Characterization of *Drosophila* Carboxypeptidase D*

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Metallocarboxypeptidase D (CPD), is a 180-kDa protein that contains three carboxypeptidase-like domains, a transmembrane domain, and a cytosolic tail and which functions in the processing of proteins that transit the secretory pathway. An initial report on the *Drosophila melanogaster* silver gene indicated a CPD-like protein with only two and a half carboxypeptidase-like domains with no transmembrane region (Settle, S. H., Jr., Green, M. M., and Burtis, K. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9470–9474). A variety of bioinformatics and experimental approaches were used to determine that the *Drosophila* silver gene corresponds to a CPD-like protein with three carboxypeptidase-like domains, a transmembrane domain, and a cytosolic tail. In addition, two alternative exons were found, which result in proteins with different carboxypeptidase-like domains, termed domains 1A and 1B. Northern blot, reverse transcriptase PCR, and sequence analysis were used to confirm the presence of the various mRNA forms. Individual domains of *Drosophila* CPD were expressed in insect SF9 cells using the baculovirus expression system. Media from domain 1B- and domain 2-expressing cells showed substantial enzymatic activity, whereas medium from domain 1A-expressing cells was no different from cells infected with wild-type virus. Domains 1B and 2 were purified, and the enzymatic properties were examined. Both enzymes cleaved substrates with C-terminal Arg or Lys, but not Leu, and were inhibited by conventional metalloprotease inhibitors and some divalent cations. *Drosophila* domain 1B is more active at neutral pH and greatly prefers C-terminal Arg over Lys, whereas domain 2 is more active at pH 5–6 and slightly prefers C-terminal Lys over Arg. The differences in pH optima and substrate specificity between *Drosophila* domains 1B and 2 are similar to the differences between duck CPD domains 1 and 2, suggesting that these properties are essential to CPD function.

CPD* was initially discovered as a 180-kDa duck protein that bound hepatitis B viral particles and was designated gp180 (1).

* This work was supported primarily by National Institutes of Health Grant R01-DK51271 and also by Research Scientist Development Award K02-DA00194 (to L. D. F.). The DNA sequencing facility of the Albert Einstein College of Medicine is supported in part by Cancer Center Grant CA13330. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF545816, AF545817, AF545818, AF545819, and AF545820.

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‡ The abbreviations used are: CPD, carboxypeptidase D; CPE, carboxypeptidase E; EST, expressed sequence tag; FA, furylacryloyl; RT, reverse transcriptase; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
are also missing key metal-binding and/or other catalytic residues and do not cleave standard carboxypeptidase substrates (17–19).

To gain insight into the significance of the various carboxypeptidase-like domains of CPD, the Drosophila genome and various expressed sequence tag (EST) data bases were analyzed for CPD-like sequences. Then Northern blot analysis was performed with exon-specific probes in order to determine the forms of mRNA and the resulting proteins. The two different first carboxypeptidase-like domains (1A and 1B) and the second carboxypeptidase-like domain were individually expressed using the baculovirus expression system, and their enzymatic properties were determined. The finding that the first carboxypeptidase-like domain (the 1B form) and the second carboxypeptidase-like domain differ in their pH optima and specificities for C-terminal Lys versus Arg indicates that this is a fundamental aspect of CPD structure/function that has been conserved from Drosophila to vertebrates.

MATERIALS AND METHODS

Analysis of the Drosophila Genome and EST Data Bases

The Berkeley Drosophila Genome Project Web site (www.fruitfly.org) and the FlyBase data base (flybase.bio.indiana.edu) were searched with a variety of carboxypeptidase sequences, including rat CPE, rat CPD, human carboxypeptidases A and B, and the Drosophila suv gene product. In addition to the genomic Drosophila sequence, data bases of EST clones were also screened with the various carboxypeptidase sequences. Five EST clones (LP08595, GH13060, LD23786, LD28490, and LP12324) were purchased (Invitrogen) and sequenced in both directions. Reverse transcriptase-PCR (RT-PCR) was also used to confirm the predicted splicing patterns of the various exons. For the predicted C-terminal region, one of the gene prediction programs in the FlyBase database (flybase.bio.indiana.edu) was searched with the predicted splicing patterns of the various exons. The presence of an N-terminal signal peptide was predicted using the MetaGene program (Research Institute of Molecular and Cellular Biology, Saint Petersburg, Russia). The Berkeley Drosophila Genome Project Web site (www.fruitfly.org) and the FlyBase data base (flybase.bio.indiana.edu) were searched with a variety of carboxypeptidase sequences, including rat CPE, rat CPD, human carboxypeptidases A and B, and the Drosophila suv gene product. In addition, the PCR product was subcloned into the pcRA-TOPO vector (Invitrogen), and ~20 clones were isolated and sequenced.

Expression of CPD Domains in Baculovirus and Enzyme Purification

Plasmid Construction—For expression in the baculovirus system, PCR was used to generate a cDNA fragment corresponding to the various carboxypeptidase domains, and this fragment was subcloned into the pVL1393 baculovirus expression vector (Pharmingen) downstream of the signal peptide sequence derived from rat CPE. This CPE signal peptide sequence has previously been found to produce high levels of secreted proteins using the baculovirus expression system (22).

The Drosophila CPD 1A and 1B constructs were created by amplifying 1.1-kb fragments using EST clones LP12324 and LD28490, respectively, as templates with Tgo DNA polymerase (Roche Molecular Biochemicals). The Drosophila CPD 2 construct was produced using Drosophila mRNA and RT-PCR SUPERScriptTM II RNAase H Reverse Transcriptase (Invitrogen) and then Phenomenex Targa DNA polymerase High Fidelity (Invitrogen) to amplify a 1.2-kb product. The 5′-end of each PCR product contained a restriction site (Xba I or Sph I) that was used to maintain the restriction site immediately following the prepro-CPE sequence in the baculovirus expression construct pCPE20 (22). The 3′-end of each PCR product contained a stop codon followed by the Not I restriction site. The PCR product was purified (PCR purification kit; Qiagen), digested with Xba I or Sph I and Not I, and ligated into XbaI/Not I-digested pCPE20. All resulting plasmids were confirmed by sequencing in both directions.

Baculovirus Expression and Protein Purification—The three baculovirus expression plasmids (2.5 μg each) were separately combined with 0.25 μg of Baculoplatinum viral DNA (Orbigen) and used to transfect 106 SF9 cells using the standard procedure recommended by Orbigen. The recombinant virus was amplified in SF9 cells as described (11). For analysis of enzyme activity and subsequent protein purification, 100–300 ml of SF9 cells at 2 × 10⁶ cells/ml were infected with recombinant virus, and after 3 days, the cells were removed by centrifugation at 30,000 × g for 30 min. The supernatant was removed and either assayed directly (described below) or purified on a 5-ml aminobenzoyl-Arg-Arg-Sephrose affinity resin column (23). For domain 1B, 100 ml of supernatant was adjusted to 100 mM NaAc, pH 5.5, and applied to a 0.5-ml aminobenzoyl-Arg-Arg-Sephrose affinity resin column. The column was washed with 50 ml of 0.1 M NaAc, pH 5.5, containing 1 mM NaCl and 1% Triton X-100 and then rinsed with 10 ml of 10 mM NaAc, pH 5.5. The resin was eluted with 5 ml of 50 mM Tris-HCl, pH 8.0, containing 0.1% CHAPS and 0.05% Triton X-100 (Elute 1) and then with 5 ml of the same buffer containing 25 mM Arg (Elute 2). For domain 1A, the 50 ml of supernatant was dialyzed against 50 mM NaAc, pH 5.5, and applied to a 0.5-ml aminobenzoyl-Arg-Arg-Sephrose affinity resin column. The column was washed with 0.1 mM NaAc, pH 5.5, containing 0.5 mM NaCl and 0.5% Triton X-100, rinsed with 10 mM NaAc, pH 5.5, and eluted with 5 ml of 10 mM Elute 1 buffer (above) and then 5 ml of Elute 2 buffer (above). Most of the enzyme was found in Elute 2. Domain 1A was also tested with the p-aminoazobenzoyl-Arg-Arg-Sephrose affinity resin but was not found to bind under any of the above conditions, even when 1 mM ZnCl₂ was included in the binding buffer (data not shown).

Enzyme Assays

Carboxypeptidase Assay with Fluorescent Substrates—Enzyme activity was typically assayed in triplicate with 200 μM dansyl-Ala-Arg in 100 mM Tris acetate, pH 7.4, 80 μM fluor (for domain 1B) or 50 μM fluor (for domain 2) containing 0.01–0.1% Triton X-100 in a final volume of 250 μl. The reaction was stopped with 100 μl of 0.5 M HCl after 30–45 min at 37°C, 2 ml of chloroform was added, and the tubes were mixed and centrifuged at 150 × g for 2 min. The amount of product (dansyl-Ala-Arg) was measured in the fluorescent phase of the chloroform extract (excitation, 350 nm; emission, 500 nm). The pH optima of purified enzymes were determined using 100 μM dansyl-Ala-Arg in 50 mM Tris acetate buffer at various pH values at 37°C. To examine the effect of inhibitors and metals, purified enzymes were preincubated with the inhibitor for 15 min at room temperature. After the preincubation, substrate (dansyl-Ala-Arg) was added at the final concentration, and the reaction was incubated at 37°C for 4–5 h. For kinetic analysis, the purified enzymes were dialyzed (Centricon 30) to remove traces of Arg and incubated with substrate (final concentration 0.1, 0.2, 0.4, 0.6, 0.8, and 1 μM) for a 1-h incubation at 37°C.
Characterization of Drosophila Carboxypeptidase D

FIG. 1. Structure of Drosophila CPD gene. The location of BamHI (B) and EcoRI (E) restriction fragments are shown, corresponding to those described previously (4). The open bars represent 5’- and 3’-untranslated regions; the length of the 3’-untranslated region was not completely determined (indicated by a question mark). The gray-shaded box indicates the region of exon 8 that is spliced as an intron in the tail-2 form of the mRNA.

and $K_{cat}$ were calculated using SigmaPlot 2001 Enzyme Kinetic Module (Jandel Scientific).

Furylacryloyl Assays—Enzymes were prepared as for dansyl-Phe-Ala-Arg kinetics. Either 0.1 μg of domain 1B or 0.5 μg of domain 2 were added to 100 μM furylacryloyl-Ala-Arg (FA-Ala-Arg), furylacryloyl-Ala-Lys (FA-Ala-Lys), or furylacryloyl-Gly-Leu (FA-Gly-Leu) in 0.1 M Tris acetate, pH 7.4 or 5.7, and 0.01% Triton X-100. The reactions were incubated at 25°C, and absorption was monitored at 336 nm in a spectrophotometer.

RESULTS

Analysis of the Drosophila genomic and EST data base sequences as well as RT-PCR and sequencing revealed that the CPD gene is comprised of eight exons (Fig. 1). There are three alternatively spliced first exons. Exon 1A encodes the sequence previously found as a partial cDNA sequence and named 1a (4), exon 1B corresponds to a cDNA sequence previously named 1b (4), and exon 1C corresponds to an EST clone (discussed further below). The original sequence report of the gene sequence was incorrect (svr). Both the 1A or 1B form of Drosophila CPD, which is consistent with the lack of a pro region in vertebrate CPD. In addition to these two forms, a third form lacking exon 1A or 1B, it is likely that all three polyadenylation sites are used or additional upstream exons may be included in some of the forms.

The various carboxypeptidase-like domains were compared with each other and to selected members of the E/N subfamily of metallo carboxypeptidases. Domains 1A and 1B have highest amino acid sequence identity with each other (82%), although this includes the common region encoded by exons 2–4. The unique regions of these two proteins show 42% amino acid identity. Both the 1A and 1B forms of Drosophila CPD have 30–40% amino acid sequence identity with Drosophila CPD domain 2 and with various vertebrate carboxypeptidases (Table I). Interestingly, Drosophila domain 1B does not show higher amino acid sequence identity to the first domain of duck
or rat CPD and has slightly higher sequence identity with the second domain of these proteins (Table I). The second domain of Drosophila CPD does show higher amino acid sequence identity to the second domains of duck and rat CPD relative to the first or third domains of these proteins (Table I).
identity to all domains of duck and rat CPD and to other members of the gene family (Table I). In addition, the third domain of Drosophila CPD lacks many of the residues required for substrate binding and enzymatic activity, although it is predicted to fold into a carboxypeptidase-like structure.Domains 1A, 1B, and 2 are also predicted to fold into carboxypeptidase-like structures. Domains 1A, 1B, and 2 contain all of the metal and substrate-binding residues in the expected positions and are predicted to encode active enzymes. Domain 1A contains nearly all of the active site residues and only lacks one of the metal-binding His residues (the His in the position equivalent to His{superscript}69{subscript}H{subscript}11001{subscript}} of bovine carboxypeptidase A, the reference numbering system for active site residues). In domain 1A, this critical His is replaced by a Gln, which is not predicted to bind Zn{superscript}2{superscript}+. The third domain of duck CPD was previously found to be inactive toward standard carboxypeptidase substrates, and because the Drosophila third domain has even fewer of the key catalytic residues, this domain was not further studied. However, carboxypeptidase domain 1A appeared to only lack a single metal-binding residue, so this was included in further studies investigating enzyme activity. The individual carboxypeptidase domains 1A, 1B, and 2 were expressed in baculovirus under the polyhedrin promoter and with the signal peptide of rat CPE (in order to provide a uniform 5′-untranslated region and signal peptide for all three constructs). Western blot analysis of medium from cells expressing wild-type virus (Fig. 4A), which corresponds to either a viral or an Sf9 cell protein. The media from cells infected with the domain 1A construct showed bands of 54 kDa, in addition to the common 64-kDa band (Fig. 4A). Similar results were obtained using an antiserum raised against purified rat CPE (data not shown). When assayed with the standard CPD substrate, dansyl-Phe-Ala-Arg, media from both domain 1B- and domain 2-expressing cells showed substantial amounts of activity at pH 5.6 and 7.4 (Table II). The domain 1B medium was more active at pH 7.4, whereas the domain 2 medium was more active at pH 5.6. Medium from domain 1A-expressing cells showed carboxypeptidase activity that was comparable with the medium from cells infected with wild-type virus (Table II).

### Table I

| Amino acid sequence identities among various metallocarboxypeptidases |
|-----------------|-----------------|-----------------|-----------------|
|                  | Drosophila CPD  | Drosophila CPD  | Drosophila CPD  |
|                  | 1A | 1B | 2 | 3 |
| Drosophila CPD 1A | 100| 82| 34| 16 |
| Drosophila CPD 1B | 82| 100| 35| 15 |
| Drosophila CPD 2 | 34| 35| 100| 17 |
| Drosophila CPD 3 | 16| 15| 17| 100 |
| Duck CPD 1 | 38| 36| 37| 15 |
| Duck CPD 2 | 39| 40| 47| 18 |
| Duck CPD 3 | 30| 31| 35| 20 |
| Rat CPD 1 | 39| 39| 39| 19 |
| Rat CPD 2 | 40| 40| 43| 18 |
| Rat CPD 3 | 28| 29| 37| 20 |
| Rat CPE | 39| 40| 38| 19 |
| Human CPM | 40| 39| 37| 18 |
| Human CPN | 38| 40| 42| 15 |
| Human CPZ | 37| 39| 33| 22 |

* The overall amino acid sequence identity between protein domains 1A and 1B is 82%, but this includes the common exons 2, 3, and 4; the amino acid sequence identity between the coding region of exon 1A and exon 1B is only 42%.

### Table II

| Activity of various Drosophila CPD domains secreted from Sf9 cells using the baculovirus expression system |
|------|------|------|
| Enzyme activity§ | pH 5.6 | pH 7.4 |
| Wild type virus | 0.22 | 0.25 |
| Drosophila CPD 1A | 0.19 | 0.17 |
| Drosophila CPD 1B | 50.5 | 173 |
| Drosophila CPD 2 | 23.2 | 11.5 |

§ Activity was determined with 200 μM dansyl-Phe-Ala-Arg. Units are nmol of product formed/ min/medium from 10{superscript}⁶ cells, with less than 10% variation between duplicate determinations.

To further study CPD domains 1B and 2, they were purified using a substrate affinity column previously used to isolate vertebrate CPD (3, 11). Both preparations of enzyme showed a major band of the expected size when analyzed by denaturing gel electrophoresis and silver staining (Fig. 4B). Drosophila domain 1B is maximally active at pH values between 7 and 8, whereas domain 2 is maximally active in the pH 5–6.5 range (Fig. 5). The pH optima of the purified enzymes are not substantially different from those of the unpurified medium (Fig. 5). Domain 1B is not inhibited by 1 mM phenylmethylsulfonyl fluoride or iodoacetamide or by 1 mM guanidinoethylmercapto succinic acid, an active site-directed inhibitor (Table III). Domain 2 shows a slight degree of inhibition by 1 mM iodoacetamide and is more strongly inhibited by 1 mM guanidinoethylmercapto succinic acid. As previously found for duck CPD (11, 12), both domains of Drosophila CPD are inhibited by the thiol-directed reagent para-chloromercuriphenyl sulfonate, with the second domain more sensitive than the first domain (Table III). The metalloproteinase-nature of Drosophila CPD is evident from the substantial inhibition observed in the presence of the Zn{superscript}2{superscript}+ chelator 1,10-phenanthroline (Table III). EDTA is less potent as an inhibitor than 1,10-phenanthroline.

Domains 1B and 2 of Drosophila CPD show differences in their sensitivity to various divalent cations. Concentrations of Zn{superscript}2{superscript}+ of 10 μM or higher inhibit domain 1B, whereas domain 2 is not substantially affected by a 10 or 100 μM concentration of this ion (Fig. 6). Both domains are activated by Co{superscript}2{superscript}+, with domain 1B slightly more sensitive to this metal than domain 2. Cu{superscript}2{superscript}+ and Hg{superscript}2{superscript}+ inhibit domain 2 more potently than they...
Characterization of Drosophila Carboxypeptidase D

Effect of inhibitors on Drosophila CPD domains 1B and 2

| Inhibitor | Concentration | Activity |
|-----------|---------------|----------|
|           |               | Domain 1B | Domain 2 |
| PMSF      | 1 mM          | 99 ± 2    | 91 ± 4   |
| Iodoacetamide | 1 mM       | 102 ± 1   | 85 ± 4   |
| GEMSA     | 10 μM         | 56 ± 2    | 12 ± 1   |
| GEMSA     | 1 μM          | 99 ± 1    | 47 ± 3   |
| GEMSA     | 0.1 μM        | 103 ± 2   | 90 ± 5   |
| pCMPS     | 0.1 mM        | 85 ± 1    | 45 ± 1   |
| 1,10-Phenan-tholine | 1 mM | 1.8 ± 0.2 | <0.5     |
| EDTA      | 1 mM          | 87 ± 1    | 35 ± 1   |

a PMSF, phenylmethylsulfonyl fluoride; GEMSA, guanidinoethylmercaptoacetic acid; pCMPS, para-chloromercuriphenyl sulfonate.

Activity was determined in triplicate with 200 μM dansyl-Phe-Ala-Arg in Tris Acetate at either pH 7.4 (domain 1B) or at pH 5.7 (domain 2). Activity is expressed as the % of control incubations performed in the absence of inhibitor (± standard error of the mean).

Kinetic analysis of cleavage of dansyl-Phe-Ala-Arg by Drosophila CPD domains 1B and 2

| Inhibitor | Concentration | Activity |
|-----------|---------------|----------|
|           |               | Domain 1B | Domain 2 |
| Zn²⁺      | 100 μM        | 208 ± 11  | 246 ± 31 |
| Cu²⁺      | 100 μM        | 39.5 ± 0.5| 4.30 ± 0.17|
| Ni²⁺      | 100 μM        | 32.6 ± 0.4| 3.86 ± 0.15|
| Hg²⁺      | 100 μM        | 157.8 ± 8 | 15.7 ± 1.8|

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Discussion

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Both domains of Drosophila CPD cleave dansyl-Phe-Ala-Arg with generally comparable Km values (Table IV). However, the Vmax for domain 1B is substantially higher than that of domain 2, and the resulting Kcat/Km values for the two domains vary 10-fold. Because comparable substrates with a C-terminal Lys residue are not available, we employed another assay using shorter substrates, FA-Ala-Arg and FA-Ala-Lys. Unfortunately, these compounds showed a large amount of substrate inhibition at concentrations greater than 200 μM, so it was not possible to evaluate kinetic parameters. Using a single concentration of 100 μM, domain 1B cleaved FA-Ala-Arg ~10 times faster than FA-Ala-Lys (Fig. 7). No detectable hydrolysis of FA-Gly-Leu was observed (Fig. 7), even upon prolonged incubation (data not shown). Domain 2 also did not show any detectable hydrolysis of the Leu-containing substrate but cleaved FA-Ala-Lys slightly faster than it cleaved FA-Ala-Arg. Domain 2 was markedly less active toward either substrate than domain 1B; to produce comparable amounts of product formation, 5-fold more domain 2 protein was used than domain 1B protein (Fig. 7). Thus, the 10-fold difference between Kcat/Km observed between domains 1B and 2 for cleavage of dansyl-Phe-Ala-Arg is comparable with the difference in activity observed for cleavage of 100 μM FA-Ala-Arg.

Figure 5. pH optima of Drosophila CPD domains 1B (D1B) and 2 (D2). CPD activity was determined using 100 μM dansyl-Phe-Ala-Arg in 50 mM Tris acetate buffer at the indicated pH, measured at 37 °C. Activity was normalized to the maximal activity detected at the optimal pH.

Table III

| Inhibitor | Concentration | Activity |
|-----------|---------------|----------|
|           |               | Domain 1B | Domain 2 |
| PMSF      | 1 mM          | 99 ± 2    | 91 ± 4   |
| Iodoacetamide | 1 mM       | 102 ± 1   | 85 ± 4   |
| GEMSA     | 10 μM         | 56 ± 2    | 12 ± 1   |
| GEMSA     | 1 μM          | 99 ± 1    | 47 ± 3   |
| GEMSA     | 0.1 μM        | 103 ± 2   | 90 ± 5   |
| pCMPS     | 0.1 mM        | 85 ± 1    | 45 ± 1   |
| 1,10-Phenan-tholine | 1 mM | 1.8 ± 0.2 | <0.5     |
| EDTA      | 1 mM          | 87 ± 1    | 35 ± 1   |

Effect of divalent cations on enzyme activity of Drosophila CPD domain 1B (solid line) and domain 2 (dashed line). Metals were preincubated with enzymes for 15 min at room temperature. Activity is expressed as a percentage of control (enzyme in the absence of ion), measured with 200 μM dansyl-Phe-Ala-Arg. Mg²⁺ and Cu²⁺ (1 mM) had no effect on enzyme activity (not shown). Error bars represent S.E. Data points without error bars had S.E. values smaller than symbol size.

Table IV
Various proteins.

The decreased in absorption at 336 nm was measured and converted to nmol of product using a standard curve generated by incubating substrate with a large excess of enzyme.

There are eight members of the N/E subfamily of carboxypeptidases in humans but only two in Drosophila; one is CPD, and the other has the highest amino acid sequence identity to CPM. The lower number of N/E-type carboxypeptidases in Drosophila is consistent with estimates that this organism contains fewer genes than humans. One way to increase the diversity from a small number of genes is by alternative splicing of the exons. The present analysis of Drosophila CPD is consistent with this concept. The long form of Drosophila CPD containing the 1B exon (form 1B long) (Fig. 3) is most like mammalian CPD in that it contains two active carboxypeptidase domains followed by a carboxypeptidase-like domain that lacks many critical active site residues and then a transmembrane domain and cytosolic tail. The Drosophila 1B short form is more like mammalian CPN or CPZ than CPD in that this form contains a single carboxypeptidase domain lacking any membrane-association domain and is maximally active at neutral pH. Because the Drosophila 1A short form lacks a critical active site residue and has no detectable enzyme activity, this form is more like the mammalian proteins CPX-1, CPX-2, and AEBP-1, all of which lack one or more critical residues and have no detectable activity toward dansyl-Phe-Ala-Arg (17–19, 24).

The second domain of Drosophila CPD has enzymatic properties that are similar to those of mammalian CPE: a pH optimum in the 5–6 range and a slight preference for C-terminal Arg over Lys (11, 12). Thus, the single Drosophila sur gene appears to encode proteins that have properties in common with seven of the mammalian members of this family: CPD, CPN, CPZ, CPE, CPX-1, CPX-2, and AEBP-1.

The proposal that the Drosophila sur gene is the functional equivalent of multiple mammalian carboxypeptidases is consistent with the degree of amino acid sequence identity among various proteins. Drosophila CPD domain 1B has essentially the same amino acid sequence identity (39–40%) with rat CPD, rat CPE, human CPM, human CPN, and human CPZ. Although the second domain of the Drosophila CPD has a slightly higher sequence identity to the corresponding domain of duck CPD, the various mammalian carboxypeptidases show a more equal level of sequence identity. Despite the moderate conservation of amino acid sequences between Drosophila and vertebrate CPD proteins, the key enzymatic properties of the first and second domains of duck CPD are highly conserved in Drosophila domains 1B and 2. This finding implies that the difference in pH optimum and substrate specificity between the first and second domains of CPD is an essential feature. Other differences between the properties of the first and second domains, such as sensitivity to divalent cations and various enzyme inhibitors, are not as well conserved between Drosophila and duck CPD. However, these features are not physiological; none of the divalent cations that affect CPD activity are present in vivo at the concentrations that influence enzyme activity. Although Ca$^{2+}$ is typically present at millimolar levels in the secretory pathway, this ion does not affect enzyme activity of either domain. Thus, the only physiological differences appear to be substrate specificity and pH, and together the first and second domains of CPD enable the enzyme to efficiently remove both Lys and Arg residues at all pH values present in the exocytic and endocytic pathways.

Other peptide-processing enzymes exist as multidomain enzymes in mammals, such as peptidyl glycine α-amidating monoxygenase and angiotensin-converting enzyme. Peptidyl glycine α-amidating monoxygenase functions in the formation of C-terminal amide residues in neuropeptides and consists of a hydroxylase and a lyase (25). Although both of these activities are present in Drosophila, they result from distinct genes (26).

Similarly, mammalian angiotensin-converting enzyme is generally found with two tandem active domains (except for the single-domain testis form), but in Drosophila two distinct genes encode the active forms (27–29). As with CPD, the functional properties of the individual peptidyl glycine α-amidating monoxygenase and angiotensin-converting enzyme domains appear to be conserved between Drosophila and mammals, suggesting that these differences are evolutionarily important. In the case of peptidyl glycine α-amidating monoxygenase, the two domains perform distinct functions, which are both required for the formation of C-terminal amide residues (25, 26).

The two angiotensin-converting enzyme domains are somewhat redundant, with only subtle differences in substrate specificity and buffer conditions for optimal activity (27, 30), as found for CPD.

The function of the third carboxypeptidase-like domain within CPD is not clear. Although a full-length third domain is present in Drosophila, the amino acid sequence identity between this region and the corresponding region of vertebrate CPD is extremely low. This contrasts with the relatively high (82%) amino acid sequence identity between the third domain of duck and rat CPD (9). The third domain of Drosophila CPD is predicted by the PSSM Web site program to fold into a metallocarboxypeptidase-like structure, and it is possible that the general structural requirement of this region is the important feature that has been conserved rather than a substrate-binding function. It is possible that the 1A domain of Drosophila CPD functions as a catalytically inactive substrate-binding domain. Metallocarboxypeptidases require Zn$^{2+}$ for enzymatic activity but not for substrate binding (31). The absence of one of the three Zn$^{2+}$-binding residues in domain 1A suggests that this protein will have a reduced affinity for Zn$^{2+}$, if it binds at all, and this will translate into a reduced catalytic activity. However, the absence of Zn$^{2+}$ may not affect substrate binding.
Characterization of Drosophila Carboxypeptidase D

further studies are needed to address this possibility and to test whether the addition of divalent cations can activate enzyme activity of domain 1A.

Previously, Settle et al. (4) found that lethality and some, but not all, features of the svr mutant phenotype could be rescued with a 13.4-kb genomic BamHI fragment that contains most of the CPD gene. This fragment could produce all of the short forms of CPD and the long forms corresponding to tail-1 and tail-3. However, the BamHI site is located within the coding region of the tail-2 form, and this fragment would therefore result in a truncated protein lacking 40 C-terminal amino acids. Within this C-terminal region are two acidic clusters and a casein kinase 2 consensus site; similar domains are present in vertebrate CPD and have been found to be important in the intracellular routing of this protein (13–15). Thus, it is possible that the 13.4-kb genomic BamHI fragment failed to correct all of the features of the mutant phenotype because of the improper intracellular trafficking of the tail-2 form of the CPD protein. One of the nonlethal svr mutants, svrpol, has a 0.9-kb insertion in the 1.5-kb EcoRI gene fragment (4). Because this corresponds to exons 5 or 6, it is likely that this mutant would express all of the short forms of CPD found in the present study. Thus, it appears that the first domain alone is sufficient for survival. Further studies are needed to define the precise role of the specific CPD forms in various phenotypes found in the different svr mutants.

Acknowledgments—We thank Dr. Nick Baker and members of his laboratory for assistance with Drosophila and for helpful ideas.

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