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# Characterization and Cloning of Tripeptidyl Peptidase II from the Fruit Fly, *Drosophila melanogaster**

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**Susan C. P. Renn‡, Birgitta Tomkinson§, and Paul H. Taghert‡¶**

*From the ‡Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, Missouri 63110 and the §Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, Biomedical Center, Box 575, 751 23 Uppsala, Sweden*

**We describe the characterization, cloning, and genetic analysis of tripeptidyl peptidase II (TPP II) from *Drosophila melanogaster*. Mammalian TPP II removes N-terminal tripeptides, has wide distribution, and has been identified as the cholecystokinin-degrading peptidase in rat brain. Size exclusion and ion exchange chromatography produced a 70-fold purification of dTPP II activity from *Drosophila* tissue extracts. The substrate specificity and the inhibitor sensitivity of dTPP II is comparable to that of the human enzyme. In particular, dTPP II is sensitive to butabindide, a specific inhibitor of the rat cholecystokinin-inactivating activity. We isolated a 4309-base pair *dTPP II* cDNA which predicts a 1354-amino acid protein. The deduced human and *Drosophila* TPP II proteins display 38% overall identity. The catalytic triad, its spacing, and the sequences that surround it are highly conserved; the C-terminal end of dTPP II contains a 100-amino acid insert not found in the mammalian proteins. Recombinant dTPP II displays the predicted activity following expression in HEK cells. TPP II maps to cytological position 49F4-7; animals deficient for this interval show reduced TPP II activity.**

Intercellular communication depends critically on both the generation and termination of biological signals. In the case of neuropeptides and neurohormones, the signaling molecules may be cleaved by extracellular enzymes to produce new active peptides (*e.g.* by angiotensin-converting enzyme, ACE[1] (1) and endothelin-converting enzyme (2)) or cleaved by them to be inactivated (*e.g.* by enkephalinase (3)). These peptidases are found in the extracellular space or on the cell surface and typically have low specificity (for example substance P, neurokinins, neurotensin, and the endothelins can all be cleaved by enkephalinase, reviewed in Refs. 4 and 5). Although a large number of peptidases have been identified in various tissues, only a handful have been shown definitively to be ectoenzymes with neuropeptide-degrading capability (reviewed in Refs. 4 and 5). These observations have led to the current hypothesis that diverse neuropeptides are inactivated by a relatively small number of enzymes. Recently Rose *et al*. (6) identified a membrane-associated variant of the enzyme tripeptidyl peptidase II (TPP II) as the peptidase responsible for cleavage and inactivation of the mammalian neuropeptide CCK-8. Intravenous injection of the potent TPP II inhibitor, butabindide, has prosatiety effects on both behavior and gastric emptying (6). Based on this pharmacology, as well as its substrate specificity, and its correlated expression in CCK-responsive tissues, Rose *et al*. (6) propose that the neuropeptidase activity of TPP II is intimately associated with CCK signaling and not broadly active on diverse neuropeptides.

TPP II was previously isolated and characterized as an extra-lysosomal peptidase that could release N-terminal tripeptides from a wide range of larger substrates (7, 8). TPP II is a serine protease with a subtilisin-like catalytic domain, but compared with other subtilases, it contains an extended C-terminal region (9). The native form of the enzyme has a remarkably high molecular mass (>1000 kDa) that suggests an oligomeric association of the ~138-kDa subunits (7, 8). The cDNAs encoding the human (10, 11), murine (12), and rat (6) enzymes have been cloned, and the genomic region encoding a putative homologue in *Caenorhabditis elegans* has been sequenced (GenBank™ accession number U23176). In both human and mouse cDNAs (12), an alternatively spliced exon encoding an additional 13 amino acids has been identified, which is involved in complex formation (13). Rose *et al*. (6) identified both cytoplasmic and membrane-associated forms of rat TPP II and suggested that an alternatively spliced TPP II mRNA was involved in the membrane association through a glycosylphosphatidylinositol anchor.

Neuropeptides are important signaling molecules in insects (for review see Refs. 14 and 15), and the enzymes involved in neuropeptide regulation appear to be highly conserved (16). Previous studies of neuropeptidases in *Drosophila* have defined enzyme activities resembling enkephalinase (17, 18), angiotensin-converting enzyme (19, 20), and aminopeptidase activity (21). In each case, the *Drosophila* activities displayed similar substrate specificities and inhibitor sensitivities to known mammalian enzymes. Furthermore, the gene encoding the *Drosophila* ACE, *AnCE*, demonstrates a high degree of sequence similarity to one form of mammalian ACE (20). We propose to use *Drosophila* genetics to investigate the extent to which TPP II enzyme function has been conserved. In the current study, we show that a TPP II-like activity is present in *Drosophila* extracts. Furthermore, we partially purify this enzyme activity and characterize it in comparison to the mammalian enzyme. We have cloned and expressed a *dTPP II* cDNA. Finally, we use

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*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF035351 for Drosophila, M73047 for human, and U23176 for C. elegans.*

¶ To whom correspondence should be addressed: Dept. of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, MO 63110. Tel.: 314-362-3641; Fax: 314-362-3446; E-mail: Taghertp@thalamus.wustl.edu.

[1] The abbreviations used are: ACE, angiotensin-converting enzyme; aa, amino acid(s); bp, base pairs; CCK, cholecystokinin; DTT, dithiothreitol; EST, expressed sequence tag; HEK, human embryonic kidney cells; HPLC, high performance liquid chromatography; kb, kilobase pairs; pNA, *para*-nitroanilide; PCR, polymerase chain reaction; suc, succinyl; TPP II, tripeptidyl peptidase II.

genetics to demonstrate that this cloned gene is largely responsible for the observed TPP II activity in tissue extracts.

## EXPERIMENTAL PROCEDURES

*Materials*—Chromogenic substrates were obtained from Bachem (Bubendorf, Switzerland) and Sigma. *N*-Ethylmaleimide, phenylmethanesulfonyl fluoride, pepstatin, and *p*-chloromercuribenzoate were purchased from Sigma; iodoacetamide was obtained from BDH, and bestatin was from Boehringer Mannheim (Bromma, Sweden). Sepharose CL-4B was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), and DEAE-cellulose (DE52) was from Whatman. A Nucleosil $C_{18}$ column (10 $\mu$m; 4 × 250 mm) for HPLC was packed by Skandinaviska GeneTec AB (Kungsbacka, Sweden). Human TPP II was purified from red blood cells as described previously (8) with modifications subsequently reported (9, 22). The inhibitor butabindide was a generous gift from Drs. Schwartz and Ganellin (6).

*Insect Culture*—Standard culture methods were used to obtain large numbers of adult *Drosophila melanogaster* (Oregon R strain) from which eggs were collected on agar-apple juice plates. Eggs were collected in 24-h intervals, washed in $H_2O$, and stored at −70 °C until needed. Flies used for enzyme purification were 14–18 days old, quieted, and collected at 4 °C and frozen to −70 °C for storage. The deficiency lines *Df(2R)vg 33 (49D;50A), Df(2R)vg 56 (49D;49F), Df(2R)vg B (49D3–4;50A1–2)* and *Df(2R)vg C (49B2–3;49E7-F1)* were mapped according to complementation analysis *inter se* and with stocks bearing mutations (23, 24). The following 12 ethyl methanesulfonate and one irradiated mutant stocks which potentially represent *dTPP II* were analyzed: *vr3-2*, *vr4-57*, *vr5-5*, *vr5-48*, *vr6-6*, *vr6-R6* (gamma ray), *vr 8-9*, *vr9-11*, *vr9,15-43*, *vr 9-23*, *vr11-14*, *vr13-24*, and *vr17-32*.

*TPP II Enzyme Activity Measurements*—TPP II-like activity was measured in a 96-well plate by combining 100 $\mu$l of sample mixed with 50 $\mu$l of 0.8 mM Ala-Ala-Phe-pNA (AAF-pNA) and 50 $\mu$l of a 0.2 M potassium phosphate buffer, pH 7.5, that contained 8 mM DTT. The plate was incubated at 37 °C and the change in absorbance at 405 nm was measured in a Multiscan PLUS reader (Labsystems). Tissue extract and cell lysate assays contained 100 $\mu$M bestatin to inhibit aminopeptidases. The enzyme preparations were diluted in a 100 mM potassium phosphate buffer, pH 7.5, that contained 30% glycerol and 1 mM DTT; this solution stabilizes the mammalian enzyme (7).

We defined 1 unit to equal a change in absorbance of 0.001/min, which under these conditions corresponds to the hydrolysis of 0.04 nmol of the substrate, using the molar absorbance of 9.6 $mM^{-1}\,cm^{-1}$ (25). One unit defined as above corresponds to about 0.01 unit defined previously (11) using the substrate Arg-Arg-Ala-[$^{32}$P]Ser-Val-Ala, as determined by measuring of the same sample of purified human TPP II under the two assay conditions.

*Enzyme Purification—Drosophila* TPP II enzyme was partially purified from embryos and from adult flies in a two-step purification process. Tissues were homogenized at 1 g/4 ml in 50 mM Tris-HCl, pH 7.5, using a probe tip homogenizer (Ultra-turrax T25) with 2 pulses (10 s each) followed by sonication (MSE Soniprep 150) with 3 pulses (5 s each). Extract samples were prepared by centrifugation at 14,000 × *g* for 30 min and filtration of the supernatant through glass wool. The filtered extract was loaded onto a Sepharose CL-4B column (Fig. 1*A*). The active fractions were pooled and further purified by DEAE-cellulose chromatography (Fig. 1*C*). The column was eluted with a potassium gradient, and fractions were collected and analyzed by the standard assay. Active fractions were pooled for further characterization. The amount of protein in the samples was measured by the modified Bradford method (27, 28) with bovine serum albumin as standard.

*Western Blot Analysis*—Samples were mixed with sample buffer to give final concentrations of 2.3% SDS, 5% β-mercaptoethanol, and 10% glycerol. The samples were heated for 5 min at 95 °C before loading onto an 8% polyacrylamide gel. The SDS-polyacrylamide gel electrophoresis and Western blot analysis were performed essentially as described previously (29) using chicken anti-human TPP II antibody. Silver staining of the polyacrylamide gel was performed according to Morrisey (30).

*Characterization of dTPP II Activity*—Assays to determine substrate specificity were conducted according to the standard procedure, but various chromogenic substrates were substituted for the standard AAF-pNA, all at a concentration of 0.2 mM. During inhibitor sensitivity assays, 0.5 mg/ml bovine serum albumin was substituted for DTT in the enzyme dilution buffer to stabilize the enzyme because the presence of 1 mM DTT would hamper the effect of the thiol-reactive compounds. The final concentration of DTT in the incubation was therefore about 50 $\mu$M. In control experiments greater than 95% of the activity was retained under these conditions. Inhibitor solutions were prepared according to manufacturers' protocols, and none of the solvents used had any effect on dTPP II activity at the concentrations used. The enzyme was preincubated with the inhibitor for 30 min at 22 °C before addition of the substrate.

For the determination of $K_m$, 2–5 units of enzyme prepared from *Drosophila* according to Table I or purified recombinant dTPP II were incubated in triplicates with AAF-pNA at concentrations of 1.0, 0.5, 0.2, 0.1, 0.05, and 0.025 mM in 0.1 M potassium phosphate buffer, pH 7.5, that contained 15% glycerol and 2.5 mM DTT and 1% $Me_2SO$. The $K_m$ was calculated from Lineweaver-Burk plots to 157 and 138 $\mu$M using two different *Drosophila* enzyme preparations and to 107 and 103 $\mu$M for two different preparations of recombinant dTPP II. For the determination of $K_i$ for butabindide, the enzyme was incubated with AAF-pNA at concentrations of 0.5, 0.1, or 0.025 mM in the absence or presence of butabindide (0.025, 0.1, or 0.4 $\mu$M) at 37 °C and pH 7.5, as described above. The $K_i$ was determined in two separate experiments to 0.12 and 0.15 $\mu$M using two different *Drosophila* enzyme preparations and 0.11 and 0.14 $\mu$M for two different preparations of recombinant dTPP II.

*HPLC Analysis*—For analysis of cleavage products, 6 units of enzyme were incubated with the nonapeptide GVLRRASVA (10 nmoles) in 0.1 M potassium phosphate buffer, pH 7.5, that contained 2 mM DTT and 3% glycerol, in a final volume of 100 $\mu$l at 37 °C. As a control the enzyme was incubated without the substrate. The reaction was interrupted by diluting the sample 10-fold with buffer A, and the sample was stored at −20 °C. An aliquot (200 $\mu$l) was loaded on a Nucleosil $C_{18}$ column that was equilibrated with a mixture containing 90% (v/v) buffer A (60 mM sodium phosphate buffer, pH 2.8, 15 mM sodium pentane sulfonate, and 17.7 mM triethylamine) and 10% buffer B (18 mM sodium phosphate buffer, pH 2.8, 15 mM sodium pentane sulfonate, 17.7 mM triethylamine, and 35% (v/v) ethanol). As per Rosén *et al*. (31), elution was performed at 1 ml/min, with a gradient of 10–90% buffer B. The gradient was started at 10 min and finished at 40 min, and the column was thereafter eluted with 90% buffer B for 20 min.

*Molecular Biology*—A partial clone, PGA9, recovered from a 9- to 12-h embryonic cDNA library (K. Zinn, CalTech) showed high sequence homology to the 5′ end of the mammalian TPP II.[2] The missing 3′ portion of the *dTPP II* gene was cloned using two PCR steps. Nested, gene-specific oligonucleotides were made in the sense direction corresponding to positions +2171 (CCAAGCAATCCTGTCGT) and +2278 (TGCATTGCCAAGTACTG) of the PGA9 clone. In the first round of PCR, oligonucleotide +2171 and the lambda gt11 reverse vector oligonucleotide were used to amplify templates from 5 $\mu$l of an amplified stock of the Zinn library. Klentaque LA (Sigma) enzyme was added prior to a 5-min 95 °C phage-denaturing step followed by 30 cycles of 20-s 96 °C denaturing, 30-s 50 °C annealing, and 3-min 68 °C extension. 5% of the unpurified product of the first PCR reaction served as template for a second round of amplification with the nested gene-specific oligonucleotide +2278 and the lambda gt11 reverse vector oligonucleotide. The same cycling conditions, without the initial denaturing step, were used and produced a distinct band. This product was cloned into the T/A vector according to manufacturer's recommendations (Promega, Madison, WI) with the exception that the Klentaque LA enzyme was used. A full-length clone, LD18681, containing EST sequence number AA538993, was obtained from Genome Systems (St. Louis, MO). Genomic clones containing *dTPPII* sequences were isolated from a Charon 4A library using the PGA9 clone and standard techniques (32). The nucleotide sequence was determined by automated sequencing (Applied Biosystems) using gene- and vector-specific oligonucleotides.

Low stringency Southern blot analysis was performed using a 50% formamide hybridization solution at 37 °C. The filter was rinsed with 2× SSC, 0.1% SDS at room temperature.

*Expression and Purification of Recombinant dTPP II*—The *dTPP II* cDNA clone LD18681 was amplified by PCR using the vector oligonucleotide T3 and a gene-specific oligonucleotide that included the stop codon and introduced a *Not*I restriction site for cloning into the expression vector pCDNA3 (Invitrogen, Carlsbad, CA). Clones were recovered in both the forward and reverse orientation. The mouse *TPP II* gene was cloned into the *Eco*RI site of the same vector (13). The constructs were introduced into HEK cells in culture by lipid transfection (TransIT™) according to manufacturer's protocol (Mirus Corp., Madison, WI) and incubated 40–60 h. Cells were harvested, rinsed in PBS, lysed in 50 mM Tris, pH 7.5, with 1% Triton X-100 (100 $\mu$l/$10^6$ cells), and centrifuged at 14,000 × *g* for 30 min. The supernatant was diluted 10-fold in 100 mM potassium phosphate buffer that contained 30% glycerol and 1 mM DTT.

[2] R. Nusse and F. Van Leeuwen, personal communication.

TABLE I
*Purification of dTPP II from Drosophila extracts*

The enzyme was purified as described under "Experimental Procedures." The data represent one purification out of four performed.

| Purification step | Total volume | Protein | Activity | Specific activity | Yield | Purification factor |
|---|---|---|---|---|---|---|
| | | *μg/μl* | *units/μl* | | % | *units/μg* |
| Extract | 2.0 | 14.8 | 7.58 | 0.51 | 100 | |
| Sepharose CL-4B | 11.5 | 0.4 | 0.94 | 2.35 | 71 | 4.6 |
| DEAE-cellulose | 9.6 | 0.022 | 0.77 | 35.0 | 49 | 69 |

TPP II activity was assayed according to standard procedure.

For the preparation of stable transformants the constructs were introduced into HEK cells in culture by the calcium phosphate precipitation method, and stable clones were selected after growing the cells in medium containing Geneticin, as described previously (13). Approximately $30 \times 10^6$ cells were harvested, lysed (10 μl lysis buffer/$10^6$ cells), centrifuged, and diluted as described above. The diluted supernatant (2.6 ml) was loaded onto a Sepharose column for chromatography and analysis as described in Fig. 1*A*. Pooled fractions were loaded as a 10-ml sample onto a DEAE-cellulose column, and chromatography was performed as described in Fig. 1*C*. The peak fractions were pooled and used for further characterization.

*Cytological Location*—Two independent strategies were used to identify the cytological location of the *dTPP II* gene. *In situ* hybridization of the genomic phage clones to polytene chromosomes of salivary glands of third instar larvae was carried out using a biotin-avidin detection system (ENZO Detek) and standard techniques (33). As the second strategy, we screened a *Drosophila* P1 library (34) (Genome Systems) with a 1-kb 3′ fragment of the *dTPP II* cDNA.

## RESULTS

*Partial Purification of Drosophila TPP II*—To investigate the endogenous TPP II-like activity in *Drosophila* extracts we used the substrate AAF-pNA (8, 13). The activity was measured in the presence of the inhibitor bestatin in order to protect the substrate from sequential aminopeptidase degradation. We found approximately 0.5 units/μg protein in *Drosophila* extracts (Table I). To achieve a partial purification of the *Drosophila* TPP II activity, we used the purification procedure that was previously designed for partial purification of TPP II from rat liver (7). Sepharose CL-4B followed by DEAE-cellulose chromatography produced a 70-fold purification, and 48% total recovery, of TPP II-like activity from the extract (Fig. 1 and Table I). In the Sepharose CL-4B chromatography, the enzyme eluted at a $K_{av}$ of 0.26, which is comparable to the value for the purified mammalian enzyme (7, 8, 13). This indicates that, like the mammalian enzyme, the native $M_r$ is above $10^6$ for the *Drosophila* enzyme and suggests that oligomeric dTPP II-included complexes are formed (Fig. 1*A*). Unlike mammalian TPP II, the *Drosophila* enzyme was not retained by the DEAE-cellulose column. Based upon the *dTPP II* gene sequence (see below) the deduced isoelectric point is 7.0 for the dTPP II enzyme, compared with 6.2 for the human enzyme. The prediction of a higher isoelectric point for dTPP II is consistent with the observation that it does not bind under these conditions (Fig. 1*C*). Nevertheless, a large portion of the contaminating proteins did bind the anion exchanger, and a significant purification factor was achieved (Table I) as illustrated by the silver-stained gel (Fig. 2).

We monitored the extent of dTPP II purification using an antibody raised against the human TPP II (29). Western blot analysis revealed a ~150,000-Da band co-eluting with the enzyme activity in the Sepharose chromatography (Fig. 1*B* and Fig. 2). Following the DEAE chromatography (Fig. 2), the active fractions retained the same ~150-kDa TPP II immunoreactive band. This size is consistent with that predicted by the *dTPP II* sequence (see below). A few additional immunoreactive bands of lower molecular weight also co-purified with the enzyme activity; these may represent partially degraded subunits still incorporated in the high molecular weight complex and/or other cross-reacting proteins. Immunoreactive bands of lower molecular weight were also seen in the purification of human TPP II (8, 22).

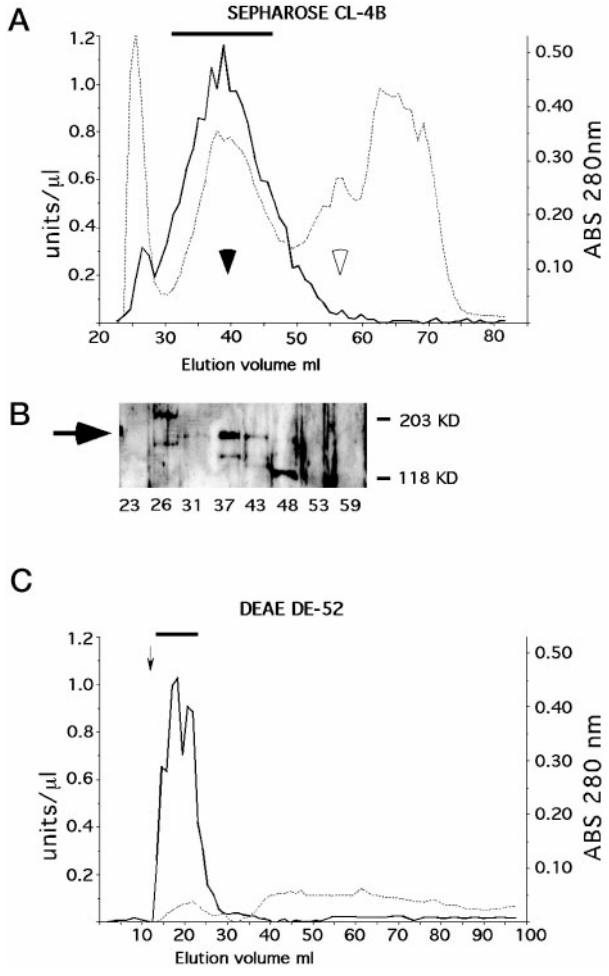



FIG. 1. **Partial purification of dTPP II.** *A*, adult *Drosophila* extract (2.0 ml, corresponding to 0.5 g wet weight), was prepared and loaded onto a Sepharose CL-4B column (1 × 89 cm); the column had a void volume of 27.5 ml and a total volume of 75.3 ml. The column was equilibrated and eluted with 100 mM potassium phosphate buffer, pH 7.5, that contained 30% glycerol and 1 mM DTT. Fractions of 0.9 ml were collected at a flow rate of 5.4 ml/h and pooled according to activity as indicated by the *bar*. The *dashed line* indicates the absorbance at 280 nm; the *solid line* indicates the peptidase activity; the *black arrowhead* indicates the $K_{av}$ corresponding to $10^6$ Da; the *white arrowhead* indicates the estimated elution point of monomeric TPP II (~150 kDa). *B*, Western blot analysis of fractions from the Sepharose column following SDS-polyacrylamide gel electrophoresis separation; samples were probed with chicken anti-human TPP II antibodies. *Arrow* indicates the predicted ~150-kDa size of dTPP II. *C*, 8.8 ml of the pooled active fractions from *A* were loaded onto a DEAE-column (1.6 × 13.6 cm) that was equilibrated with 100 mM potassium phosphate buffer, pH 7.5, containing 30% glycerol and 1 mM DTT. Fractions eluted at a flow rate of 6 ml/h with a linear gradient (100 + 100 ml) of 100–750 mM potassium phosphate, pH 7.5, that contained glycerol and DTT. Fractions of 1.3 ml were collected and analyzed by the standard assay. The *arrow* indicates where the gradient was started. Fractions were pooled as indicated by the *bar* and used for further characterization.

We found a higher specific activity in extracts from embryos (0.83 units/μg) as compared with adult *Drosophila*. The same purification scheme applied to the embryonic extracts produced an overall purification of only 40-fold. In preliminary experiments, the enzyme preparation from the embryo and adult tissues behaved identically with respect to substrate specificity and inhibitor sensitivity (data not shown). Due to its greater purity, the enzyme preparation derived from adult tissue was

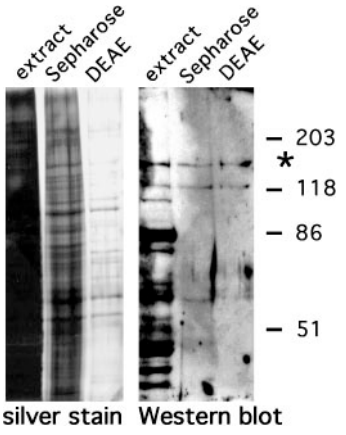



FIG. 2. **Silver stain and Western blot analysis of dTPP II purification.** Samples of approximately equivalent TPP II activity were prepared and electrophoresed according to "Experimental Procedures." The samples are as labeled: original extract, pool of active Sepharose column fractions, pool of active DEAE-cellulose column fractions. The *asterisk* indicates the expected size for dTPP II, ~150 kDa.

selected for further characterization.

*Characterization of dTPP II*—In order to characterize further the partially purified dTPP II, we investigated its pH optimum, substrate specificity, inhibitor sensitivity, and kinetics. The optimal pH for cleavage of the standard substrate AAF-pNA was between 7.5 and 7.8 (data not shown). For comparison, the mammalian enzyme has a pH optimum of 7.5 with the hexapeptide Arg-Arg-Ala-Ser(P)-Val-Ala as the substrate (7, 8). This feature differentiates TPP II from the lysosomal enzyme TPP I that prefers an acidic environment (35, 36).

The sequential cleavage of a larger substrate to form a series of tripeptides is the defining nature of a tripeptidyl peptidase. The tripeptidyl peptidase specificity of the partially purified dTPP II was confirmed by cleavage of the nonapeptide Gly-Val-Leu-Arg-Arg-Ala-Ser-Val-Ala. Fig. 3 shows that the major products corresponded to tripeptides cleaved sequentially from the N terminus of the nonapeptide. No additional cleavage products were detected.

To pursue the hypothesis that the activity contains a true tripeptidyl peptidase, we used the chromogenic substrates Ala-pNA, Phe-pNA, and Suc-Ala-Ala-Phe-pNA (Table II). None of these substrates were cleaved at a detectable rate by the partially purified dTPP II. Failure to cleave suc-Ala-Ala-Phe-pNA demonstrated the need for a free N terminus and showed that the observed cleavage of the standard substrate was not due to a chymotrypsin-like activity. Failure to cleave the aminopeptidase substrates Ala-pNA and Phe-pNA demonstrated that the cleavage of AAF-pNA was not due to sequential cleavage by contaminating aminopeptidase activity. We studied the substrate specificity of dTPP II compared with that of the human enzyme by use of five additional chromogenic tripeptidyl-pNA substrates (Table II). From these experiments we conclude that various peptide bonds are cleaved at different rates and that the enzymes from human and *Drosophila* show similar specificity. Ala-Ala-Ala-pNA and Phe-Pro-Ala-pNA were cleaved at an appreciable rate by human and *Drosophila* enzymes. The cleavage of Val-Leu-Lys-pNA could only be detected when a relatively high enzyme concentration of either enzyme was used (data not shown). Ala-Phe-Pro-pNA and Pro-Leu-Gly-pNA were not cleaved by either the *Drosophila* or the human enzyme.

Table III describes the sensitivity of dTPP II to various inhibitors. As expected, the *Drosophila* enzyme was inhibited by the serine peptidase inhibitor phenylmethanesulfonyl fluoride but not by the aspartic peptidase inhibitor pepstatin, the metallopeptidase inhibitor EDTA, or the aminopeptidase inhibitor bestatin. Similar to the human enzyme (8), dTPP II was inhibited by some thiol-reactive compounds (*p*-chloromercuribenzoate and *N*-ethylmaleimide) but not by others (*e.g.* io-

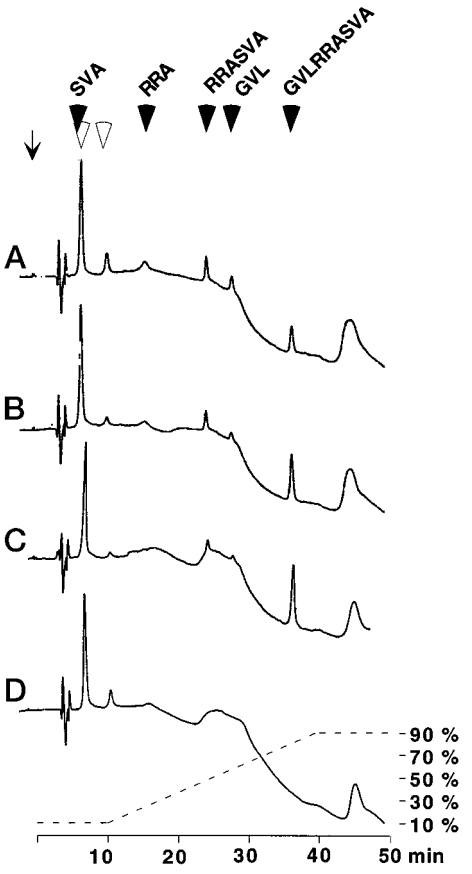



FIG. 3. **HPLC analysis of cleavage of the nonapeptide GVLRRASVA.** The enzyme (6 units prepared as in Fig. 1) was incubated at 37 °C with the nonapeptide GVLRRASVA for 90 min (*A*), 30 min (*B*), and 10 min (*C*). As a control the enzyme was incubated without the substrate (*D*). The *solid line* indicates absorbance at 210 nm; the *dashed line* indicates percent of buffer B. The elution positions of standard peptides are indicated by *black arrowheads*. The tripeptide Ser-Val-Ala cannot be detected in the HPLC chromatogram because it is obscured by a peak originating from the buffer or from the enzyme preparation. The *white arrowheads* indicate main peaks originating from the enzyme preparation or the buffer. The *arrow* indicates time of sample injection. The data represent one of two experiments performed.

TABLE II

*Cleavage of chromogenic substrates by partially purified dTPP II, purified human TPP II, and partially purified recombinant dTPP II*

Two different preparations of dTPP II, one preparation of human TPP II, and two different preparations of recombinant dTPP II were used. The data represent mean ± S.D. from four (*Drosophila*), two (human), or four (recombinant) experiments, with duplicates. The rates with the substrate AAF-pNA were 0.51 ± 0.05 units/μl (*Drosophila*), 0.44 ± 0.04 units/μl (human), and 0.26 ± 0.03 units/μl (recombinant).

| Chromogenic substrate | Activity | | |
|---|---|---|---|
| | Purified dTPP II | Human TPP II | Recombinant dTPP |
| | *% AAF-pNA* | | |
| Ala-Ala-Phe-pNA | 100 | 100 | 100 |
| Ala-pNA | <2 | <2 | <2 |
| Phe-pNA | <2 | <2 | <2 |
| Suc-Ala-Ala-Phe-pNA | <2 | <2 | <2 |
| Ala-Ala-Ala-pNA | 24 ± 2 | 31 ± 6 | 22 ± 1 |
| Phe-Pro-Ala-pNA | 12 ± 2 | 25 ± 3 | 12 ± 1 |
| Ala-Phe-Pro-pNA | <2 | <2 | <2 |
| Pro-Leu-Gly-pNA | <2 | <2 | <2 |
| Val-Leu-Lys-pNA | <2 | <2 | <2 |

TABLE III
*Investigation of inhibitor sensitivity*

The activity was measured by the standard assay as described under "Experimental Procedures." The final concentration of inhibitor is indicated in the table. The experiment was performed four times in duplicates with two different enzyme preparations, and the data represent means ± S.D. The rate in the absence of inhibitor was 0.43 ± 0.02 units/$\mu$l. PMSF is phenylmethanesulfonyl fluoride; NEM is *N*-ethylmaleimide; pCMB is *p*-chloromercuribenzoate.

| Inhibitor | Concentration | Remaining activity |
|---|---|---|
| | | % |
| None | | 100 |
| PMSF | 1 mM | <2 |
| Pepstatin | 1 $\mu$g/ml | 101 ± 8 |
| EDTA | 2 mM | 95 ± 3 |
| DTT | 1 mM | 93 ± 3 |
| Bestatin | 100 $\mu$M | 98 ± 3 |
| Butabindide | 2 $\mu$M | 16.5 ± 0.5 |
| NEM | 1 mM | 13 ± 1 |
| pCMB | 0.2 mM | 3 ± 3 |
| Iodoacetamide | 1 mM | 94 ± 4 |

doacetamide). The specific TPP II inhibitor butabindide is a competitive inhibitor designed against the CCK-8 degrading activity of TPP II activity in rat brain (6). It was an efficient inhibitor of the *Drosophila* TPP II enzyme. However, the $K_i$ for butabindide determined as described under "Experimental Procedures" was about 0.1 $\mu$M, *i.e.* an order of magnitude lower for the *Drosophila* enzyme than for the rat enzyme (7 nM) (6).

*Cloning of dTPP II*—A 2.5-kb clone fortuitously isolated by Drs. F. Van Leeuwen and R. Nusse from an expression library provided the 5′ portion of the *Drosophila TPP II* cDNA (PGA9). The Berkeley *Drosophila* Genome Project (BDGP) has described several *dTPP II* ESTs (clot 474) containing *TPP II* sequences, among these, the EST produced from cDNA clone LD18681 indicates additional 5′-untranslated region sequences. By use of PCR a complete cDNA was isolated as described under "Experimental Procedures." The complete cDNA sequence consists of 4309 bp that contains a single long open reading frame encoding 1354 aa (Fig. 4). The putative initiator methionine, deduced by alignment with the human protein, is preceded by the sequence GCAGG which does not correspond well with the consensus for *Drosophila* mRNAs (37). The cDNA has an untranslated 5′ end of 126 bp, containing no other AUGs. The 107-bp 3′-untranslated region ends with 15 A bases and displays the consensus polyadenylation signal AATAAA 12 bp upstream.

*TPP II* was previously cloned from rat, human, and mouse species (6, 10–12). In addition, a putative homologue has been identified in *C. elegans* by the genome sequencing project (38). The deduced amino acid sequence of the proposed *Drosophila TPP II* homologue was aligned with the corresponding proteins from human and *C. elegans* using ClustalW method (39) and manual adjustment (Fig. 4). dTPP II is more similar to the mammalian enzyme (38% identity) than it is to the *C. elegans* TPP II (28%). The high degree of conservation is notable in the subtilisin-like catalytic domain (aa 1–526) (44%). A special feature of TPP II, in comparison with other subtilases, is the large 200-aa insert between the Asp and His of the catalytic triad (12). Only one other subtilase, pyrolysin, also has a large insert in this area (40). This region (amino acids 50–270) shows a high degree of identity (33%) to human TPP II, whereas the identity to pyrolysin is only 17%. Together these observations support the hypothesis that the cloned *Drosophila* cDNA is a true *TPP II* homologue and not a different subtilase gene.

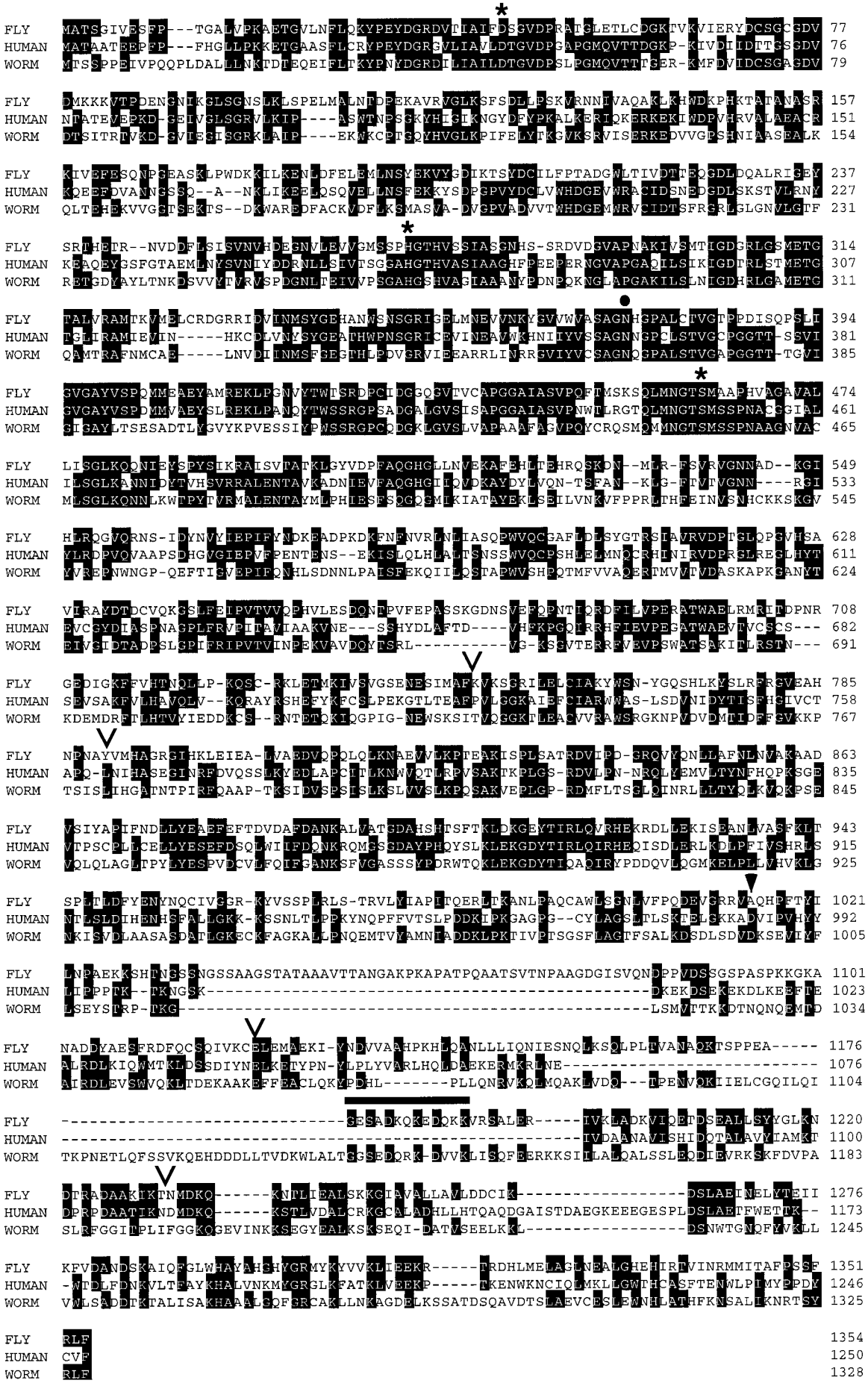
The C-terminal half of the deduced dTPP II protein (amino acids 925–1354) is less well conserved with human TPP II than is the N-terminal. When comparing the human and murine enzymes, the entire subunit was well conserved (96% overall identity), with the exception of residues 1140–1183 which only showed 70% identity (12). The region corresponding to *Drosophila* aa 1033–1195 differs from those of the aligned human or *C. elegans* proteins in both sequence and number of amino acids. The size of this region in the *C. elegans* protein is more similar to that of *Drosophila*.

By using the PGA9 cDNA clone, we recovered three independent phage clones that represent overlapping portions of the *dTPP II* genomic locus. By blot and partial sequence analysis, we found that the entire 4309-bp cDNA maps to a genomic region not greater than 6 kb. Sequence analysis of the 3′ end of the gene (corresponding to deduced aa 740–1354) revealed the presence of four introns. None of the introns are greater than 100 bp, and they lie at deduced amino acids 752, 789, 1121, and 1231 (Fig. 4). The positions of *Drosophila* and deduced *C. elegans* TPP II introns are not similar. Furthermore, there is no potential for additional amino acids in the *Drosophila* sequence at the position aligned to the alternatively spliced site of the mammalian enzyme.

*Expression and Characterization of Recombinant dTPP II*—To test the activity of the putative *dTPP II* cDNA, we used transient heterologous expression in HEK-293 cells. Transfection with the *dTPP II* sequence produced a 1.5–11-fold increase of AAF-pNA cleavage activity in cell extracts (Fig. 5). The mouse sequence produced a 2–7-fold increase, under these same conditions. Butabindide produced approximately 80% inhibition of the measured activity which is consistent with the induction of a TPP II-like activity. Transfection with the *dTPP II* clone in reverse orientation produced no increase in TPP II activity.

Cells with a stable expression of recombinant dTPP II were selected as described under "Experimental Procedures." The highest expressing clones had 6- and 8-fold higher AAF-pNA cleaving activity than the control cells transfected with dTPP II in the reverse orientation. Control experiments demonstrated that the majority of the activity was cytoplasmic and not membrane-bound or secreted from the cells. The two-step purification procedure (*cf.* Fig. 1) was used to purify recombinant dTPP II from the cells (Fig. 6). The recombinant enzyme eluted from the Sepharose column with a $K_{av}$ of 0.26 (*i.e.* the same as the purified *Drosophila* enzyme). The recombinant dTPP II did not bind to the anion exchanger and could therefore be separated by this purification step from the endogenous human TPP II expressed by the HEK-293 cells (Fig. 6). The presence of an immunoreactive band of lower molecular weight co-purifying with the active enzyme indicated that, similar to the purified *Drosophila* enzyme (Fig. 2), the recombinant dTPP II was partly degraded. Upon prolonged storage at +4 °C, the *Drosophila* enzyme could be completely converted into the lower molecular weight product without any loss of activity (data not shown), thus indicating that the partial degradation was not detrimental to the activity of the enzyme. The recombinant enzyme had the same substrate specificity as the purified dTPP II (Table II) and was as sensitive to the inhibitor butabindide, *i.e.* in the presence of 2 $\mu$M butabindide only 15 ± 1% of the activity remained (*cf.* Table III). The $K_i$ for this inhibition was 0.1 $\mu$M, consistent with that for the partially purified enzyme. In addition, the $K_m$ for the cleavage of the chromogenic substrate AAF-pNA, determined as described under "Experimental Procedures," was approximately 0.1 mM similar for both the purified and recombinant dTPP II.

*dTPP II Cytological Location and Genetic Analysis—In situ* hybridization of a *dTPP II* genomic phage clone to polytene chromosomes produced a major hybridizing band at *49F* (Fig. 7*A*) and a minor band at *95F*. A *dTPP II*-specific probe, repre-


FLY MATSGIVESFP---TGADVPKAETGVLNFLQKYPEYDGRDVTIAIFDSGVDPRATGLETLCDGKTVKVIERYDCSGCGDV 77
HUMAN MATAATEEPFP---FHGLLPKKETGAASFLCRYPEYDGRGVLIAVLDTGVDPGAPGMQVTTDGKP-KIVDIIDTTGSGDV 76
WORM MTSSPPEIVPQQPLDALLLNKTDTEQEIFLTKYPNYDGRDILIAILDTGVDPSLPGMQVTTTGER-KMFDVIDCSGAGDV 79
FLY DMKKKVTPQENGNIKGLSGNSLKLSPELMALNTDPEKAVRVGLKSFSDLLPSKVRNNIVAQAKLKHWDKPHKTATANASR 157
HUMAN NTATEVEEKD-GEIVGLSGRVLKIP----ASWTNPSGKKYHIGIKNGYDFYPKALKERIQKERKEKIWDPVHRVALAEACR 151
WORM DTSITRIVKD-GVIEGISGRKLAIP----EKWKCPTGQYHVGLKPIFELYTKGVKSRVISERKEDVVGPSHNIAASEALK 154
FLY KIVEFESQNPGEASKLPWDKKILKENLDFELEMLNSYEKVYGDIKTSYDCILFPTADGWLTIVDTTFQGDLDQALRIGEY 237
HUMAN KQEEFDVANNGSSQ--A--NKLIKEELQSQVELLNSFEKKYSDPGPVYDCLVWHDGEVWRACIDSNEDGDLSKSTVLRNY 227
WORM QLTEHEKVVGGTSEKTS--DKWAREDFACKVDFLKSMASVA-DVGPVADVVTWHDGEMNRVCIDTSFRGRLGLGNVLGTF 231
FLY SRTHEIR--NVDDFLSISVNVHDEGNVLEVVGMSSPHGTHVSSIASGNHS-SRDVDGVAPNAKIVSMTIGDGRLGSMETG 314
HUMAN KEAQEYGSFGTAEMLNYSVNIYDDRNLLSIVTSGGAHGTHVASIAAGHFPEEPERNGVAPGAQILSIKIGDTRLSTMETG 307
WORM RETGDYAYLTNKDSVVYTVRVSPDGNLTEIVVPSGAHGSHVAGIAAANYPDNPQKNGLAPGAKILSLNIGDHRLGAMETG 311
FLY TALVRAMTKVMELCRDGRRIDVINMSYGEHANWSNSGRIGELMNEVVNKYGVVWVASAGNHGPALCTVGTPPDISQPSLI 394
HUMAN TGLIRAMIEVIN-----HKCDLVNYSYGEATHWPNSGRICEVINEAVWKHNIIYVSSAGNNGPCLSTVGCPGGTT-SSVI 381
WORM QAMTRAFNMCAE-----LNVDIINMSFGEGTHLPDVGRVIEEARRLINRRGVIYVCSAGNQGPALSTVGAPGGTT-TGVI 385
FLY GVGAYVSPQMMEAEYAMREKLPGNVYTWTSRDPCIDGGQGVTVCAPGGAIASVPQFTMSKSQLMNGTSMAAPHVAGAVAL 474
HUMAN GVGAYVSPDMMVAEYSLREKLPANQYTWSSRGPSADGALGVSISAPGGAIASVPNWTLRGTQLMNGTSMSSPNACGGIAL 461
WORM GIGAYLTSESADTLYGVYKPVESSIYPWSSRGPCQDGKLGVSLVAPAAAFAGVPQYCRQSMQMVNGTSMSSPNAAGNVAC 465
FLY LISGLKQQNIEYSPYSIKRALSVPATKLGYVDPFAQGHGLLNVEKAFEHLTEHRQSKDN--MLR-FSVRVGNNAD--KGI 549
HUMAN ILSGLKANNIDYTVHSVRRALENTAVKADNIEVFAQGHGILQVDKAYDYLVQN-TSFAN--KLG-FTVTVGNN----RGI 533
WORM MLSGLKQNNLKWTPYTVRMALENTAYMLPHIESFSQGQGMIKIATAYEKLSSILVNKVFPPRLTHFEINVSNHCKKSKGV 545
FLY HLRQGVQRNS-IDYNVYIEPIFYNDKEADPKDKFNFNVRLNLIASQPWVQCGAFLDLSYGTRSIAVRVDPTGLQPGVHSA 628
HUMAN YLRDPVQVAAPSDHGVGIEPVFPENTENS--EKISLQLHLALTSNSSWVQCPSHLELMNQCRHLNIRVDPRGLREGLHYT 611
WORM YVREPNWNGP-QEFTIGVEPIFQNHLSDNNLPAISFEKQIILQSTAPWVSHEQTMFVVAQERTMVVTVDASKAPKGANYT 624
FLY VIRAYDTDCVQKGSLFEIPVTVVQPHVLESDQNTPVFEPASSKGDNSVEPQPNTIQRDFILVPERATWAELRMRITDPNR 708
HUMAN EVCGYDLASPNAGPLFRVPLTAVIAAKVNE---SSHYDLAFTD----VHFKPGQLRRHFIEVPEGATWAEVTVCSCS--- 682
WORM EIVGIDTADPSLGPIFRIPVTVINPEKVAVDQYTSRL----------VG-KSGVTERRFVEVPSWATSAKITLRSTN--- 691
FLY GEDIGKFFVHTNQLLP-KQSC-RKLETMKIVSVGSENESIMAFKVKSSRILELCIAKYWSN-YGQSHLKYSLRFRGVEAH 785
HUMAN SEVSAKFVLHAVQLV--KQRAYRSHEFYKFCSLPEKGTLTEAFPVLGGKAIFFCIARWWAS-LSDVNIDYTTSFHGLVCT 758
WORM KDEMDRFTLHTVYIEDDKCS--RNTETQKIQGPIG-NEWSKSITVQGGKTLEACVVRAWSRGKNPVDVDMTIDFFGVKKP 767
FLY NPNAYVMHAGRGIHKLEIEA-LVAEDVQPQLQLKNAEVVLKPTEAKISPLSATRDVIPD-SRQVYQNLLAFNLNVAKAAD 863
HUMAN APQ-LNIHASEGINRFDVQSSLKYEDLAPQTTLKNWVQTVRPVSAKTKPLGS-RDVLPN-NRQLYEMVLTYNFHQPKSGE 835
WORM TSISLIHGATNTPIRFQAAP-TKSIDVSPSISLKSLVVSLKPQSAKVEPLGP-RDMFLTSSLQINRLLLTYQLKVQKPSE 845
FLY VSIYAPIFNDLLYEAEFEFTDVDAFDANKALVATGDAHSHTSFTKLDKGEYTIRLQVRHEKRDLLEKISEANLVASFKLT 943
HUMAN VTPSCPLLCELLYESEFDSQLWILFDQNKRQMGSGDAYPHQYSLKLEKGDYTIRLQIRHEQISDLERLKDLPFIVSHRLS 915
WORM VQLQLAGLTPYLYESPVDCVLFQIFGANKSFVGASSSYPDRWTQKLEKGDYTIQAQIRYPDDQVLQGMKELPLLVHVKLG 925
FLY SPLTLDFYENYNQCIVGGR-KYVSSPLRLS-TRVLYIAPLTQERLTKANLPAQCAWLSSNLVFPQDEVGRRVAQHPFTYI 1021
HUMAN NTLSLDIHENHSFALLGKK-KSSNLTLPPKYNQPFFVTSLPDDKLPKGAGPG--CYLAGSLTLSKTELGKKADVIPVHYY 992
WORM NKISVDLAASASDATLGKECKFAGKALLPNQEMTVYAMNTADDKLPKTIVPTSGSFLAGTFSALKDSDLSDVDKSEVIYF 1005
FLY LNPAEKKSHTNGSSNGSSAAGSTATAAAVTTANGAKPKAPATPQAATSVTNPAAGDGISVQNDPPVDSSGSPASPKKGKA 1101
HUMAN LIPPPTK--TKNGSK----------DKEKDSEKEKDLKEEFTE 1023
WORM LSEYSTRP-TKG----------LSMVTTKKDTNQNQEMTD 1034
FLY NADDYAESFRDFQCSQIVKCELEMAEKI-YNDVVAAHPKHLQANLLLIQNIESNQLKSQLPLTVANAQKTSPPEA----- 1176
HUMAN ALRDLKIQWMTKLDSSDIYNELKETYPN-YLPLYVARLHQLDAEKERMKRLNE---------- 1076
WORM AIRDLEVSWVQKLTDEKAAKEFFEACLQKYPDHL-------PLLQNRVKQLMQAKLVDQ---TPENVQKIIELCGQILQI 1104
FLY ----------SESADKQKEDQKKVRSALER-----IVKLADKVIQETDSEALLSYYGLKN 1220
HUMAN ----------IVDAANAVISHIDQTALAVYIAMKT 1100
WORM TKPNETLQFSSVKQEHDDDLLTVDKWLALTGGSEDQRK-DVVKLISQFEERKKSIILAQALSSLEQDIEVRKSKFDVPA 1183
FLY DTRADAAKIKTNMDKQ------KNTLIEALSKKGIAVALLAVLDDCIK----------DSLAEINELYTEII 1276
HUMAN DPRPDAATIKNDMDKQ------KSTLVDALCRKGCALADHLLHTQAQDGAISTDAEGKEEEGESPLDSLAETFWETTK-- 1173
WORM SLRFGGILPLIFGGKQGEVINKKSEGYEALKSKSEQI-DATVSEELKKL----------DSNWTGNQFYVKLL 1245
FLY KFVDANDSKAIQFGLWHAYAHSHYGRMYKYVVKLIEEKR-----TRDHLMELAGLNEALGHEHIRTVINRMMITAFPSSF 1351
HUMAN -WTDLFDNKVLTFAYKHALVNKMYGRGLKFATKLVEEKP-----TKENWKNCIQLMKLLGWTHCASFTENWLPLMYPPDV 1246
WORM VWLSADDTKTALISAKHAAALGQFGRCAKLLNKAGDELKSSATDSQAVDTSLAEVCESLPWNHLATHFKNSALIKNRTSY 1325
FLY RLF 1354
HUMAN CVF 1250
WORM RLF 1328

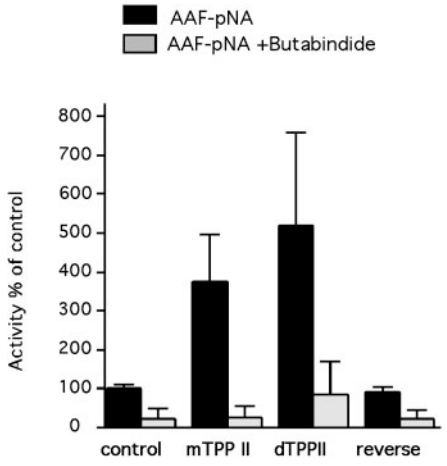



FIG. 5. **Relative activity of transiently expressed dTPP II and mTPP II.** HEK-293 cells were transiently transfected with either *mTPP II*, *dTPP II*, or reversed *dTPP II*, as described under "Experimental Procedures." The activity of cell lysates was assayed: *black bars* represent the standard peptidase assay ($n = 4$) and *gray bars* represent activity in the presence of 2 $\mu$M butabindide ($n = 2$). For each experiment, relative activity is normalized to activity in lysates from mock-transfected HEK-293 cells in the absence of butabindide. *Error bars* indicate standard error.

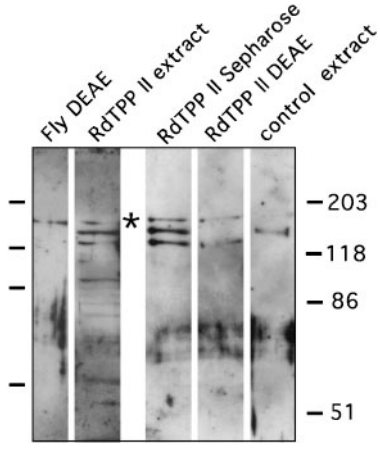



FIG. 6. **Western blot analysis of purification of recombinant dTPP II.** Samples of approximately equivalent AAF-pNA cleaving activity were prepared and electrophoresed according to "Experimental Procedures." The samples from purification steps of recombinant dTPP II (RdTPP II) are as labeled: cell extract, pool of active Sepharose column fractions, pool of active DEAE-cellulose column fractions. The partially purified *Drosophila* sample from the pool of DEAE-cellulose column fractions is included for comparison, and the control extract prepared from lysate of HEK-293 cells transfected with the vector alone displays the endogenous TPP II protein. This figure presents lanes from two gels and the appropriate standards are labeled for each. The *asterisk* indicates expected size for dTPP II, ~150 kDa.

senting the 3′ portion of the gene, produced a strong hybridization signal on the genomic P1 clone array (34) corresponding to the clone DS01087. This P1 clone has been assigned to the Mp20 contig and maps to the chromosomal interval 49F14–59A1. Low stringency Southern blot analysis of *D. melanogaster* genomic DNA gave a hybridization pattern corresponding to the recovered genomic clone and no additional bands (data not shown). Together these observations indicate the presence of a single *dTPP II* gene at position 49F of chromosome 2R. We attribute the weak hybridization at 95F to sequences in the genomic phage clone unrelated to *dTPP II*.

We related the observed enzyme activity to the cloned *dTPP II* gene through the analysis of available mutations of the 49F region. Southern blot analysis of heterozygous deficiency lines using a *dTPP II*-specific probe in comparison to an unrelated control probe revealed reduced relative hybridization in three lines. This result reflects the heterozygous deletion of the *dTPP II* coding region in the lines *Df(2R)vg 33, Df(2R)vg 56*, and *Df(2R)vg B* but not in *Df(2R)vg C* (Fig. 7*B*). TPP II activity of extracts from the three *dTPP II*-deficient lines was reduced ~50–60% compared with wild type and to the non-*dTPP II*-compromised stock *Df(2R)vg C* (Fig. 7*C*). For each line, the cleavage rate of the control substrate, AFP-pNA, was not significantly different from that of wild type; this demonstrates that there is not a general reduction in peptidase activity. The ratios of AAF:AFP cleavage rates among these lines were significantly different (one-way analysis of variance: $F(4,19) = 21.06$, $p = 0.0001$). As compared with wild type, the ratio of AAF:AFP cleavage was significantly reduced for the lines *Df(2R)vg 33*, *Df(2R)vg 56*, and *Df(2R)vg B* but not for *Df(2R)vg C* (Scheff's *S* test $p = 0.0001$, $p = 0.0001$, $p = 0.0005$, $p = 0.6134$, respectively). The correlation between reduced TPP II activity and elimination of *dTPP II* DNA sequences suggests that the 49F *dTPP II* gene is largely responsible for the observed activity.

The deficiency lines are homozygous lethal, and display reduced vitality even as heterozygotes.[3] Within the region defined by deficiency analysis, there are at least 11 lethal complementation groups (23). This complexity precludes analysis of the deficiency lines for dTPP II-specific phenotypes. Two of the 11 lethal mutations have been correlated with specific genes (see "Discussion" for details); the remaining 9 complementation groups represent candidate mutations of the *dTPP II* locus. We analyzed dTPP II enzyme levels in one or more representative alleles from each of these complementation groups to determine if any represent mutations in the *dTPP II* locus. Two alleles of one complementation group, *vr6*, showed reduced AAF-pNA cleavage. However, we found no evidence for decreased dTPP II (AAF-pNA cleavage) as compared with control peptidase activity (AFP-pNA cleavage) in any of the stocks tested (Fig. 8) (one-way analysis of variance: $F(13,47) = 0.231$, $p = 0.996$). Thus, none of these alleles are likely to be specific mutations in the *dTPP II* gene.

## DISCUSSION

This study has assigned a TPP II identity to a *Drosophila* gene located at cytological position 49F. This assignment derives from results of biochemical, molecular, and genetic experiments. Antibodies against the human enzyme cross-react with a *Drosophila* protein of expected size that is enriched through the partial purification of activity. The partially purified activity shows similar pH optimum, substrate specificity, and inhibitor sensitivity compared with the mammalian TPP II. The

[3] S. C. P. Renn, and P. H. Taghert, unpublished observations.

FIG. 4. **Comparison of the deduced amino acid sequences of *Drosophila*, human, and *C elegans* TPP II.** Sequences were aligned by the ClustalW program (29) and manual adjustment. Identical amino acids are *boxed*. The *asterisks* indicate the Asp, His, and Ser residues that form the catalytic triad; the *dot* indicates the Asn that stabilizes the tetrahedral intermediate. The *arrowhead* marks the site at which alternate splicing of the human RNA results in the inclusion of the sequence GQSAAKRQGKFKK. The *black bar* indicates the region of the *Drosophila* protein that is similar to the additional 13 aa of the human splice form. The *V* symbols indicate the sites corresponding to introns identified for the 3′-half of the gene. The GenBank™ accession number for the *Drosophila* sequence is AF035351, that for the human sequence is M73047, and that for the *C. elegans* sequence is U23176.

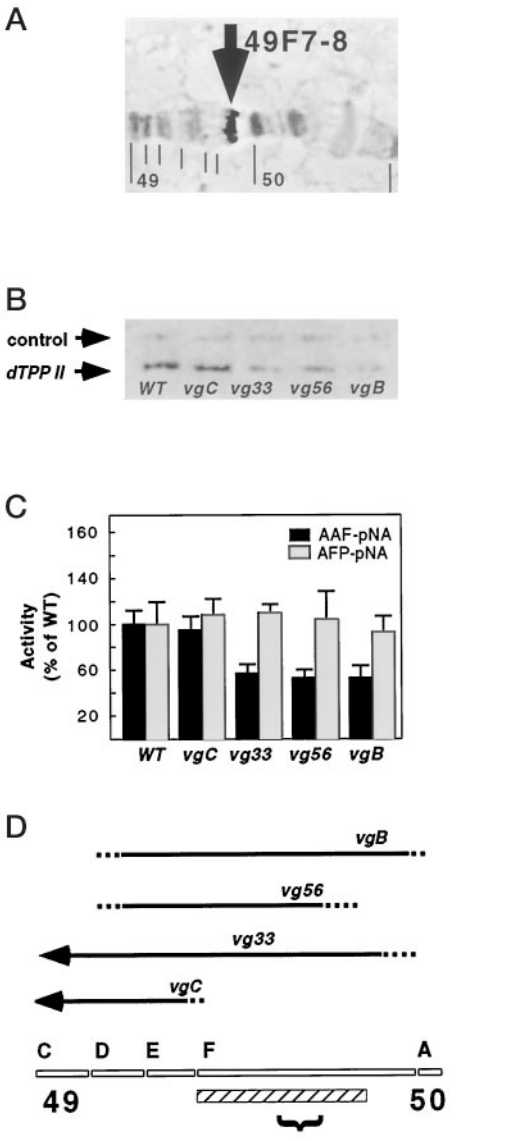



FIG. 7. **Analysis of four genetic deficiencies of the 49F polytene region.** *A*, *in situ* hybridization of a single genomic phage clone to polytene chromosomes produces a major band at *49F7-8* (*black arrow*) and a minor band at 95F (not shown). *B*, high stringency Southern blot analysis of five distinct deficiency stocks. For each lane, DNA of 10 flies was restricted with the enzyme *Pst*I. The filter was hybridized with a *dTPP II*-specific probe (bp 1–913) and an unrelated, control probe (~500 bp) from the 83E polytene region, each at a specific activity of $\sim 10^6$ cpm/ml. *C*, enzyme activity assay. Extracts of 10 flies from each of the five distinct, genetic stocks were prepared and assayed according to "Experimental Procedures." The cleavage rates for AAF-pNA (*black bars*) and AFP-pNA (*gray bars*) are expressed as a percentage of the wild type cleavage rate on that substrate. The data represent the mean of three experiments ± S.D. For each experiment wild type activity levels were determined as the mean of four samples. *D*, schematic representation of genomic regions deleted in the various deficiency stocks. *Hatched bar* indicates minimal region of *dTPP II* gene as determined from data of *B* and *C*. *Bracket* marks the *dTPP II* localization by *in situ* hybridization (49F7-8, *A*).

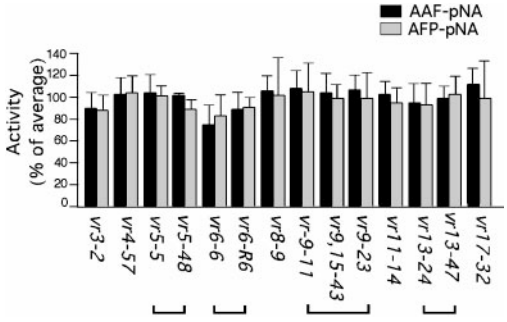



FIG. 8. **Analysis of stocks with recessive lethal mutations in the 49F cytogenic region that includes *dTPP II*.** Extracts of 10 flies from each of the 13 distinct genetic stocks were prepared and assayed according to "Experimental Procedures." Results are expressed as the percentage of the average cleavage activity for either AAF-pNA substrate (*black bars*) or for AFP-pNA substrate (*gray bars*). *Brackets* indicate multiple alleles comprising a complementation group. The results represent the mean of five experiments ± S.D.

*Drosophila* and mammalian enzymes display a high percentage of identical and similar amino acids not only around the catalytic triad but also in the extra insert of 200 aa within the catalytic domain that is characteristic of the TPP II. The analysis of deficiency animals correlated the absence of *dTPP II* gene sequences with a reduction of dTPP II activity. Finally, transfection of HEK-293 cells with the *dTPP II* sequence results in an induction of TPP II activity, and the recombinant enzyme shows similar biochemical characteristics to enzyme preparations from *Drosophila* extracts.

Lacking an endogenous substrate, we used AAF-pNA to assay TPP II activity from *Drosophila* because this chromogenic peptide has been shown to be a good substrate (8). This assay revealed a high level of TPP II-like activity in both adult and embryonic *Drosophila* extracts, 0.5 and 0.85 units/$\mu$g, respectively. For comparison, when using the substrate RRAS($^{32}$P)-VA, the TPP II activity in rat liver homogenate was 1.2 units/$\mu$g (8); this corresponds to about 0.1 units/$\mu$g under the present assay conditions (see "Experimental Procedures").

Our two-step chromatographic purification strategy follows that used for the initial characterization of rat TPP II (7). We have used the partially purified material to begin defining the properties of the insect enzyme in comparison to those of mammalian TPP II. The *Drosophila* enzyme, both partially purified from tissue and recombinant, cleaved the substrate AAF-pNA with a $K_m$ of 0.1 mM, whereas the $K_m$ for the human enzyme is 0.02 mM when measured under identical conditions.[4] This value corresponds to previously reported $K_m$ values for cleavage of a similar substrate AAF-7-amido-4-methylcoumarin by mammalian TPP II: 0.025 mM for rat TPP II (6) and 0.016 mM for human TPP II (8). Based on these results, we hypothesize that human TPP II cleaves AAF-pNA more efficiently than does the *Drosophila* enzyme. A deviating $K_m$ value (0.148 mM) has, however, been reported for human TPP II (41).

TPP II displays low substrate specificity *in vitro*, as evidenced by the release of tripeptides of no apparent similarity. However, the peptidase is not indiscriminate in that different peptide substrates are cleaved at different rates (7–8, 41). The cleavage rate is sensitive to the N-terminal tripeptide sequence and also to the C-terminal sequences and to the phosphorylation state of the substrate (7). However, the tripeptidyl nature of TPP II is absolute. Our results using a nonapeptide substrate (Fig. 3) as well as the chromogenic tripeptide substrates (Table II) demonstrate that the *Drosophila* enzyme activity adheres to the strict tripeptidyl peptidase characteristic. Furthermore, although the exact rate of cleavage may vary between the mammalian and the *Drosophila* enzymes, good substrates for one are also cleaved well by the other. Proline residues within the N-terminal tripeptide sequence affect the cleavage rate by the mammalian enzyme (8). Among the chromogenic tripeptide substrates tested, a proline is accepted only in the second position by both the *Drosophila* and mammalian enzymes (Table II), although a proline in the first position is accepted by the human enzyme in some peptide substrates (41).

[4] B. Tomkinson, unpublished data.

In conclusion, even though the general pattern of acceptable substrates is conserved between the enzyme from human and *Drosophila*, there are small differences in the relative rates of cleavage.

The results of the inhibitor sensitivity for dTPP II (Table III) are consistent with those observed previously for human TPP II (8) as well as with our own preliminary comparison to human TPP II. The inhibitor sensitivities define the enzyme activity as that of a serine protease. Furthermore, dTPP II is sensitive to the same thiol-reactive compounds as is the mammalian enzyme (8), suggesting a similarity in tertiary structure and accessibility to essential cysteine residues. This property is quite remarkable since only 3 out of 16 cysteine residues in the predicted *Drosophila* enzyme are conserved in the human enzyme (aa 207, 599, and 760). Of these three, only Cys-760 is also conserved in the predicted *C. elegans* sequence (Fig. 4).

The *Drosophila* EST project has produced 9 ESTs (clot 474) containing *TPP II* sequences, all of which derive from the *dTPP-II* gene we have mapped to 49F. The *C. elegans* genome project has identified 5 cDNAs which correspond to a predicted *C. elegans TPP II* gene. The longest *C. elegans* clone, yk15e7, contains sequence that predicts an initiator methionine 46 amino acids upstream from the methionine which begins the region of high homology. There is no evidence for alternate initiation sites for the mammalian or *Drosophila* enzyme.

The Sepharose column provides an estimation of the very high molecular weight of the native dTPP II enzyme (Fig. 1*A*). Thus similar to mammalian TPP II, the *Drosophila* enzyme is active in a large, oligomeric complex. In addition to this abundant active complex, mammalian TPP II can form an even larger complex, eluting at the void volume of the Sepharose column, as judged by Sepharose chromatography of human erythrocytes (8) and expression experiments of alternate splice forms (13). Since an activity shoulder can be seen in this position also for the *Drosophila* enzyme (Fig. 1*A*), it is possible that the *Drosophila* enzyme can form a similar larger complex. Two features of the mammalian sequence have been implicated in the formation of the oligomeric TPP II complex. The first is a KEKE domain (13); such domains are thought to mediate protein-protein interactions (42). There is no apparent KEKE motif in the *Drosophila* protein. The second domain implicated in oligomer formation is a 13-amino acid sequence (GQSAAKRQGKFKK, separated from the KEKE domain by 20 amino acids in the human sequence) (13). The *Drosophila* enzyme does not contain a sequence resembling the 13-amino acid domain at a position aligned to the mammalian splice site (aa 985). In addition, the possibility of an alternative exon at this position is precluded because the genomic sequence for this region corresponds directly to that of the cDNA without interruption. However, dTPP II does contain a sequence closer to the C terminus (GESADKQKEDQKK, aa 1175) that shows some similarity to the 13-amino acid domain. When expressed in a human cell line, the recombinant dTPP II, including the similar *Drosophila* 13-aa domain, did not give rise to the higher molecular weight complex that elutes in the void volume of the Sepharose column. This suggests that either this 13-amino acid region does not serve a similar complex-forming function in the *Drosophila* enzyme or that the complex requires additional *Drosophila* proteins not present in the transfected cell line. These differences in the behavior of the dTPP II enzyme indicate a need for further structure-function studies. Such studies will be facilitated by the ability to perform them *in vivo* through efficient generation of transgenic *Drosophila*. Furthermore, the hypothesis that TPP II is involved in CCK physiology (6) raises several questions that can best be addressed by genetic experiments.

Another advantage of using *Drosophila* is the ability to create and isolate mutants. Toward this goal we have identified three deficiency lines that display reduced TPP II activity due to loss of the gene. Embryos homozygous for the deficiencies *Df(2R)vg 56*, *Df(2R)vg 33*, or *Df(2R)vg B* display abnormal mitosis soon after fertilization (43), and development proceeds until the germ band retraction stage when the embryos die, presumably due to loss of multiple gene functions. Therefore, accurate analysis of a *dTPP II* null phenotype is not possible with these large deficiencies. These lines define a cytogenetic region that contains 11 known lethal complementation groups (23, 44)[5] suggesting the presence of at least 11 vital gene functions. Two of these have been identified as follows: one, *vr10*, is the dDP transcription factor involved in cell cycle regulation (44), and the other, *vr 14*, is *Su(z)2*, a DNA-binding protein (45, 46). We assayed the AAF-pNA cleavage activity for stocks representing one or more alleles from each of the (as yet) unidentified complementation groups. None of the lines showed significant reduction of TPP II-like activity, relative to the AFP-pNA cleavage activity. From this we conclude that the *dTPP II* gene is not mutated in any of these defined lethal stocks. Further analysis is required to determine if dTPP II is necessary for survival.

The genetic analysis initiated in this study provides the basis for future studies of TPP II function. *TPP II* is a single copy gene in *Drosophila*, and deficiencies of the *dTPP II* locus significantly reduce the enzyme activity. Our results now provide the means to identify single gene mutations of *dTPP II* and analyze its loss-of-function phenotypes. By using germ line transformation, it will also be possible to test partial restoration and gain-of-function TPP II phenotypes to address the hypothesis that this enzyme is involved in neuropeptide metabolism.

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