The pro region is not required for the expression or intracellular routeing of carboxypeptidase E

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Carboxypeptidase E (CPE) is initially synthesized as a larger precursor containing an additional 14-residue propeptide that is highly conserved between human and rat. Previous studies have established that the proenzyme is enzymically active and that deletion of the pro region does not affect the expression of the active enzyme. In the present study the function of the pro region was examined both by deleting this region from CPE and by attaching this region to the N-terminus of albumin. CPE lacking the pro region is sorted into the regulated secretory pathway in AtT-20 cells, based on confocal microscopy and examination of the stimulated secretion of the protein. Stimulation of AtT-20 cells with either forskolin or phorbol 12-myristate 13-acetate induces the secretion of wild-type CPE and of CPE lacking the pro region to similar extents, indicating a similar efficiency of sorting of the mutant. When the pro region of proalbumin is replaced with the pro region of CPE followed by expression in AtT-20 cells, the protein is not sorted into the regulated pathway, based on the lack of stimulated secretion. Confocal microscopy suggests that the proCPE/albumin protein is retained in the endoplasmic reticulum to a greater extent than is proalbumin. Pulse–chase analysis indicates that the pro region of CPE is not efficiently removed from the N-terminus of albumin, and the small amount of propeptide cleavage that does occur takes place soon before secretion of the protein. In contrast, confocal microscopy indicates that the majority of the propeptide is removed from CPE, and that this cleavage occurs in the *trans*-Golgi network or soon after sorting into the secretory vesicles. Taken together, these results suggest that the pro region of CPE is not required for the expression or intracellular routeing of this protein.

INTRODUCTION

Carboxypeptidase E (CPE) is involved in the biosynthesis of peptide hormones and neurotransmitters [1,2]. CPE functions after an endopeptidase has initially cleaved the peptide precursor at specific basic amino acid-containing cleavage sites [3–5]. These cleavages occur primarily in secretory vesicles, consistent with the subcellular localization of CPE to the peptide-containing vesicles [1,2]. In addition to CPE, carboxypeptidase D may also participate in peptide processing, although the role of this second enzyme is not entirely clear [6]. Carboxypeptidase activity is necessary to remove the basic amino acids from the C-terminus of the processing intermediates [1,2]. A deficiency of CPE activity caused by a naturally occurring mutation of Ser^{202} to Pro in the Cpe^{fat}/Cpe^{fat} mouse results in the incomplete processing of peptide hormones such as insulin [7].

As with most peptidases, CPE is initially produced as a precursor, proCPE, which contains 14 additional N-terminal amino acids that are not present on the major forms of CPE found in tissues [8–10]. The pro regions of other peptidases are generally involved in the regulation of enzyme activity, the folding of the protein and/or the routeing of the protein to the correct intracellular destination. Carboxypeptidases A and B, which are in the same gene family as CPE, are produced as precursors that are inactive until their N-terminal pro regions have been cleaved [11]. Subtilisin, an endopeptidase in the same gene family as the peptide-processing endopeptidases, requires its N-terminal pro region for correct folding of the protein during biosynthesis [12]. Cathepsin D, a lysosomal endopeptidase, requires its N-terminal pro region for transport to the lysosome [13].

The 14-amino-acid pro region has been completely conserved among human, rat and mouse proCPE [9,14]. This pro sequence is moderately conserved in bovine CPE, but is absent from Anglerfish CPE [8,15]. The lack of the pro region in Anglerfish CPE suggests that this region is not essential for the production of enzyme activity or routeing of the protein into the secretory pathway. Alternatively, another region of Anglerfish CPE may compensate for the absence of the pro region. The complete conservation of this sequence between human and rat suggests that it plays a critical function.

The pro region does not function as an inhibitor of CPE; bovine proCPE has been found to be enzymically active [10,16]. In addition, this region does not appear to be needed for the folding or expression of human or rat CPE [17,18]. Manser et al. [17] deleted the pro region from human CPE and expressed the protein in the C6 glioma cell line. Interestingly, although the protein was enzymically active, it was not secreted from the cell and remained largely membrane-associated [17]. This finding suggests that the pro region is required for the transport of CPE through the secretory pathway. However, the construct used by Manser and colleagues involved the deletion of the signal peptide cleavage site, and so the aberrant secretion could merely have been due to the absence of the removal of the hydrophobic N-terminal signal peptide [17]. Recently rat CPE with a deletion of the pro region (but not the signal peptide cleavage site) was found to be secreted at high levels from the Sf9 insect cell line following baculovirus infection [18]. However, it is possible that

Abbreviations used: CPE, carboxypeptidase E; wt, wild type; HA, haemagglutinin; WGA, wheat-germ agglutinin; PMA, phorbol 12-myristate 13-acetate ('TPA'); DMEM, Dulbecco's modified Eagle's medium; BNPS-skatole, 3-bromo-3-methyl-2-(2-nitrophenylmercapto)-3*H*-indole; PAM, peptidylglycine α-amidating mono-oxygenase.

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this difference reflects the species of cell line rather than differences in the mutant construction. Furthermore, neither study used a cell line with a regulated secretory pathway, and so the effect of this deletion on the routeing of CPE could not be investigated.

The pro region has previously been hypothesized to function in the intracellular routeing of CPE, based on (i) the high conservation of this sequence among human and rat, but the lack of a clear function in folding or enzyme activity, and (ii) the finding that propeptide removal does not occur until the *trans* Golgi network or an early post-Golgi compartment, based on pulse–chase analysis [19]. In the present study we have investigated the role of this pro region in the routeing of CPE in the AtT-20 mouse corticotrophic cell line.

MATERIALS AND METHODS

Construction of plasmids

Full-length rat CPE cDNA was subcloned into *Bam*HI and *Eco*RI sites of the vector pGEM7zf (Promega). The haemagglutinin (HA) epitope (YPYDVPDYA) that is recognized by the 12CA5 monoclonal antiserum was inserted into a *Pst*I site located immediately downstream of the pentabasic propeptide cleavage site of proCPE to generate HA–CPE (Figure 1). In addition to inserting the HA epitope, an *Xba*I site (TCTAGA) was included at the 5′ end of the sequence; this added a Ser-Arg to the insert (Figure 1). PCR was performed with rat proCPE cDNA as a template to generate a 150 bp product with an *Xba*I site immediately following the signal peptide cleavage site. The 5′ region of the HA–CPE construct (*Bam*HI/*Xba*I) was replaced with this PCR fragment to generate the epitope-labelled CPEΔpro construct (Figure 1). For mammalian cell expression, the cDNAs were subcloned into the *Bam*HI/*Eco*RI sites of the pcDNA3 vector.

For construction of CPE/Alb (Figure 1), PCR was used to create an *Nsi*I site in the human albumin cDNA sequence immediately downstream of the propeptide cleavage site. Then the 5′ *Apa*I/*Pst*I fragment encoding the preproCPE sequence was subcloned into the *Apa*I and *Nsi*I sites of the modified albumin cDNA. For eukaryotic expression, the CPE/Alb cDNA was subcloned into pcDNA3. Dideoxynucleotide sequencing was performed to confirm the sequences of the mutated regions of all constructs and all regions produced by PCR.

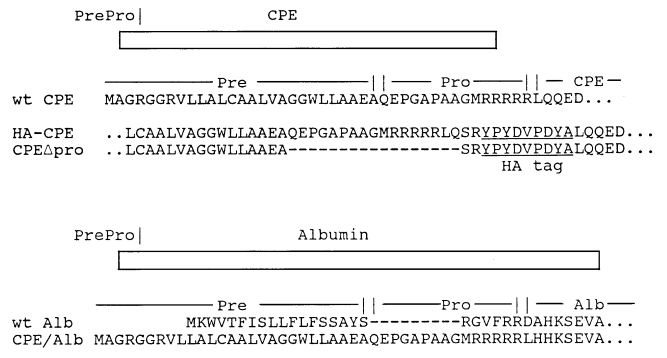

Figure 1 Diagram of wt preproCPE (wt CPE) and preproalbumin (wt Alb)

The partial N-terminal amino acid sequences are indicated for rat wt CPE, epitope-tagged CPE (HA–CPE), CPE lacking the pro region (CPEΔpro), wt human albumin (wt Alb) and a construct containing the signal peptide and pro region of CPE attached to the N-terminus of human albumin (CPE/Alb).

Transfection of AtT-20 cells

The expression plasmids were transfected into AtT-20 cells using the standard calcium phosphate procedure, as described in [20]. Stable cell lines were selected using 0.6 mg/ml geneticin (G418) as previously described [20]. For each construct, approx. 48–72 colonies were isolated and grown in 35 mm plates. Cell lines expressing the desired protein were detected by Western blot analysis using antisera raised against the HA epitope tag (a gift from Dr. Jon Backer, Department of Molecular Pharmacology, Albert Einstein College of Medicine) or human albumin (Cappel Organon Teknika Corp., Durham, NC, U.S.A.). For each construct, two or three separate subclones were selected and used for further analysis.

Immunofluorescence

Transfected and wild-type (wt) AtT-20 cells were cultured on 18 mm coverslips precoated with 1 mg/ml polylysine (Sigma). Cells were washed with PBS, fixed in 4 % paraformaldehyde for 15 min and then permeabilized for 15 min in 0.1 % Triton X-100 in PBS. After 1 h of blocking in 3 % BSA, the cells were immunostained for 1 h with the primary antisera: HA-epitope-labelled proteins were detected using a 1:10000 dilution of the mouse monoclonal antiserum 12CA5, CPE was detected using a 1:5000 dilution of a rabbit polyclonal antiserum directed against the C-terminal nine amino acids of CPE [21], and human albumin was detected using a 1:5000 dilution of a rabbit polyclonal antiserum. Cells were washed three times with PBS containing 0.2 % Tween 20 and then incubated for 1 h with a 1:200 dilution of fluorescein-labelled anti-mouse or anti-rabbit secondary antibody (Vector Laboratories Inc.) and a 1:300 dilution of rhodamine-labelled wheat-germ agglutinin (WGA). After extensive washing in PBS, immunofluorescent staining was examined using a Nikon confocal microscope.

Western blot analysis

To examine whether the CPEΔpro and CPE/Alb fusion proteins are secreted via the regulated pathway, cells were treated with 0.1 μg/ml phorbol 12-myristate 13-acetate (PMA; 'TPA'), 5 μM forskolin or control medium. After 30 min at 37 °C, the media were removed, cooled on ice and then concentrated using a Centricon microfiltration apparatus (Amicon). Protein samples were combined with SDS/polyacrylamide gel loading buffer, heated to 95 °C for 5 min, cooled, fractionated on a denaturing SDS/10 %-polyacrylamide gel and then transferred to nitrocellulose. The nitrocellulose was then probed with a 1:1000 dilution of either the 12CA5 monoclonal antiserum raised against the HA epitope tag or rabbit polyclonal antisera directed against the N-terminal region of CPE [22] or albumin. The primary antisera were detected using the standard enhanced chemiluminescence method (Amersham).

Pulse–chase analysis

Cells expressing HA–CPE or CPEΔpro were grown in 25 cm^2 flasks until 50–80 % confluent. Cells were washed twice with PBS and then incubated in Dulbecco's modified Eagle's medium (DMEM) lacking methionine for 60 min. This medium was removed and replaced with DMEM lacking non-radioactive methionine but containing 200–300 μCi of [^{35}S]methionine. Following a 30 min incubation with the radiolabel, the medium was removed and the cells were incubated in DMEM for 30, 60, 180 or 360 min. Cells were washed with PBS and then frozen in

10 mM sodium acetate, pH 5.5, with 1 mM PMSF. The cells and the media were then subjected to immunoprecipitation using the 12CA5 monoclonal antiserum. To examine the processing of CPE/Alb in AtT-20 cells, cells expressing CPE/Alb were labelled as described above and chased for the indicated times. Cells were then extracted and the albumin isolated by immunoprecipitation with an antiserum raised against human albumin (Calbiochem). After immunoprecipitation, the samples were treated with 2 mM 3-bromo-3-methyl-2-(2-nitrophenylmercapto)-3*H*-indole (BNPS-skatole) at room temperature for 48 h, loaded on a 10% polyacrylamide gel and exposed to X-ray film (Kodak). Quantification of the autoradiograms was performed using an image analysis system as previously described [23].

RESULTS

Confocal microscopy of the distribution of epitope-tagged wt CPE (HA–CPE) in non-transfected AtT-20 cells showed a pattern consistent with the expression of this protein in secretory vesicles (Figure 2, top row). CPE was enriched in the cell processes (arrow), and showed punctate staining throughout the cytoplasm. Slight accumulation of CPE was detected in a perinuclear structure that resembles the Golgi apparatus. Co-labelling of the cells with WGA, a Golgi marker [24], showed substantial overlap with the perinuclear staining of CPE (Figure 2, top right). The pattern of distribution of epitope-tagged CPE was very similar to that previously observed with endogenous CPE in AtT-20 cells [21], indicating that the epitope tag does not interfere with the distribution of CPE in these cells. The construct lacking the pro region (CPEΔpro) showed a distribution similar to that of the epitope-tagged wt CPE (Figure 2, middle row), indicating that the pro region is not required for correct intracellular distribution. An antiserum raised against the pro region of rat CPE showed intense staining of a Golgi-like structure (arrowhead), with no significant staining of the cell processes (Figure 2, bottom row). The Golgi marker WGA showed perinuclear staining that

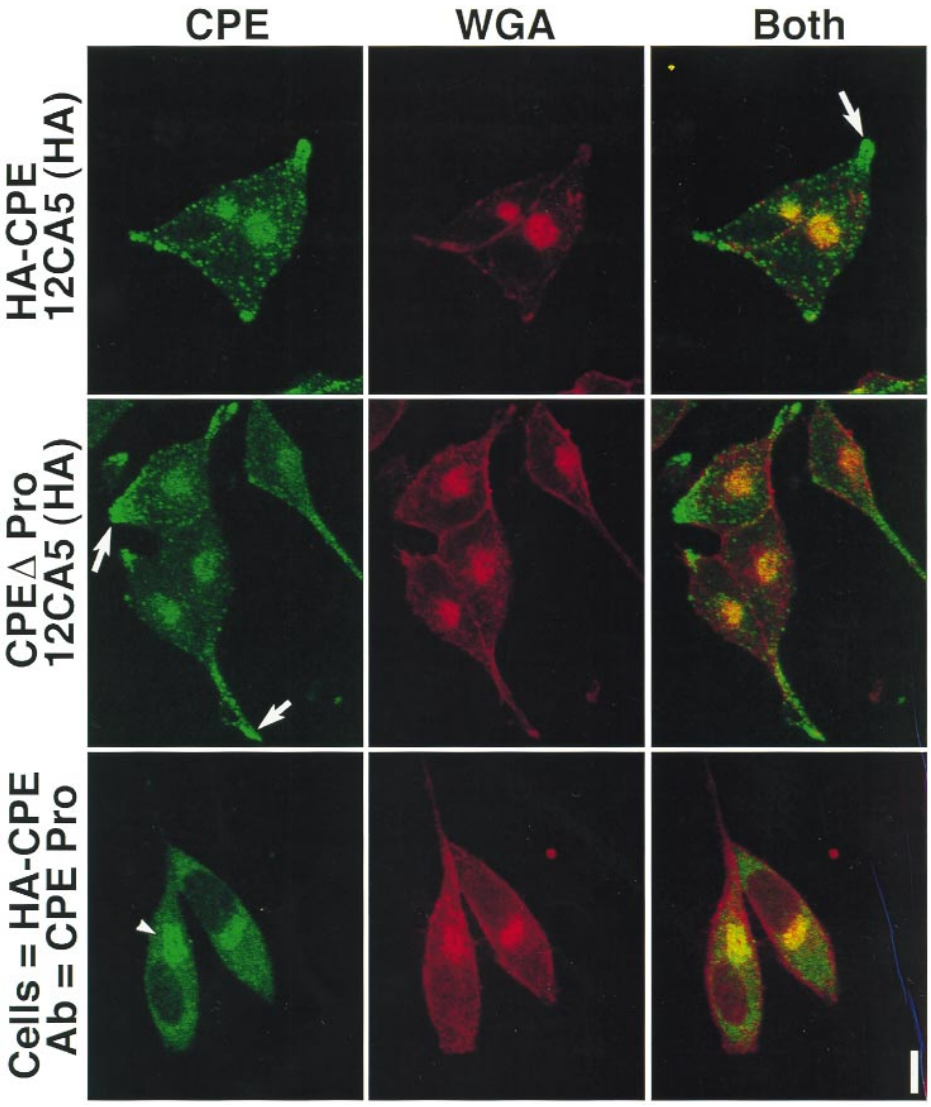

Figure 2 Confocal microscopy of AtT-20 cells expressing HA–CPE or CPE*Δ*pro

Cells were stained with the indicated antiserum against HA (12CA5) or the CPE pro region and with rhodamine-labelled WGA as described in the Materials and methods section. Images show single optical sections of the indicated antisera in green (left), WGA in red (middle) and combined images of the two (right). Areas of overlap are in yellow. Scale bar indicates 10 μm.

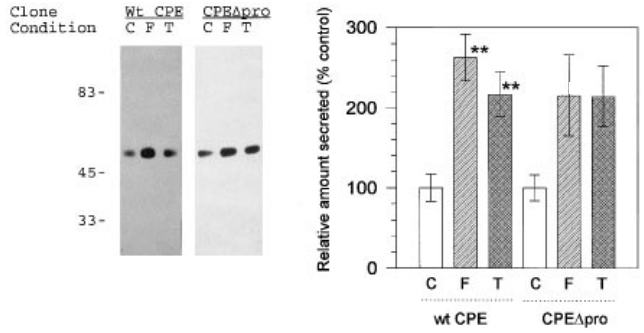

Figure 3 Western blot analysis of secretion of CPE from wt AtT-20 cells and from AtT-20 cells expressing CPE*Δ*pro

Cells were treated with 100 ng/ml PMA (T), 5 μM forskolin (F) or control medium (C) for 30 min, and the media were analysed on a Western blot as described in the Materials and methods section. Left panel: representative autoradiogram. The positions and molecular masses (in kDa) of protein standards (Bio-Rad) are indicated. Right panel: quantification of results from several experiments using three different clones of CPEΔpro-expressing cells. Error bars show S.E.M.; $n = 5$ for CPEΔpro and $n = 7$ for wt CPE. The significance of differences (Student's t test) from control media values is indicated: $^{**}P < 0.01$.

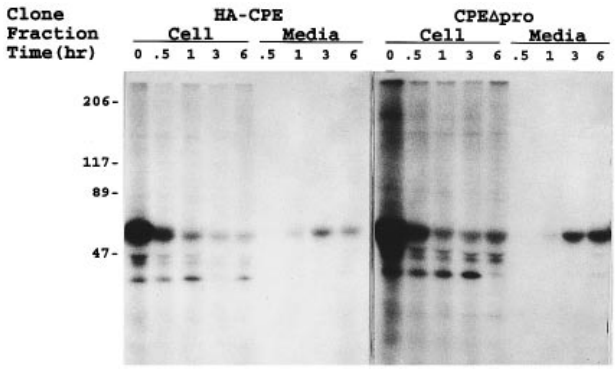

Figure 4 Pulse–chase analysis of HA–CPE and CPE*Δ*pro in AtT-20 cells

Cells were labelled with [^{35}S]Met for 30 min and chased for the indicated times, as described in the Materials and methods section. Radiolabelled CPE was immunoprecipitated using the monoclonal antiserum 12CA5, which recognizes the HA epitope. The positions and molecular masses (in kDa) of protein standards (Bio-Rad) are indicated.

overlapped substantially with that of proCPE (Figure 2, bottom right). This suggests that the pro region is removed from the epitope-tagged CPE either in the Golgi or soon after packaging into secretory vesicles.

To confirm that the CPEΔpro construct is correctly sorted into the regulated pathway, the effects of secretagogues on the secretion of CPE were examined. The secretion of CPE from wt AtT-20 cells was stimulated approx. 150 % by forskolin and 100 % by PMA (Figure 3). These secretagogues each produced a 100 % increase in the secretion of CPEΔpro (Figure 3). To investigate whether the pro region is required for the efficient transport of CPE through the secretory pathway, the cells were labelled with [^{35}S]Met and then chased in the presence of unlabelled Met. Immediately after the 30 min pulse, a large amount of radiolabelled CPE (and/or proCPE) was detected for the HA–CPE construct (Figure 4). The intensity of this labelled protein decreased substantially during the first 60 min of the chase, and then decreased less rapidly over the remainder of the chase period (Figure 4). Radiolabelled CPE was first detected in the medium after 1 h of chase, and the amount increased further with longer chase periods (Figure 4). Pulse–chase analysis of the CPEΔpro construct showed a similar profile of disappearance of the radiolabelled CPE from the cell, and its appearance in the medium (Figure 4). Similar results were obtained with an additional CPEΔpro-expressing AtT-20 cell line (not shown). These results indicate that the sorting and efficiency of transport of CPEΔpro is similar to that of epitope-tagged wt CPE. Both constructs showed a greater loss of radioactive CPE during the chase than in previous studies on endogenous CPE in AtT-20 cells [23], suggesting that the overexpression of the constructs leads to greater degradation of the protein.

The deletion analysis only shows that the pro region of CPE is not essential for the sorting of CPE into the regulated pathway; this analysis does not reveal whether the pro region contributes to the sorting. To investigate this further, the pro region of CPE was attached to the N-terminus of albumin (Figure 1) and the protein expressed in AtT-20 cells. Wt albumin has been found previously to be sorted into the constitutive pathway in this cell line [20]. Confocal microscopy showed the wt albumin to be enriched in a Golgi-like perinuclear compartment (Figure 5, top left, arrowhead). Co-staining with WGA gave a similar pattern, with substantial overlap of the perinuclear staining (Figure 5, top right). In contrast with the distribution of wt albumin, the CPE/Alb construct showed a diffuse endoplasmic reticulum-like pattern of staining in the cytoplasm in addition to the Golgi-like pattern (Figure 5, bottom left, arrowhead). Co-labelling with WGA showed overlap with the perinuclear distribution of the CPE/Alb construct, but not with the diffuse endoplasmic reticulum-like pattern (Figure 5, bottom right).

The effects of secretagogues on the release of wt albumin and CPE/Alb were examined in order to test whether either protein is sorted into the regulated pathway. Stimulation of the cells with either forskolin or PMA failed to elevate the amount of wt albumin or CPE/Alb in the medium (Figure 6). Quantification of autoradiograms from three separate determinations using separate clones of the CPE/Alb-expressing cells showed no significant increase with either secretagogue (results not shown), indicating that these proteins are not sorted into the regulated pathway. Pulse–chase analysis was used to determine the efficiency of secretion, and to test whether the pro region of CPE was removed from the albumin prior to secretion. Since the molecular masses of albumin with or without the pro region of CPE attached are too similar to be resolved on polyacrylamide gels, the immunoprecipitated protein was digested with BNPS-skatole, which cleaves proteins at Trp residues [25]. Human albumin has a single Trp residue, and should be cleaved by BNPS-skatole into a 24841 Da N-terminal fragment and a 41868 Da C-terminal fragment. If the pro region of CPE is not cleaved, the N-terminal fragment would be 26376 Da. Following a 30 min pulse with [^{35}S]Met, CPE/Alb was detected in AtT-20 cells (Figure 7). The amount of radiolabelled CPE/Alb in the cells did not change much over the first 20 min of the chase, and then decreased by over 90 % during the subsequent 40 min (Figure 7). After treatment with BNPS-skatole, fragments of approx. 26 and 42 kDa were detected (Figure 7), suggesting that the majority of this protein contains an uncleaved pro region of CPE. CPE/Alb was detected in the medium after only 10 min of chase (Figure 7). The amount of CPE/Alb in the medium increased rapidly over the first 1 h of chase. Interestingly, whereas the cellular protein only showed the presence of uncleaved propeptide, approx. 10 % of the protein in the medium appeared to have had the propeptide removed (Figure 7). The ratio of CPE/Alb to albumin (without the pro region) in the medium was

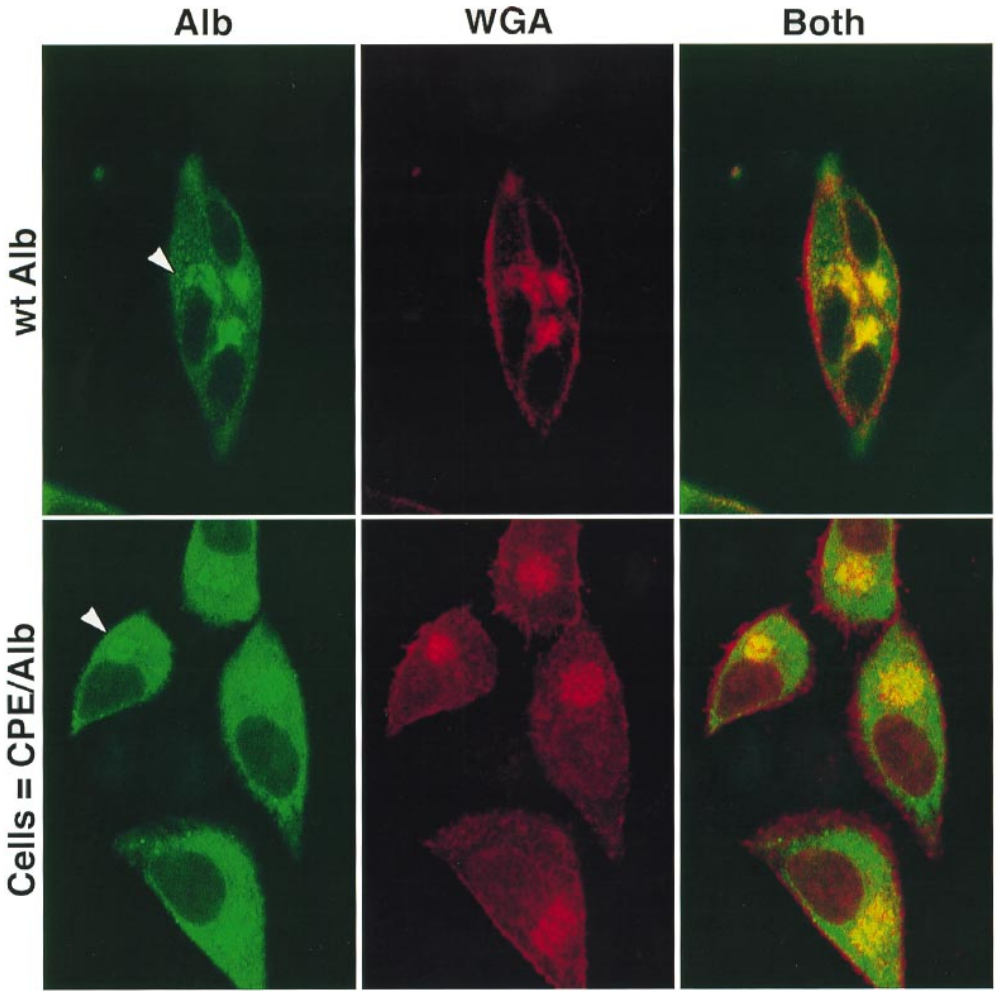

Figure 5 Confocal microscopy of AtT-20 cells expressing wt albumin (top) or CPE/Alb (bottom)

Cells were stained with an antiserum to human albumin (Alb) and with rhodamine-labelled WGA, as described in the Materials and methods section. Images show single optical sections of the antiserum in green (left), WGA in red (middle) and combined images of the two (right). Areas of overlap are in yellow. The magnification relative to the frame size is identical to that shown in Figure 2.

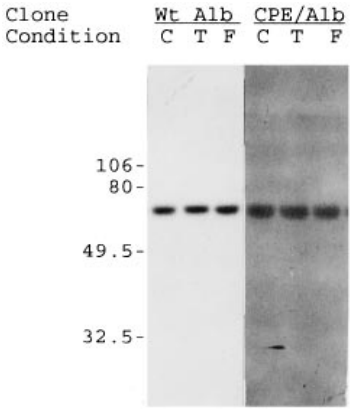

Figure 6 Western blot analysis of the secretion of albumin from AtT-20 cells expressing wt albumin (wt Alb) or CPE/Alb

Cells were treated with 100 ng/ml PMA (T), 5 μM forskolin (F) or control medium (C) for 30 min, and the media were analysed on a Western blot as described in the Materials and methods section. The positions and molecular masses (in kDa) of protein standards (Bio-Rad) are indicated.

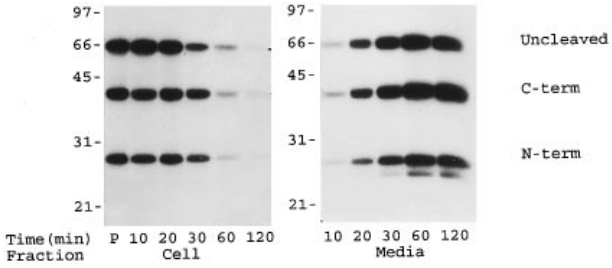

Figure 7 Pulse–chase analysis of the processing of CPE/Alb in AtT-20 cells

Cells were labelled with [^{35}S]Met for 30 min (P) and chased for the indicated times (in minutes). Cells were extracted and the albumin isolated by immunoprecipitation. The immunoprecipitated material was treated with BNPS-skatole and fractionated on a denaturing polyacrylamide gel, as described in the Materials and methods section. The positions and molecular masses of prestained standards (Bio-Rad) are indicated. The positions of the undigested protein and of the two fragments [N-terminal (N-term) and C-terminal (C-term)] of the BNPS-skatole-digested protein are indicated. The heterogeneity of the N-terminal fragment in the media samples presumably reflects variation in the removal of the propeptide.

not affected by the duration of the incubation, suggesting that processing does not occur after secretion.

DISCUSSION

The major finding of the present study is that the pro region is not required for the correct sorting of CPE. Furthermore, this region does not redirect the constitutively secreted albumin into the regulated pathway. The combination of negative (deletion) and positive (CPE/Alb fusion protein) analysis is more powerful than either approach alone. The deletion method only tests whether a region is essential, whereas the fusion protein method tests whether a region is sufficient. Based on our results, it appears that the pro region is neither required nor sufficient for the sorting or transport of CPE through the cell.

The pro region of another peptide processing enzyme, peptidylglycine α-amidating mono-oxygenase (PAM), also has no clear function in protein folding, expression or routeing to secretory vesicles [26]. Deletion of the 10-residue propeptide from PAM did not influence the amount of PAM sorted into the regulated secretory pathway in AtT-20 cells [26]. However, PAM lacking the pro region took longer to be secreted from either AtT-20 or hEK-293 cells, suggesting that the pro region of PAM plays a role in the transit of the protein through the cell [26]. When the pro region of PAM was attached to the N-terminus of the pro region of prohormone convertase 2, the resulting protein exited the endoplasmic reticulum more rapidly than prohormone convertase 2 alone [26]. This result implies that the pro region of PAM plays a role in the transit of proteins from the endoplasmic reticulum to the Golgi.

Similarity between the pro region of PAM (FRSPLSVFKR) and the pro region of albumin [RGVFRR; consensus is (G/S)VF(K/R)R] was noted previously by Mains et al. [26]. Although the pro region of CPE (QEPGAPAAGMRRRR) does not have substantial similarity to either sequence, the consensus (G/S)(V/M)X(R/K)R is present. The pro region of albumin, like that of PAM, is involved in determining the rate of secretion of the expressed protein. When rat albumin lacking the pro region was expressed in COS cells, the protein was secreted at a reduced rate compared with the rate of a wt proalbumin construct in the same cell line [27]. While our data (Figure 4) show a slight decrease in the rate of secretion of the CPEΔpro construct compared with the construct containing the pro region, this difference was not substantial. Also, the small difference was not detected in an additional experiment with the same cell line (results not shown).

The immunostaining of AtT-20 cells expressing proCPE/Alb showed accumulation of the protein in the Golgi and in a diffuse pattern typical of the endoplasmic reticulum, whereas the wt albumin was present mainly in the Golgi (Figure 5). This difference implies that the pro region of CPE cannot substitute for the pro region of albumin. In some experiments a small amount of albumin immunoreactivity was detected in the tips of cells transfected with the wt proalbumin construct (results not shown). Although the presence of immunoreactivity in the cell processes is typically taken as evidence for localization to secretory vesicles, this distribution is also found for constitutive proteins expressed in AtT-20 cells [28]. Matsuuchi et al. [28] expressed the immunoglobulin kappa light chain in AtT-20 cells and found that the protein was distributed in a *trans*-Golgi network-like structure and cell processes, but was not secreted via a regulated pathway. Thus the distribution alone cannot prove whether a protein is sorted into the regulated pathway. The strongest evidence that CPE/Alb and wt albumin are not secreted via the regulated pathway is the lack of secretagogue-stimulated secretion (Figure 6).

The propeptide is removed from the majority of the endogenous proCPE in AtT-20 cells, based on the immunofluorescence results shown in Figure 2 and on previous studies using pulse–chase methods [19]. However, when this pro region is transferred to the N-terminus of albumin, the efficiency of cleavage is greatly reduced (Figure 7). This reduced efficiency could be due to differences within the protein to which the pro region is attached, or to the failure of the protein to enter the regulated secretory pathway. Our previous pulse–chase analysis suggested that the pro region of CPE was removed soon after the protein exited the Golgi apparatus [19]. This interpretation is consistent with the distribution of immunoreactive proCPE in the present study (Figure 2). If cleavage of proCPE occurs in the regulated pathway, then the constitutively expressed CPE/Alb would not be exposed to the same processing enzymes and/or conditions (pH, ions) that are present in the regulated pathway. The finding that some processing of the pro region of CPE occurs for the CPE/Alb construct suggests that enzymes present in the constitutive pathway are able to perform a limited amount of removal of the pro region of CPE. This interpretation is consistent with our previous finding that furin, an endopeptidase present in the *trans*-Golgi network, can slowly convert proCPE into CPE [19].

The function of the pro region of CPE remains unknown. Since this sequence is completely conserved among human, rat and mouse proCPE, it is likely that it has an important role. Although it is possible that the pro region could function as a bioactive peptide if it were secreted, the lack of propeptide immunoreactivity in the secretory-vesicle-containing cell processes (Figure 2) suggests that this peptide is not secreted. It is also possible that the pro region of CPE is involved in an intracellular process such as sorting, activation or inhibition of other secretory pathway proteins. Recent studies on the Cpe^{fat}/Cpe^{fat} mouse have revealed that the absence of CPE and/or proCPE influences the prohormone processing endopeptidases [7]. These mice have a point mutation within the coding region of the CPE gene that causes the protein to be inactive and to be degraded within the early secretory pathway [7,21]. The level of proinsulin is higher in Cpe^{fat}/Cpe^{fat} mice than in control mice, indicating a deficiency of the endopeptidase cleavage step [7]. Further studies are needed to examine whether it is the absence of CPE activity or proCPE protein that is responsible for the decreased efficiency of the proinsulin processing endopeptidases in the Cpe^{fat}/Cpe^{fat} mouse.

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REFERENCES

1 Fricker, L. D. (1988) Annu. Rev. Physiol. **50**, 309–321
2 Fricker, L. D. (1991) in Peptide Biosynthesis and Processing (Fricker, L. D., ed.), pp. 199–230, CRC Press, Boca Raton
3 Docherty, K. and Steiner, D. F. (1982) Annu. Rev. Physiol. **44**, 625–638
4 Eipper, B. A., Mains, R. E. and Herbert, E. (1986) Trends Neurosci. **9**, 463–468
5 Steiner, D. F. (1991) in Peptide Biosynthesis and Processing (Fricker, L. D., ed.), pp. 1–16, CRC Press, Boca Raton
6 Song, L. and Fricker, L. D. (1995) J. Biol. Chem. **270**, 25007–25013
7 Naggert, J. K., Fricker, L. D., Varlamov, O., Nishina, P. M., Rouille, Y., Steiner, D. F., Carroll, R. J., Paigen, B. J. and Leiter, E. H. (1995) Nature Genet. **10**, 135–142
8 Fricker, L. D., Evans, C. J., Esch, F. S. and Herbert, E. (1986) Nature (London) **323**, 461–464
9 Fricker, L. D., Adelman, J. P., Douglass, J., Thompson, R. C., von Strandmann, R. P. and Hutton, J. (1989) Mol. Endocrinol. **3**, 666–673
10 Parkinson, D. (1990) J. Biol. Chem. **265**, 17101–17105

11 Rinderknecht, H. (1986) in The Exocrine Pancreas: Biology, Pathobiology, and Diseases (Go, V. L. W., ed.), pp. 163–183, Raven Press, New York
12 Inouye, M. (1991) Enzyme **45**, 314–321
13 Fortenberry, S. C. and Chirgwin, J. M. (1995) J. Biol. Chem. **270**, 9778–9782
14 Manser, E., Fernandez, D., Loo, L., Goh, P. Y., Monfries, C., Hall, C. and Lim, L. (1990) Biochem. J. **267**, 517–525
15 Roth, W. W., Mackin, R. B., Spiess, J., Goodman, R. H. and Noe, B. D. (1991) Mol. Cell. Endocrinol. **78**, 171–178
16 Fricker, L. D. and Devi, L. (1993) in Innovations in Proteases and their Inhibitors (Aviles, F. X., ed.), pp. 259–277, Walter de Gruyter, Berlin
17 Manser, E., Fernandez, D. and Lim, L. (1991) Biochem. J. **280**, 695–701
18 Varlamov, O. and Fricker, L. D. (1996) J. Biol. Chem. **271**, 6077–6083
19 Song, L. and Fricker, L. D. (1995) J. Neurochem. **65**, 444–453
20 Mitra, A., Song, L. and Fricker, L. D. (1994) J. Biol. Chem. **269**, 19876–19881
21 Varlamov, O., Leiter, E. H. and Fricker, L. D. (1996) J. Biol. Chem. **271**, 13981–13986
22 Fricker, L. D., Das, B. and Angeletti, R. H. (1990) J. Biol. Chem. **265**, 2476–2482
23 Fricker, L. D. and Devi, L. (1993) J. Neurochem. **61**, 1404–1415
24 Milgram, S. L., Mains, R. E. and Eipper, B. A. (1993) J. Cell Biol. **121**, 23–36
25 Allen, G. (1989) in Sequencing of Proteins and Peptides, 2nd edn. (Burdon, R. H. and Knippenberg, P. H., eds.), pp. 73–104, Elsevier, Amsterdam
26 Mains, R. E., Milgram, S. L., Keurmann, H. T. and Eipper, B. A. (1995) Mol. Endocrinol. **9**, 3–13
27 McCracken, A. A. and Kruse, K. B. (1989) J. Biol. Chem. **264**, 20843–20846
28 Matsuuchi, L., Buckley, K. M., Lowe, A. W. and Kelly, R. B. (1988) J. Cell Biol. **106**, 239–251

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