Induced and Spontaneous Mutations at Ser202 of Carboxypeptidase E

EFFECT ON ENZYME EXPRESSION, ACTIVITY, AND INTRACELLULAR ROUTING*

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Carboxypeptidase E (CPE) is involved in peptide processing in the brain and various neuroendocrine tissues. In mice homozygous for the Cpe<sup>fat</sup> mutation, the virtual absence of CPE activity in islets of Langerhans and pituitary was associated with a missense mutation effecting a Ser<sup>202</sup> to Pro shift (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) Nat. Genet. 10, 135-142). To examine the importance of Ser<sup>202</sup> in CPE function, several mutations in this position were generated (Pro<sup>202</sup>, Ala<sup>202</sup>, Gly<sup>202</sup>, and Phe<sup>202</sup>). When the mutant prototypes were expressed in a Baculovirus system, both Phe<sup>202</sup> and Pro<sup>202</sup>CPE were enzymatically inactive, were unable to bind to a substrate affinity column, and were not secreted from Sf9 cells. In contrast, Ala<sup>202</sup>CPE or Gly<sup>202</sup>CPE exhibited enzymatic properties similar to those of wild-type CPE and were secreted from Sf9 cells.

When expressed in ATT-20 cells, a mouse pituitary-derived cell line, CPE with Pro<sup>202</sup> and Phe<sup>202</sup> were not secreted. Pulse-chase analysis with [<sup>35</sup>S]Met indicated that Pro<sup>202</sup>CPE was degraded in ATT-20 cells within several hours. This degradative process was blocked by incubation at 15 °C but not by brefeldin A or by lysosomotropic drugs. Pulse-chase analysis using dispersed pituitary cells from C57BLKS/Lt-fat/fat<sup>−</sup> mutant mice shows similar results; Pro<sup>202</sup>CPE produced in these cells was not secreted but rather was degraded within 5 h. Immunofluorescence analysis of epitope-tagged CPE revealed Ser<sup>202</sup>CPE to be present primarily in secretory vesicles, whereas Pro<sup>202</sup>CPE was localized to the endoplasmic reticulum and not the secretory vesicle-like structures. These results support the previous finding that Cpe<sup>at</sup>/Cpe<sup>fat</sup> mice are defective in CPE activity because of the point mutation producing the Ser<sup>202</sup> to Pro substitution. Furthermore, these results are consistent with a model that Ser<sup>202</sup> is important for the intracellular folding of CPE.

Most peptide hormones and neurotransmitters are produced from larger precursors by limited proteolysis. Initially, endopeptidases such as prohormone convertase 1, prohormone convertase 2, and furin cleave the prohormone precursor at multiple basic amino acid cleavage sites (1-5). Then, a carboxypeptidase removes the basic amino acids from the C terminus of the peptide to generate either the bioactive product or a precursor for the formation of the C-terminal amide group (6, 7). The carboxypeptidase step is important for the production of numerous bioactive peptide in many tissues (6, 7). Carboxypeptidase E (CPE,<sup>3</sup> also known as carboxypeptidase H and enkephalin convertase) is the major carboxypeptidase involved with the processing of many peptides (6, 7). A second enzyme, carboxypeptidase D, also may participate in the intracellular processing of peptide hormones and neurotransmitters, although the exact role of carboxypeptidase D is not yet clear (8). Both CPE and carboxypeptidase D are members of the metallo-carboxypeptidase gene family, which includes carboxypeptidases A, B, M, and N and AEBP-1, a recently described transcription repressor (9-16).

The importance of CPE in peptide processing is evident from the finding that C57BLKS/Lt-fat/fat mice have a reduced ability to convert proinsulin into insulin (17). The mutation responsible for the defect in these mice has been localized to a single point mutation within the coding region of the Cpe gene on chromosome 8. The mutant allele, now designated Cpe<sup>fat</sup>, contains proline in place of serine at residue 202. Ser<sup>202</sup> is conserved in human, bovine, rat, mouse, fish, and Aplysia (18-21) and is also conserved in human carboxypeptidase M, human carboxypeptidase N, and human, rat, duck, and Drosofila carboxypeptidase D (11, 13, 22, 23). However, in the digestive enzymes (CPA and carboxypeptidase B), the amino acid in the corresponding position is either Phe (CPA) or Tyr (carboxypeptidase B). Furthermore, in the bacterial metallo-carboxypeptidases, the amino acid in a comparable position is Ala (Streptomyces griseus), His (Streptomyces capreolus), Leu (Thermoactinomyces vulgaris), and Val (Apllysia cera). The crystal structures of carboxypeptidases A, B, and T (the T. vulgaris carboxypeptidase) have been determined (24-26) and are generally similar despite the moderate amount of amino acid homology between the various sequences (approximately 50% amino acid identity between CPA and carboxypeptidase B and approximately 25% amino acid identity between CPA and carboxypeptidase T). In all structures, the residue in the position that corresponds to Ser<sup>202</sup> of CPE (Phe<sup>202</sup> of bovine CPA) is present in a b sheet. Substitution of a Pro into this position

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1 The abbreviations used are: CPE, carboxypeptidase E; CPA, carboxypeptidase A; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.

2 G. Nagle, personal communication.

3 O. Varlamov, X. Xin, and L. Fricker, unpublished observation.
of CPA is predicted to perturb the structure.4 There is virtually no CPE activity in Cpeαβ/Cpeαβ mouse pituitary or pancreatic islets. Although a small amount of CPE-like activity is detected in these tissues, this action is due entirely to other metallocarboxypeptidases5 such as carboxypeptidase D (8). The absence of CPE in Cpeαβ/Cpeαβ mouse tissues is consistent with our previous finding that CPE with a substitution of Pro202 is not active when expressed in the Baculovirus system (17). In this study, we investigated whether other amino acids can substitute for Ser in position 202. The amino acids chosen for this substitution are those that are present in a comparable position in other metallocarboxypeptidases (Phe, Ala, Gly). In addition, we examined CPE with a Pro202 in further detail. The results of this analysis indicate that Ser202 is not essential for the production of enzymatically active CPE since Ala or Gly can replace the Ser. Analysis of the Ser202 CPE mutants, the cell homogenates were adjusted to 10 ml with 1M NaCl, 1% Triton X-100 in 100 mM sodium acetate buffer, pH 5.5. Aliquots of cell homogenate and media were subjected to immunoprecipitation as described, using an antisera raised against the N-terminal region of CPE (29, 31).

Materials and Methods

Generation of Constructs—Site-specific mutations of the Ser202 in CPE were generated using the Altered Sites I in vitro mutagenesis system (Promega). Rat CPE cDNA was subcloned into the BamHI and EcoRI sites of the vector pALTER-1, and the mutagenic oligonucleotides with different codon changes in position 202 were used to generate CPE with the single amino acid substitutions. The constructs were subcloned into the BamHI and EcoRI sites of the Baculovirus transfer vector pVL1393 or into eukaryotic expression vector pcDNA-3. For eukaryotic expression, the epitope recognized by the 12CA5 monoclonal antiserum (YPYDVPDYA) was inserted into a PsI site located immediately downstream of the pentabasic propeptide cleavage site, with the resulting amino acid sequence RRRLRSLQPPDYPDYLQQED ... (the gap indicates the propeptide cleavage site). Diexoydodeotide sequencing was performed to confirm the sequence of the mutated region of CPE.

Expression of Proteins in AT-20 Cells—The expression plasmids containing the sequences encoding CPE Ser202 mutants were transfected into AT-20 cells using the standard calcium phosphate procedure (27). Stably transfected cells were selected using 0.6 mg/ml of genetin (G418). Cell expression was detected by Western blot analysis and by immunoprecipitation using the 12CA5 monoclonal antiserum raised against HA epitope tag (a gift of Dr. J. jonathan Backer, Molecular Pharmacology, Albert Einstein College of Medicine).

Expression of Mutant Proteins in Baculovirus—Recombinant Baculovirus expressing different constructs was generated using the Baculovirus system (Pharmingen) as described (17). The cells from 25 ml of the culture were recovered by centrifugation at 1000 × g for 10 min. The cells were homogenized (Polytron, Brinkmann Instruments) in 5 ml of 100 mM sodium acetate buffer, pH 5.5. Aliquots of cell homogenate and media were assayed for carboxypeptidase activity using 0.2 mM dansyl-Phe-Ala-Arg in 100 mM sodium acetate buffer, pH 5.5, as described (28). Protein was determined using the Bradford reagent. Aliquots were also analyzed on a Western blot as described (17). To purify wild-type CPE and Ser202 CPE mutants, the cell homogenates were adjusted to 10 ml with 1 M NaCl, 1% Triton X-100 in 100 mM sodium acetate buffer, pH 5.5; sonicated; and centrifuged at 30,000 × g for 30 min. The supernatants containing cellular CPE were subjected to purification on a p-aminobenzoylarginine-Sepharose 6B affinity column as described (29). The CPE was recovered in 2 ml of 50 mM Tris buffer, pH 8, containing 150 mM NaCl and 0.01% Triton X-100. The ability of the various CPE constructs to bind the column was assessed by Western blot analysis using an antiserum against the CPE C-terminal sequence KMMSETLNF, as described (30).

Pulse-Chase Analysis of AT-20 Cells at Different Conditions—AT-20 cells expressing wild-type CPE and Pro202 were labeled (pulse) with [35S]Met (60 μCi/ml) for 20 min and then washed twice with DMEM (5.5 mM glucose) and the media replaced with 5 ml of serum-free DMEM. Some of the plates were incubated without radiolabel (chase) for 30 min in control medium or for 3 h under different conditions: incubation at 15 °C or at 37 °C in media containing breflidin A (5 μg/ml), chloroquine (100 μM), or NH4Cl (10 mM) or no addition. These conditions have been used previously to study the processing of proCPE in the AT-20 cell line (38). Media were removed and the cells were washed with phosphate-buffered saline (PBS) and then frozen in 10 ml sodium acetate buffer, pH 5.5, with 1 M phenylmethylsulfonyl fluoride. The cells and media were subjected to immunoprecipitation as described (31) using the 12CA5 antiserum against the HA epitope.

Immunofluorescence—Transfected and wild-type AT-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% bovine serum albumin, the cells were immunostained for 1 h with the primary antisera: mouse monoclonal antisera 12CA5 against the HA epitope tag (a gift from Dr. Sarah Klumperman, Albert Einstein College of Medicine) or rabbit polyclonal antiserum directed against the C-terminal region of CPE (29, 31).

Immunofluorescence—Transfected and wild-type AT-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% bovine serum albumin, the cells were immunostained for 1 h with the primary antisera: mouse monoclonal antisera 12CA5 against the HA epitope tag (1:10,000 dilution); rabbit polyclonal antisera directed against the C-terminal region of CPE (1:5,000 dilution); or rabbit polyclonal antisera to calcinein (1:1000). Cells were washed three times with PBS containing 0.2% Tween 20 and then incubated with fluorescein-labeled anti-mouse or anti-rabbit secondary antibody (Vector Laboratories Inc., 1:200 dilution) for 1 h followed by extensive PBS washing. Immunofluorescence staining was examined using a Nikon confocal microscope.

Results

On expression in Sf9 insect cells using the Baculovirus system, CPE with Ser202, Gly202, Ala202, Phe202 or Pro202 was expressed at relatively high levels in the cells (Fig. 1, left). However, the amount of CPE protein detected by Western blot analysis does not correlate with the amount of CPE activity present in the same cell extracts; only CPE with Ser202, Gly202, and Ala202 showed carboxypeptidase activity greater than the amount of activity detected in cells infected with control Baculovirus (Table I). Analysis of the media from infected cells revealed that only the forms of CPE that showed enzyme activity (Ser202, Gly202, and Ala202) were secreted in detectable amounts (Fig. 1, middle). CPE protein could not be detected by Western blot analysis of media from cells expressing the Phe202 or Pro202 mutants (Fig. 1, middle), and the amount of CPE-like enzyme activity in these fractions was comparable to the level in media from cells infected with control virus (Table I). In two separate experiments, the level of CPE-like activity in the cells and media varied as much as 50% for the same construct, which is presumably due to variables associated with the viral infection. In both experiments, cells expressing Ser202, Gly202, or Ala202 showed high levels of CPE activity,
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and cells expressing Pro$^{202}$ or Phe$^{202}$CPE showed background levels. To investigate which of the CPE variants in the cell extracts were capable of binding to a C-terminal Arg, extracts were purified on a p-aminobenzoylarginine-Sepharose affinity column. Only the enzymatically active forms of CPE (Ser$^{202}$, Gly$^{202}$, and Ala$^{202}$) were bound by the substrate affinity resin (Fig. 1, right).

The stability of CPE to thermal denaturation was investigated by preincubating the purified CPE at various temperatures for 1 h. The reaction mixture was then combined with dansyl-Phe-Ala-Arg and sodium acetate buffer (final pH 5.5), and the samples were incubated at 37°C. Product was detected using the standard procedure (28). Error bars, standard error (n = 3).

TABLE I

| Fraction | Activity* | CPE mutation          | Control virus |
|----------|-----------|-----------------------|---------------|
|          | Ser$^{202}$ | Gly$^{202}$ | Ala$^{202}$ | Phe$^{202}$ | Pro$^{202}$ |
| Cells    | 15.7      | 15.2      | 12.5      | 0.3        | 0.2        |
| Media    | 51.0      | 29.5      | 36.3      | 0.1        | <0.1       |

* Assayed with dansyl-Phe-Ala-Arg (32).

endogenous wild-type CPE present in AtT-20 cells. The epitope tag in the N-terminal position does not interfere with enzyme activity, thermostability, or sorting to the regulated secretory pathway. As found for Baculovirus-mediated expression in Sf9 cells, epitope-tagged CPE with Ser$^{202}$ mutated to Pro, Gly, or Phe was expressed in the cells, and the Ser$^{202}$ and Gly$^{202}$ forms of CPE were secreted (Fig. 3). Similar results were found when the various forms of CPE were transiently expressed in COS cells and examined by immunoprecipitation and Western blot analysis (not shown). In addition to the 58-kDa epitope-tagged CPE, the 12CA5 antisera detected several 40- to 50-kDa proteins in the AtT-20 cells; since these proteins were also present in the untransfected AtT-20 cells, they are not related to the epitope-tagged CPE. When AtT-20 cell extracts expressing the various mutations were purified on a substrate affinity resin, only the Ser$^{202}$CPE and Gly$^{202}$CPE were bound to the resin (data not shown). AtT-20 cells expressing epitope-tagged Ser$^{202}$CPE or Pro$^{202}$CPE were examined using [35S]Met pulse-chase analysis. As previously found for wild-type CPE, [35S]Met-labeled epitope-tagged Ser$^{202}$CPE shows a major band around 58 kDa after a 20-min pulse (Fig. 4, left). After a 3-h chase, this band is substantially reduced, which is due to secretion into the media and proteolytic processing which removes N- and C-terminal Met residues (33). When the chase is performed at 15°C, which blocks transport between the endoplasmic reticulum and Golgi, or performed in the presence of brefeldin A, the amount of epitope-tagged Ser$^{202}$CPE is slightly reduced, compared with the level in the "no-chase" control (Fig. 5, middle). Compounds that interfere with the acidification of granules and lysosomes (ammonium chloride, chloroquine) had little effect on the decrease in levels of cellular Ser$^{202}$CPE (Fig. 5, middle).

The Pro$^{202}$ form of CPE also showed a major band of approximately 58 kDa after labeling for 20 min with [35S]Met (Fig. 4, right). Unlike Ser$^{202}$CPE, the apparent size and the intensity of the labeled Pro$^{202}$CPE did not change following 30 min of chase. After 3 h of chase, the intensity of this band was considerably reduced, although in contrast to Ser$^{202}$CPE, no radiolabeled Pro$^{202}$CPE could be detected in the media (Fig. 4, right). Incubation of the cells at 15°C prevents the degradation of Pro$^{202}$CPE (Fig. 5, right). Densitization of these data, and

6 L. Song and L. Fricker, unpublished observations.
data from two additional experiments showed the 15°C group to be approximately 90% of the 'no chase' control group. In contrast, brefeldin A, chloroquine, and ammonium chloride had no effect on the stabilization of Pro202CPE. Similar results were observed in two additional experiments. These results are consistent with previous studies on a variety of unfolded proteins that have been found to be degraded by a temperature-sensitive endoplasmic reticulum or Golgi process that is not sensitive to lysosomotropic agents (34–37).

Pulse-chase analyses of pituitaries from control and Cpefat mice were performed to determine whether the Pro202CPE, which occurs naturally in the Cpefat mice, was also degraded before secretion. After labeling for 1.5 h, strong bands of radiolabeled CPE (approximately 52-55 kDa) were detected after immunoprecipitation in both the genotype-normal control and Cpefat pituitary cell suspensions (Fig. 6). Whereas the Cpefat pituitary cells showed a single band of 55 kDa for CPE, the genotype-normal cells showed two bands of 52 and 55 kDa, consistent with previous reports of the size of CPE in cell lines and tissues (29, 38). A 5-h chase was associated with a large reduction in the level of labeled CPE in pituitaries from both mice. However, CPE is detected in the media only for the genotype control cells and not from the Cpefat pituitary cells (Fig. 6).

To examine the intracellular distribution of Ser202 and Pro202CPE in the AtT-20 cells, the stably transfected cells were fixed, permeabilized, and probed with an antiserum to the HA epitope. Epitope-tagged Ser202CPE showed punctate staining throughout the cell body, with dense staining at the tips of the cell processes (Fig. 7). In addition, some staining was observed in a perinuclear region (Fig. 7). This distribution of epitope-tagged Ser202CPE is similar to the distribution of endogenous CPE in wild-type AtT-20 cells, as visualized with an antiserum directed against the C-terminal region (Fig. 7). The endogenous CPE is concentrated in the tips of the cell processes and shows punctate staining throughout the cell body (Fig. 7). This distribution of CPE is consistent with the distribution of other secretory vesicle proteins in AtT-20 cells (39, 40). In contrast, Pro202CPE shows a diffuse, reticular network-like distribution throughout the cell body (Fig. 7). This distribution is similar to that of calnexin (Fig. 7), a resident endoplasmic reticulum protein (41). Wild-type AtT-20 cells showed negligible staining when probed with the antiserum directed against the HA epitope (Fig. 7). Also, approximately 80% of the cells expressing either Ser202 or Pro202CPE were not labeled with the HA epitope-directed antiserum (shown for Pro202CPE in Fig. 7), suggesting that the population of stably transfected cells included many cells that did not express the CPE construct. In contrast, all of the cells were labeled with antiserum directed against CPE or calnexin.

DISCUSSION

A major finding of this study is that Ser202 is not essential in the function of CPE since this residue can be replaced with Gly or Ala. The enzymatic properties of these two mutant forms of CPE are comparable to the properties of wild-type (Ser202)
CPE. This finding suggests that Ser<sup>202</sup> is not an essential residue in the folding of CPE, despite the conservation of this residue in CPE from various species and in other related carboxypeptidases such as carboxypeptidase M and carboxypeptidase N. In contrast, replacement of Ser<sup>202</sup> with Pro or Phe causes the complete loss of CPE activity. This effect is presumably due to misfolding of the protein since incorrectly folded proteins are typically degraded in the cell and not secreted. The finding that CPE with a Pro<sup>202</sup> substitution is inactive is consistent with our previous finding that this mutation, which is found in the Cpe<sup>fat</sup> mouse, leads to a loss of enzyme activity (17). The inability of Phe to substitute for Ser<sup>202</sup> is not surprising since the Phe is much larger than the Ser. Although a Phe is found in the comparable position of CPA and B, there are numerous other differences between these enzymes and CPE; the amino acid identity is only 20% between CPE and either CPA or carboxypeptidase B.

Our finding that Ser<sup>202</sup> can be mutated to Gly or Ala without adversely affecting the enzyme activity implies that if this Ser is a site for post-translational modification (phosphorylation or glycosylation) these modifications are not essential. It is unlikely that Ser<sup>202</sup> is a phosphorylation site since the consensus sequence for phosphorylation of secretory pathway proteins is Ser-X-Asp/Glu (42), which is not present in this region of CPE. The region of CPA, carboxypeptidase B, and carboxypeptidase T that is comparable to Ser<sup>202</sup> of CPE is a β sheet that is buried in the interior of the protein (24–26). If CPE has a similar structure for this region, Ser<sup>202</sup> would be an unlikely site for phosphorylation or glycosylation.

The finding that Pro<sup>202</sup>CPE is inactive in both Sf9 cells and AtT-20 cells is consistent with the identification of a single Ser<sup>202</sup> to Pro point mutation within CPE as the defect associated with the Cpe<sup>fat</sup> mutation (17). These mice have essentially no CPE activity; the small amount of CPE-like activity detected in pituitary and pancreatic islets is entirely due to carboxypeptidase D and other enzymes. However, the effect of the CPE mutation on prohormone processing is complicated. A defect in CPE would be predicted to interfere only with the step catalyzed by CPE, the removal of C-terminal Lys and Arg residues from intermediates formed by the action of prohormone convertases 1 and 2 on the prohormone (43). However, proinsulin accumulates in very high levels in Cpe<sup>fat</sup> mice, suggesting that the defect in CPE affects the previous enzymatic step (17). In considering the mechanism by which the defect in CPE interferes with the endopeptidase processing of proinsulin, there does not appear to be a dominant negative effect of mutant CPE. Animals that are heterozygous for the Cpe<sup>fat</sup> mutation and that presumably produce Pro<sup>202</sup>CPE in addition to wild-type CPE have levels of proinsulin in the normal range (17).

There are several possible mechanisms by which the defect in CPE could interfere with the previous endopeptidase step. The high intragranular levels of C-terminally extended forms of insulin conversion intermediates (resulting from the absence of CPE) could mediate feedback inhibition on the prohormone convertase activities. A related possibility is that an endogenous inhibitor of the endopeptidases is inactivated by CPE, and the absence of CPE then leads to a decrease in prohormone convertase activity. Evidence for this possibility has recently been reported, although the inhibitor (7B2) blocks only prohormone convertase-2 activity and not prohormone convertase 1 (44). Another possibility is that CPE is directly involved in the activation of the prohormone convertases, perhaps by cleaving the C-terminal basic residues from the propeptide of these endopeptidases. Alternatively, it is possible that CPE plays a role in the transport and/or sorting of the endopeptidases into the regulated secretory pathway. From this study, the finding that Pro<sup>202</sup>CPE appears to be degraded in an endoplasmic reticulum or Golgi compartment and not sorted into the regulated pathway is important for understanding the defect of the Cpe<sup>fat</sup> mice. Thus, the defect is not merely in the absence of CPE activity but also in the absence of CPE protein from the regulated secretory pathway. CPE has recently been found to aggregate at acidic pH in the presence of Ca<sup>++</sup>, and this may contribute to the sorting of CPE into the regulated pathway (45). It is possible that aggregation of CPE is important for the sorting of other proteins, and the absence of CPE protein, rather than CPE activity, is responsible for the apparent defect in the endopeptidase cleavage step.

The finding of comparable translation of CPE in control and Cpe<sup>fat</sup> pituitary cells immediately after the [<sup>35</sup>S]Met pulse is consistent with the results of Pro<sup>202</sup>CPE expressed in AtT-20 cells and with the previous report that levels of CPE mRNA are not reduced in endocrine tissues of Cpe<sup>fat</sup> mice (17). This result is also consistent with the results of Pro<sup>202</sup>CPE expressed in Sf9 cells; in all cases, there are large amounts of mutant CPE found in the cells. Thus, the defect appears to be one of protein stability, presumably due to an altered structure caused by the Ser<sup>202</sup> to Pro mutation.
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