Glu300 of Rat Carboxypeptidase E Is Essential for Enzymatic Activity but Not Substrate Binding or Routing to the Regulated Secretory Pathway*

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Several recently discovered members of the carboxypeptidase E (CPE) gene family lack critical active site residues that are conserved in other family members. For example, three CPE-like proteins contain a Tyr in place of Glu300 (equivalent to Glu270 of carboxypeptidase A and B). To investigate the importance of this position, Glu300 of rat CPE was converted into Gln, Lys, or Tyr, and the proteins expressed in Sf9 cells using the baculovirus system. All three mutants were secreted from the cells, but the media showed no enzyme activity above background levels. Wild-type CPE and the Gln300 point mutant bound to a p-aminobenzoyl-Arg-Sepharose affinity resin, and this binding was competed by an active site-directed inhibitor, guanidinoethylmercapto-succinic acid. The affinity purified mutant CPE protein showed no detectable enzyme activity (<0.004% of wild-type CPE) toward dansyl-Phe-Ala-Arg. Expression of the Gln300 and Lys300 mutant CPE proteins in the NIT3 mouse pancreatic beta-cell line showed that these mutants are routed into secretory vesicles and secreted via the regulated pathway. Taken together, these results indicate that Glu300 of CPE is essential for enzyme activity, but not required for substrate binding or for routing into the regulated secretory pathway.

Metallocarboxypeptidases perform many physiological functions. Pancreatic carboxypeptidases A and B (CPA and CPE) are primarily involved with the digestion of food, whereas CPA and related family members perform more selective cleavages and are involved in the biosynthesis of peptides and proteins (1). Altogether, there are 13 members of this gene family, which is subdivided into two groups based on homology and size. Each group has 30–70% amino acid sequence identity with other family members of the same group, but only 15–25% amino acid sequence identity with members of the other group. One group includes pancreatic CPA1, CPA2, CPB, mast cell CPA, and carboxypeptidase U (also known as plasma CPB) (2–6). These family members are all enzymatically active, and are 30–40-kDa proteins. The other group includes CPE, carboxypeptidase M (CPM), carboxypeptidase N (CPN), carboxypeptidase D (CPD), carboxypeptidase Z (CPZ), and three proteins designated CPX1, CPX2, and AEBP1 (7–17). Except for CPD, which is 180 kDa and contains 3 distinct carboxypeptidase-like domains, the members of this family contain a single carboxypeptidase domain of approximately 400 amino acids. Only CPE, CPM, CPN, CPZ, and the first two domains of CPD have been demonstrated to have enzymatic activity; the third domain of CPD and the other proteins in this group do not appear to cleave the standard carboxypeptidase substrates (13–15, 18).

The observation that CPX2, AEBP1, and the third domain of CPD are not active toward standard substrates is consistent with a comparison of active site residues of the various members of the family. CPX2, AEBP1, and the third domain of CPD lack a Glu in the position equivalent to Glu270 of CPA and CPE. Instead, these novel members of the gene family contain Tyr in this position (Fig. 1). Glu270 in CPA/B is a critical catalytic residue that is thought to contribute a proton to the leaving group following hydrolysis of the amide bond (19). Theoretically, it is possible that a Tyr in this position could also function as a proton donor, but the catalytic activity would be predicted to be greatly reduced. However, substrate binding may not be greatly affected by the absence of a Glu in this position. These observations led to the prediction that the novel members of the metallocarboxypeptidase gene family that lack the Glu270 equivalent residue are binding proteins, rather than active enzymes.

In the present study, we tested the importance of Glu300 in rat CPE. In addition to testing the affect on enzyme activity and binding to a substrate affinity resin, we also tested whether the mutant CPE could be correctly sorted into the regulated secretory pathway in a CPE-deficient pancreatic beta cell line. Recently, CPE has been proposed to be involved in the sorting of neuroendocrine peptides into the regulated secretory pathway (20). Thus, we were interested in testing whether mutations that inactivate CPE had an influence on the sorting of CPE in a neuroendocrine cell line. For this analysis, we used the NIT3 cell line (21), which was derived from Cpe+/Cpe+/ mice and does not contain active CPE due to a point mutation of Ser205 to Pro in the coding region of the CPE gene (22). This cell line is an ideal test system to examine the sorting of CPE because it contains a regulated secretory pathway without endogenous CPE (the Pro205 mutant CPE is degraded before entry into the Golgi) (21). Our findings that mutations of Glu300 eliminate CPE activity but not binding to a substrate affinity column supports the hypothesis that the novel family members lacking a Glu in the equivalent position are binding proteins rather than active enzymes. Furthermore, our finding that the CPE proteins with mutations of Glu300 are sorted into the regulated pathway in the NIT3 cells indicates that neither

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enzyme activity or substrate binding are required for this sorting.

MATERIALS AND METHODS

Generation of Constructs—To generate point mutants, Altered Sites II in vitro mutagenesis system (Promega) was used. Briefly, full-length CPE cDNA was subcloned into the vector pAlter-1. Oligonucleotides with the desired codon changes were used to generate single amino acid substitutions. The cDNA fragments with point mutations were subcloned into the BamHI and EcoRI sites of the baculovirus transfer vector pVL-1393. Dideoxynucleotide sequencing was performed to confirm the sequences of the mutated region of CPE.

For expression in NIT3 cells, a SacI/EcoRI fragment of the CPE cDNA (which contains the point mutant) was combined with a BamHI/SacI fragment from a construct containing the hemagglutinin (HA) epitope tag between the pro region of CPE and the mature form of the protein (23). These fragments were subcloned into the pcDNA3 vector (Invitrogen). The resulting proteins contain the N-terminal HA epitope tag and the various mutations of Glu300.

Prodynorphin cDNA in the pcDNA1/Amp expression vector was obtained from Dr. Lakshmi Devi (Dept. of Pharmacology, New York University).

Expression of Proteins in the Baculovirus System—CPE proteins were expressed using the recombinant baculovirus expression system (PharMingen) as described previously (24). For large scale protein expression, SF9 cells were grown to 2 x 10^7/ml in shaker flasks and infected with the baculovirus. Cells were harvested by centrifugation at 10,000 x g for 30 min. Cell pellets were homogenized (Polytron, Brinkman Instruments) in 0.1 M NaAc, pH 5.5, buffer containing 1 M NaCl and 1% Triton X-100 and centrifuged at 50,000 x g for 1 h. Aliquots of media and cell extracts were assayed for carboxypeptidase activity as described below.

Western blot Analysis—Aliquots of expression cell extracts and media were resolved on a 10% denaturing polyacrylamide gel and electro-photographically transferred onto nitrocellulose membrane. The blot was probed with an antiserum to the CPE C-terminal region (24), followed by horseradish peroxidase-labeled goat anti-rabbit antiserum. The enhanced chemiluminescence method (Amersharm Pharmacia Biotech) was used to detect the bound antiserum.

Enzyme Activity Assay—In a typical assay, 25-μl aliquots of media or cell extracts were combined with 225 μl of 0.1 M NaAc, pH 5.5, 0.01% Triton X-100, and 0.22 mM dansyl-Phe-Ala-Arg. After incubation at 37°C for 1 h, a product was detected by measuring fluorescence after extraction into chloroform from an acidified aqueous phase (25). A similar assay was used for the affinity purified proteins.

Affinity Chromatography—To test the binding ability of the CPE mutants, 50 ml of baculovirus-infected SF9 media or cell extracts was adjusted to pH 5.5 and 0.15 M NaCl and applied to a 0.5-mL column of p-aminobenzoyle-Arg-Sepharose (25). The columns were washed with 50 mM NaAc, pH 5.5, buffer containing 0.5 M NaCl and 0.5% Triton X-100. In some experiments, bound proteins were eluted with 5 mL of 50 mM Tris-Cl buffer, pH 8.0, containing 100 mM NaCl and 0.01% Triton X-100. Following this treatment, the columns were then eluted with 5 μL of 25 mM Arg in the same buffer. In other experiments, bound proteins were competitively eluted with increasing concentration of guanidinoethylmercaptoacetic acid (GEMSA) in 0.6 mL of washing buffer. The GEMSA solution was applied to the column, and the mixture was recycled 2 times over a 30-min period of time at 4°C. Following this step, the remaining CPE was eluted with 0.6 mL of 50 mM Tris-Cl, pH 8.0, buffer with 100 mM NaCl. The various column fractions were analyzed on denaturing polyacrylamide gels. For quantitation of the amount of CPE recovered by the various concentrations of GEMSA, the protein was detected using the silver staining method (26). The protein gel was densitized using the Java Image analysis program (Jandel Scientific). The percentage of the bound CPE eluted by GEMSA was determined by calculating the amount of CPE protein in the GEMSA elute versus the total amount of CPE recovered from the column (i.e. the combined amount of the GEMSA and pH 8.0 elutes).

Expression of CPE in NIT3 Cells—For the studies investigating whether different mutants of CPE co-localize with prodynorphin, NIT3 cells were transiently transfected with CPE cDNA and prodynorphin cDNA in expression vectors and co-stained with the HA-tag and CPE. Briefly, cells were co-transfected using the standard calcium phosphate procedure (27) with equal amounts of the two constructs. Two days after transfection, the cells were replated on glass growth-supporting coverslips (Fisher Scientific), cultured for one more day, and then fixed in 4% paraformaldehyde for 10 min. The cells were rinsed in phosphate-buffered saline (PBS), permeabilized in 0.1% Triton X-100 for 1 h, and then blocked in 5% bovine serum albumin for 1 h. After blocking, the cells were immunostained with 1:2,500 dilution of monoclonal antibodies to the HA tag (a gift of Dr. Jonathan Backer, Molecular Pharmacology, Albert Einstein College of Medicine), and 1:1,000 dilution of polyclonal antiserum “135” raised against dynorphin B-13 (a gift of Dr. Lakshmi Devi). This antiserum to dynorphin recognizes both prodynorphin and processed peptides (28). Following the incubation with primary antibodies, fluorescein-labeled goat anti-rabbit IgG and rhodamine-labeled goat anti-mouse IgG (1:200 dilutions) were added, and the cells were incubated in the dark for 1 h. The coverslips were extensively washed, mounted on glass slides in 50% glycerol in PBS and examined using a Bio-Rad confocal microscope. The images represent a single plane of focus. The transfections and analysis were performed twice with similar results.

Secretion of CPE from NIT3 Cells—To examine whether different CPE mutants undergo regulated secretion, NIT3 cells were transfected as described above using CPE cDNA in the pcDNA3 expression vector. Stably transfected cells were selected using 0.8 mg/ml of geneticin (G418). Typically, 2–3 separate clones expressing different CPE mutants were examined for the regulated secretion. Cells were cultured in 6-well plates. Medium was removed, and the cells were washed three times with PBS and then incubated in Krebs Ringer buffer containing no addition or 45 mM KCl (ionic strength was maintained by reducing the NaCl concentration). After 40 min of incubation, the medium was removed and subjected to Western blot analysis using antibodies to the HA epitope-tag (1:1,000 dilution).

RESULTS

Wild type CPE has previously been expressed in high levels in SF9 cells using the baculovirus system (24). Mutation of the Glu300 into Lys, Gln, or Tyr does not affect the relative levels of CPE protein expression in the baculovirus system (Fig. 2). High levels of CPE protein are detected in both cells and media for all three point mutants. However, enzymatic activity toward...
dansyl-Phe-Ala-Arg is detectable only for the wild-type CPE (Table I). The level of enzyme activity of the wild-type CPE is similar to that determined in previous studies (13). The three point mutants show only background activity that is comparable with controls with nonexpressing virus (Table I). Similar results were observed in three separate experiments. Because of the presence of low levels of endogenous activities in the Sf9 cells, studies on crude cells or media cannot rule out the possibility that the mutant proteins have low levels of enzyme activity. To investigate this further, purification of the proteins were attempted on a substrate affinity resin.

When applied to a Sepharose column containing p-aminobenzoyl-Arg, the majority of the wild-type CPE is bound and only trace levels are detected in the flow through fraction (Fig. 3, F). Raising the pH to 8.0 causes most of the bound CPE to elute from the column (Fig. 3, E1). Similar analysis of the three point mutants showed that only the Glu300 form of CPE was able to bind to the affinity resin, and subsequently elute with high pH buffer (Fig. 3). Both the Lys300 and the Tyr300 mutants were unable to bind to the affinity resin. To test whether a small amount of protein bound to the column but could not be eluted by the high pH, the columns were also eluted with a high concentration of Arg after the high pH treatment. This treatment, which elutes other carboxypeptidases such as CPD, did not elute any detectable amount of Lys300 or Tyr300 CPE (Fig. 3, E2). Thus, these two mutants do not appear able to bind to the substrate affinity resin, whereas the Glu300 mutant is able to bind.

To ensure that the binding of the Glu300 form of CPE to the p-aminobenzoyl-Arg-Sepharose represents binding to the active site of the protein, we tested whether an active site-directed inhibitor could compete for this binding. GEMSA is a competitive inhibitor of CPE with Ki around 10 nM (29). When affinity column-bound wild-type or Glu300 CPE was incubated with 0.1 mM GEMSA, nearly all of the bound protein was eluted. To quantitate this, the experiment was performed with various concentrations of GEMSA. Approximately 50% of the wild-type CPE is eluted from the p-aminobenzoyl-Arg column with 6 μM GEMSA (Fig. 4). Similar analysis of the Glu300 form of CPE shows that 2 μM GEMSA elutes 50% of the bound protein (Fig. 4). Thus, both wild-type and Glu300 CPE bind to the affinity column through their active sites, and this binding is competed by generally similar concentrations of an active site-directed inhibitor.

Following purification of the Glu300 form of CPE on the affinity columns, the activity toward the standard carboxypeptidase substrate dansyl-Phe-Ala-Arg was tested. For this analysis, the affinity column-bound protein was eluted by raising the pH to 8.0 (as done in Fig. 3) rather than with GEMSA (as done in Fig. 4) because GEMSA would interfere with the enzyme activity measurement. Wild type CPE showed a Vmax of 20 μmol/min/mg protein, whereas no activity could be detected for the Glu300 form of CPE. Based on the sensitivity of the assay and the amount of purified protein tested, the Glu300 CPE has less than 0.004% of the activity of wild-type CPE. To test whether CPE activity or substrate binding are required for sorting of CPE into the regulated pathway, we expressed the proteins in the NIT3 pancreatic cell line. For this analysis, the HA epitope was added to the N terminus of the CPE so that the expressed protein could be detected above the background of endogenous CPE, which is only detected in the endoplasmic reticulum of these cells due to the naturally occurring point mutation (21). The cells were co-transfected with a plasmid expressing rat prodynorphin so that the distribution of the CPE could be compared with that of a prohormone. Prodynorphin has been previously found to be routed into the secretory pathway of neuroendocrine cells (30, 31). When expressed in NIT3 cells, the epitope tagged wild-type CPE (Glu300) showed a punctate pattern throughout the cytoplasm (Fig. 5). Co-staining of the cells with a rabbit polyclonal antiserum to dynorphin showed a similar pattern of distribution (Fig. 5). This pattern is consistent with localization of the CPE to secretory vesicles in this cell type. The distribution of the Lys300 and Glu300 forms of CPE also showed overlap to the prodynorphin (Fig. 5), suggesting that they were also routed to the secretory vesicles.

To directly examine if the expressed CPE was routed to the regulated secretory pathway, the media of the cells were examined following stimulation with 45 mM KCl. In the absence of stimulation, extremely low levels of CPE protein are detected in the media (Fig. 6). Following stimulation with KCl, there is
In this study, replacement of the Glu\(^{300}\) of CPE with Tyr resulted in an inactive enzyme that did not bind to the substrate affinity column. It is possible that the bulky side chain of the Tyr caused changes in the active site that precluded substrate binding. The substitution of Glu for Glu is relatively minor, changing only the -COOH into a -CONH\(_2\). This smaller change preserved binding to the substrate affinity column, indicating that binding does not require Glu\(^{300}\). Even though the Tyr\(^{300}\) and Lys\(^{300}\) forms of CPE were not able to bind to the affinity resin, these forms were secreted from both the Sf9 cells, and the Lys\(^{300}\) CPE was secreted from NIT3 cells (Tyr\(^{300}\) CPE was not examined in NIT3 cells). In contrast, forms of CPE containing deletions of 33 or more C-terminal residues, or point mutation of Ser\(^{202}\) into Pro are not secreted from either cell type and are degraded before protein transport into the Golgi (24, 33). The failure of these other mutations of CPE to be secreted presumably reflects the improper folding of these proteins. Because the Tyr\(^{300}\) and Lys\(^{300}\) forms of CPE were secreted in levels comparable with wild-type CPE, these point mutants are unlikely to be completely misfolded.

The regulated secretion of Glu\(^{300}\) and Lys\(^{300}\) CPE in the NIT3 cells indicates that neither enzyme activity or substrate binding are required for entry into the regulated pathway. Recently, Y. P. Loh and colleagues (20, 34) have proposed that CPE is a hormone-sorting receptor. This proposal is based on studies examining the sorting of prohormones in the fat/fat mouse, and in cell lines that have a reduced level of CPE protein due to the expression of antisense RNA. However, proinsulin sorting is not dependent on CPE, based on studies of primary cultures of pancreatic beta cells (35) or the NIT3 beta cell line (21). The sorting of proinsulin and insulin into the regulated pathway of the beta cells proceeds in the complete absence of CPE from this pathway. Also, previous studies have found that prodynorphin is routed to the regulated secretory pathway in the NIT3 cell line.\(^2\) Thus, CPE is not essential for the sorting of prohormones in pancreatic beta cells, although it is possible that CPE contributes to this process in other cell types. The mechanism by which CPE is sorted into the regulated pathway has not been elucidated. The C-terminal region of CPE appears to contain an element that contributes to the sorting into the regulated pathway; attachment of the C-terminal 50 residues to albumin causes a portion of the fusion protein to be secreted via the regulated pathway (33). This region is distinct from the membrane-binding motif also present in the C-terminal region of CPE (36). CPE aggregates at slightly acidic pH values, and this aggregation is enhanced by Cu\(^{2+}\) (37, 38); it is possible that this aggregation contributes to the routing of CPE. Models of co-aggregation of the secretory vesicle constituents have been proposed to account for the routing of the proteins into the regulated pathway (39, 40). Because the mutants of CPE that do not bind to the substrate affinity column are correctly routed in the NIT3 cells, the mechanism of CPE sorting presumably does not involve substrate binding to the active site.

The finding that Glu\(^{300}\) is not essential for binding of CPE to the affinity column supports the hypothesis that the new members of the CPE gene family that lack a Glu in the comparable position function as binding proteins rather than active enzymes. These three proteins (CPX2, AEBP1, and the third CP-like domain of CPD) have not been found to have enzyme activity upon expression in Sf9 cells using the baculovirus system (13, 14, 18). Although AEBP1 was reported to have detectable activity upon expression in bacteria (16), this activity was extremely low (0.02 absorption units at 256 nm with the

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\(^2\) L. Devi, personal communication.
substrate hippuryl-Arg after 10 min with 2 μg of enzyme). Furthermore, attempts to repeat the experiment using the identical constructs, expression system, and assay were unsuccessful. However, it remains possible that these proteins are active enzymes. The substitution of the Tyr for Glu300 of CPE was created with the hope that the protein would still bind to the affinity column and thus be able to be purified and tested with substrate in the absence of other enzymes present in the Sf9 cells and media. This was done for the Glu300 mutant, with the finding that this variant had less than 0.004% of the activity of wild-type CPE. However, as discussed above, the bulky side chain of the Tyr may have altered the active site and prevented binding to the affinity resin. Although alternative purification strategies could have been used to isolate the enzyme, the failure to bind to the substrate affinity resin suggests that the protein would not bind substrates and would therefore be unlikely to possess enzyme activity. The lack of enzyme activity in the media or cell extracts of the Sf9 cells infected with Tyr300 CPE-expressing baculovirus indicates that this mutant has less than 0.6% of the wild-type CPE activity.

In summary, Glu300 of CPE is essential for enzyme activity but not substrate binding. The recent identification of three members of the CPE gene family that lack a Glu in a comparable position highlights the importance of this residue. Interestingly, several other enzyme families contain members with substitutions of critical active site residues that affect enzyme activity but not substrate binding. For example, aspartyl protease-like proteins that lack the critical Asp have been found in bovine and sheep placenta (41, 42), and disintegrin-like proteins that lack the consensus site for metal binding have been found in human brain, testis, and other tissues (43, 44). In addition to these inactive members of protease gene families, several phosphatase-like proteins that lack one or more residues critical for catalytic activity have been discovered (45). Taken together with the present study, these results suggest that the evolution of proteins that lack enzyme activity but not substrate binding is a widespread mechanism for the generation of binding proteins.

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