Processing and secretion of human carboxypeptidase E by C6 glioma cells

Edward MANSER,*† Denise FERNANDEZ* and Louis LIM*†

*Molecular Neurobiology Laboratory, Institute of Molecular Cell Biology, National University of Singapore, Kent Ridge, Singapore 0511, and †Department of Neurochemistry, Institute of Neurology, 1 Wakefield Street, London WC1N 1PJ, U.K.

Carboxypeptidase E (CPE) catalyses the removal of C-terminal basic amino acids and is implicated in the processing of peptides and hormones in secretory vesicles. The enzyme has been isolated in multiple forms from both soluble and membrane-bound compartments, and has been demonstrated to be co-secreted with peptides from pancreatic and adrenal cells. To address the question regarding the origin of the multiple forms of the enzyme, we have transfected a construct containing the cDNA for human CPE under the control of the murine-sarcoma-virus enhancer and metallothionein promoter into the C6 rat glioma cell line, which itself has extremely low levels of CPE expression. Within transfectants, multiple forms of the enzyme have been detected by Western blotting, and their sizes are comparable with that in pituitary. Fractionation of the intracellular forms of CPE with Triton X-114 at various pH values indicates that the membrane-bound, but not the soluble, form is amphipathic; this difference probably arises from post-translational modification of the enzyme. Interestingly, the secreted enzyme is found to have the amphipathic characteristics of the membrane-bound form. By modification of the cDNA sequence, enzyme lacking N-terminal and C-terminal domains have been expressed: deletion of the 'pro' region of CPE, leading to loss of the signal cleavage site, gives a more hydrophobic but active enzyme which is not efficiently secreted from the cell; deletion of a C-terminal region gives a secreted form of the enzyme which no longer exhibits amphipathic behaviour. Our data indicate that a single mRNA species can give rise to the multiple forms of CPE enzyme that have been identified and that the C6 cells are able to carry out the intracellular processing events to generate these forms.

INTRODUCTION

One of the enzymes implicated in the processing of propeptides and prohormones is carboxypeptidase E (CPE; also known as carboxypeptidase H; EC 3.4.17.10). The activity of the enzyme is detected in a number of tissues and has been localized to secretory granules in adrenal and pituitary cells (Fricker & Snyder, 1982; Hook & Loh, 1984). Additional evidence for the role in peptide processing is that the enzyme is co-secreted with peptide hormones in a regulated manner (Guest *et al.*, 1989). The mRNA expression of CPE is highest in the brain, although the major site of transcription may be in astrocytes rather than peptidergic neurons, as was originally proposed (Vilijn *et al.*, 1989). The enzyme is detected in endocrine tissues including testis, heart, ovary and adrenal (Strittmatter *et al.*, 1984; Lynch *et al.*, 1988) and its activity is characterized by an acidic pH optimum, stimulation of cobalt and inhibition by the substrate analogue guanidinoethylmercaptosuccinic acid (GEMSA).

Within secretory granules CPE is present as soluble and membrane-associated forms (Supattapone *et al.*, 1984; Laslop *et al.*, 1986), with the smaller form having a more acidic pI. The soluble forms are released by hypo-osmotic lysis at neutral pH and membrane forms by alkaline lysis or detergent extraction in the presence of high salt (Fricker, 1988a). Some studies suggested that a size difference between the soluble and membrane CPE was due to the presence of a 'membrane-association domain' that could be removed by post-translational modification or alternative splicing at the RNA level. Since sequence analysis of various CPE cDNAs (Fricker *et al.*, 1986; 1989; Rodriguez *et al.*, 1989; Manser *et al.*, 1990) have to date indicated a single

mRNA species, it is important to establish whether this RNA can generate one or both forms of the enzyme when introduced into CPE-negative cells.

One region which may participate in membrane binding includes the last 25 amino acid residues at the C-terminus, whose sequence is markedly divergent from two related carboxypeptidases (M and N), and is predicted to be an amphipathic α -helix. Antibodies raised to this region were reported not to recognize the soluble form of pituitary enzyme (Fricker *et al.*, 1990), but more recently the opposite result has been obtained (Parkinson, 1990).

In the present study the full-length human cDNA has been transfected into a rat glial-cell line (C6). These cells secrete active enzyme, and analysis of intracellular forms suggest that soluble and membrane-bound forms of CPE analogous to those observed in tissues are generated. Therefore a single CPE mRNA is capable of producing a polypeptide which, by post-translational modification in these cells, generates different forms of the enzyme. We have also investigated the effect of the removal of two domains from CPE; affinity chromatography of these modified expressed forms of the enzyme indicate that removal of the 'pro' region from the enzyme or 25 residues from the C-terminus does not interfere with enzyme activity, but modifies the surface properties of the protein.

MATERIALS AND METHODS

Construction of expression vectors

The full-length human CPE cDNA described previously (P1622; Manser *et al.*, 1990) was cut at a 5' *Xma*III site (position

Abbreviations used: MSV, murine sarcoma virus; (h) CPE, (human) carboxypeptidase E; GEMSA, guanidinoethylmercaptosuccinic acid; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline, 137 mM-NaCl/2 mM-KCl/8 mM-Na₂HPO₄/1.58 mM-KH₂PO₄, pH 7.3; SSPE, 0.18 M-NaCl/10 mM-sodium phosphate-buffer, pH 7.4, containing 1 mM-EDTA; CPM, carboxypeptidase M.

† To whom reprint requests should be addressed.

267) and 3' *HpaI* site (position 2118) which includes all of the coding region, 'filled-in' with Klenow fragment of DNA polymerase I, and cloned into the polylinker *XhoI* site (filled) of the expression vector pMMTN (W. J. Hong, unpublished work). This plasmid contains the *neo* gene, conferring resistance to the antibiotic G418 (geneticin; Gibco-BRL) driven by the simian-virus-40 early promoter, and a polylinker site downstream of the mouse metallothionein promoter and murine-sarcoma-virus (MSV) enhancer. The deletion of 16 amino acids in hCPEΔPRO (see the legend to Fig. 1 below) was achieved by digestion of the plasmid P1622-Bluescript with *NarI* (which cuts at three closely spaced sites), blunting with mung-bean nuclease and re-ligation with T4 DNA ligase. An *XmaIII*-*BamHI* 2.1 kb fragment of this plasmid was blunted and cloned as described above. The C-terminal deletion (hCPEΔCOOH) construct was achieved by limited *Bal31* nuclease treatment of the 1.1 kb *DraI* coding fragment (whose extreme 3' end corresponds to amino acid residue 474), digestion with *BgIII*, and ligation into the parent Bluescript-P1622 plasmid cut with *BgIII* and *HpaI*.

The resulting clones were analysed by sequencing of the resulting plasmids after single-strand phagemid rescue according to the Stratagene protocol. The expression vector pMMTN-hCPE is under control of the metallothionein promoter and can be induced by the presence of metal ions. The hCPE sequence is followed by the small T-antigen splice and poly(A) addition sites; however, for the two deletion constructs hCPEΔPRO and hCPEΔCOOH the 3' CPE poly(A) site is also present and is preferentially used as judged by the size of the corresponding mRNA (Fig. 2 below).

Cell culture, transfection and Northern blotting

C6 glioma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal-bovine serum (Hyclone Laboratories) and gentamycin (50 µg/ml; Sigma). The cells were transfected by calcium phosphate precipitation (Chen & Okayama, 1987) using plasmid (20 µg/ml) followed by glycerol shock. After 24 h, cells were put under selection in G418 (400 µg/ml) for 2 weeks, and thereafter maintained at a concentration of 200 µg/ml of the drug. Induction of the metallothionein promoter was achieved by addition of 10 µM-CdCl₂ or 100 µM-ZnSO₄ to complete media for 16 h.

For RNA preparation cells were harvested in phosphate-buffered saline (PBS) and frozen. Cell pellets were lysed in 10 mM-Tris (pH 7.3)/0.15 M-NaCl/0.5% Nonidet P40 (BRL) and nuclei removed by centrifugation (Microfuge; 12000 rev./min; 11 000 g; 5 min). The supernatant was made 0.5% in SDS, extracted with phenol/chloroform (1:1, v/v) (three times), with chloroform, then ethanol-precipitated. The standard protocols described by Maniatis (1982) were used; RNA samples were run on denaturing gels and blotted on to Hybond N (Amersham International). The ³²P-labelled probe was hybridized overnight at 55 °C in 7% SDS/0.5 M-NaHPO₄/1 mM-EDTA and washed in 0.1 × SSPE (0.18 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4, containing 1 mM-EDTA) at 60 °C.

Cell fractionation, enzyme assays and CPE purification

Procedures were carried out at 4 °C. Cells were washed with PBS, scraped from plates, pelleted in a Microfuge for 2 min and frozen in Solid CO₂. In order to obtain soluble and membrane-bound fractions, cells were homogenized in a hypo-osmotic buffer (10 mM-Tris, pH 7.0, containing 0.5 mM-phenylmethanesulphonyl fluoride) and sonicated for 20 s (MS50; Microson); after 30 min the extract was clarified by centrifugation at 150 000 g for 10 min. (Beckman airfuge; A100 rotor; 200 µl tubes). The resultant pellet was re-extracted under the same conditions and the two supernatants pooled (final protein

concentration 2–4 mg/ml). Membrane-bound CPE was solubilized in an equivalent volume of 0.5% Triton X-100/50 mM-Mes (pH 5.5)/250 mM-NaCl, and clarified at 12000 rev./min in a Microfuge. Protein concentrations were determined in duplicate using the Bio-Rad protein assay kit.

CPE was purified by affinity chromatography on columns of *p*-aminobenzoylarginine coupled to CNBr-activated Sepharose 6 MB (1–2 mg/ml). Hypo-osmotic extracts (soluble protein) was acidified with 50 mM-Mes, pH 5.5 (with addition of 0.1% Triton X-100) and precipitated proteins removed by centrifugation at 12000 rev./min in a Microfuge. The acidified extracts were circulated for 2 h on small columns (0.4–2.0 ml) of affinity matrix corresponding to one-fifth of the extract volume. Non-specific proteins were washed with 5 column vol. of 20 mM-acetate (pH 5.6)/1 M-NaCl/0.1% Triton X-100, then with 20 mM-acetate, pH 5.6, alone. CPE was specifically eluted in 2 column vol. of high-pH buffer [50 mM-Tris (pH 8.0)/100 mM-NaCl/0.1% Triton X-100]. Standard CPE assays were carried out essentially as described by Stack *et al.* (1984) in 100 µl containing 100 µM-benzoyl-Phe-Ala-Arg (containing 100 000 c.p.m. of tritiated peptide; NEN) buffered at pH 5.5 with 50 mM-Mes/50 mM-acetate. After a 30 min incubation at 37 °C, the samples were acidified with 200 µl of 0.2 M-HCl, and 200 µl of chloroform was added. The reaction product quantitatively partitioned into the organic layer, and 150 µl was taken for counting radioactivity; the radioactivity equivalent to 100% hydrolysis was established by adding a saturating level of purified enzyme.

CPE hydropathicity was determined by partitioning of the enzyme in Triton X-114 as described by Bordier (1981). The affinity-purified protein from cell extracts or media containing the secreted protein was made 1% in Triton X-114 at 4 °C; 60 µl aliquots were added to tubes containing 10 µl of 1 M-buffer at the relevant pH to give a final concentration of 140 mM (for the pH range 4–5.5, sodium acetate was used; for pH 5.5–7.0, Mes). Samples were clarified in a Microfuge for 3 min at 12000 rev./min to remove any precipitate and layered on to a 15 µl cushion of 0.25 M-sucrose containing 50 mM-buffer (at the relevant pH) in 0.5 ml Eppendorf tubes. After a 5 min incubation at 35 °C to induce the phase change, the samples were spun for 3 min in the Microfuge at room temperature. The upper phase and sucrose cushion were removed and combined; the dense detergent pellet remaining was redissolved at 4 °C in an equal volume of PBS; 10 µl samples of these solutions were taken for assay.

Immunological analysis

Antibodies were raised against *Escherichia coli*-expressed human CPE using the T7 expression system (Studier & Moffatt, 1986) in the plasmid pAR3038. A 2 kb *SacII* DNA fragment from the P1622 cDNA was cloned into the polylinker site to give expression of amino acid residues 34–476 (after a 15-amino-acid leader from the vector) in the *E. coli* strain BL21-DE3. The CPE was gel-purified from inclusion bodies and antisera raised as described previously (Manser *et al.*, 1990). Anti-CPE antibodies were purified by absorbing out *E. coli*-reacting material on a CNBr-activated Sepharose 6 MB column of insoluble material from induced *E. coli* with the insert in the reverse orientation. The flow-through was passed over a second Sepharose column of immobilized expressed CPE, washed with PBS+0.5 M-NaCl/0.05% Tween 20, and eluted with PBS+100 mM-glycine, pH 2.5, and immediately neutralized with 1 M-Tris, pH 10. Antibodies were stored with 0.05% azide or frozen in 5% (v/v) glycerol.

After semi-dry blotting of proteins from SDS/polyacrylamide gels on to nitrocellulose, the Western blots were blocked for 1 h with 3% skimmed milk (Carnation) and probed overnight with 1:100 dilution of the purified antibodies in PBS containing 1% BSA/0.05% Tween 20. The blots were washed (3 × 10 min;

PBS/0.05% Tween 20) and bands revealed by incubating with peroxidase anti-rabbit IgG (Amersham 1:10,000), washed again, and luminol reagent added to the filter. This was wrapped in cling film and exposed to film (Kodak XAR 5) for times varying from 10 s to 10 min.

RESULTS

Expression of human CPE mRNA in C6 cells

Plasmid constructs for CPE transfection contained coding and some flanking sequences of human CPE (hCPE) were cloned into a eukaryotic expression vector (see the Materials and methods section for details). The two modified cDNA sequences were created (Fig. 1) that generate (a) deletion of 16 amino acids in the *N*-terminal region between the signal peptidase site and a polyarginine sequence which can be removed *in vivo* by post-translational cleavage ('pro' region) and (b) deletion of 26 amino acids from the C-terminus of the protein with addition of five novel amino acids. The expression of the insert is under the control of the metallothionein promoter and MSV enhancer; the plasmid also confers resistance to G418. After 4 weeks selection with 200 μ M-G418, transfected cells were assayed for carboxypeptidase activity. Since the full-length construct gave greater than 10-fold higher CPE activity than for untransfected cells (cf. Fig. 3 below), individual clones were not selected, but the pool of transfected cells (derived from 100–500 clones) were used in the experiments described (referred to as 'C6:hCPE'). In the absence of any treatment the transfected cells showed high levels of mRNA expression by Northern blotting (Fig. 2) when compared with total RNA from brain cortex (lane 12), which has one of the highest levels *in vivo*. The two deletion constructs contained additional 3' non-coding sequence including the CPE poly(A) addition site, which appears to be preferentially used compared with that of the vector simian-virus-40 site, since the smaller bands (large arrow) are the major mRNA species in this case. Addition of Zn to the media produced little induction in pMMTN-hCPE-derived message and yielded only a 25% increase in measured CPE activity. With Cd addition there may be a more pronounced toxic effect, since the cells showed altered morphology. Since in all three constructs the constitutive level of enzyme in transfected cells was already sufficient for the analysis described, the cells were not incubated with metals, which avoided

N-Terminus

bCPE MARRGGCALL VLCGLAACA WLLGAEA RGP GGPVAGARRR RRP-OED.
hCPE MAGRGGCALL ALCGALAACG WLLGAEA QEP GAPAGMRRR RRLQOED.
hCPE Δ PRO MAGRGGCALL ALCGALAACG WLLG----- RRLQOED.

C-Terminus

hCPE DFELE SFSEKKEEK EELMEWKKMM SETLNF. 476
hCPE Δ COOH DFELE HYLKV. 455

Fig. 1. Corresponding protein sequence derived from hCPE cDNA and deletion constructs in the *N*- and C-terminal regions of the protein

The transfected hCPE sequence is aligned at the *N*-terminus with the bovine sequence (bCPE) from the corresponding cDNA (Fricker *et al.*, 1989). The putative signal-peptide cleavage site is by reference to the bovine soluble enzyme (Parkinson, 1990); subsequent processing gives a 'mature' polypeptide reported for both membrane and soluble forms of bovine CPE (Fricker *et al.*, 1986). CPE Δ PRO was generated by digestion with endonuclease *NarI*, and CPE Δ COOH by exonuclease *Bal31* digestion (see the Materials and methods section). The three cDNA constructs, containing 20 bp of 5' non-coding sequence, but various amounts of 3' non-coding sequence (see the Materials and methods section), were cloned downstream from the metallothionein promoter (MT-1) and MSV enhancer.

Fig. 2. Expression of human CPE in transformed C6 cells by Northern blotting

RNA (20 μ g) from wild-type C6 cells and polyclonal transformed cells was fractionated on a denaturing agarose gel, blotted on to Hybond N and hybridized with a 1.1 kb *DraI* fragment of human CPE cDNA described previously (Manser *et al.*, 1990; positions 611–1711). Autoradiograms corresponding to C6:hCPE cells (lanes 1–3) were exposed for 6 h; the remainder are 24 h exposures. Cells were treated with 100 μ M-ZnSO₄ or 10 μ M-CdCl₂ in order to induce the metallothionein promoter. The pMMTN-hCPE construct was spliced and polyadenylated by using the simian-virus-40 sequence derived from the vector, whereas the hCPE Δ PRO and hCPE Δ COOH constructs are predominantly polyadenylated using a site introduced with the insert (major bands marked with large arrows). The minor bands (small arrows) would contain the simian-virus-40 non-coding sequences. Total RNA from rat brain cortex (B) was used as a positive control. Positions of the 18 S and 28 S ribosomal bands are shown.

Fig. 3. CPE activity in soluble and membrane-bound pools of wild-type C6 cells and transfectants

Soluble and Triton X-100 extracts of the cells were prepared as described in the Materials and methods section. Activities are normalized to total protein (soluble + detergent extract) and measured at pH 5.5 in the presence of 100 μ M-benzoyl-Phe-Ala-Arg. Secreted activity was also measured and expressed relative to 1 mg of total cell protein/h. Values were determined from three experiments and expressed as means $\pm$ S.E.M. Symbols: $\blacksquare$, C6; $\square$, C6:hCPE; $\blacksquare$, C6:hCPE Δ COOH.

the complication of such treatment on general cell metabolism. The level of endogenous CPE mRNA expression in C6 cells was barely detectable (overnight exposure; lane 10 and 11) and this

Fig. 4. (a) Western blots of intracellular and secreted CPE in transfected C6 cells and (b) comparison of C6:hCPE and pituitary (pit.) forms of CPE by Western blotting

(a) Samples were run on a 9% gel, blotted on to nitrocellulose and probed with polyclonal CPE antibodies. Lanes 1–4 contain 60 μ g of total protein from control C6 cells (lane 1), hCPE transfected cells (lane 2) and deletion constructs [hCPE Δ PRO (lane 3) and hCPE Δ COOH (lane 4)]. Serum-free medium was collected from cells corresponding to lanes 1–4 after conditioning for 5 h (2 ml on 6 cm plate), and the equivalent of 30 μ l (lanes 5, 7 and 8) or 3 μ l (lane 6) loaded on the gel. Pre-stained molecular-mass markers (Sigma) were co-blotted for standards. (b) Cell and tissue homogenates were fractionated as described (see the Materials and methods section) into pH-5.5-soluble (s) and detergent extracts (m); material which precipitated upon acidification of the pH 7 hypo-osmotic fraction was dissolved in $\frac{1}{3}$ equiv. vol. to give approximately the same total protein concentration (p; 40 μ g/lane). Affinity-purified CPE from the C6:hCPE cells from soluble (lane 7) and detergent-extracted protein (lane 8) are clearly different in size. Medium conditioned by HepG2 cells (lane 9; 18 h) and C6:hCPE cells (lane 10; 1 h) in the absence of serum.

correlated with the low level of enzymic activity detected in the soluble compartment (Fig. 3).

Intracellular and secreted forms of CPE

Higher intracellular carboxypeptidase activity was detected in the transfected than control cells (Fig. 3) in both soluble and membrane-bound pools. This additional activity, due to the presence of CPE, was more than 10-fold higher than in untransfected cells. Approx. 40% of the enzymic activity arising from the transfected hCPE cDNA could be released from intracellular compartments after rapid freezing and two rounds of hypo-osmotic lysis at pH 7. We have previously shown in brain microsomes that a remaining membrane-bound form of the enzyme is released at high pH (Manser *et al.*, 1990); therefore CPE does not exist as an integral membrane form. The enzymic activity in C6:hCPE cells shows an acidic pH optimum, is stimulated in the presence of Co and potentially inhibited by 1 μ M-GEMSA, as expected for CPE (Supattapone *et al.*, 1984). An activity in the membrane-bound compartment of C6 cells (Fig. 3)

also showed this behaviour, but is probably not due to CPE, since there is little secretion into the medium; further, after being solubilized with Triton X-100, the enzyme did not bind to the affinity column under conditions used for CPE purification. Since CPM has been shown to exhibit similar enzymic properties at low pH (Deddish *et al.*, 1989), it is probable that the intracellular activity detected in C6 cells is due to CPM or a related enzyme. This correlated with a lack of immunoreactivity in membrane fractions of C6 cells probed with anti-CPE antibodies on Western blots (Fig. 4). Intracellular levels of CPE were high in the membrane-bound compartment of C6:hCPE Δ PRO cells, but relatively low for the C6:hCPE Δ COOH cells; conversely, the activity released into the medium by C6:hCPE Δ PRO cells was not significantly higher than control but substantial for C6:hCPE Δ COOH.

Intracellular forms of CPE in transfected cells were readily detected by Western blotting (Fig. 4a). The transfected CPE polypeptides were recognized by an anti-human antibody preparation which also cross-reacts with the rat form of the

Fig. 5. Effect of pH on the partitioning of various forms of CPE into Triton X-114

(a) Intracellular forms of the enzyme were purified from transfected cells or whole rat pituitary as described in the Materials and methods section, except that Triton X-114 was used in the elution buffer. Protein from soluble pituitary extract (○) or C6:hCPE extract (◇) remained in the upper phase independent of pH. By contrast, enzyme from the membrane fraction of C6:hCPE (◆) shows a pH-dependence of partitioning, and hCPEΔPRO enzyme (▲) is largely detergent-associated. CPE activity for equivalent volumes of both phases was assayed in duplicate. Each experiment was performed at least twice at each pH with a difference of less than 5%; data points shown are averages. (b) Behaviour of CPE secreted by transfected cells. C6:hCPE (○) and C6:hCPEΔCOOH (■) enzyme was treated according to the protocols described in (a).

enzyme. In the C6:hCPE cells a 55 kDa band and smaller unresolved bands were observed; the CPEΔPRO product (lane 3) is observed to be of the same size, despite the deletion of 16 amino acids from the sequence; this suggests that either the signal peptide is not removed or there is altered mobility due to post-translational modifications, since the primary translation products *in vitro* derived from corresponding synthetic mRNA are of two different sizes (results not shown). The presence of an uncleaved signal peptide would also be consistent with the increased hydrophobicity of the hCPEΔPRO. The C6:hCPEΔCOOH cells contain the expected smaller 50 kDa form of the protein, but, in addition, both the modified polypeptides are subject to proteolysis to give a band of 40 kDa (arrow). When we look at the medium (lanes 5–8), this smaller band is not present; this cleavage may therefore occur by diversion from the secretory pathway (to lysosomes), or the

cleaved product may be further degraded after secretion. Cells which harbour the hCPEΔPRO construct secrete very little enzyme compared with the intercellular level, therefore the majority of enzyme does not enter the secretory pathway or fails to be released at the cell surface. The level of activity detected in these cells can be attributed to the 55 kDa form (retaining the signal peptide), and we conclude that *N*-terminal processing is not required for activation of CPE. This altered polypeptide does not behave as an integral membrane protein, since a proportion can be extracted by hypo-osmotic lysis, and all is released in carbonate buffer at pH 11 (results not shown).

To compare the transfected CPE with 'in vivo' forms of the enzyme, rat pituitary (posterior and anterior lobes) was fractionated and blotted under the same conditions (Fig. 4b, lanes 1–3). Although the luminol detection system is extremely sensitive, band intensity is not linearly related to the amount of antigen. Loadings were chosen to allow resolution of bands rather than to demonstrate relative levels in the various compartments. The predominant form in the tissue is a soluble enzyme of 53 kDa, and only 20–30% of the activity is membrane-bound (Fricker, 1988b). The C6:hCPE cells, which have roughly equivalent activity in proportion to total protein as pituitary, contain mainly the larger 55 kDa form of the enzyme, most of which is released only by high salt and detergent (lane 6). When the soluble protein fraction obtained by hypo-osmotic lysis at pH 7 is acidified to pH 5.5 (in order to load the material on to the affinity column), approx. 30% of the proteins precipitate and some 55 kDa band is found in the pellet after centrifugation (lane 5); this leads to a removal from this fraction of approx. 10% of the activity. The remaining 'soluble' enzyme has the same molecular mass as observed for pituitary enzyme isolated under the same conditions (lane 1). Comparison of the immunoreactivity of the soluble and membrane fractions of intracellular hCPE with the enzymic activity suggested that both 53 kDa and 55 kDa forms were active. In order to confirm this, the soluble protein and detergent-extracted protein fractions at pH 5.5 were passed over affinity columns of *p*-aminobenzoylarginine coupled to Sepharose 6B. The columns were washed extensively and CPE specifically eluted at pH 8.0 (Grimwood *et al.*, 1989). Lanes 7 and 8 show that both 53 kDa and 55 kDa intracellular forms bound and were purified by these means. The secreted enzyme for these cells is detected as two forms by Western blotting (Fig. 4, lane 10). The molecular mass of these correlate with two forms observed intracellularly. For comparison we also looked at the conditioned medium from HepG2 cells containing CPE that is reported to be larger than the bovine pituitary enzyme (Grimwood *et al.*, 1989). Since these cells have a lower rate of CPE secretion, a longer conditioning period was required in order to detect the protein in the media by Western blotting, but under these conditions two forms of the enzyme are evident and do appear to be slightly larger than hCPE forms. The larger 65 kDa immunoreactive band which has been observed with other polyclonal sera on Western blots (Hook *et al.*, 1990), is also present in media of control C6 cells and therefore probably represents an immunologically related protein.

Triton-X114 fractionation of CPE

It has been reported that the membrane-binding properties of CPE are due to the amphipathic behaviour of the protein (Fricker *et al.*, 1990). This can be assayed by association of the protein with the detergent-rich phase after the temperature-induced micelle formation of Triton X-114 (Bordier, 1981). The affinity-purified enzyme is relatively stable between pH 4.5 and 8.0, thus one can measure the ratio of CPE activity remaining in the upper 'soluble' phase to that in the detergent pellet after incubation at 37 °C and centrifugation. At pH 5 a large pro-

portion of the 'membrane-bound' form of CPE partitions into the detergent phase, but at pH 7 most remains in the supernatant (Fig. 5). Neither the pituitary 'soluble' form nor that released from C6:hCPE cells show this pH-dependent amphipathic behaviour. The membrane-bound enzyme isolated from the cells does exhibit hydrophobicity at acidic pH; in control experiments using Triton X-100 (which does not undergo a phase change) no activity was lost from the supernatant after centrifugation. These experiments were conducted with affinity-purified enzyme, but identical results were obtained with total extracts diluted to 1 mg of protein/ml. Partitioning into the detergent-rich phase at low pH may be due to the C-terminal residues, as has been suggested (Fricker *et al.*, 1990), or related to charge neutralization near the pI of the protein (calculated value without signal peptide = 4.9). Fig. 5(a) shows the behaviour of intracellular hCPEΔPRO enzyme; as expected for a protein retaining signal peptide, it is overall more hydrophobic than the unmodified enzyme. In addition, hCPEΔPRO enzyme shows little pH-dependence of partitioning into the detergent phase. Interestingly the secreted hCPE enzyme, which is predominantly of M_r 55 kDa, has the characteristics of the intracellular membrane-associated form of the enzyme (Fig. 5b). In contrast the enzyme from C6:hCPEΔCOOH conditioned medium had characteristics of the 'soluble' enzyme. After affinity chromatography of secreted hCPE enzyme, electrophoresis and staining of bands with Coomassie Blue or silver, only two protein bands were visible (results not shown); we therefore conclude that the amphipathic behaviour is not the result of an association with other proteins.

DISCUSSION

All CPE cDNAs isolated to date from bovine, rat and human libraries (Rodriguez *et al.*, 1989; Manser *et al.*, 1990) predict a precursor polypeptide that includes a short *N*-terminal propeptide region after the signal peptide (Fig. 1), compared with the mature soluble and membrane-bound forms of the bovine enzyme (Fricker *et al.*, 1986). This region of the protein was postulated to be involved with inactivation of the enzyme, as has been observed for other proteinases (Fricker, 1988a). Recently it was reported that a larger unprocessed form of the enzyme can be isolated by affinity purification from bovine pituitary provided that the purification protocol is rapid (Parkinson, 1990); it also appears that the extent of this *N*-terminal processing differs between tissues (Hook *et al.*, 1985). Two bands have been described after immunoprecipitation of pancreatic-cell extracts (Guest *et al.*, 1989). In cell cultures some studies have failed to detect the forms of enzyme by Western blotting, even with high levels of enzymic activity (Vilijn *et al.*, 1989). CPE-like activity is certainly detected in all cell lines that we have looked at. In considering a suitable cell type for these transfection experiments we screened a number of cell lines of neuro-endocrine origin by Northern blotting; mRNA was found in all the cells, but at much lower levels than that in brain tissue, and C6 cells had one of the lowest levels. This cell line is characterized by a number of biochemical markers specific for astrocytes. Since primary astrocytes are known to express CPE at relatively high levels (Vilijn *et al.*, 1989) our observations regarding CPE may also be relevant to processing *in vivo*.

The high level of mRNA expression observed with the pMNTN:hCPE vector in the absence of metal induction is probably the result of efficient use by C6 cells of the MSV enhancer. These cells secrete hCPE into the media at a level comparable with other endogenous C6 secreted proteins, as determined by the intensity of 35 S-labelled bands on SDS polyacrylamide gels (results not shown).

Because of previous reports that suggest CPE modification

after extraction, protocols were established for rapid processing of cell pellet and tissues using 10 min ultracentrifugation steps in an Airfuge. Hypo-osmotic lysis of the cell homogenate is more effective at neutral pH, and this was confirmed by the almost complete release of the 53 kDa form of CPE from the C6:hCPE cell pellet (Fig. 4) compared with lysis at pH 5.5 (results not shown); it has been shown that lysis of neurosecretory granules is most effective above pH 7 (Morris & Cannata, 1973). The most abundant membrane-bound form of CPE in pituitary has the same molecular mass as the soluble form, we therefore conclude that the difference in molecular mass is not related to the membrane association *per se*. In tissues the enzyme is located in storage granules (which are also seen in some cell lines, for example AtT-20), but such a compartment has not been described in astrocytes. It appears that, in the C6:hCPE cells, the generation of 'soluble' CPE correlates with a lower-molecular-mass species. Further experiments will be needed to establish the relationship between the larger 'membrane' and smaller 'soluble' form of the enzyme in C6:hCPE cells. This system is more amenable to experimentation than previous studies with tissues, since the cells can be studied in isolation and enzyme is derived from a defined mRNA species. The secreted enzyme is revealed as two bands on gels, with a predominance of the larger form; differences in the extent of processing could be related to cell type or intracellular compartmentation, and the relationship between the transfected cell type, the level of transgene expression and the secreted forms of the enzyme requires investigation. HepG2 cells, which also generate two CPE forms (as reported previously; Grimwood *et al.*, 1989) appear slightly larger than bands from the transfected cells. This cannot be explained on the basis of a difference in primary sequence, because HepG2 cells are of human origin, and may rather relate to differences in glycosylation. *N*-Terminal processing at the polybasic site is probably carried out by the trypsin-like endopeptidase activity which is required for processing prohormones (Mains *et al.*, 1990). Recently proteinases have been cloned (PC2; Smeekens & Steiner, 1990; PACE; Wise *et al.*, 1990) on the basis of sequence similarity to the yeast enzyme KEX-2 (which is responsible for α -factor processing; Mizuno *et al.*, 1988), and PACE has been shown to correctly cleave von-Willebrand-factor precursor (Wise *et al.*, 1990). At present it is not possible to speculate which enzyme is responsible for the observed processing of hCPE in C6 cells or *in vivo*.

Both deletion constructs described produce enzymically active proteins, indicating that neither the 'pro' region nor the C-terminus are essential for activity. In the former case, cleavage of the signal peptide probably does not occur, as judged by the mobility on SDS/polyacrylamide gels and the increase in hydrophobicity of the protein product, thus affecting the secretion of the enzyme. One consequence of this would be an intracellular accumulation of enzyme requiring its subsequent removal; this may result in a proteolytic intermediate of 40 kDa. [If the signal peptide is removed from this protein with cleavage at a site close to that of the N-terminus of the mature 53 kDa form, then its properties should not be so markedly different from those of the wild-type CPE.] Conversely, hCPEΔCOOH transfected cells have intracellular carboxypeptidase levels only 2-fold higher than control cells, but secrete substantial amounts of CPE. The lack of amphipathic behaviour for the secreted enzyme suggests that Fricker *et al.* (1990) are correct in implicating the C-terminus in membrane binding. The possibility exists in *in vivo* that such an interaction with membrane in the secretory pathway is regulated by modification of the C-terminus or another part of the protein. Indeed, Western blots of the C6:hCPE enzyme compared with the primary soluble forms (whose *N*-termini have been sequenced and retain immunoreactivity to C-terminus-specific antibodies; Parkinson, 1990) suggest that generation of a 'soluble' form of

the enzyme does not involve cleavage at the C-terminus (i.e. at dibasic residues, position 456). Our results show further that the amphipathic forms of the enzyme can be released from the cell, and therefore modification of the protein is not required for secretion.

At present the significance of the C-terminal amphipathic helix is not clear; such domains are implicated in protein-protein interactions (e.g. calmodulin-binding domains; DeGrado, 1988), in protein-membrane interactions (e.g. apolipoproteins; Segrest *et al.*, 1974) and in polypeptide hormones (e.g. β -endorphin; Taylor & Kaiser, 1986). Since this region is divergent from two closely related carboxypeptides, M and N (Manser *et al.*, 1990), but well conserved between species, we suggest that it is required for the specific functions of CPE. If this region is solely responsible for the membrane-binding of CPE, then our data indicate that, during intracellular routing, it is possible to block this function.

We thank Dr. Hong Wanjin for the expression vector pMMTN. L.L. acknowledges support from the Brain Research Trust.

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Received 22 April 1991/18 June 1991; accepted 25 June 1991