raft lipids. Three prohormone processing enzymes, CPE [6], PC2 [8] and PC1/3 [14], have been shown to be raft-associated membrane proteins. Indirect evidence through *in vivo* cholesterol or sphingolipid depletion has suggested that raft association may be necessary for the sorting of CPE [6] and PC2 [8] to the RSP. However, cholesterol and sphingolipid depletion can also interfere with secretory granule biogenesis [21]. Therefore we have in the present study generated deletion mutants of CPE and a N-Tac–CPE chimaeric protein to examine the role of raft association in the sorting of CPE in cells with an intact RSP. These experiments have also allowed us to define a minimum sequence within CPE that is required for its raft association and sorting.

The requirement for raft association in the sorting of CPE was examined using mutants of CPE harbouring deletions within the C-terminal membrane-binding region. Deletion of the final four residues of the cytoplasmic tail of CPE (CPE-Δ4) or disruption inclusive of the transmembrane domain (CPE-Δ15) resulted in a lack of association of the mutant CPE proteins with rafts, as measured by sucrose gradient floatation. Both CPE-Δ4 and CPE-Δ15 also were not efficiently sorted to the RSP and did not sort POMC–RFP to the RSP. These results indicate that the transmembrane domain of CPE is necessary for the correct sorting of both CPE and its prohormone cargo, POMC. In addition, proper sorting of CPE requires its association with lipid rafts at the TGN, strengthening our previous results showing that cholesterol depletion *in vivo* inhibited the sorting of CPE and POMC to the RSP [6]. Since cholesterol depletion can affect secretory granule formation, an important conclusion from the present study is that non-raft-associated CPE is not sorted to the RSP even in the presence of secretory granules (as evidenced by the immunostaining pattern of the granule marker CGA). Therefore, in the presence of an intact RSP, CPE requires raft association for efficient sorting, and for its function as a sorting receptor.

Our results also demonstrate that as few as the final four residues of CPE (Thr^{431}-Leu-Asn-Phe^{434}) are necessary for its raft association and correct targeting to the RSP. Recently we showed that the C-terminal 22 amino acids of CPE form an amphipathic α-helix which is inserted in a transmembrane fashion into membranes of secretory granules, with the last four to six residues exposed from the cytoplasmic leaflet of the membrane [11]. These four residues may constitute a signal for directing CPE into lipid rafts, and may facilitate the insertion of the transmembrane domain of CPE through the lipid bilayer. Residues within transmembrane domains, as well as within cytoplasmic tails, of proteins have been shown to direct proteins into lipid rafts. It has been proposed that a stretch of amino acids showing identity with the gastrointestinal peptide sorbin can serve as a motif for the targeting of signalling proteins to lipid rafts [25]. Certain conserved residues within the transmembrane domain of influenza virus haemagglutinin are critical for the apical sorting of haemagglutinin via association with rafts [26]. Interestingly, there appears to be a strict requirement for residues in contact with the inner leaflet of the plasma membrane [27]. The motif Leu-Ile-Arg-Trp in the C-terminus of the proteolipid protein MAL (VIP17) directs this protein into rafts at the plasma membrane [28]. The B cell signalling protein CD20 is directed to rafts via a membrane-proximal four-residue sequence in the cytoplasmic C-terminal tail [29]. These results suggest that there is no apparent consensus sequence for directing proteins into rafts. Rather, residues in contact with or in close proximity to the membrane may determine interactions with lipid microdomains. We propose a similar mechanism of raft association for CPE, whereby the final four residues of the cytoplasmic tail, Thr-Leu-Asn-Phe, which are also proximal to the outer leaflet of the TGN and secretory granule membranes, direct CPE to lipid rafts in the TGN.

Our results show that raft association of CPE via its C-terminal transmembrane domain is a necessary prerequisite in the sorting of CPE. In order to demonstrate that this region of CPE alone is sufficient for raft association and sorting, a fusion protein was produced consisting of N-Tac and the C-terminal 25 residues of CPE (N-Tac–CPE25). N-Tac has been used as a reporter protein to study the trafficking of the prohormone processing enzymes peptidylglycine α-amidating monooxygenase [30], furin [31] and PC5/6B [32]. Our fusion protein, N-Tac–CPE25, was raft-associated in Neuro2A cells. Sorting of N-Tac–CPE25 was observed in 41% of cells, compared with 12% of cells expressing N-Tac alone. Therefore the C-terminal region of CPE, which contains a transmembrane domain and a short cytoplasmic tail [11], can itself mediate raft association, and is both necessary and sufficient for sorting of CPE to the RSP. However, N-Tac–CPE25 appeared to be sorted less efficiently compared with wild-type, full-length CPE. These results are similar to those obtained with an albumin–CPE fusion protein containing the final 51 residues of CPE, which was also sorted at a reduced efficiency compared with full-length CPE [12]. Taken together, results from both studies suggest that other mechanisms, in addition to raft/membrane association, may be required for efficient sorting of CPE, such as pH-dependent aggregation [3,33]. Such aggregation may be dependent on the luminal domain of CPE, and therefore may not occur to the same extent with the N-Tac–CPE25 fusion protein. Such a mechanism has been proposed for the apical sorting of placental alkaline phosphatase: association with rafts is mediated by the glycosylphosphatidylinositol anchor, followed by stabilization of the protein into rafts via its ectodomain [34]. Similarly, the transmembrane domain of CPE may be responsible for raft association, and its luminal domain may play a role in the subsequent stabilization of CPE into rafts, possibly through aggregation.

In conclusion, we have shown that interactions with lipid rafts play a critical role in the sorting of CPE, a prohormone sorting receptor and processing enzyme, to the RSP. Raft association of its C-terminal region is both necessary and sufficient to direct CPE to the RSP. Two observations support this conclusion: first, deletion of the transmembrane domain removes CPE from rafts and impairs its sorting; secondly, addition of the transmembrane domain of CPE to N-Tac results in raft association and targeting of the latter to the RSP. The mechanism of raft association may involve interactions between the juxtamembrane C-terminal four amino acids of the cytoplasmic tail of CPE and lipids in the outer leaflet of the TGN and secretory granule membranes.

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