Characterization of the Molecular Basis of the Drosophila Mutations in Carboxypeptidase D

EFFECT ON ENZYME ACTIVITY AND EXPRESSION

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Carboxypeptidase D (CPD) functions in the processing of proteins and peptides in the secretory pathway. Drosophila CPD is encoded by the silver gene (svr), which is differentially spliced to produce long transmembrane protein forms with three metallocarboxypeptidase (CP)-like domains and short soluble forms with a single CP domain. Many svr mutants have been reported, but the precise molecular defects have not been previously determined. In the present study, three mutant lines were characterized. svrPG33 mutants do not survive past the early larval stage. These mutants have a P-element insertion within exon 1B upstream of the initiation ATG, which greatly reduces mRNA levels of all forms of CPD. Both svr and svrPG33 mutants are viable, with a silvery body color and pointed wings. The wing shape is generally similar between these two mutants, although svrPG33 mutants have smaller wings. The svr gene has a three-nucleotide deletion in exon 6, removing a leucine in a region of the protein predicted to function as a folding domain for the second CP-like domain. svrPG33 has a 1072-bp duplication of the gene that introduces a stop codon into the open reading frame, causing the truncation of the protein in the middle of the second CP-like domain. Both deletions eliminate enzyme activity of the second CP-like domain and appear to cause the misfolding of the protein. This greatly reduces the levels of the long forms of CPD protein but do not affect the levels of the short forms. Taken together, these findings suggest that lethal and viable svr alleles differ in which protein forms are affected. Flies that retain the short form are viable, whereas flies that are missing all forms of CPD do not survive past the early larval stages.

Metallocarboxypeptidases (CPs) perform many physiological functions, ranging from the digestion of food to the biosynthesis of neurotransmitters (1–10). CPs are divided into three subfamilies based on amino acid sequence conservation. The A/B subfamily is primarily involved in protein digestion, either in the digestive tract or elsewhere in the body. In contrast, the N/E subfamily plays more of a biosynthetic role by selectively removing specific residues from peptide processing intermediates, and this step often affects the biological properties of the substrate. In humans and mice, there are eight members of this N/E family, of which five show enzymatic activity (CPE, CPN, CPM, CPZ, and CPD); the remaining members of this subfamily (CPX1, CPX2, and AEBP-1/ACLP) are not active toward standard substrates (4–8, 10, 12–15). In contrast, Drosophila contains only two members of this subfamily, one that has high homology to CPM and another that is a CPD homolog (16).

CPD is unique among CPs in that it contains multiple CP-like domains, a transmembrane domain, and a short cytosolic tail (5). Humans, rats, mice, duck, and Drosophila CPD all contain three CP-like domains, of which the first two are enzymatically active and the third is missing key catalytic residues but still appears to retain the basic CP-like structure (16–27). The functional significance of the third CP-like domain in CPD is not clear. One proposal is that the distinct pH optima of the first two domains enable CPD to be active throughout the secretory pathway, which ranges from neutral to acidic pH values (17, 28). In both duck and Drosophila, the first domain is more active at neutral pH, whereas the second domain is optimally active at pH 5.5–6 (16, 17, 28).

Protein containing both domains together showed a broader pH optimum (17, 28).

There are several known mutations for CPD in Drosophila that are collectively known as the silver, or svr, mutants based on the silvery body color of the viable mutants (27, 29). In addition to the body color, the viable svr mutants have been reported to show altered wing shape and mating behavior in light (30, 31), although these results have not been adequately described in the literature. In addition to the viable svr mutants, there are a number of svr mutants that have been reported to be embryonic lethal (27, 30, 31). Through genetic complementation studies, the viable and lethal mutants were shown to represent the same gene, but the molecular basis of the defects was not characterized. The purpose of the present study was to first evaluate the phenotypic differences between CPD mutants and then to determine the molecular basis for each mutant. The results of these analyses provide insights into the function of the various domains of CPD.

MATERIALS AND METHODS

Drosophila Strains—Flies were maintained on standard cornmeal-agar Drosophila medium at 18 or 25 °C. Wild type stock (w) and the mutants svr and svrPG33 were obtained from Bloomington Stock Center. The mutant svrPG33 was obtained from Dr. Alain Vincent (32). The svrPG33 line was propagated as w[P33G33w]/FM7. For selection of svrPG33 homozygous embryos, w[P33G33w] flies were balanced over the FM7, actGFP chromosome (obtained from Bloomington Stock...
Embryos for RNA preparation were collected on agar-apple juice plates at 16–20 h after egg laying. Nonfluorescent svr1, svrP6, and svrG3 homozygous embryos were selected using a Leica MZFLIII microscope with the GFP2 fluorescent filter.

**Wing Shape Analysis**—Wings were cut from 3–4-day-old females and mounted in DPX mounting medium (Fluka BioChemika). Images were collected with a Nikon SMZ-U microscope with ×6 optical magnification. The relative length of each wing segment was determined by tracing the outline of the segment, as shown in Fig. 1, using Image J 1.33u (National Institutes of Health).

**The Effect of Light on Reproduction**—Nine vials of each of the two viable silver mutants (svr1, svrP6) and nine vials of wild type (w) flies were used. Three vials of each strain were exposed to constant bright light by exposing the vials within 10–20 cm of a light box containing two 20-watt fluorescent bulbs. Three vials were wrapped in foil and kept in darkness for the same time period, and three vials were housed under regular conditions (ambient room light). Each vial contained 1–2 virgin males and 5–6 virgin females, which were put into the test within 1–8 h from eclosion. Flies were kept in vials for 2–5 days, the adults were discarded, and their progeny were counted. The entire experiment was repeated several times with comparable results.

**PCR and Sequencing**—Genomic DNA for PCR amplification was prepared using the Qiagen DNeasy tissue kit. Pairs of oligonucleotides located 1–3 kb apart were used as PCR primers to determine if there were any large changes in the CPD gene in the svr mutants, compared with wild type flies. Each PCR product overlapped so that the entire 11-kb svr gene was covered. When an insert was identified within the svrP6 mutant, several additional oligonucleotides were used to narrow down the insertion point. PCR product containing the insert was amplified, purified (Qiagen), introduced into the pCR4-TOPO vector (Invitrogen), and sequenced. For the svr1 mutant, this analysis did not reveal any detectable insertion or deletion. Therefore, the PCR was repeated using the Roche Expand HiFi PCR system to amplify all of the exons and introns of the svr1 gene. PCR products from three independent reactions were gel-purified (Qiagen), and sequenced. The sequences were analyzed by the MegAlign program (DNA-Star).

**Southern Blot**—To confirm the position of the P-element insertion in yw[P33svr]/FM7 flies, the Southern blot method was employed. DNA was prepared by overnight digestion of 50 adult animals at 55 °C with 20 mg/ml Proteinase K (Invitrogen) in TNE buffer (0.1 M EDTA, 0.4 M NaCl, 0.1% SDS, pH 8). Approximately 3 g/ml denatured salmon sperm DNA at 50 °C over-night. After hybridization, the blot was washed with 2 bottles containing 0.1% SSC, 1× SSC containing 0.1% SDS, and then 0.1× SSC containing 0.1% SDS buffer at 50 °C. The blot was dried and exposed to x-ray film (X-Omat Blue XB-1; Eastman Kodak Co.) for 1 day at −80 °C with an intensifying screen.

**Protein Extraction, Purification, and Western Blot**—To obtain the membrane fraction for Western blot analysis, frozen adult flies were homogenized (Polytron; Brinkman) in 0.1 M Na2CO3 (pH 11–12) and centrifuged at 15,000–30,000 × g for 20–30 min, and the pellet was resuspended and heated at 95 °C in gel loading buffer containing 4% SDS. To examine the forms of CPD in whole tissue extracts, the central nervous system (CNS) was dissected from third instar larvae. Dissections were done in 0.1 M phosphate buffer, pH 7.4, containing a protease inhibitor mixture (Roche Applied Science), transferred to loading buffer containing 4% SDS, sonicated, heated at 95 °C, and loaded onto a denaturing polyacrylamide gel. The gel was transferred to nitrocellulose and probed as described below.

For purification of the soluble forms of CPD, adult frozen flies were homogenized (Polytron) in 20 mM NaAc, pH 7.4, filtered through mesh, and centrifuged at 800 × g for 5 min. The supernatant was centrifuged at 4 °C for 40 min at 40,000 × g. The second supernatant was adjusted to 100 mM NaAc, pH 5.5, and applied to a 0.5-mL p-aminobenzoyl-Arg-Sepharose affinity resin column as described (16). The column was first washed with 0.5 M NaCl and 1% Triton X-100 at pH 5.5 and then washed with 50 mM Tris containing 100 mM NaCl and 0.01% CHAPS at pH 8.0. CPD was eluted with 25 mM Arg in the Tris/NaCl, pH 8, wash buffer.

**Western Blot**—Proteins were fractionated on SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose membrane (Optitrap; Scheicher and Schuell). The nitrocellulose blots were blocked as described (16). Blots were probed with 1:1000 dilutions of polyclonal rabbit or mouse antisera raised against peptides corresponding to the N-terminal region of exon 1A, the N-terminal region of exon 1B, or the C-terminal region of exon 8, which corresponded to the long tail 2 form of Drosophila CPD (16). Following exposure of the blots to the primary antisera, the blots were washed and exposed to horseradish peroxidase-labeled goat anti-rabbit antiserum or sheep anti-mouse antiserum (Amersham Biosciences). The enhanced chemiluminescence method (Pierce) was used for detection of bound antisera.

**Expression Constructs**—For expression of different forms of CPD in the baculovirus system, reverse transcription-PCR was used to generate the wild type or mutant form of domain 2. These were then attached to fly CPD1 containing either exon 1A or 1B; the creation of the 1A and 1B forms of CPD has been previously described (16). CPD domain 2 was amplified as a 1.3-kb band for both wild type and svr1 mutant flies using the Qiagen One Step reverse transcription-PCR kit. The 3′ oligonucleotide introduced a NotI restriction site (downstream of the coding region) to facilitate subsequent cloning steps. PCR products were digested with Stul and NotI, gel-purified (Qiagen), and ligated into Stul/NotI-digested CPD1A or CPD1B constructs, as described (16). To generate the svrP6 mutant form of CPD domain 2, PCR was used to introduce the break point and short insert found in the svrP6 gene sequence into the corresponding region of fly CPD cDNA. The oligonucleotide used for the mutagenesis included a NotI site downstream of the coding region, and the CPD1A/2wt construct described above was used as a template. After PCR with Platinum Taq (Invitrogen), the 780-bp product was purified (Qiagen) and subcloned into pCR-Blunt II-TOPO cloning vector (Invitrogen). Then the resulting plasmid was
digested with Stul and NotI, and the 780-bp band was ligated into Stul/NotI-digested CPD1A or CPD1B. All of the constructs were confirmed by sequencing in both directions.

**Baculovirus Expression and Protein Purification**—Baculovirus expressing only the short 1B form or wild type domain 2 were previously created (16). The new constructs containing either the 1A or the 1B form and one of three different sequences of domain 2 (wild type, svr1, or svrpos) were generated using a similar procedure. For expression, 10^8 Sf9 cells growing in solution were infected with one of the baculovirus constructs. After 72 h, the cell suspensions were pelleted at 300 g, and the media were removed. Cells were resuspended in 50 mM Tris buffer. All fractions were assayed for carboxypeptidase activity at pH 5.6 and 7.4, as described above.

**RESULTS**

Although the original *Drosophila svr* mutation was described many years ago and a number of other mutations in the *svr* gene have been reported (27), no previous reports have quantified the difference in wing shape between the viable *svr* mutants and wild type flies. To gain a more precise understanding of the phenotype of the viable *svr* mutants, the length of various wing veins was determined for *Drosophila svr* mutants with *svr*pos wings, and many of the individual segments are shorter in *svr*pos wings, and many of the individual segments are shorter in general, the wings of the *svr*pos mutants are smaller than either the wild type or *svr*1 wings, and many of the individual segments are shorter in the *svr*pos mutants compared with the other two.

The above observations are consistent with the previous literature describing the phenotype of *svr*1 and *svr*pos (27, 31). However, it is possible that another mutation has spontaneously arisen in these lines and that this contributes to the observed phenotype. To ensure that the wing shape is solely due to the *svr* mutation, we crossed homozygous viable *svr* mutants with *svr*posPG33 to create *svr*1/*svr*posPG33 and *svr*pos/*svr*posPG33 lines. The same general tendency was observed in these two lines as found in the homozygous lines: decreased length of proximal wing parts, increased length of distal parts of the third, fourth, and fifth longitudinal veins, and decreased length of cross-veins (data not shown).
Previously, svr<sup>1</sup> males were reported to be unable to mate when exposed to constant light (30). In order to test this, naive svr<sup>1</sup>, svr<sup>poi</sup> and w<sup>−</sup> males were introduced to virgin females for several days under conditions of constant bright light, constant dark, or regular ambient light. All groups produced the same number of offspring, indicating that there was no substantial effect of the lighting conditions on mating ability (data not shown).

The phenotype of the svr mutant svr<sup>PGE3</sup>, which was previously reported to be embryonic lethal (32), was also further characterized. Using a balancer gene expressing a fluorescent reporter protein, homozygous svr<sup>PGE3</sup> eggs were selected and allowed to develop. Although none of the embryos developed into adults, a number of dead larvae were detected. Analysis of these larvae indicated an age around the first or second instar stage. Thus, the svr<sup>PGE3</sup> mutant is not embryonic lethal but dies early in the larval stage, around the first larval molt. A similar lethal phase was previously reported for another lethal svr<sup>1</sup> allele, even when maternal gene function was eliminated, and may reflect the null phenotype for svr (33).

The svr<sup>PGE3</sup> mutant was previously reported to result from the insertion of a P-element several nucleotides upstream of the initiation ATG in exon 1B of the Drosophila CPD gene (32). However, the molecular defect of the viable svr mutants has not been reported. To identify the precise defect, several strategies were employed. First, PCR and Northern blots were used to screen for large inserts or deletions within the Drosophila CPD gene. This analysis revealed a 1.1-kb insert in exon 6 of the CPD gene in svr<sup>poi</sup> (Fig. 2A). Upon sequence analysis, the insert was found to consist of seven nucleotides of novel DNA together with a 1072-bp duplication of the CPD gene that spans the region from the intron upstream of exon 5 to the middle of exon 6 (Fig. 2A). The insert and duplication introduces a stop codon in the middle of the second CP-like domain in CPD (Fig. 2A).

Similar analysis of svr<sup>1</sup> failed to find a detectable difference in the size of any of the PCR fragments, which spanned the entire CPD transcription unit and included a portion of the upstream region. Therefore, the PCR fragments corresponding to the coding region of each exon and the exon/intron junctions were sequenced. From this analysis, the svr<sup>1</sup> gene was found to be identical to the wild type sequence in Flybase except for a three-nucleotide deletion in exon 6 (Fig. 2A and C). This deletion eliminates a Leu residue located in the transthyretin-like subdomain within the second CP-like domain in CPD (Fig. 2A, B, and D). The transthyretin-like domain is present in every member of the N/E subfamily of carboxypeptidases and has been proposed to function in the folding of the protein. Biochemical evidence that this amino acid deletion is the basis for the svr<sup>1</sup> phenotype is presented below.

Northern blot analysis was performed to determine the forms of CPD mRNA in the various mutants. Previously, multiple forms of CPD mRNA were found, including a 6.5-kb species that coded for the long forms of CPD (with either exon 1A or 1B) and numerous forms of 1.5–3.4 kb that coded for the short forms (also with either exon 1A or 1B) (16). In the present analysis, similar forms of CPD mRNA were found in the wild type flies (Fig. 3) with no major difference between the embryonic, larval, or adult stages (Fig. 3). In all three stages, the exon 1A, 1B, and 3 probes detected bands from 1.5 to 7 kb, whereas the exon 6 probe detected only the 7-kb form. The pattern of bands detected with the four probes was similar between the wild type and svr<sup>1</sup> mutant at all
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During expression analysis by Northern blot, RNA from embryos, larvae, and adult wild type (wt) and svr1 and svr poin mutants Drosophila was hybridized with RNA probes corresponding to the indicated exons, as described under "Materials and Methods." The positions of RNA size standards (Invitrogen) are indicated.

The mutations in Drosophila carboxypeptidase D (CPD) have been identified in two non-fluorescent (svr PG33) and non-fluorescent (svr poi) mutants. The presence of RNA from svr PG33 and svr poi mutants was confirmed by hybridization to RNA probes specific for the corresponding exons. RNA from svr PG33 and svr poi mutants were detected with all four probes at all three stages (Fig. 3). In contrast, the svr poin mutant did not show a detectable band for the 7-kb form of CPD mRNA that was present in the wild type or svr1 flies. The anomalous bands in the svr poin mutants were detected with all four probes at three stages (Fig. 3).

RNA from the svr PG33 mutant was also analyzed by Northern blot. For this analysis, fluorescent (i.e. heterozygous and balancer-homozygous) and nonfluorescent (i.e. mutant) embryos that were 16–20 h old were selected, and RNA was prepared. Several separate analyses were performed from different fluorescent and nonfluorescent pools of embryos with similar results. A faint band of ~8 kb that was detectable with the probe 6 probe (Fig. 3). The svr poin mutants also expressed mRNA species of ~2.5–3 kb that were not present in the wild type or svr1 flies. The anomalous bands in the svr poin mutants were detected with all four probes at three stages (Fig. 3).

For the analysis of fluorescent (i.e. heterozygous and balancer-homozygous) and nonfluorescent (i.e. mutant) embryos that were 16–20 h old, we selected and RNA was prepared. Several separate analyses were performed from different fluorescent and nonfluorescent pools of embryos with similar results. A faint band of ~6–7 kb was detected in the svr PG33 RNA with the exon 3 or 6 probes (Fig. 4A). Levels of most of the 1.5–3.4 kb bands detected with the various probes were also greatly reduced in the svr PG33 mutants, compared with wild type flies, with the exception of a 3.4-kb band detected with the exon 1A probe (Fig. 4A). However, it is not clear if this band represents the same form as wild type flies or if it represents another mRNA form that has only the exon 1A portion of the CPD gene spliced to other exons. This latter interpretation would explain the failure to detect elevated levels of this band with the other CPD probes (Fig. 4A). Southern blotting was used to confirm that the svr PG33 flies used in our analysis have a large insert in the N-terminal region of the 1A form and the C-terminal region of the tail-2 form of CPD. After exposure of the blots to primary antiserum, the enhanced chemiluminescence method (Pierce) was used to detect bound antiserum. The titers of CPD were varied and no immunoreactive material was detected in the pH 8 eluate in the absence of Arg (not shown). There were no differences in the levels of soluble CPD between wild type and svr mutant flies (Fig. 5A).

The membrane forms of endogenous CPD could be detected in carbamylated membranes by Western blot analysis. The level of the 150-kDa band detected with the 1A N-term probe was greatly reduced in both the svr PG33 and svr poi mutants (Fig. 5A). Because in svr poin the CPD is performed. To detect the short forms of CPD, it was necessary to affinity-purify the soluble extracts on benzoyl-Arg-Sepharose. This column has been previously used to purify both CPE and CPD from mammalian tissue; CPE elutes when the pH is raised from 6 to 8, whereas CPD remains bound even at pH 8 (19). CPD can subsequently be eluted by the addition of an arginine elution buffer. CPD is eluted when the pH is raised from 6 to 8, whereas CPD remains bound even at pH 8 (19). CPD can subsequently be eluted by the addition of an arginine elution buffer. CPD is eluted when the pH is raised from 6 to 8, whereas CPD remains bound even at pH 8 (19). CPD can subsequently be eluted by the addition of an arginine elution buffer. CPD is eluted when the pH is raised from 6 to 8, whereas CPD remains bound even at pH 8 (19). CPD can subsequently be eluted by the addition of an arginine elution buffer. CPD is eluted when the pH is raised from 6 to 8, whereas CPD remains bound even at pH 8 (19). CPD can subsequently be eluted by the addition of an arginine elution buffer. CPD is eluted when the pH is raised from 6 to 8, whereas CPD remains bound even at pH 8 (19). CPD can subsequently be eluted by the addition of an arginine elution buffer. CPD is eluted when the pH is raised from 6 to 8, whereas CPD remains bound even at pH 8 (19). CPD can subsequently be eluted by the addition of an arginine elution buffer. CPD is eluted when the pH is raised from 6 to 8, whereas CPD remains bound even at pH 8 (19). CPD can subsequently be eluted by the addition of an arginine elution buffer. CPD is eluted when the pH is raised from 6 to 8, whereas CPD remains bound even at pH 8 (19).
truncated in the middle of the second domain and therefore does not contain the transmembrane domain, the absence of a membrane form of CPD was expected. However, in svr<sup>1</sup>, the CPD is simply missing a single Leu from the second CP domain, and therefore the protein was expected to contain the transmembrane domain. The reduction in the level of the membrane form of CPD in the svr<sup>1</sup> mutant therefore suggested a defect with the folding of the protein and a subsequent effect on the stability. To test this, we performed Western blots with whole tissue extracts of larval CNS. The long form of CPD was detected in wild type tissue extracts with an antisem against the 1A form (Fig. 5C). The short CPD 1A form was not detected in the total protein extract from larval CNS, indicating that this short form is not abundant in CNS. As expected from the Western blots of membrane extracts of mutant flies, the long CPD 1A forms were not detectable in whole protein extracts of unglycosylated forms (Fig. 6A). Bands of the appropriate size were detected in cells, 110 kDa for the svr<sup>1</sup> mutant protein and 74 kDa for the svr<sup>poil</sup> mutant protein (Fig. 6B). As previously found, constructs containing a single CP domain, either 1B alone or wild type domain 2 alone, were secreted into the media at high levels relative to the amount detected in the cells (Fig. 6B).

To quantify the amount of CPD activity in cells and media, an enzymatic assay was employed at two pH values: 5.6 (which preferentially detects CP domain 2) and 7.4 (which preferentially detects CP domain 1). At both pH values, only background levels of enzyme activity were detected with the 1A/2<sup>svr<sup>1</sup></sup> and 1A/2<sup>svr<sup>poil</sup></sup> constructs (Table 1; compare with wild type virus). Because the 1A domain was previously found to be inactive (16), this result indicates that both mutant forms of CP domain 2 are inactive. Relatively high levels of activity were detected in the media from cells expressing either the 1A/2<sup>wt</sup> or the 1B/2<sup>wt</sup> forms (Table 1), consistent with the Western blot result. In contrast, the 1B/2<sup>svr<sup>1</sup></sup> and 1B/2<sup>svr<sup>poil</sup></sup> constructs showed greatly reduced medium levels but roughly similar cellular levels relative to the wild type construct (Table 1). This result is consistent with the Western blot result and shows that the mutant forms of Drosophila CPD are made in the cells but are not secreted efficiently.

The above results could possibly be explained by the hypothesis that the mutations affect protein folding, which in turn affects the trafficking of the protein through the secretory pathway. Because unfolded proteins usually are less soluble than correctly folded proteins, the ability of the various CPD forms to be extracted from the Sf9 cells was examined. Approximately 90% or more of the forms of CPD that represented wild type CP domains (1A/2<sup>wt</sup>, 1B/2<sup>wt</sup>, 1B alone, and 2wt alone) could be extracted from the cells with a combination of salt and/or detergent (Table 2). In contrast, only 70% of the 1B/2<sup>svr<sup>poil</sup></sup> construct and 54–67%
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**TABLE 1**

| Activity of various Drosophila CPD forms expressed in SF9 cells |
|---------------------------------------------------------------|
| Activity was measured in triplicate with dansyl-Phe-Ala-Arg at either pH 5.6 (left columns) or at pH 7.4 (right columns). Units are nmol of dansyl-Phe-Ala product formed/min of incubation/ml of medium or per 2 million cells (i.e. the number of cells/ml of medium prior to centrifugation, removal of the medium, and homogenation of the cells). |

| CPD form | pH 5.6 | pH 7.4 |
|----------|--------|--------|
|          | Medium | Cells | Medium | Cells |
|          | nmol/min/ml | nmol/min/2 × 10⁶ cells | nmol/min/ml | nmol/min/2 × 10⁶ cells |
| 1A/2wt   | 25     | 8     | 6      | 2.5   |
| 1A/2svr⁻ | 0.4    | 0.4   | 0.2    | 0.1   |
| 1A/2svr⁺⁻ | 0.4    | 0.4   | 0.2    | 0.1   |
| 1B/2wt   | 37     | 9     | 120    | 9     |
| 1B/2svr⁻ | 1.7    | 2.5   | 4      | 7     |
| 1B/2svr⁺⁻ | 7.1    | 3.5   | 25     | 15    |
| 1B       | 86     | 65    | 637    | 228   |
| 2wt      | 64     | 32    | 21     | 7     |
| Wild type virus | 0.8    | 0.4   | 0.4    | 0.2   |

**DISCUSSION**

A major finding of the present study is that Drosophila svr mutants that are viable lack the full-length forms of CPD but still express short forms containing an active CP domain. Thus, the single active CP domain present in the short 1B form is sufficient for survival of the organism. In addition, the CPD long forms appear to play a role in wing and cuticle development for which the short form is insufficient.

Previously, svr⁺⁻⁻⁺⁻ was reported to be embryonic lethal (32). Larval lethality for other svr allele was reported as well (33). Careful analysis in the present study revealed that the svr⁺⁻⁻⁺⁻ mutants die as late embryos; in those mutants with a strong hypomorphic allele svr, it was proposed that this svr⁻⁻⁻⁻ mutant causes a large truncation of the protein that is expected to affect folding. The apparent folding defect for the svr⁻⁻⁻⁻ mutant implies that the single amino acid deletion causes a change in conformation. The Leu residue that is deleted in the svr⁻⁻⁻⁻ mutant is located in a subdomain of the CP domain that has structural homology to transthyretin. The transthyretin-like subdomain of CPD displays a rodlike shape; its seven strands are connected by short loops and form a β-sandwich or β-barrel of pre-albumin-like folding topology (25, 26). A transthyretin-like subdomain is present in all members of the N/E subfamily of carboxypeptidases, including those with enzymatic activity as well as those that appear to be inactive (such as CPX-1, CPX-2, ACLP/AEBP1, and the third CP-like domain of CPD). Interactions between the catalytic subdomain and the transthyretin-like subdomain are mediated both by hydrophobic and ionic interactions (25, 26). Members of A/B subfamily of CPs lack the transthyretin-like subdomain. Instead, the A/B subfamily of CPs have an N-terminal propeptide domain, which serves in folding as well as enzyme inactivation (I, 37, 38). Because members of the N/E subfamily lack a prodomain, and all contain the transthyretin domain, it was proposed that this transthyretin domain functions in place of the prodomain in the folding of the catalytic domain. Interestingly, the basic structure of the transthyretin-like subdomain of N/E subfamily CPs has some similarities to the P domain of prohormone/proprotein convertases, which is also located immediately to the C-terminal side of the catalytically active domain (39–41). The P domain consists of an eight-stranded β-sandwich, forming a β-barrel. Interdomain interactions between the P domain and catalytic domain are mediated mainly by hydrophobic central patch and polar salt bridge interaction (39–41). As proposed for the transthyretin-like domain of the CPs, the P-domain of the PCs is thought to function in the folding of the catalytic domain.

Based on the distribution of CPD in the secretory pathway and its localization to compartments that contain the endopeptidase furin, it was proposed that CPD plays a role in the processing of growth factors (5, 42, 43). The lethality of the svr⁺⁻⁻⁺⁻ mutants and the altered wing shape in both svr⁻⁻⁻⁻ and svr⁻⁻⁻⁻ are consistent with a role for CPD in the biosynthesis of growth factors. Because the short forms of CPD containing only the first CP domain are produced in normal amounts in the svr⁻⁻⁻⁻ and svr⁻⁻⁻⁻ mutants, it is likely that the long forms perform a function that is not completely compensated for by the presence of the short forms. One major difference between these forms is that the short forms of Drosophila CPD are secreted from cells, whereas the long forms are not. However, it is not completely clear whether this difference is responsible for the lethality of the svr⁻⁻⁻⁻ and svr⁻⁻⁻⁻ mutants.
retained in a trans-Golgi-like compartment. In addition, the long membrane-bound forms of CPD traffic to the cell surface and are internalized back to a perinuclear compartment (44, 45). Thus, it is likely that the long forms of CPD function in the same pathway as furin, removing basic residues from the C terminus of the intermediates formed by furin either in the Golgi or in the endocytic pathway. Although the short forms will transiently be present in Golgi and may contribute to processing in this compartment, the lack of a membrane anchor in the short forms prevents their accumulation within the cell, so the endocytic vesicles will contain only the long and not the short forms of CPD.

There are several Drosophila growth factors that require furin for activity and therefore are likely substrates of CPD. One Drosophila growth factor that is cleaved by pro-BMP-4 by furin at the S1 site adjacent to the mature ligand domain is required for subsequent cleavage at an upstream S2 furin site within the prodomain (49, 50). It was proposed that sequential cleavage of pro-BMP-4 regulates the range of action of mature ligand (50). The pro-BMP-4 cleavage sites are conserved in the Drosophila ortholog Dpp (50). Notch is involved in processes of cell-cell signaling that determine cell fate and regulate pattern formation. Newly synthesized Notch is also known to be processed by furin in the trans-Golgi network (51). Another Drosophila growth factor that contains consensus sites for furin is Gbb (glass bottom boat), which is a homolog of BMP5/6/7/8 (52). Gbb is thought to function as a retrograde ligand in the neuromuscular junction (53). Gbb mutants have reduced larval neuromuscular junctions and diminished neurotransmission (54, 55). Gbb is involved in proliferation and vein patterning in wing disk (52). The wings of Gbb mutant flies are reduced in size and show specific loss of the posterior cross-vein as well as truncation of distal tips of longitudinal veins 4 and 5 (52). Clonal analysis of gbb mutants demonstrated that dorsalventral gbb clones that occupy the entire posterior compartment show loss of the posterior cross-vein and the distal part of longitudinal vein 5, whereas anterior clones show the reduction of overall wing size by ~30% (52, 56). Misexpression of the extracellular Dpp antagonist Sog (short gastrulation), the Drosophila ortholog of Chordin, causes a phenotype that is very similar to those of viable Gbb mutants. Sog is type II transmembrane protein, although at least a fraction of Sog protein is released from cells (57). Sog is known to be processed at three sites by Tolloid, an astacin-like zinc metalloprotease related to BMP-1 (57). Sog may also be processed by other enzymes; Sog contains numerous furin and PC consensus sites, and if it is cleaved by these enzymes, then it is likely that CPD would further process the peptides. Truncated forms of Sog can inhibit vein formation when misexpressed during pupal wing development (58). Misexpressed Sog reduces the space between longitudinal veins 3 and 4, which is similar to the phenotype of the Drosophila CPD mutants (58). Because CPD is able to remove C-terminal Lys and Arg residues from a large number of peptides (5, 28) and is present in the same compartments as furin (43, 45, 59), it is likely that CPD also processes those proteins initially cleaved by furin and related endopeptidases. Although it is not known if the removal of C-terminal basic residues is required for the production of the active form of any of these peptides, the fact that svr<sup>PG33</sup> mutants die in the larval stage and the svr<sup>r</sup> and svr<sup>env</sup> mutants have altered wing shape suggests that at least some of the peptides processed by CPD require this step for the generation of the biologically active form.

Because the general three-CP-like domain structure of CPD has been conserved from Drosophila to humans, it was assumed that all of these CP-like domains play an important role. From the present study, it is not possible to determine whether the observed phenotypes of the viable Drosophila CPD mutants are due to the loss of CP-like domain 2, CP-like domain 3, or all transmembrane forms. Further studies using transgenic flies will be necessary to distinguish among these possibilities and shed light on the role of the individual CP-like domains of Drosophila and presumably human CPD.

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