The opt1 gene of Drosophila melanogaster encodes a proton-dependent dipeptide transporter

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Roman, Gregg, Victoria Meller, Kwok Hang WU, and Ronald L. Davis. The opt1 gene of Drosophila melanogaster encodes a proton-dependent dipeptide transporter. Am. J. Physiol. 275 (Cell Physiol. 44): C857–C869, 1998.—We have cloned and characterized the opt1 gene of Drosophila melanogaster. This gene encodes a protein with significant similarity to the PTR family of oligopeptide transporters. The OPT1 protein is localized to the apical epithelial membrane domains of the midgut, rectum, and female reproductive tract. The opt1 message is maternally loaded into developing oocytes, and OPT1 is found in the α-yolk spheres of the developing embryo. It is also found throughout the neuropil of the central nervous system, with elevated expression within the α- and β-lobes of the mushroom bodies. Transport activity was examined in HeLa cells transiently expressing OPT1. This protein is a high-affinity transporter of alanylalanine; the approximate Kₘ constant is 48.8 μM for this substrate. OPT1 dipeptide transport activity is proton dependent. The ability of selected β-lactams to inhibit alanylalanine transport suggests that OPT1 has a broad specificity in amino acid side chains and has a substrate requirement for an α-amino group. Together these data suggest an important role for OPT1 in regulating amino acid availability.

PT transporters; oligopeptide transport; protein metabolism; yolk; nutrient uptake

The cellular uptake of small peptides is fundamental to nutrition and the economy of amino acids in many organisms. This process occurs primarily through saturable carrier proteins. Many bacteria and yeast actively take up short peptides directly from the environment and are capable of using these peptides as their sole source of nitrogen (61, 63). Carrier proteins within the roots of Arabidopsis thaliana can also transport di- and tripeptides from the growth media, thereby providing an additional source of fixed nitrogen for this plant (79). In mammals, as much as 60% of digested proteins is absorbed into the intestinal epithelium as di- or tripeptides (14, 57). Furthermore, the loss of small peptides from the mammalian glomerular filtrate is minimized by a saturable oligopeptide transport system found in the renal proximal tubule (28).

Small peptide transport may have additional importance in the early development of many organisms. Many plants and animals stockpile proteins in their oocytes or associated maternal tissue as a supply of amino acids for developing embryos. In Arabidopsis, di- and tripeptides are transported from the protein stores of the endosperm into the cotyledons of the developing embryo (77). In most invertebrates and many vertebrates, yolk proteins are stored in membrane-limited compartments within the oocyte. These protein stores are digested, primarily by a cathepsin B-like peptidyl dipeptidase, and used as a source of amino acids by the developing embryo. The mechanism by which these amino acids are released from yolk vesicles remains largely unexamined.

The transport and metabolism of small peptides may have a particularly profound role in the nutrition and physiology of insects. In Drosophila melanogaster, di- and tripeptides are found in body fluids at unusually high concentrations, constituting up to 30% of the total amino acids in adults (15, 18, 54). It has been proposed that these peptides may function in osmoregulation, as has been shown in some marine invertebrates (15, 18). Most dietary protein digestion in Drosophila occurs within the midgut; the end products of this proteolytic digestion are thought to be quickly absorbed into the epithelia (40, 45, 75). The formation of primary urine in insects occurs within the Malpighian tubules (48, 64). The secretions of this organ are deposited into the hindgut, where amino acids are reabsorbed into the rectal epithelia (64). Despite the abundance of peptides within Drosophila, virtually nothing is known of their fate within the digestive or excretory systems.

Recently, several genes encoding a family of peptide transporters have been cloned from mammals, yeast, plants, and bacteria (PTR family; Refs. 59, 78). The characterized PTR proteins transport di- and tripeptides with little specificity for amino acid composition (10, 25, 46, 47, 63, 69). Activity has been shown to be coupled to proton symport for several of the family members (9, 25, 34, 47). The identified mammalian PTR proteins are subdivided into two types: pepT1 and pepT2. This classification derives from the sequence similarity, biochemical activity, and expression pattern similarities of these proteins (9, 25, 47). The pepT1 proteins from human, rabbit, and rat are high-capacity, low-affinity transporters. These proteins are expressed abundantly in the small intestine and at lower levels in the kidney (25, 46, 69). The human, rabbit, and rat pepT2 proteins are low-capacity, high-affinity transporters. The pepT2 proteins are expressed predominantly in the kidney proximal tubules without detectable expression in the intestines (9, 47, 53).

Here we report the characterization of opt1, a Drosophila member of the PTR family of transporters. This gene was originally identified as a transcript expressed preferentially in females, located adjacent to the roX1 untranslated nuclear RNA (3, 52). We show that the opt1 locus encodes a high-affinity di- and tripeptide transporter. Furthermore, our experiments suggest that the OPT1 transport activity is proton dependent.
Opt1 mRNA is expressed in germlinal and somatic tissue of both genders but is most highly expressed in the nurse cells of the female ovary. OPT1 protein is found on distinct membrane domains in neurons and epithelial cells of the midgut, rectum, and female reproductive tract. The biochemical activity and the sites of OPT1 expression suggest an integral role for this protein in governing amino acid availability in Drosophila.

METHODS AND MATERIALS

Strains. Fly stocks were raised on cornmeal agar food at 22–25°C. Wild-type control strains Canton-S or ry606 were used. The isolation of the MB710 line has previously been described (36). The relevant genotypes of the three sex determination lines are as follows: 1) w SxM1;J sn(C1)DX y/Y; 2) y cm SxM1;J c8 v(C1)DX y/BY; and 3) X;B/Y;th st;ra1 cpr p/pr/TM3.

Molecular biology. The isolation of the opt1/rox1 genomic clones has previously been described (52). The opt1 transcribed region was identified by hybridizing isolated genomic clones to Northern blots containing 5 µg poly(A) purified RNA from whole flies. Selected genomic fragments were then used to isolate cDNA clones from head-specific libraries kindly supplied by P. Salvaterra (City of Hope, Duarte, CA), T. Schwarz (Stanford, CA), and C. Hall (Baylor College of Medicine, Houston, TX). Two partial cDNAs and one full-length cDNA were sequenced entirely. For the preparation of sex-specific RNAs, ~28,000 ry606 flies between 0 and 4 days old were harvested and sexed by hand. Heads and bodies were separated, and RNA was isolated using Trizl reagent [Bethesda Research Laboratories (BRL), Rockville, MD). Both formaldehyde and glyoxal-DMSO methods were used in the Northern analysis (70).

For the developmental RT-PCR analysis, ~200 mg of mRNA from each stage were isolated by hand. The animals from each stage were homogenized in a 1.5-ml Eppendorf tube, and RNA was extracted with Trizol reagent according to the manufacturer’s recommendations (Pierce, Rockford, IL). The methods for the preparation of sex-specific RNAs, ~28,000 ry606 flies between 0 and 4 days old were harvested and sexed by hand. Heads and bodies were separated, and RNA was isolated using Trizl reagent [Bethesda Research Laboratories (BRL), Rockville, MD). Both formaldehyde and glyoxal-DMSO methods were used in the Northern analysis (70).

For the developmental RT-PCR analysis, ~200 mg of animals from each stage were isolated by hand. The animals were homogenized in a 1.5-ml Eppendorf tube, and RNA was extracted with Trizl reagent according to the manufacturer’s protocols. Total RNA (2 µg) from each stage was directly reverse transcribed with Superscript II RT (BRL). Control reactions were digested with 10 µg DNase-free RNase for 1 h at 37°C before reverse transcription. PCR reactions were performed with 1% of total reverse transcription reaction. PCR amplicons for opt1 long and short shared the same antisense primer and utilized exon-specific sense primers (sequence available on request). PCR conditions were 94°C for 20 s, 64°C for 20 s, and 72°C for 40 s, with 30 cycles for opt1 long and 40 cycles opt1 short.

Computer analysis. DNA and protein sequence analyses were performed with the GCG suite of programs (23) and DNA Strider (version 1.2). Additional opt1 splice variants were sought in the genomic DNA sequence with the GeneFinder program (76). The MAR Finder algorithm was used to identify regions with high probability of forming matrix attachment sites; for this analysis, all six rules were utilized (71). The TOPPREDICT and MEMSAT programs were used for membrane topology prediction (38, 72). The FASTA (62) and BLAST (1) algorithms were utilized to identify homologous sequences in the GenBank and EMBL databases. Protein alignments were performed with a PAM250 matrix specific for integral membrane proteins (39). The phylogenetic tree was generated by the TreeGen web server (http://cbrg.inf.ethz.ch/subsection3_1_6.html; Ref. 32). The accession numbers for the proteins used in sequence comparisons are as follows: rabbit pepT2 (U32507; Ref. 9), human pepT2 (S78203; Ref. 47), rat pepT2 (D63149; Ref. 69), rabbit pepT1 (U06467; Ref. 25), human pepT1 (U21936; Ref. 47), rat pepT1 (D50664; Ref. 53), Caenorhabditis elegans ORF2 (g1246435; Ref. 87), C. elegans ORF1 (1049410; Ref. 87), cucumberchloroplast (Z69370), Arabidopsis Chl1 (L10357; Ref. 85), AtPtr2B (L39082; Ref. 77), Candida Ptr2 (U09781; Ref. 8); AtPtr2A (U01171; Ref. 79), Saccharomyces Ptr2 (L11954; Ref. 63), Escherichia coli YH1 (178991; Ref. 53), and Lactococcus lactis DtpT1 (U05215; Ref. 34), and E. coli Y D (1786927).

Antibodies. The Histag outer loop fusion protein was generated by inserting the 0.65-kb Bgl II-PstI fragment from the opt1 cDNA into the pRETSA vector (Invitrogen, Carlsbad, CA). The resulting Histag-Opt1 fusion protein was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in BL21(DE3)pLysS and purified over a nickel-agarose column (Qiagen, Santa Clara, CA). The anti outer loop (α-OL) antibody was raised against this purified fusion protein. The glutathione S-transferase fusion to the Opt1L (677–A743) COOH-terminal peptide was generated by inserting the 0.3-kb Nhel fragment from the opt1 cDNA into the Xba I site of the pGEX-KG vector. Production of the fusion protein was induced with 0.4 mM IPTG in XL-1 Blue and purified over glutathione-agarose according to the manufacturer’s recommendations (Pharmacia, Uppsala, Sweden). The anti-COOH-terminal (α-CO) antibody was raised against this fusion protein. Two New Zealand White female rabbits were injected for each fusion protein. Fusion proteins were coupled to Reacti-Gel 6X CDI-agarose according to manufacturer’s recommendations (Pierce, Rockford, IL). The methods of Smith and Fisher (74) were used to purify the antibodies from the fusion protein-agarose columns.

Standard procedures were used for Western blotting experiments (70). Drosophila proteins were isolated by using Trizol. Proteins from transfected HeLa cells were extracted in 1% SDS. The affinity-purified 2141 α-Cterm antibody was used at 1:100 dilution. We used a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody from Vector Laboratories (Burlingame, CA) at a 1:10,000 dilution.

Transport assays. HeLa cells were seeded at 2 × 105 cells per 35-mm well and incubated for 24 h before transfections. For each transfection, either 1 µg of pCMV/OP1 or 1 µg of pCMV2 was mixed with 6 µl of lipofectamine (BRL) according to the manufacturer’s recommendations. Transfection proceeded for 6 h, after which cells were cultured in DMEM (BRL) for an additional 18 h. Transport assays were performed directly in the 35-mm wells. All data points represent three independent transfections. For these transport assays, transfected cells were rinsed twice with transport buffer [25 mM MES-Tris (pH 6.0), 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, and 5 mM glucose] and then incubated with assay buffer at 22°C; this is also the incubation temperature at which we raise Drosophila. Assay buffer consisted of 1 ml of L-[3H]Jalan (Moravek Biochemicals, Brea, CA) diluted at the specified concentration in transport buffer. Specific activity of L-[3H]Jalan was 1 Ci/mM, with the exception of the 400 µM alanine transport assays, which were at 0.5 Ci/mM. Transport was stopped by the addition of 5 ml of ice-cold 1× PBS (pH 7.5), followed immediately by a second rinse in the same buffer. Cells were lysed in 1 ml of 1% SDS, and L-[3H]alanine was measured by scintillation counting. In the time course experiment, assay buffer contained 400 µM alanine. In most of the inhibition experiments presented in Table 1, 10 µM alanine was incubated in the presence of 10 mM peptide or peptidomimetics competitors (Sigma, St. Louis, MO). The proline-phosphoryl carboxyl cyanide p-trifluoromethoxyphenyl hydrazide (FCCP) (Sigma) was applied at 25 µM...
Table 1. Inhibition of OPT1-dependent transport

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Velocity</th>
<th>% of Control</th>
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<tr>
<td></td>
<td>pmol/2 min for 10⁶ cells</td>
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<tr>
<td>Experiment 1</td>
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<tr>
<td>None</td>
<td>35.9±3.5</td>
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<tr>
<td>Ala</td>
<td>26.6±2.7</td>
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<td>pH 7</td>
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<tr>
<td>Carbeneicillin</td>
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<tr>
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<tr>
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</tr>
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</tr>
<tr>
<td>Ala</td>
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<tr>
<td>Glutathione</td>
<td>1.8±0.9</td>
<td>51.2±5.5</td>
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Values are means ± SE. FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone.

RESULTS

Identification and primary structure of opt1. The MB710 enhancer detector line was identified in a screen for genes preferentially expressed in the mushroom bodies of the female Drosophila brain (36). The P element in MB710 was inserted at cytological position 3F (36, 52). Two genes were identified at this locus by Northern analysis: roX1 and opt1 (Fig. 1A) (52). No additional RNAs within 15 kb on either side of the P element were detected by hybridization to poly(A)⁺ RNA isolated from whole flies (data not shown). The MB710 element interrupts and greatly reduces roX1 expression (52). RoX1 expression in wild-type adult flies is principally limited to the male central nervous system (3, 52). Therefore, the LacZ activity in MB710 does not reflect this gene's expression pattern. The opt1 gene was examined further as a potential gene expressed in mushroom bodies.

Eight independent opt1 cDNAs were isolated from several Drosophila head-specific libraries. We estimate the abundance of opt1 within the head to be ~1 in 23,000 transcripts as determined by the hybridization to an unamplified cDNA library. Selected cDNA and genomic clones were completely sequenced and compared. The longest cDNA was 2.85 kb; this cDNA starts 18 bp 3' to a consensus transcription start site and ends in a poly(A) tail. Both opt1 and roX1 are transcribed in simultaneously with 10 µM alanylalanine. Uptake was measured at 2 min in both dose-response and inhibition experiments.

Histology. Cryosections (10 µm) for LacZ staining, in situ hybridizations, and immunohistochemistry were as described by Han et al. (35). Paraffin sections (5 µm) of female and male abdomens were processed as described by Skoulakis and Davis (73). The collection, dechorionation, and fixation of embryos were as described (82). RNA in situ hybridization to embryos was as described (82). The collection, dechorionation, and fixation of embryos were as described (82). RNA in situ hybridization to sectioned material was essentially as described by Skoulakis and Davis (73). Riboprobes were generated by in vitro transcription of portions of the opt1 coding region from the c5 cDNA using digoxigenin-UTP (Boehringer Mannheim, Indianapolis, IN). In situ hybridizations to embryos were as previously described (52). OPT1 digoxigenin-labeled DNA probes for these experiments were generated by random prime labeling of cDNA fragments.

Immunohistochemistry procedures were as previously described (73). The 1476 α-OL affinity-purified antibody was used at a 1:800 dilution for immunohistochemistry of frontal head cryosections. Immunohistochemistry on the paraffin abdominal sections was performed with either a 1:100 dilution of 2141 α-Cterm affinity-purified antibody or a 1:10 dilution of 1476 α-OL affinity-purified antibody. A 1:2,000 dilution of 2141 α-Cterm affinity-purified antibody was used in immunohistochemistry experiments. The Vectastain ABC kit (Vector) was used for signal detection in all immunohistochemistry experiments. Yolk spheres from stage 10 and 14 oocytes were measured at ×100 magnification under oil immersion with an ocular reticle calibrated with a stage micrometer. Ten neighboring spheres from two oocytes were measured for each stage.
the same direction, with just 529 bp separating the two distances, and small circle represents weighted centroid of tree.

Fig. 2. OPT1 is a member of proton-dependent oligopeptide transporter family. Phylogenetic tree representing family of proton-coupled oligopeptide transporters was generated by a least-squares dynamic programming algorithm (32). Numbers are calculated PAM distances, and small circle represents weighted centroid of tree.

Fig. 3. Amino acid sequence comparison of animal proton-dependent oligopeptide transporter subfamily. Caenorhabditis elegans ORF1, C. elegans ORF2, human pepT2, rabbit pepT2, rat pepT2, human pepT1, rat pepT1, rabbit pepT1, and OPT1 proteins are optimally aligned. Conserved 28 amino acid NH2-terminus of C. elegans ORF1 is poorly conserved in the nonanimal family members (26). The size, but not the sequence, of the large fifth outer loop of OPT1 is well conserved in this branch of the family tree, but the length of this domain is poorly conserved in the nonanimal family members (data not shown). The strong sequence identity and similar topology among these animal transporters present cogent evidence that they arose from a common ancestor (49).

OPT1 has proton-dependent oligopeptide transport activity. Because OPT1 shares significant sequence similarity to nitrate as well as oligopeptide transporters, we investigated the transport properties of this protein in transfected HeLa cells. The kinetic parameters were examined by measuring [3H]alanine influx as a function of time and substrate concentration.
Fig. 4. OPT1 facilitates cellular uptake of alanylalanine. A: alanylalanine uptake was measured in pCMV.OPT1-transfected HeLa cells or control cells transfected with empty vector as a function of time. Transfected HeLa cells were incubated in presence of 50 µM [3H]alanylalanine (Ci/mm) for indicated times. Assay buffer was pH 6. SE bars are shown. B: [3H]alanylalanine uptake was examined at substrate concentrations ranging from 5 to 400 µM. Assays were conducted at pH 6 for 2 min. Inset: Eadie-Hofstee plot. From this plot, K_m and V_max were determined to be 48.8 µM and 384 pmol/2 min for 10^6 cells, respectively. SE bars are shown.

(Fig. 4). OPT1-transfected HeLa cells demonstrated significant alanylalanine uptake; this activity was found to be linear at 2 min for substrate concentrations ranging from 5 to 400 µM (Fig. 4A, data not shown). Additionally, after 2 min, we failed to detect any degradation of [3H]alanylalanine within the HeLa cell extracts by TLC (data not shown). These data indicate that the substrate was not significantly processed within the first 2 min of the assay and that we could measure the rate of alanylalanine uptake.

OPT1-dependent alanylalanine transport activity displayed Michaelis-Menten saturation kinetics with an apparent K_m and V_max of 48.8 µM and 384 pmol/2 min for 10^6 cells, respectively (Fig. 4B). We further investigated whether this transport process is driven by a proton motive force by either increasing the pH of the transport buffer or by removing the proton gradient. Alanylalanine transport is reduced ~10-fold at pH 7 and 20-fold at pH 8 (Table 1). When the protonophore FCCP (25 µM) was used to collapse the proton gradient, alanylalanine uptake was almost eliminated (Table 1). Together, these data strongly suggest a proton dependence for OPT1-driven dipeptide transport.

The potential substrate specificity of OPT1 was examined by measuring uptake of 10 µM [3H]alanylalanine in the presence of 10 mM competitor (Table 1). The capability of OPT1 to transport peptides of different lengths was examined with a series of alanine peptides. Alanine and tetraalanine did not significantly inhibit uptake (P > 0.05, Student’s t-test), whereas di- and trialanine peptides practically eliminate measurable uptake (Table 1). The D-enantiomer of alanylalanine failed to significantly inhibit uptake, indicating that OPT1 is also stereoselective in substrate recognition (Table 1). Glutathione is one of the most abundant peptides in Drosophila (58). Nevertheless, this γ-glutamyl-linked tripeptide was a poor inhibitor of alanylalanine uptake, suggesting that it is an unlikely substrate for OPT1 transport in vivo (Table 1). We also examined the ability of several peptidomimetic drugs to inhibit alanylalanine transport. For this comparison, we used the closely related β-lactams, ampicillin, carbenicillin, and benzylpenicillin. These drugs have identical backbones but differ at the α-substituent; ampicillin has an α-amino group, whereas carbenicillin has a carboxyl moiety and benzylpenicillin has a hydrogen. Of these three, only ampicillin significantly inhibited transport, suggesting that the α-amino group may be essential for substrate recognition (Table 1). Consistent with this hypothesis, the peptidomimetic angiotensin-converting enzyme inhibitor captopril, which has an α-sulfhydryl group, was a poor inhibitor, and the aminoccephalosporin cefadroxil was an effective inhibitor of transport (Table 1).

Distribution of OPT1. We examined the distribution and timing of opt1 expression by Northern analysis and RT-PCR (Fig. 5). The full-length opt1 cDNA hybridized to a single 3.0-kb message in the heads and bodies of both adult male and females, with most of the signal located in the female body (Fig. 5A). Additionally, both opt1 long and short splice variants are present throughout development (Fig. 5B). The short splice form, however, was barely detectable during larval stages.

We further identified the foci of opt1 expression with both in situ hybridization and immunohistochemistry. To ensure that we detected authentic OPT1 immunoreactivity, we used four independent affinity-purified antibodies raised against either the COOH-terminus or a polypeptide containing most of the large fifth outer loop (see METHODS AND MATERIALS). On Western blots, one of the antibodies raised against the COOH-terminus could specifically recognize OPT1 expressed in both HeLa cells and Drosophila (Fig. 6). The other three antibodies failed to reproducibly recognize OPT1 from Drosophila extracts but specifically recognized OPT1 expressed in HeLa cells (data not shown). Nevertheless, we had an excellent correlation between the patterns of expression detected by in situ hybridization and all four antibodies.

Opt1 is expressed in both somatic and germinal tissues. The opt1 message and protein are detected within the epithelia of the midgut, rectum, and the oviducts, seminal receptacles, and spermathecal ducts of the female reproductive tract (Fig. 7). OPT1 immunoreactivity is clearly limited to the apical membranes of the midgut and rectal epithelium (Fig. 7, A and B). OPT1 is detected along the entire length of the midgut, beginning at the cardia and ending at the junction with the anterior intestine. We also detected OPT1 immunoreactivity within the basal portions of all four rectal
papilla, but no signal is found in the more apical regions, suggesting the presence of specific domains within this tissue (Fig. 7C). The oviducts, seminal receptacles, and spermathecal ducts of the female reproductive tract also stain specifically with anti-OPT1 antibodies, and a low amount of OPT1 is detected within the uterus (Fig. 7C). OPT1 may be expressed at very low levels within almost all neuropil regions of the central nervous system (Fig. 7D). An increase in signal is seen in the α- and β-lobes of the mushroom bodies, consistent with a modest preferential expression in this area. There was no increase in protein levels detected in the γ-lobes, suggesting that α- and β-lobe mushroom body neurons may require or benefit from more peptide transport activity than the γ-lobes. OPT1 is also detected in the antennal nerve (data not shown). Nevertheless, no sexually dimorphic staining patterns are found in the central nervous system. In contrast to the weak expression in the brain, a strong signal is seen within the fat bodies surrounding the central nervous system (Fig. 7D). It is therefore probable that opt1 expression in the fat bodies accounts for the majority of transcripts present the head RNA population (Fig. 5A).

Hybridization of antisense riboprobes to female abdomens revealed that copious levels of opt1 are synthesized within the nurse cells and deposited in the developing oocyte (Fig. 8, A and B). The earliest visible message is perinuclear to the oocyte, and, by early stage 10, the nurse cells are filled with transcript that is excluded from their nuclei (Fig. 8, A and B). In stage 11 follicles, the nurse cell-produced message is seen transported through the ring canal into the oocyte in a pattern characteristic for maternally loaded messages (Fig. 8B). Because the expression of the adjacent roX1 gene is controlled by the dosage compensation system, we examined whether ectopically expressed msl-2 could eliminate opt1 expression in the ovaries. Females carrying the msl-2 transgene have smaller ovaries than wild-type females, but the few developing follicles that they produce contain opt1 in the same pattern as seen in wild-type follicles (data not shown). This suggests that msl-2 does not downregulate opt1 in the female germ line.

In early stage 9 follicles, immunohistochemical staining for OPT1 produces an even staining throughout the developing oocyte. By late stage 9, an additional punctate staining at the nurse cell-oocyte boundary near the germinal vesicle is detected (Fig. 8C). In slightly older oocytes, this vesicular pattern spreads through the subcortical regions, and the even background staining...
Fig. 7. OPT1 expression in somatic membrane domains. A: immunohistochemical staining of a sagittal section of boundary between thorax and 1st abdominal segment. α-Cterm antibody was used to detect OPT1 in sectioned tissue. OPT1 is found within apical membranes of midgut. Magnification: ×40. B: immunohistochemical staining of a sagittal section of posterior abdomen of a wild-type female. OPT1 was detected within apical membranes of rectal epithelium with α-Cterm antibody. Magnification: ×40. C: immunohistochemical staining of a sagittal section of posterior abdomen of a wild-type female. α-Cterm antibody was used to detect OPT1. OPT1 immunoreactivity is detected within rectal papilla (rp), oviducts (od), seminal receptacle (sr), spermathecal ducts (sd), and oocytes (oo). Magnification: ×10. D: immunohistochemical staining of a frontal section of a wild-type female head. Anti-outer loop (α-OL) antibody was used to detect OPT1 within head cavity. fb, Fat bodies; bl, β-lobes of mushroom bodies. Magnification: ×20.

Fig. 8. Maternal OPT1 is localized to embryonic yolk spheres. In situ hybridizations with opt1 antisense riboprobe and immunohistochemical staining with α-Cterm antibody are shown to developing oocytes and embryos. A: in situ hybridization to a sagittal section of a wild-type female abdomen. oo, Oocyte; ncn, nurse cell nucleus. B: in situ hybridization to whole mount ovaries. C: immunohistochemical staining of a sectioned late stage 9 oocyte. Punctate staining is seen surrounding germinal vesicle (gv). D: immunohistochemical staining of a sectioned stage 14 oocyte. Punctate OPT1 staining is now spread throughout central ooplasm. E: lateral view of a 0- to 1-h embryo after in situ hybridization. F: lateral view of a 1- to 2-h embryo after in situ hybridization. Signal is gone from cortical regions. G: lateral view of a 3-h-old embryo (blastocele) after in situ hybridization. Opt1 message is no longer detectable. H: dorsal view of a stage 15 embryo after immunohistochemical staining. α-Cterm antibody was used to detect OPT1. Staining is present in central yolk mass.
begins to fade from the ooplasm. By stage 14, OPT1-containing vesicles are detected throughout the ooplasm but not in the cortex; the general ooplasm staining has disappeared by this stage (Fig. 8D). We measured the diameter of these vesicles under ×100 magnification. Vesicles from late stage 9 oocytes are 3.8 ± 0.3 µm in diameter, and stage 14 oocyte vesicles were 4.3 ± 0.4 µm. The appearance and size of these vesicles correspond well with mature α-yolk spheres (20, 29, 30). At ×100 magnification, many smaller vesicles are distinguishable within the cortex of stage 10 oocytes. These smaller vesicles range in size from ~100 nm close to the oocyte membrane to 1 µm in diameter near the central ooplasm boundary (data not shown). Stage 10 follicle cells occasionally display OPT1 immunoreactivity (data not shown). This infrequent staining may denote a very transient expression of OPT1 within these cells.

Opt1 transcripts remain abundant in wild-type embryos <2 h old, but, during the formation of the blastoderm, transcripts are eliminated from the cortical regions of the embryo, and by the completion of cellularization, opt1 can no longer be detected (Fig. 8, E–G). In the late stages of embryogenesis, the developing midgut encircles and engulfs the central yolk mass. We detected OPT1 in the central yolk mass during these late stages (Fig. 8H). Although the opt1 message is gone by 3 h after egg laying, the protein perdures for at least 16 h and remains associated with the yolk spheres.

Opt1 message is also detected in premeiotic germ cell cysts of the testes (Fig. 9). The opt1 message and protein are limited to a small number of cysts in wild-type males, suggesting that the gene product is tightly regulated and limited to a specific stage of germ cell development in this tissue. No significant differences are seen in opt1 expression patterns in Canton-S, MB710, Sx(M1,F1), or Sx(M1,F7,M1) males (Fig. 9; data not shown). In XX pseudomales produced by mutations of tra or Sxl, testicular development and spermatogenesis is initiated but never completed; the result is incomplete gonads referred to as pseudotestis. Pseudotestes from XX;tra1 pseudomales are smaller than those of wild-type males, and development of germ cells is abnormal (Fig. 9D). Opt1 probes stain cysts darkly; however, occasional abnormally large cysts located in a more basal region of the testis are also seen hybridizing with opt1 (Fig. 9D).

**DISCUSSION**

In this paper, we present the characterization of the D. melanogaster opt1 gene and a functional analysis of the OPT1 protein. We also define the loci of OPT1 expression through in situ hybridization and immunohistochemistry. The opt1 gene encodes a proton-dependent oligopeptide transporter found at cytological position 3F immediately adjacent to the roX1 nuclear RNA gene (3, 52). OPT1 is expressed in several epithelia including the apical membranes of the midgut, rectum, and female reproductive tract. OPT1 is also expressed at low levels throughout the central nervous system and in the α-yolk spheres of the oocyte and developing embryo.

Opt1 gene structure. We have shown that the opt1 gene contains two alternative 5′-exons. The most upstream exon was identified by Northern analysis and RT-PCR and found within four full-length cDNAs. The second exon was identified by the GeneFinder algorithm and subsequently verified by Northern analysis (76). During the preparation of this paper, Amrein and Axel (3) reported the sequence of an opt1 cDNA that contained this second intron. The opt1 long transcript, containing the upstream alternative exon, is the most abundant in both head and body. The promoter for this transcriptional start site is therefore the most active.

OPT1 has proton-dependent dipeptide transport activity. The opt1 gene product shares significant sequence similarities to the PTR family of carrier proteins (59, 78). The greatest similarities were to the mammalian pepT1 and pepT2 proteins. These proteins transport di-
and tripeptides across membranes energized by an electrochemical proton gradient (9, 25, 46, 47, 69). We utilized a transient expression assay to examine the biochemical activity of the OPT1 protein. A similar assay system was previously used for the characterization of human pepT1 and pepT2 (46, 47). We have shown that OPT1 has a high-affinity dipeptide transport activity. OPT1-dependent alanlyalanine uptake is also severely affected by the pH of the cis-compartment; active transport is seen at pH 6, severely reduced uptake at pH 7, and almost absent at pH 8. When the proton gradient was collapsed with FCCP, very little transport occurred. Taken together, these data support the proton dependence of dipeptide transport by OPT1. The length of the peptides transported also appears to be selective; single amino acids and tetraalanine are incapable of competing for alanlyalanine uptake in our assay. These data strongly suggest that OPT1 transports primarily di- and tripeptides in vivo.

To examine possible substrate specificities, we utilized β-lactam antibiotics. The benzylpenicillin family of antibiotics includes ampicillin, carbenicillin, and penicillin G. These peptidomimetics have almost identical structures, differing only at the ω-substituent. The ability of ampicillin and cefadroxil to inhibit alanlyalanine transport suggests that these molecules are substrates for OPT1 transport. The side chains of these molecules are very dissimilar chemically from alanlyalanine and from each other. OPT1 may therefore have little specificity for amino acid side chains. In contrast, the failure of carbenicillin and benzylpenicillin to inhibit alanlyalanine transport suggests that these molecules are substrates for OPT1 transport. OPT1’s ability to transport alanlyalanine and the weak inhibition found with captopril are consistent with a requirement for an ω-amino group in the peptide substrate of OPT1. This necessity for the ω-amino group is similar to the rabbit pepT2, which shares this requirement; the rabbit pepT1 protein does not have a strict requirement for an ω-amino group (9, 10, 25, 27). Glutathione is an extremely abundant dietary and cellular peptide (33, 58). Because reduced glutathione is a γ-glutamyl-linked tripeptide, in theory, it was possible for this peptide to be a substrate for OPT1 transport. Glutathione, however, is a poor inhibitor of alanlyalanine transport and is therefore an unlikely substrate in vivo. Consistent with this finding, dietary and interorgan glutathione uptake in humans is not mediated by either pepT1 or pepT2 but through a distinct Na+-dependent carrier protein (33).

OPT1 and protein metabolism. The presence of OPT1 within several epithelial membranes suggests a general role in protein metabolism. The expression of OPT1 in the midgut is consistent with a role in the absorption of dietary peptides. The midgut is the site of almost all dietary protein digestion and absorption (75). A carboxypeptidase, a trypsinlike activity, and at least two dipeptidases have been identified in the Drosophila midgut (40, 44, 86). The position of OPT1 on the apical membrane would suggest that this protein is organized in the membrane for uptake of peptides, generated by the digestive proteases, from the lumen of the midgut into the epithelia cells. The absence of OPT1 in the basolateral membrane intimates that the transported peptides are processed intracellularly, presumably by the dipeptidase A and B activities previously identified in this tissue (45, 44).

In most insects, including Drosophila, the formation of primary urine by filtration and the active secretion of selected substances occurs within the Malpighian tubules (48, 65). The excreta are then deposited into the hindgut, where many filtered metabolites are reabsorbed by the rectum (64). Small peptides in the Drosophila hemolymph represent a significant portion of the total amino acids in adults (15, 18, 54). OPT1 present in the rectal epithelia is probably involved in the reabsorption of some of these peptides that are filtered through the Malpighian tubules.

There are few indications of protein digestion within the female reproductive tract of Drosophila. During copulation, the male transfers several proteins and small peptides, produced in his accessory glands, into the female reproductive tract. These proteins elicit several changes in female physiology and behavior, including decreases in life span and mating receptivity and increases in egg laying and the efficient storage and utilization of sperm in the seminal receptacles and spermatheca (12, 16, 37). At least 85 distinct accessory gland proteins and peptides have been identified by two-dimensional electrophoresis (19, 81). One such protein, Acp26Aa, has been shown to be proteolytically processed within the female reproductive tract (55, 60). The cleavage of Acp26Aa requires at least one additional product of the male accessory glands, although cleavage does not occur until deposition in the female reproductive tract (60). The Acp76Aa protein is also transferred into the uterus during copulation; by 6 h after transfer, Acp76Aa is barely detectable in the female reproductive tract (17). This protein is a member of the serine protease inhibitor superfamily (17). Taken together, these results demonstrate that, after copulation, digestion of accessory gland proteins occurs within the female reproductive tract. OPT1 may remove the resulting peptides from the sites of proteolysis into the epithelium. It is worth noting that the female reproductive tract of Drosophila has four times more soluble dipeptidase activity than the alimentary tract (44).

Besides epithelia, OPT1 is also expressed in cells of the fat body and the neurons of the central nervous system. The fat bodies have the function in flies analogous to that of the vertebrate liver. The human pepT1 and both the rabbit pepT1 and pepT2 genes are expressed in the liver, consistent with a conserved function (9, 46, 47). The role of peptide transport in the central nervous system is poorly understood. Interestingly, rabbit pepT1 and pepT2 messages are expressed in the brain, suggesting a general role for PTR proteins within the central nervous system (9, 25). Saturable uptake systems have been described for several neuropeptides in the blood-brain barrier, although the proteins have not yet been isolated (5, 7, 80). Most of these neuropeptides appear to be too large for transport by PTR family members. However, many neuropeptides
are metabolized in the brain on the cell surface (6, 13, 41). OPT1 may function in the absorption of the metabolites. A modest increase in OPT1 expression is seen in the α- and β-lobes of the mushroom bodies. These lobes are a subset of the axonal projections of the mushroom bodies (21). There are currently few characterized neuropeptides in Drosophila. Nevertheless, the amnesiac gene encodes a PACAP-like peptide that may effect the cAMP-dependent physiology of mushroom bodies (22, 24). Thus metabolites of the amnesiac gene product represent possible substrates for OPT1 transport within the mushroom body axons.

OPT1 in early development. The OPT1 protein is located on the α-yolk spheres from their formation in stage 10 oocytes throughout embryogenesis. The α-yolk spheres are membrane-limited vesicles containing crystalline arrays of the three distinct yolk proteins (11). In the developing embryo, these yolk spheres are the primary source of amino acids for protein synthesis. An aspartic proteinase is active in the yolk spheres of mature oocytes (51). The aspartic proteinase is thought to activate a cathepsin B-like proteinase found within the spheres at the start of embryogenesis (50). In contrast to the aspartic proteinase, this cathepsin B-like proteinase readily cleaves the yolk proteins at pH 6, and its activity increases throughout embryogenesis (50). The cathepsin B proteins are endoproteases with peptidyl dipeptidase activity (4). OPT1 is probably required to transport the dipeptides generated by this protease into the developing embryo.

An interesting question is how OPT1 is placed on the yolk sphere membrane. The insect oocyte remains an excellent model cell for the observation of intercellular trafficking. Yolk proteins enter the oocyte from the hemolymph through receptor-mediated endocytosis (68, 83, 84). The dachner-coated vesicles containing yolk protein are then trafficked through tubular, transient vesicles, where the yolk proteins disassociate from receptor (31, 67). Small vesicles containing free yolk proteins will pinch off these tubular compartments and fuse with immature or transitional α3- yolk spheres (31, 67). The maturation of these spheres involves fusion with Golgi vesicles and the formation of yolk protein crystals (20, 29–31). The mature α3-sphere moves from the cortex into the central regions of the oocyte. The α3-yolk spheres of Drosophila are ~3 µm in size and number close to 10^4 in a stage 14 oocyte (20). The small 0.1- to 1-µm OPT1-containing vesicles within the stage 10 oocyte cortex are correctly sized and positioned to be the early transitional yolk spheres. It is probable that OPT1 is deposited in these spheres by the fusion of Golgi vesicles, and the larger 4-µm OPT1 vesicles are the maturing α1- and α2-yolk spheres that have left the oocyte cortex and are awaiting proteinase activation. The orientation of OPT1 on these yolk spheres would be appropriate for the transport of peptides out of the vesicle and into the developing embryo.

We have presented data that are consistent with OPT1 being an authentic orthologue of both pepT1 and pepT2. The primary sequence similarities between OPT1 and the pepT1 and pepT2 proteins are significant throughout their entire lengths, and the predicted topologies are also very well conserved. The phylogenetic tree suggests the pepT1-pepT2 split occurred after divergence from OPT1. The kinetic properties of OPT1 are more like the pepT2 than pepT1 proteins. Specifically, the high-affinity for dipeptides and the apparent requirement for an α-amino group for substrate recognition are properties shared with the renal pepT2 but not pepT1 proteins. The expression of OPT1 in the apical membranes of the midgut and rectum is directly analogous to the expression of pepT1 on the brush-border membranes of the small intestine and pepT2 in the renal proximal tubules, respectively. Thus it is likely that OPT1 has the cognate pepT1 and pepT2 biological functions in Drosophila.

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