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Drosophila S2 Cells Produce Multiple Forms of Carboxypeptidase D With Different Intracellular Distributions

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Abstract Carboxypeptidase D (CPD) functions in the processing of proteins that transit the secretory pathway, and is present in all vertebrates examined as well as *Drosophila.* Several forms of CPD mRNA were previously found in *Drosophila* that resulted from differential splicing of the gene. In the present study, Northern blot, reverse transcriptase PCR, and Western blot analysis showed that each splice variant occurs in a single cell type, the *Drosophila*-derived Schneider 2 (S2) cell line. The short forms containing a single carboxypeptidase domain were secreted from the S2 cells while the long forms containing three carboxypeptidase domains, a transmembrane domain, and one of two different cytosolic tails were retained in the cell. To investigate the role of the two different C-terminal tail sequences (tail-1 and tail-2) that result from the differential splicing within exon 8, constructs containing a reporter protein (albumin) attached to the transmembrane domain and tail-1 or tail-2 of CPD were expressed in S2 cells and a mouse pituitary cell line (AtT20 cells). Immunofluorescence analysis revealed different intracellular distributions of the two constructs, with the tail-2 construct showing considerable overlap with a Golgi marker. The two C-terminal tail sequences also resulted in different internalization efficiencies from the cell surface in both cell lines. Interestingly, the distribution and routing of the tail-2 form of *Drosophila* CPD in the AtT20 cells are similar to the previously characterized endogenous mouse CPD protein, indicating that the elements for this trafficking have been conserved between *Drosophila* and mammals. J. Cell. Biochem. 99: 770–783, 2006.

Key words: peptide processing; metallocarboxypeptidase; furin; *trans* Golgi network; silver gene

Metallocarboxypeptidases (CPs) serve a large number of physiological functions ranging from the digestion of food to the selective biosynthesis of signaling molecules [Reznik and Fricker, 2001]. In all animal species examined, the CPs can be divided into one of two major subfamilies based on amino acid sequence homology and domain structure; the A/B subfamily and the N/E subfamily. In mammals, members of the N/E family typically have selective roles in the specific processing of proteins and peptides either within cells (CPE, CPD), in plasma (CPN), attached to the cell surface (CPM), or as part of the extracellular matrix (CPZ) [Fricker, 2004a,b,c; Skidgel, 2004; Skidgel and Erdos, 2004]. Three mammalian members of the N/E gene family appear to encode proteins that do not have CP activity: Carboxypeptidase-like protein X1 (CPX1), carboxypeptidase-like protein X2 (CPX2), and a protein designated both aortic carboxypeptidase-like protein (ACLP) and adipocyte enhancer binding protein 1 (AEBP1) [He et al., 1995; Layne et al., 1998; Xin et al., 1998; Lei et al., 1999]. The function of these proteins is not currently known, although mice lacking ACLP/AEBP1 have a phenotype that suggests this protein plays a role in cell–cell interactions [Layne et al., 2001]. Whereas humans and other mammals that have been examined have eight members of the N/E family of CPs, *Drosophila melanogaster* has only two genes for members of this family; one with

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highest homology to CPM and another that is clearly the CPD homolog. This *Drosophila* CPD was identified as the *svr* gene product in 1995 [Settle et al., 1995], independently of the discoveries of mammalian CPD [Song and Fricker, 1995] and duck CPD [Kuroki et al., 1995] in the same year.

Mammalian and duck CPD have broad tissue distributions and within cells are enriched in the *trans* Golgi network (TGN) [Song and Fricker, 1996; Varlamov and Fricker, 1998]. As found for other TGN proteins, CPD cycles from the TGN to the cell surface and back to the TGN [Varlamov and Fricker, 1998; Eng et al., 1999; Varlamov et al., 1999a]. Sequences within the 58-residue cytosolic tail of rat and duck CPD are responsible for the intracellular routing of this protein [Eng et al., 1999; Varlamov et al., 1999b; Kalinina et al., 2002]. Based on its broad tissue distribution, abundance in the TGN, and ability to remove basic residues (Lys, Arg) from the C-terminus of a number of diverse peptides, CPD is thought to play a role in the processing of peptides and proteins that are products of furin and other TGN endopeptidases that cleave proteins at basic amino acids [Fricker, 2002, 2004c]. In addition, CPD also appears to contribute to the processing of neuroendocrine peptides in the absence of CPE activity (i.e., in the $Cpe^{fat/fat}$ mouse, which lacks functional CPE due to a naturally occurring point mutation) [Naggert et al., 1995; Fricker and Leiter, 1999].

In mammals and duck, CPD is a multidomain protein that contains three CP-like domains followed by a transmembrane domain and a 58-residue cytosolic tail [Kuroki et al., 1995; Tan et al., 1997; Xin et al., 1997; Ishikawa et al., 1998]. The first two CP-like domains of CPD are enzymatically active; the third CP-like domain is missing key catalytic residues and has no detectable CP activity [Eng et al., 1998; Novikova et al., 1999]. Although *Drosophila* CPD was originally described as containing only two CP-like domains [Settle et al., 1995], subsequent analysis found that a sequencing error had been made and that *Drosophila* CPD does exist in a form with three CP-like domains and a transmembrane domain [Sidyelyeva and Fricker, 2002]. The first two CP-like domains are enzymatically active but the third lacks a number of critical active site residues needed for CP activity [Sidyelyeva and Fricker, 2002]. Following the transmembrane domain, *Drosophila* CPD has one of two possible cytosolic domains, depending on whether splicing occurred within exon 8 [Sidyelyeva and Fricker, 2002]. In addition, two other sites of the *Drosophila* CPD gene undergo differential splicing (Fig. 1, panel A). One of these splicing differences produces a form with either an inactive CP domain 1 (the 1A form) due to the absence of a critical Zn^{2+}-binding residue or an enzymatically active domain 1 (the 1B form) [Sidyelyeva and Fricker, 2002]. Another splice site determines whether the protein contains only a single CP domain (the "short" forms) or three CP-like domains followed by a transmembrane domain and cytosolic tail (the "long" forms) [Sidyelyeva and Fricker, 2002]. Mammalian and duck CPD mRNA have not been reported to undergo differential splicing, and mammalian CPD protein predominantly exists as the full-length membrane-bound form with a smaller amount of a soluble form presumably produced by proteolysis [Song and Fricker, 1996].

Because *Drosophila* has only two members of the N/E subfamily of CPs, it is possible that differential splicing of the *Drosophila* CPD gives rise to multiple CP activities that have properties of the mammalian CPs that are "missing" from *Drosophila* (i.e., CPE, CPZ, CPN, CPX1, CPX2, and ACLP/AEBP1). To examine whether a single cell type is capable of generating the myriad forms of CPD, we determined the forms present in S2 cells, a *Drosophila* cell line derived from a primary culture of late stage (20–24 h) *D. melanogaster* embryos [Schneider, 1972]. The finding that this cell line produced the same forms as adult *Drosophila* suggests a role for multiple forms in individual cells. The routing of the different forms within the cell was examined and found to show large differences, suggesting that the various forms play specific roles within individual cells.

MATERIALS AND METHODS

Northern Blot Analysis and RT-PCR

Total RNA from adult *D. melanogaster* and from S2 cells (Invitrogen) was prepared using the RNeasy protect minikit (Qiagen). Northern blot analysis was performed as described [Sidyelyeva and Fricker, 2002]. Enzymatic amplification of RNA by PCR using a combination

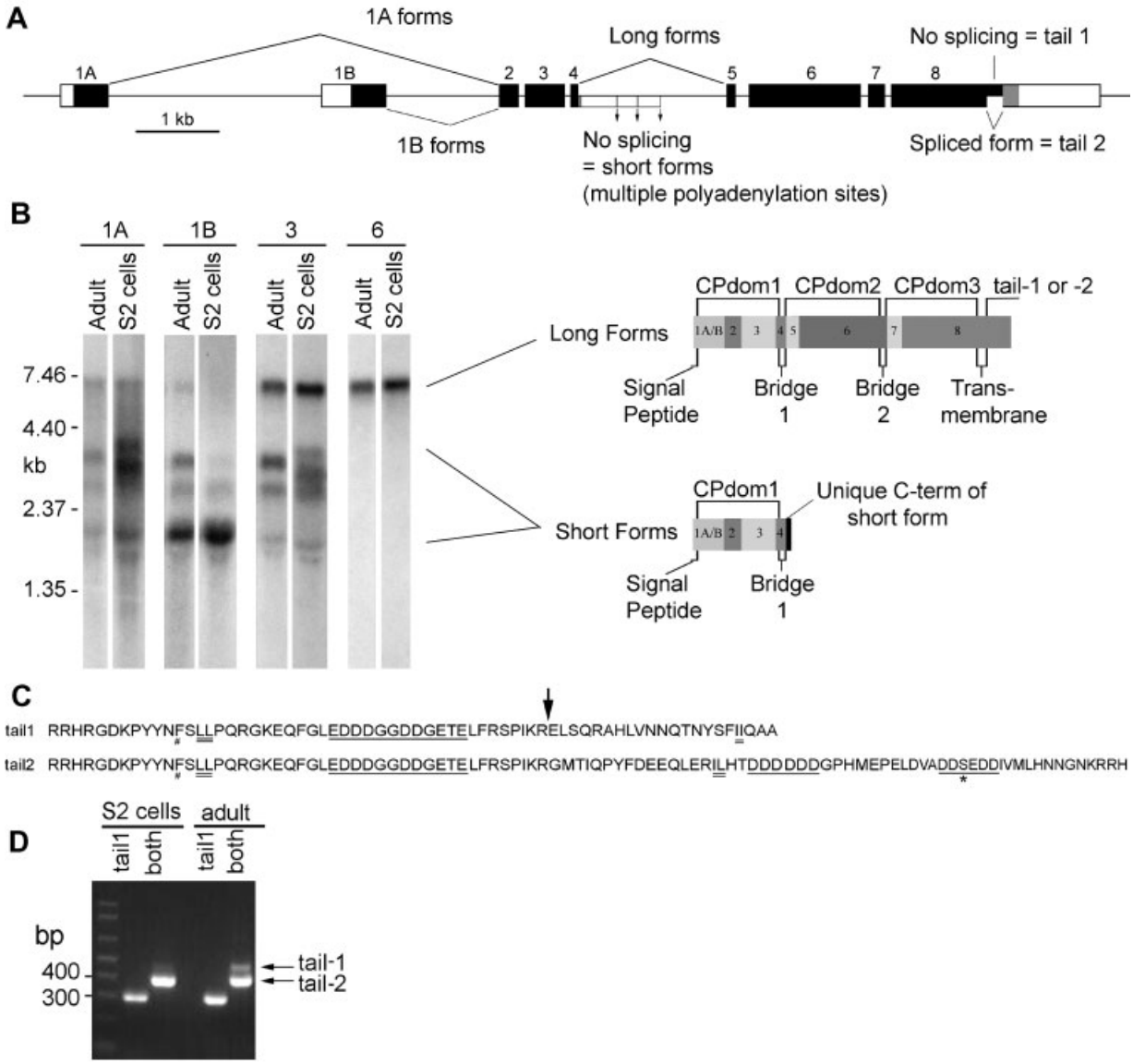

Fig. 1. Analysis of the forms of CPD RNA in adult *Drosophila* and S2 cells. **A**: Gene structure of *Drosophila* CPD, showing the location of the splice sites previously determined for adult *Drosophila* [Sidyelyeva and Fricker, 2002]. Exons 1A/B encode the signal peptide and N-terminal region of the first carboxypeptidase domain; this region includes two of the metal-binding residues critical for enzymatic activity. If the intron between exons 4 and 5 is spliced out, the resulting mRNA has a long open reading frame that contains three carboxypeptidase-like domains. If this intron is not spliced, there are multiple polyadenylation sites within this region that gives rise to a variety of short mRNA forms; the resulting protein contains a single carboxypeptidase-like domain followed by a unique C-terminal sequence (encoded by the intron) that is not present in the long forms. Another variable splice site is located within exon 8 and affects the sequence of the cytosolic region of the protein. If unspliced, the protein form corresponds to tail-1; if spliced, there is a change in reading frame and the protein form corresponds to tail-2. **B**: Northern blot analysis of RNA from adult wild-type *Drosophila* and S2 cells, hybridized with ^{32}P-labeled cRNA probes corresponding to either exon 1A, 1B, 3, or 6, as described in Materials and Methods. The positions of RNA size standards (Invitrogen) are indicated. The exons contained in the long and short forms and the location of the carboxypeptidase domains (CP dom) are indicated in the **right panel**. **C**: Amino acid sequences of the cytosolic regions of the tail-1 and tail-2 forms of *Drosophila* CPD [Sidyelyeva and Fricker, 2002]. Acidic clusters are indicated by a single underline, di-Leu and di-Leu-like motifs are indicated by a double underline, # indicates the Phe of the FxxL motif, * indicates the casein kinase II site within the acidic cluster of tail-2, and the arrow indicates the splice site within exon 8. **D**: Analysis of the tail-1 and tail-2 forms of CPD in S2 cells and in adult *Drosophila*. Reverse transcription of 1 μg RNA samples extracted from adult wild-type *Drosophila* and S2 cells was performed followed by PCR amplifications using the OneStep RT-PCR kit (Qiagen) and a 3′ oligonucleotide either specific for the tail-1 form (located in the region that is spliced out to form tail-2) or common to both tail-1 and tail-2 forms (located downstream of the splice site). Arrows indicate the size of the expected product from this common oligonucleotide that corresponds to the tail-1 and tail-2 forms. The positions of DNA size standards (Invitrogen) are indicated.

of Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase, and HotStarTaq DNA Polymerase (RT-PCR) was performed using the OneStep RT-PCR kit (Qiagen) with an oligonucleotide corresponding to the region flanking the transmembrane domain (5′-GCGAATTCCTTTTTGTTCATTTTGTGCGCCA) and another oligonucleotide corresponding to either the region of tail-1 that is unique to this form (5′-GCGGGCCCTCATGCCGCCTGAATAATGAA) or an oligonucleotide that is located downstream of the splice site and is therefore common to both tail-1 and tail-2 forms (5′-GCGGGCCCCTAGTGCCGTCTCTTGTTCCCGT).

Antisera Production

For antisera production, the synthetic peptides corresponding to the appropriate region of *Drosophila* CPD (described below) contained an additional Cys residue on the N- or C-terminus, and the peptides were coupled to maleimide activated keyhole limpet hemocyanin (Pierce). The peptide corresponding to the N-terminal 14 amino acids (after signal peptide removal) of the domain 1A form of CPD (EYSEVRVIQEEDNFC) was used to produce antisera in a rabbit (AE688) and in 5 mice. The peptide corresponding to the N-terminal 14 residues of *Drosophila* CPD form 1B (YTIKEDESFLQQPHC) was used to produce rabbit antiserum (AE689). Another rabbit antiserum (AE 690) was raised to the C-terminal 12 residues of the short forms of *Drosophila* CPD (CNFDGISSFYSPYYF).

For the production of the rabbit antisera, the standard 80-day protocol was used (Covance Research Products). For the production of the mouse polyclonal antisera, the antigen in phosphate-buffered saline, pH 7.4 (PBS) was blended with 0.75 ml Freund's complete adjuvant and injected i.p. Booster injections of antigen in Freund's incomplete adjuvant were given, also i.p., on days 15 and 30. On day 41, the mice were sacrificed and serum collected.

Western Blot Analysis

Proteins were fractionated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrophoretically transferred to nitrocellulose membrane (Optitran; Schleicher & Schuell). The blots were blocked with 5% nonfat milk in 10 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20 for 1 h at room temperature and then probed with 1:1,000 dilutions of polyclonal rabbit antisera or 1:2,000 dilutions of polyclonal mouse antisera described above. The nitrocellulose was washed, exposed to the secondary antiserum, and bound antiserum was detected with the enhanced chemiluminescence method (Pierce).

Affinity Column Purification of *Drosophila* CPD From S2 Cells

S2 cells were grown in shaker flasks at 27°C to a density of 2.2×10^6 cells/ml and 300 ml were centrifuged at $30{,}000 \times g$ and 4°C for 30 min. The medium was saved for Western blot analyses and for purification (below). The pellet was homogenized (Polytron, Brinkmann) in 10 ml of 0.1 M sodium acetate (NaAc) buffer, pH 5.5, containing a protease inhibitor cocktail (Sigma). The homogenate was centrifuged at $30{,}000 \times g$ for 30 min at 4°C, and the pellet was resuspended in 10 ml of 0.1 M NaAc buffer, pH 5.5, containing 1 M NaCl and the protease inhibitor cocktail and centrifuged as above. Finally, the pellet was homogenized in the same buffer containing additionally 1% Triton X-100 to extract a membrane bound fraction of proteins and centrifuged at $30{,}000 \times g$ for 30 min at 4°C. The membrane extract and medium from S2 cells were loaded onto two separate *p*-aminobenzoyl-Arg Sepharose 6B substrate affinity columns, as described [Fricker and Snyder, 1982]. The flow-through fractions were recycled several times, and then the columns were washed with 0.1 M NaAc buffer, pH 5.5, containing 1 M NaCl and 1% Triton X-100, followed by a rinse with 10 mM NaAc, pH 5.5. The columns were then washed with 50 mM Tris, pH 8.0, containing 100 mM NaCl and 0.01% CHAPS and the CPD was eluted with 25 mM Arg in the same pH 8 buffer.

Plasmid Construction and Recombinant Protein Production

The transmembrane and the cytoplasmic domains of *Drosophila* CPD were attached in-frame to the C-terminus of human albumin in the pcDNA3 expression plasmid, using the *Bs*u36I site within the coding region of albumin in a strategy similar to that previously used for other albumin constructs [Mitra et al., 1994]. Briefly, two complementary oligonucleotides were ligated and subcloned into the *Bs*u36I/*Ap*aI sites of Alb/pcDNA3 to create a linker containing internal *Xh*oI and *Afl*II sites and encoding a cleavage site for thrombin. FLAG

(DYKDDDDK) and HA (YPYDVPDYA) linkers were generated using synthetic oligonucleotides that were annealed and then subcloned into XhoI/*Eco*RI sites of Alb/pcDNA3. The KpnI/EcoRI fragment of this plasmid was subcloned into the pMT-V5 vector (Invitrogen). PCR fragments encoding the transmembrane domain and the tail-1 or tail-2 cytoplasmic domains of *Drosophila* CPD were ligated into the EcoRI/ApaI sites of either the pMT-V5 plasmid containing albumin and FLAG or HA epitopes (to transfect S2 cells) or into the Alb/pcDNA3 plasmid also containing FLAG or HA epitopes (to transfect AtT-20 cells). The constructs were transfected into AtT-20 cells using the Lipofectamine transfection kit (GIBCO BRL). Stable cell lines were selected using 1 mg/ml Geneticin (G418). For transient transfection of S2 cells, Cellfectin reagent (Invitrogen) was used.

Immunofluorescence Microscopy and Antibody Uptake Experiments

S2 cells were grown in *Drosophila* SFM (Invitrogen) with added Gln (18 mM) in shaker flasks at 27°C. For steady-state distribution of transiently co-transfected constructs in S2 cells a rabbit anti-HA antiserum (Sigma) and anti-FLAG M2 mouse monoclonal antibody (Sigma) were used. A monoclonal mouse antibody raised against *Drosophila* Golgi proteins was used as a Golgi marker (Calbiochem). For co-localization with this Golgi marker, the transiently transfected constructs were detected using a rabbit polyclonal antiserum raised against human albumin (Calbiochem).

AtT-20 cells expressing the albumin fusion proteins were analyzed by immunofluorescence as described for CPD [Varlamov and Fricker, 1998] using an antiserum to human albumin (dilution 1:1,000) and anti-syntaxin 6 monoclonal mouse antibody (1:5,000; StressGen). Antibody uptake experiments were performed as described for duck CPD/gp180 [Eng et al., 1999]. Briefly, AtT-20 cells stably expressing the albumin fusion proteins or S2 cells transiently expressing the albumin fusion proteins were incubated for 40 min at 4°C in medium containing a rabbit antibody to human albumin (1:150 dilution), 5 mg/ml nonfat milk, and 20 mM HEPES, pH 7.4. Unbound antibody was removed by washing with cold medium and the cells were incubated at 37°C for the indicated times to allow internalization from the cell surface. The cells were fixed and stained with Cy2-conjugated secondary antibody to rabbit IgG (H + L) (dilution 1:200). For comparison of the internalized Alb-containing protein to a TGN marker, cells were stained with a 1:5,000 dilution of mouse monoclonal antibody to syntaxin-6 in the experiment with AtT-20 cells, and then 1:800 dilution of Cy3-labeled anti-mouse IgG. In some studies, AtT-20 cells expressing the reporter constructs were preincubated with 5 μg/ml brefeldin A or with 100 μg/ml cycloheximide (CHX) and then subjected to immunofluorescence.

Pulse-Chase Analysis

To measure the turnover rate of the CPD-containing constructs, AtT-20 cells stably expressing the albumin fusion proteins were labeled for 20 min in Dulbecco's Modified Eagles Medium (DMEM) with ^{35}S[Met/Cys], chased in non-radioactive DMEM medium for the indicated time, and analyzed as described for duck CPD/gp180 [Eng et al., 1999], except that albumin fusion proteins were immunoprecipitated using an antiserum to human albumin (Calbiochem).

RESULTS

Northern blot analysis of RNA from S2 cells shows many of the same forms of CPD mRNA as those detected in adult *Drosophila* (Fig. 1). When probed with cRNA corresponding to exon 6, a single band of approximately 7 kb was observed for both S2 cells and adult *Drosophila* (Fig. 1B). This band was also detected with probes to exons 1A, 1B, and 3 (Fig. 1B). In addition, several other mRNA species ranging from 2 to 4 kb were detected with the exon 1A, 1B, and 3 probes. The 7 kb band corresponds to the long forms of CPD, and the smaller mRNA species correspond to the short forms of CPD based on previous sequence analysis of *Drosophila* CPD cDNAs [Sidyelyeva and Fricker, 2002]. Thus, while there are some differences in the apparent size of the 2–4 kb bands between S2 and adult *Drosophila* (Fig. 1B), these differences would not be expected to have an effect on the molecular weight of the protein forms because the 2–4 kb bands all encode the short form of CPD. In addition to the splice variants that give rise to the long versus short forms and the forms with exon 1A versus exon 1B, a third splice variant produces long forms that differ in the amino acid sequence within the

C-terminal cytosolic tail (Fig. 1C) [Sidyelyeva and Fricker, 2002]. The mRNAs that produce these two forms are similar in size and cannot be resolved on Northern blots [Sidyelyeva and Fricker, 2002]. To test whether mRNAs corresponding to both forms exist in the S2 cells, RT-PCR was performed. Oligonucleotides specific for the tail-1 form showed a single band of approximately 300 bp, consistent with the expected size of the PCR product (Fig. 1D). When the RT-PCR was performed with oligos that are common to both tail-1 and tail-2 sequences, two bands were detected of the expected sizes (Fig. 1D). The tail-2 form is more abundant than the tail-1 form in both S2 cells and adult *Drosophila* (Fig. 1D).

Western blotting was used to examine the forms of CPD proteins in the S2 medium and cells. A rabbit polyclonal antiserum raised against the N-terminus of the 1A form detected a major band of 50 kDa in the medium (Fig. 2A). This 50 kDa band was largely absent from the cells, and instead the major cellular form was approximately 150 kDa (Fig. 2A). Minor lower molecular weight forms were detected, which may represent degradation products of the 150 kDa band. Using an antiserum raised against the N-terminus of the 1B form, a major band of 50 kDa was detected in the medium (Fig. 2A). This antiserum did not show any detectable signal for the cells, consistent with the Northern blot which showed much greater levels of the short form of 1B-containing mRNA relative to the levels of the long forms (Fig. 1). The short forms of CPD were predicted to have a C-terminal sequence that is translated from the intronic region between exons 4 and 5; this region is not present in the long forms of CPD because it is spliced out. To test if this unique sequence is present in the short forms of CPD, an antiserum was raised against a synthetic peptide corresponding to this sequence and used to probe S2 medium and cells. This antiserum showed a major band in the S2 cell medium of 50 kDa, and no detectable signal in the cell extracts. A substrate affinity column was used to enrich the CPD from the cells and medium; this affinity column was previously used to purify mammalian CPD [Song and Fricker, 1995]. After affinity purification, a low level of the short 1A form was detected on a Western blot, relative to the higher levels of the short 1B form (Fig. 2B). This presumably reflects the low binding of the catalytically inactive short 1A form to the substrate affinity column. In addition to the major band detected for the 1B form in medium, a detectable signal of 150 kDa is seen with the 1B-directed antiserum after purification of the cell extracts (Fig. 2B, middle panel). Taken together, these results indicate that S2 cells express both short and long forms of CPD containing either the 1A or the 1B domain, and that the short forms are secreted while the long forms remain cellular.

The cellular distribution of CPD was examined using immunocytochemistry with a mouse polyclonal antiserum to the 1A form. This antiserum showed a major band of 150 kDa when tested with S2 cellular extracts on a Western blot (data not shown). Immunofluorescence analysis with this antiserum showed punctate staining throughout the cytoplasm (Fig. 3A). This punctate staining was similar to that previously reported for a Golgi-specific protein in S2 cells [Stanley et al., 1997]. Attempts to raise antisera specifically to the tail-1 and tail-2 sequences were not successful. In order to further study these two forms in the S2 cells, we created fusion proteins that combined human albumin and one of two epitope tags (HA or FLAG) with the transmembrane domain and tail-1 or tail-2 sequence of *Drosophila* CPD (Fig. 3B). In addition to these two constructs, two additional constructs were made in which the epitope and tail sequences were reversed so that the tail-1 sequence was together with the FLAG epitope and the tail-2 sequence was together with the HA epitope (not shown). When co-transfected into S2 cells, the tail-1 and tail-2 constructs showed largely distinct subcellular localizations (Fig. 3C). Although the distribution of both the tail-1 and tail-2 constructs appeared punctate, only the tail-2 construct appeared to substantially overlap the distribution of a Golgi marker (Fig. 3C). Similar results were obtained when the reverse-labeled constructs described above were tested (data not shown) indicating that the observed results are due to the presence of the specific tail sequence and not the epitope tag.

Previously, the cytosolic tail of mammalian CPD was found to direct the re-uptake of this protein from the cell surface to the TGN. To examine whether the tail-1 and tail-2 forms of *Drosophila* CPD are also re-internalized with comparable efficiencies, S2 cells separately transfected with the two different reporter

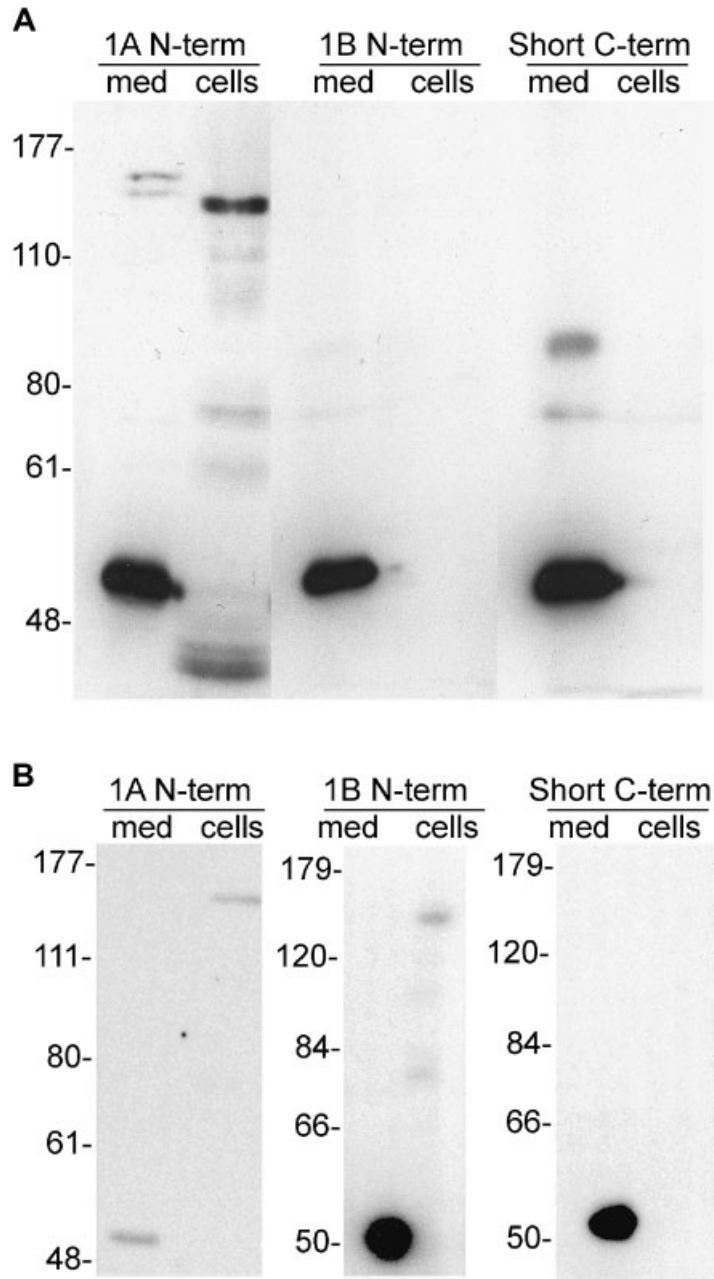

Fig. 2. Western blot analysis of CPD in S2 medium and cells. Antisera were raised against the N-terminal region of *Drosophila* CPD 1A or 1B domains, or the C-terminal region of the short form of *Drosophila* CPD, as described in Materials and Methods. Positions and molecular masses (kDa) of prestained standards are indicated. **A**: S2 cells were separated from medium by centrifugation and the membrane fraction was extracted with 0.1 M NaAc, pH 5.5, containing 1 M NaCl and 1% Triton X-100. **B**: The extracts from cell membranes and medium were purified on a p-aminobenzoyl-Arg Sepharose 6B substrate affinity column.

constructs were incubated with antiserum to albumin and then incubated at 37°C for 15 or 60 min. After 15 min, the tail-1 construct was located either on, or in close proximity to the cell surface whereas the tail-2 construct was largely present in an intracellular compartment (Fig. 4, top). The tail-1 construct was able to move to an intracellular vesicular compartment after 60 min (Fig. 4, bottom) although based on the steady state distribution (Fig. 3) it is unlikely that this is the same compartment as that containing the tail-2 construct.

Because there is extremely low amino acid sequence identity between *Drosophila* CPD

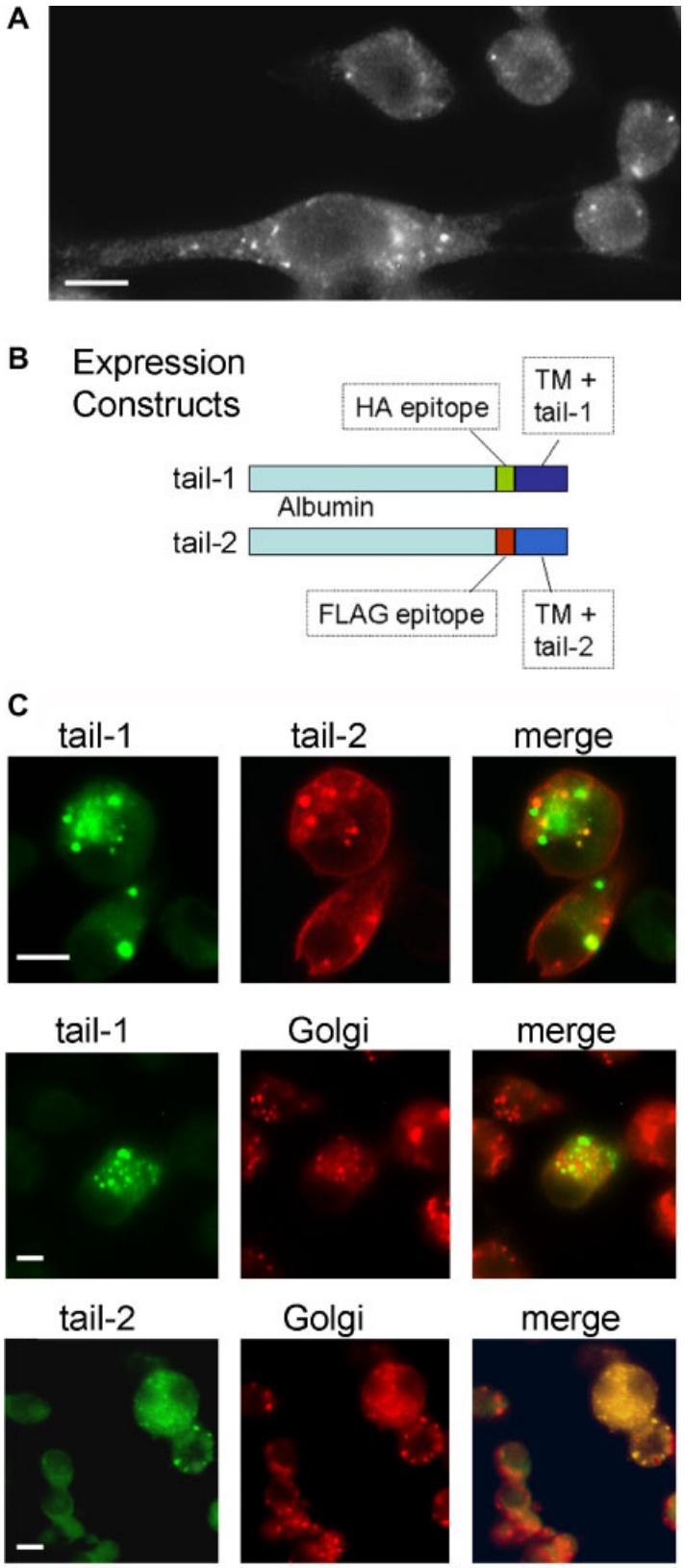
A
B
Expression
Constructs
HA epitope
TM +
tail-1
tail-1
Albumin
tail-2
FLAG epitope
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Fig. 3.

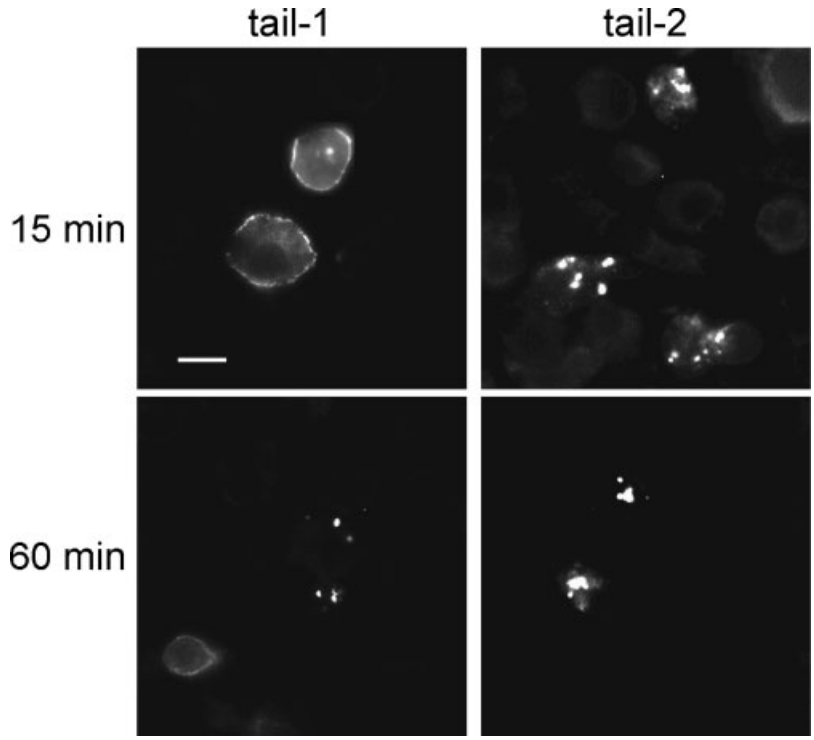

Fig. 4. Uptake of reporter constructs containing the tail-1 and tail-2 sequences of *Drosophila* CPD in S2 cells. Transiently transfected S2 cells were preincubated on ice with antibodies to albumin for 40 min, washed, incubated for the indicated time at 37°C, and then subjected to immunofluorescence analysis using Cy 2-conjugated donkey anti-rabbit IgG. Bar, 5 μm.

tail-1 or tail-2 sequences and the tail sequence of mammalian CPD, it was of interest to test the function of *Drosophila* CPD tail-1 and tail-2 in a mammalian cell line. For this analysis, we chose the AtT-20 cell line, which was previously used to examine the distribution and routing of endogenous mouse CPD as well as fusion proteins containing the duck CPD tail attached to human albumin [Varlamov and Fricker, 1998; Eng et al., 1999; Varlamov et al., 1999a]. The two reporter constructs containing the tail sequences of *Drosophila* CPD were transfected into AtT-20 cells and stable clones expressing comparable levels of each albumin construct were selected for further analysis. The steady state distribution of albumin with the *Drosophila* tail-1 sequence showed a diffuse distribution throughout the cytoplasm, although staining was concentrated in a perinuclear compartment that showed some overlap with syntaxin 6, a TGN marker (Fig. 5A). The *Drosophila* tail-2 sequence showed considerable overlap with the TGN marker and less staining throughout the cytoplasm (Fig. 5A). To distinguish between localization in Golgi versus post-Golgi compartments (such as TGN), the S2 cells were treated with brefeldin A; this compound has previously been shown to cause the redistribution of Golgi proteins but not TGN proteins such as vertebrate CPD [Varlamov and Fricker,

Fig. 3. Analysis of CPD in S2 cells. **A**: Immunofluorescence of endogenous CPD in S2 cells. The cells were fixed, permeabilized, and stained with a mouse polyclonal antiserum raised to the N-terminal region of the 1A form, followed by Cy 2-conjugated donkey anti-rabbit IgG. Bar, 5 μm. **B**: Schematic diagram of reporter constructs containing human proalbumin (including the signal peptide) followed by one of two different epitopes (FLAG or HA), the transmembrane domain (TM) of the long form of *Drosophila* CPD (common to both tail-1 and tail-2 forms), and then either the tail-1 or tail-2 sequence. **C**: S2 cells transiently co-expressing both the tail-1 and tail-2 constructs were stained with a mouse monoclonal antibody to the FLAG epitope and a rabbit antiserum to the HA epitope and then visualized using a Cy 2-conjugated donkey anti-rabbit IgG and Cy 3-conjugated donkey anti-mouse IgG. For co-localization with the Golgi compartment, S2 cells transiently expressing either the tail-1 or the tail-2 constructs alone were labeled with a mouse monoclonal antibody raised against *Drosophila* Golgi proteins and a rabbit anti-albumin antiserum. Bar, 10 μm.

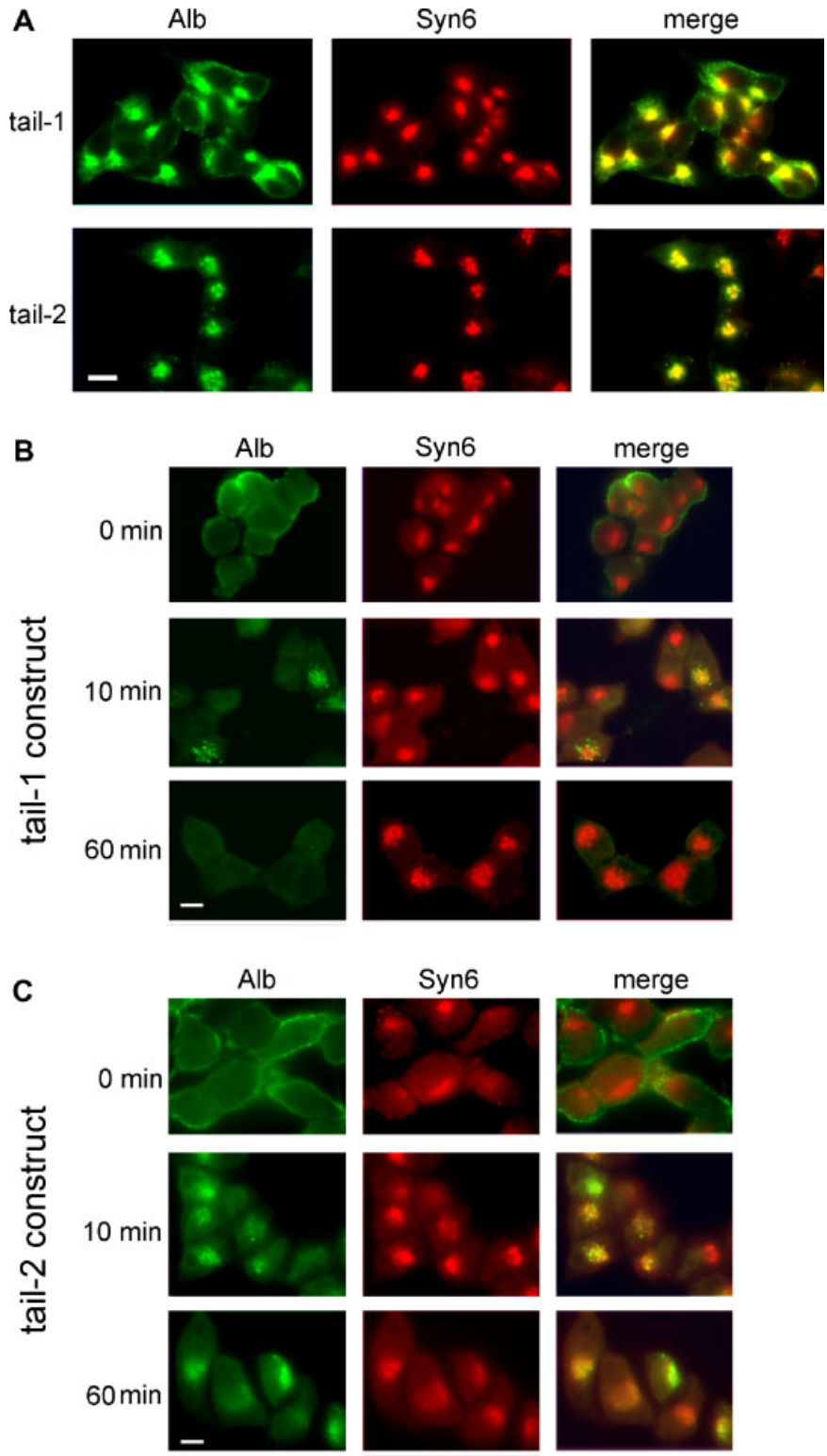

Fig. 5. Immunofluorescence analysis of the reporter constructs expressed in mouse AtT-20 cells. **A**: Steady state distribution. AtT-20 cells stably expressing either the tail-1 or the tail-2 construct were co-stained with a rabbit antiserum to albumin (Alb) and a mouse monoclonal antibody to syntaxin 6 (syn 6) and visualized using Cy 2-conjugated donkey anti-rabbit IgG and Cy 3-conjugated donkey anti-mouse IgG. Bar, 10 μm. **B** and **C**: Uptake of the reporter constructs into AtT-20 cells. AtT-20 cells stably expressing either the tail-1 (top panels, B) or the tail-2 construct (bottom panels, C) were preincubated on ice with antibodies to albumin for 40 min, washed, incubated for the indicated times at 37°C, and then subjected to dual immunofluorescence analysis using a mouse monoclonal antibody to syntaxin 6 (Syn6) followed by Cy 2-conjugated donkey anti-rabbit IgG and Cy 3-conjugated donkey anti-mouse IgG. Bar, 10 μm.

1998; Eng et al., 1999]. When treated with brefeldin A for short time periods, the staining of both tail-1 and tail-2 constructs remained in a perinuclear distribution, whereas a Golgi maker (Cab 45) changed from a perinuclear to a diffuse pattern of expression upon brefeldin A exposure (data not shown). This result suggests that at least some of the perinuclear staining observed with the tail-1 and tail-2 constructs represents post-Golgi compartments that are resistant to treatment with brefeldin A.

To examine if the *Drosophila* CPD tail sequences function in the retrieval of the protein to the TGN, as previously found for rat and duck CPD tails [Varlamov and Fricker, 1998; Eng et al., 1999], AtT-20 cells expressing each of the two constructs were incubated with antiserum to albumin at 4°C and then warmed to 37°C for various lengths of time. Immediately after the 4°C incubation, all of the albumin staining is detected on the cell surface, as expected (Fig. 5B). After 10 min at 37°C, the albumin staining in both the tail-1 and tail-2 constructs shows a punctate perinuclear staining that partially overlaps with the TGN marker (Fig. 5B). After 60 min at 37°C, the internalized tail-1 construct is largely gone from the cells, while the tail-2 construct still shows a strong perinuclear distribution that partially overlaps with syntaxin 6 (Fig. 5B).

The half-lives of the two forms of *Drosophila* CPD in AtT-20 cells were determined using pulse-chase analysis with ^{35}S-[Met/Cys]. For this analysis, several lines expressing comparable levels of each form were compared. The tail-1 construct had a half-life of about 3 h, while the tail-2 construct's half-life was about 4 h (Fig. 6A). However, there was a much larger difference in the rate of disappearance of the constructs between 2 and 8 h; the tail-1 construct decreased from 80% to 10% over this time, whereas the tail-2 construct decreased from 75% to only 40% during this same time period (Fig. 6A). The rate of disappearance of the two constructs was also evaluated by immunofluorescence after treatment of the cells with CHX to inhibit protein synthesis. Both tail-1 and tail-2 constructs showed comparable staining prior to CHX treatment, but after 4 h with this inhibitor of protein synthesis, only the tail-2 construct was detected with the antisera (Fig. 6B).

DISCUSSION

One finding of the present study is that S2 cells produce the major splice variants of CPD seen in adult *Drosophila*. The different forms of *Drosophila* CPD are routed to different parts of the cell; the short forms are secreted and the

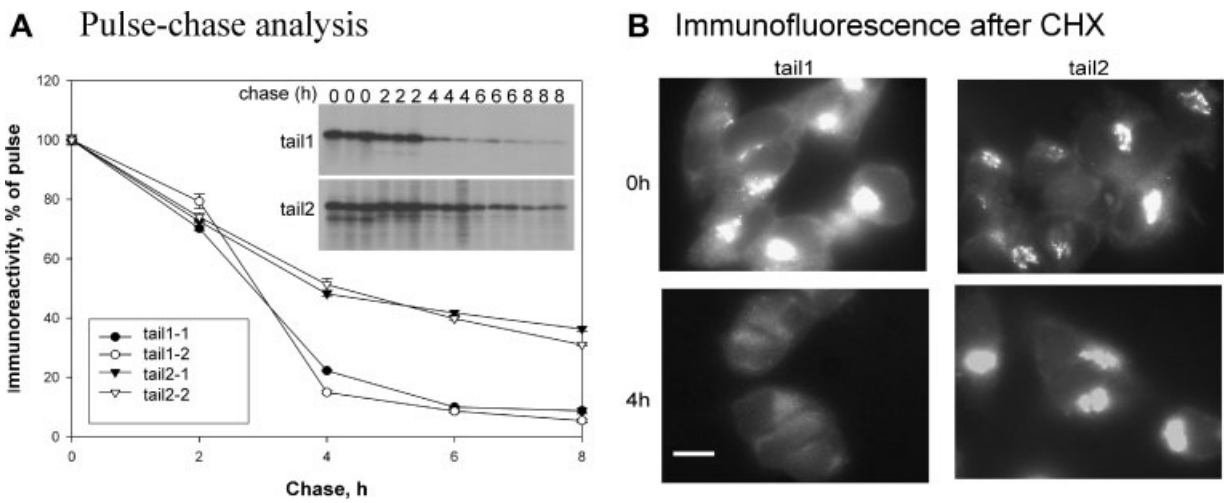

Fig. 6. Stability of the reporter constructs in AtT-20 cells. **A**: AtT-20 cells stably expressing the albumin fusion protein containing either tail-1 or tail-2 were labeled for 15 min with [^{35}S]-methionine/cysteine, and then chased for the indicated periods of time. Proteins were isolated by immunoprecipitation with an antiserum to human albumin. Error bars indicate standard error of the mean of triplicate determinations (from three distinct clonal lines of cells) and results are shown for two separate experiments. Data points without error bars had a standard error of the mean smaller than the symbol size. **B**: AtT-20 cells stably expressing similar levels of either the tail-1 or the tail-2 reporter construct were treated with cycloheximide (CHX) for the indicated time at 37°C, fixed, permeabilized, and stained with antiserum to human albumin followed by Cy 2-conjugated donkey anti-rabbit IgG. Bar, 10 μm.

long forms are retained in the cell. There are some differences between the intracellular distribution and routing of the two long forms that differ only in the C-terminal portion of their cytosolic tail sequences. Together, the various forms have a broader range of distribution throughout the cell than any single form, and this is likely to be important for the function of each form.

The various forms of *Drosophila* CPD have different enzymatic properties, based on the previous finding that the short form with exon 1A has little or no CP activity, the short form with exon 1B has CP activity and is maximally active at neutral pH, and the second CP domain is maximally active at pH 5–6 [Novikova et al., 1999; Sidyelyeva and Fricker, 2002]. Therefore, the short form with exon 1A of *Drosophila* CPD will be most like mammalian CPX1, CPX2, and ACLP, all of which are secreted and do not have detectable CP activity [Xin et al., 1998; Lei et al., 1999; Layne et al., 2001]. The short form of *Drosophila* CPD with exon 1B will be most like mammalian CPN and CPZ, both of which are secreted and have neutral pH optima [Skidgel, 1988; Novikova et al., 2000]. Of the two long forms of *Drosophila* CPD, the one with the tail-2 sequence more closely resembles mammalian CPD in terms of subcellular localization and routing, both in the *Drosophila* S2 cell line and in the mouse AtT-20 cell line [Varlamov and Fricker, 1998]. The analysis of CPD in the S2 cell lines was limited by the lack of antisera available for TGN-specific proteins, and only a Golgi marker was available. However, these two structures have the same general intracellular distribution and cannot be resolved at the light microscopic level. Thus, the co-localization of the tail-2 form of *Drosophila* CPD and the Golgi marker in S2 cells is consistent with a localization of CPD to the TGN. In AtT-20 cells, the use of brefeldin A to disrupt the Golgi is an effective method to resolve Golgi from post-Golgi compartments such as the TGN [Varlamov and Fricker, 1998]. Our finding that both tail-1 and tail-2 forms of *Drosophila* CPD show brefeldin A-resistant vesicular distributions in the AtT-20 cell line is consistent with localization to the TGN or other post-Golgi compartments. Despite the similar localization of tail-1 and tail-2 *Drosophila* CPD constructs in the mammalian AtT-20 cell line, there were differences in the routing of these two proteins that were generally consistent with those observed in the S2 line. In both AtT20 and S2 cells, protein containing the tail-2 sequence was internalized from the cell surface and transported back to a TGN-like compartment whereas the protein containing the tail-1 sequence was internalized and then largely degraded. This difference indicates that the amino acid sequences that differ between tail-1 and tail-2 contribute to the routing of the protein.

Several sequences in the cytosolic tail of mammalian and duck CPD have been previously noted to correspond to elements that facilitate the intracellular trafficking of other proteins. These elements include two acidic clusters, a tyrosine-like motif that has a Phe in place of a Tyr (FxxL in CPD, YxxL in most other proteins), a di-Leu sequence, and a casein kinase II (CKII) site within an acidic cluster [Eng et al., 1999; Varlamov et al., 2001; Kalinina et al., 2002]. Previous studies have demonstrated that all of these elements contribute to the trafficking of CPD in mammalian cells [Eng et al., 1999; Varlamov et al., 2001; Kalinina et al., 2002]. The entire cytosolic tail is highly conserved among vertebrates; there are no amino acid differences within the 58-residue C-terminal tail among human, rat, or mouse CPD, and only one conservative change (Asp for Glu) between duck and mammalian CPD [Kuroki et al., 1995; Tan et al., 1997; Xin et al., 1997; Ishikawa et al., 1998]. Although there is no recognizable amino acid sequence identity between the cytosolic tail sequences of *Drosophila* and mammalian CPD, the critical elements of the mammalian CPD tail are all present within the *Drosophila* tail-2 sequence. Specifically, the tail-2 sequence contains an FxxL sequence, multiple acidic clusters, a Leu-Leu and an Ile-Leu sequence (which may function as a di-Leu motif) [Bonifacino and Traub, 2003], and a potential CKII site within one of the acidic clusters (Fig. 1). In contrast, the tail-1 sequence is identical to the tail-2 sequence only for the first 45 residues and then the two sequences diverge due to alternative splicing (Fig. 1). The shared region contains the FxxL motif, the Leu-Leu sequence, and a single acidic cluster. Although the tail-1 sequence has an Ile-Ile sequence instead of the Ile-Leu sequence in tail-2, there are not multiple acidic clusters or CKII sites in the tail-1 sequence. Previous studies on mammalian and duck CPD found that deletions of the second acidic cluster and CKII site reduced the rate of uptake from the

cell surface [Eng et al., 1999; Kalinina et al., 2002]. The difference in the routing of the tail-1 and tail-2 forms of *Drosophila* CPD found in the present study is consistent with those previous studies. Furthermore, the finding that the *Drosophila* tail sequences function similarly in S2 and AtT-20 cells implies that the machinery for the routing of proteins in the secretory pathway has been highly conserved through evolution.

The different forms of protein produced from the *Drosophila* CPD gene that were found in the present study have implications for understanding the various naturally occurring mutations of this *Drosophila* gene. A number of mutations of the CPD gene have been described, and are collectively known as the *silver* or *svr* mutations. Some *svr* mutants are embryonic lethal [Settle et al., 1995]. Based on the site of insertion of the transposable element used to create the mutation, these mutants presumably have a defect in the expression of all forms of CPD [Settle et al., 1995]. Viable mutants include svr^{1}, svr^{poi}, and svr^{73L12} [Settle et al., 1995]. All of these viable mutants appear to have inserts in the region of the CPD gene that correspond to the second or third CP-like domain, or to introns within this region. Thus, these viable mutants are likely to produce the short forms of CPD containing the 1A and the 1B exons found in the present study to be secreted from S2 cells, but not the full length forms. This implies that the full length CPD protein is involved in the control of wing shape and body color, both of which are affected in the viable *svr* mutants [Settle et al., 1995]. This proposal is supported by the previous finding by Settle and colleagues that the embryonic lethality of the severe *svr* mutations could be corrected by P-element-mediated germline transformation of a 13.4 kb BamHI fragment that included most of the *Drosophila* CPD gene [Settle et al., 1995]. However, this 13.4 kb BamHI fragment did not completely rescue the visible pigmentation phenotype of the *svr* mutants [Settle et al., 1995]. Interestingly, 13.4 kb BamHI fragment cannot produce the full length tail-2 form of CPD due to a BamHI site located within the coding region of the unique portion of the tail-2 sequence [Sidyelyeva and Fricker, 2002]. The tail-1 form is fully contained within the 13.4 kb BamHI fragment. Thus, the failure of this DNA fragment to fully correct the pigmentation defect implies that the tail-2 form is crucial for the proper function of CPD in *Drosophila* and that the tail-1 form cannot fully compensate for the missing tail-2 CPD in the viable *svr* mutants. This is consistent with the differences in the distribution of the tail-1 and tail-2 forms found in the present study. Further studies investigating the expression of CPD in wild type and *svr* mutant flies, and in *svr* mutant flies transformed with CPD gene fragments, are needed to further investigate the physiological role of the various forms of CPD in *Drosophila*.

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