Drosophila Photoreceptors Express Cysteine Peptidase Tan

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ABSTRACT

The *Drosophila* mutant *tan* (*t*) shows reciprocal pigmentation defects compared with the *ebony* (*e*) mutant. Visual phenotypes, however, are similar in both flies: Electroretinogram (ERG) recordings lack "on" and "off" transients, an indication of impaired synaptic transmission to postsynaptic cells L1 and L2. Cloning of *tan* revealed transcription of the gene in the retina, apparently in photoreceptor cells. We expressed Tan in *Escherichia coli* and confirmed by Western blotting and mass spectroscopic analyses that Tan is expressed as preprotein, followed by proteolytic cleavage into two subunits at a conserved —Gly—Cys— motif like its fungal ortholog isopenicillin-N N-acyltransferase (IAT). Tan thus belongs to the large family of cysteine peptidases. To discriminate expression of Tan and Ebony in retina and optic neuropils, we raised antisera against specific Tan peptides. Testing for colocalization with *GMR*-driven n-Syb-GFP labeling revealed that Tan expression is confined to the photoreceptor cells R1–R8. A close proximity of Tan and Ebony expression is evident in lamina cartridges, where three epithelial glia cells envelop the six photoreceptor terminals R1–R6. In the medulla, R7/R8 axonal terminals appeared lined up side by side with glial extensions. This local proximity supports a model for *Drosophila* visual synaptic transmission in which Tan and Ebony interact biochemically in a putative histamine inactivation and recycling pathway in *Drosophila*. J. Comp. Neurol. 500:601–611, 2007. © 2006 Wiley-Liss, Inc.

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In the fruit fly *Drosophila melanogaster*, photoreceptor cells R1–R8 encode visual information. R1–R6 terminate in the first optic neuropil, the lamina. R7/R8 innervate different layers of the second neuropil, the medulla. The sites of synaptic contact in the lamina were characterized in great detail, particularly by the work of Meinertzhagen and Fischbach and their collaborators (Fischbach and Dittrich, 1989; Meinertzhagen, 1989; Meinertzhagen and Pyza, 1996; Meinertzhagen and Sorra, 2001): R1–R6 axons are organized in cartridges, modular structures that form a crystal-like lattice in the lamina. Three epithelial glia cells surround each cartridge. Photoreceptor response to light is a nonspiking depolarization, recorded as sustained negative component of the ERG (Juusola et al., 1996). Upon depolarization, the neurotransmitter histamine is released tonically at tetrad synapses to postsynaptic cells L1 and L2 (Coombe, 1986; Hardie, 1987, 1989; Stuart, 1999; Uusitalo et al., 1995). They are hyperpolarized by the activation of histamine-gated chloride channels, resulting in a block of signal transmission (Gengs et al., 2002; Gisselmann et al., 2002). Reduction of histamine concentration in the cleft leads to a depolarization of L1/L2. Consequently, a precise regulation of transmitter concentration is mandatory. Therefore, flies must have efficient means not only to release histamine but also to clear it from the synaptic cleft and terminate its action. Elaborate investigations on histamine metabolism in the *D.*

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melanogaster and *Sarcophaga bullata* eye after injection of histamine into fly heads and after feeding histamine and its β-alanyl conjugate carcinine (Arnould, 1987) revealed a fast conversion of histamine into carcinine; the hydrolysis of this compound back to histamine and β-alanine is blocked in *tan* mutants (Borycz et al., 2002). Based on these investigations and on results from our laboratory (Richardt et al., 2002, 2003), a working model for histamine neurotransmitter metabolism in the eye emerges. It takes into account that replenishment of the photoreceptor's histamine neurotransmitter pool by decarboxylation of histamine is a slow process that represents merely a minor pathway (Morgan et al., 1999). As a consequence, histamine must be recycled (Melzig et al., 1996, 1998; Stuart et al., 1996). This requires functional Tan and Ebony proteins and a transport system for histamine and its metabolite carcinine. The model implies that histamine is removed from the synaptic cleft into lamina glia cells by a histamine transporter that has yet to be identified. There, it might be locked by conversion into carcinine by Ebony. For reuse as a neurotransmitter, carcinine has to be hydrolyzed to β-alanine and histamine to allow histamine to be pumped back into synaptic vesicles in photoreceptor terminals. The cellular site of hydrolytic cleavage of carcinine by Tan is unknown. Early mosaic studies, however, indicated that *tan* acts autonomously within or close to the eye (Hotta and Benzer, 1970). In situ hybridization to *tan* transcripts in cryosections of the *Drosophila* head confirmed these genetic data by revealing expression in the retina and here most likely in photoreceptor cells (True et al., 2005).

The enzymatic activities of Ebony and Tan, which have recently been determined, support their role in a putative histamine/carcinine cycle: Ebony activates β-alanine by binding it as thioester to a 4′-phosphopantethein cofactor in a process closely related to fungal nonribosomal peptide synthetases (NRPS; Richardt et al., 2003). Activated β-alanine is then released from the enzyme by conjugation to biogenic amines such as histamine. The cloning of *tan* has revealed that the encoded protein is an ortholog of isopenicillin-N N-acyltransferase (IAT) of *Penicillium chrysogenun* (True et al., 2005), which catalyses the conversion of isopenicillin-N to penicillin-G in a two-step reaction (Barredo et al., 1989). First, an amide bond is cleaved to release adipic acid from isopenicillin-N; in the next step, a phenylacetyl group is transferred to yield penicillin-G (Alvarez et al., 1993). Tan's capacity to hydrolyze carcinine as well as β-alanyl-dopamine in vitro by hydrolytic cleavage of a carboxyamide bond corresponds to the first step of IAT activity.

All but one IATs are enzymes, which are processed from their preproteins to their mature, active form by an autocatalytic cleavage process (Fernandez et al., 2003; Tobin et al., 1993). The cleavage site is a conserved —Gly—Cys— motif about 100 amino acids (aa) behind the NH_2-terminus. The *Drosophila* Tan —Gly_{121}—Cys_{122}— motif is located at a comparable distance from the amino-terminus. We have speculated that Tan also activates itself by a processing step from a preprotein. Consequently, we expressed Tan as His_6 tag fusion protein in *Escerichia coli* and could show that it is indeed split at the conserved —Gly—Cys— motif into two subunits, most likely by self-cleavage of the precursor protein. We also raised antisera against Tan-specific peptides and used them to determine the cellular and subcellular localiza-

tion of the protein in the eye. Tan expression is specific to photoreceptor cells, where it is found in high concentration in the cytosol. In lamina and medulla, it was found to be closely associated with glial Ebony expression. Our experimental results thus support the idea of a putative histamine-carcinine cycle between photoreceptor and glia.

MATERIALS AND METHODS
Fly stocks

D. melanogaster were cultured at 24°C on standard corn meal/agar medium with a 12-hour:12-hour light:dark cycle. The wild-type strain examined was *Canton S*. To target expression of n-synaptobrevin-GFP (n-Syb-GFP) fusion protein to photoreceptor cells, the *UAS-n-Syb-GFP* construct carrying line *w[*];3* (Ito et al., 1998) was crossed to the *Glass Multiple Reporter-gal4* (*GMR-gal4*) line *w[*]; P{w[+mC] = longGMR-GAL4}2/CyO*. The P-insertion line *y w[1,118] P{Mae-UAS.6.11} CG12120[g1557]* (*P{g1557}*); Beinert et al., 2004) served as *tan* null control in the eye.

Plasmid constructs and Tan expression

tan cDNA was obtained from the *Drosophila* Genomics Resource Center (http://dgrc.cgb.indiana.edu/) as clone *RH41996* (bar code 17763) in plasmid vector *pFLC-1*. For cloning of a carboxy-terminal His_6-tag-*tan* fusion construct, an NcoI site has been introduced at the start codon by in vitro mutagenesis according to the QuikChange site-directed mutagenesis procedure (Stratagene, La Jolla, CA). The NcoI-BamHI fragment containing the 5′-portion of the *tan* reading frame was then subcloned into *pQE60* (Qiagen, Hilden, Germany). The missing 3′-end of the reading frame has been PCR amplified with a 3′-end reverse primer that introduced an artificial BamHI site right after the stop codon. The amplified fragment was cut with BamHI and introduced into the BamHI opened *pQE60* containing the 5′-portion of the *tan* reading frame. Finally, the NcoI cloning site was reverted by in vitro mutagenesis to yield the original *tan* coding sequence. For cloning of an aminoterminal His_6-tag fusion construct, a 935-bp BglII-XhoI partial digest fragment of *tan pFLCI-RH41996* cDNA has been inserted into the SalI-BamHI opened *pQE82L* vector (Qiagen). The fragment contained the amino-terminal portion and two additional codons in front of the *tan* start UAG, which extend the *pQE82L* His_6-tag linker. For the remaining carboxy-terminal cDNA fragment, a 321-bp BamHI-HindIII fragment was PCR amplified, creating an artificial HindIII site next to the stop codon. The fragment was cloned behind the amino-terminal portion of the *tan* cDNA. The constructs were transformed into *BL21-Rosetta* cells (Novagen-Merck, Darmstadt, Germany). His_6-Tan fusion protein expression was induced for 3 hours at 28°C by adjusting the growth medium to 0.1 mM IPTG. *E. coli* cells were broken up with 10 1,000-bar cycles in a microfluidizer M-110L (Microfluidics, Newton, MA). Cell debris was spun down, and the clear supernatant was used for affinity purification with Protino 2000 columns (Macherey & Nagel, Dassel, Germany).

Electrospray ionization mass spectrometry

HPLC-MS was performed with a Thermo Finnigan LCQ Deca mass spectrometer connected to an Agilent 1100 HPLC. The proteins were separated on a C4 reverse-phase

column with a pore size of 300 Å, employing a gradient of H_2O/acetonitrile in the presence of 0.1% formic acid. The resulting mass spectra (measured between m/z 700 and m/z 2,000) were deconvoluted with the "Bioworks browser," which is Thermoquest proprietary software. To avoid deconvolution artifacts, the software allows for the selection of (real) masses yielding a spectrum without additional peaks (shown in Fig. 2). The absolute deviation of the resulting masses was smaller than 2 mass units in 10,000.

Antiserum against Tan peptides

The peptides NH_2-*SSGKILPRRQAVPVL*C-$CONH_2$ and NH_2-C*PSETEPHCRLPLLYK*-COOH, each containing 15 amino acids of the *tan* reading frame as shown in italics, were covalently coupled by an additional cysteine to keyhole limpet hemocyanine (KLH) carrier. The first injection of a mixture of both peptide-KLH conjugates into rabbits was followed by boost injections after 2, 4, 8, and 12 weeks. After 3 months, the animals were killed to collect blood. Affinity purification of sera was performed with both peptides coupled concurrently to Sepharose (GE Healthcare Europe, München, Germany). In this study, we used the serum of rabbits ap61 and ap63, which gave the strongest immune response according to ELISA tests against the peptides (data not shown). In addition, we raised the rat antiserum SA2 against full-length Tan expressed as amino-terminal His_6-tag fusion protein in *E. coli BL21-Rosetta* cells. The affinity-purified His_6-Tan was injected into rats, and boost injections were given after 2, 4, and 8 weeks before the final blood was taken after 3 months.

Fly head fractionation

We used a modified version of a published procedure (Schulze et al., 1995) to prepare subcellular fractions of fly head homogenates: Flies were frozen in liquid nitrogen, and heads were separated from bodies and small parts of tissue by two rounds of sieving. Heads were ground with a mortar and pestle immersed in liquid nitrogen. The fine powder was suspended in ice-cold 0.2 M sucrose in 20 mM Hepes/NaOH (pH 7.4), 0.1 M NaCl, and homogenized in a glass homogenizer with 20 strokes of a Teflon pestle. Coarse debris and nuclei were removed by centrifugation at 1,000*g* for 10 minutes. The supernatant was diluted 1:3 with ice-cold 20 mM Hepes/NaOH (pH 7.4), 0.1 M NaCl. It was placed on top of an ice-cold sucrose step consisting of a layer of 3.5 ml of 0.4 M sucrose and a layer of 3.5 ml of 0.2 M sucrose. Synaptic vesicles were separated from the cytosol fraction by centrifugation in a SW41 rotor for 2 hours at 87,000*g*, and 0.5-ml gradient fractions were collected from the bottom.

Western blot analysis

Aliquots of the *Drosophila* head extract gradient fractions and samples of affinity-purified *E. coli*-expressed His_6-Tan fusion protein were analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) in a minigel apparatus (Hoefer Scientific, San Francisco, CA). For detection of the synaptic vesicle marker cysteine string protein (CSP; Zinsmaier et al., 1990), 200-μl aliquots of the sucrose gradient fractions were precipitated with methanol to concentrate the loaded sample tenfold. Protein transfer was performed by electroblotting onto nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany; Kyhse-Andersen, 1984).

After blocking with 1% milk powder, the nitrocellulose membrane was incubated for 1 hour at room temperature with the combined affinity-purified anti-Tan peptide antisera ap61 and ap63 at a 1:10,000 dilution, with the mouse monoclonal antibody PentaHis against a His_5-tag (Qiagen; lot 10917464) at 1:1,000 or with mab ab49 against CSP (Buchner et al., 1988; a kind gift of E. Buchner, Biozentrum, Universität Würzburg) at a 1:50 dilution in Tris-buffered saline/0.02% Tween 20 (TBST). As secondary antibody, peroxidase-coupled goat anti-rabbit IgG (H+L; Dianova, Hamburg, Germany; lot 13397) or goat anti-mouse IgG (H+L; Dianova; order 115-035-003) was used at a 1:10,000 dilution. Bands were visualized via enhanced chemiluminescence (Roche, Mannheim, Germany). Alternatively, alkaline phosphatase-coupled goat anti-mouse IgG (H+L; Dianova; lot 63580) or alkaline phosphatase-coupled goat anti-rabbit IgG (H+L; Dianova; lot 67845) was used at a 1:1,000 dilution, followed by stain development with NBT/BCIP.

Immunocytochemistry

The probosces were removed from 1–3-day-old flies in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C and fixed for 3 hours. For labeling with antihistamine antiserum, a modified fixation procedure was applied: flies were fixed in 4% 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) in 0.1 M phosphate buffer, pH 7.4, for 3 hours. Fixative was then replaced by 25% sucrose in *Drosophila* Ringer's solution overnight (Buchner et al., 1988). Fly heads were embedded in Tissue-Tek (Sakura Finetek, Zoeterwoude, The Netherlands), frozen in liquid nitrogen, and sectioned at 10 μm. Blocking of tissue was accomplished with 1% normal goat serum (Dianova; lot 23893) in TBST. The affinity-purified rabbit antiserum ap63 against the Tan peptides was used at a 1:1,000 dilution; the rat SA2 anti-His_6-Tan antiserum was diluted 1:100. A rabbit anti-Ebony antiserum against a 221-aa peptide spanning aa Gly438 to aa Asp658 of the Ebony protein (Richardt et al., 2002) was employed at a dilution of 1:750 to reveal glial Ebony expression in the optic lobes. Antichaoptin antibody mab 24B10 (Zipursky et al., 1984; Developmental Studies Hybridoma Bank) was diluted 1:100. To visualize green fluorescent protein (GFP) expression, specimens were incubated with an mab against GFP (3E6; Molecular Probes, Eugene, OR; lot 71C1-1) diluted 1:200 in PBST. EDAC-fixed histamine was detected by using a rabbit antihistamine IgG (H+L; ImmunoStar, Hudson, WI; lot 352002) at a 1:500 dilution. Primary antisera were applied overnight at 4°C. After extensive washing, secondary Cy2- or Cy3-conjugated antibodies were incubated for 2 hours at room temperature at a dilution of 1:200 to 1:1,000. For antihistamine, anti-Tan ap63 and anti-Ebony immunolabeling Cy3-conjugated affinity-pure goat anti-rabbit IgG (H+L; Dianova; lot 62035) was employed. Cy2-conjugated affinity-pure goat anti-rat IgG (H+L; Dianova; lot 47615) was used for anti-Tan SA2 immunolabeling. Cy2-conjugated affinity-pure donkey anti-mouse IgG (H+L; Dianova; lot 58162) was used for antichaoptin mab24B10 and anti-GFP mab3E6 immunolabeling. Slices were mounted in DakoCytomation Glycergel (DakoCytomation, Hamburg, Germany) and imaged via confocal microscopy with a Leica TSC2 microscope (Leica Microsystems, Wetzlar, Germany) with HCX PL APOCS 63 × 1.4 optics. The size, contrast, and brightness of the resulting images were adjusted in Photoshop 7.0 (Adobe Systems, San Jose, CA).

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RESULTS

Tan consists of two subunits generated by endoproteolytic cleavage

The Tan sequence is strongly conserved between insects and related to fungal IATs, e.g., the *penDE* gene product of *P. chrysogenum* (True et al., 2005). IAT is usually generated by autocleavage of a preprotein into a small subunit of 11 kDa and a large subunit of 29 kDa. Since *Drosophila* Tan reveals 50% amino acid sequence similarity with *P. chrysogenum* IAT, we investigated whether Tan expressed as His_6-tag fusion protein in *E. coli* is also processed into two subunits. As deduced from the encoded reading frame, Tan has a calculated molecular weight of 43.7 kDa (True et al., 2005). Together with the amino-terminal His_6-tag containing linker of 1.6 kDa, this adds up to a 45.3-kDa fusion protein. The shorter carboxy-terminal His_6-tag linker of 1.3 kDa gives rise to a 45.0-kDa fusion protein. After expression and affinity purification, fusion proteins were separated on denaturing polyacrylamide gels (SDS-PAGE). By Coomassie brilliant blue staining, we observed a faint band representing the entire fusion protein. However, the majority of the affinity-purified protein banded at smaller molecular weight comparable to that of IAT autoprocessing products (Fig. 1B, lanes 1, 2). To verify the putative cleavage of His_6-Tan, we probed Western blots with anti-His_5 antiserum. Depending on the site of His_6-tag fusion with the Tan reading frame, e.g., amino-terminal or carboxy-terminal, we detected a distinct banding pattern. The unprocessed amino-terminal His_6-Tan fusion protein band at 45.3 kDa was not detected under our experimental conditions, whereas the processed 15.4-kDa amino-terminal His_6-Tan cleavage product was strongly labeled (Fig. 1B, lane 3). Conversely, the carboxy-terminal His_6-tag fusion protein blot revealed the 31.2-kDa carboxy-terminal His_6-Tan cleavage product, in addition to a faint band representing the 45.0-kDa unprocessed precursor (Fig. 1B, lane 4). In each case, the 29.9-kDa and 13.8-kDa fragments lacking the His_6-tag re-
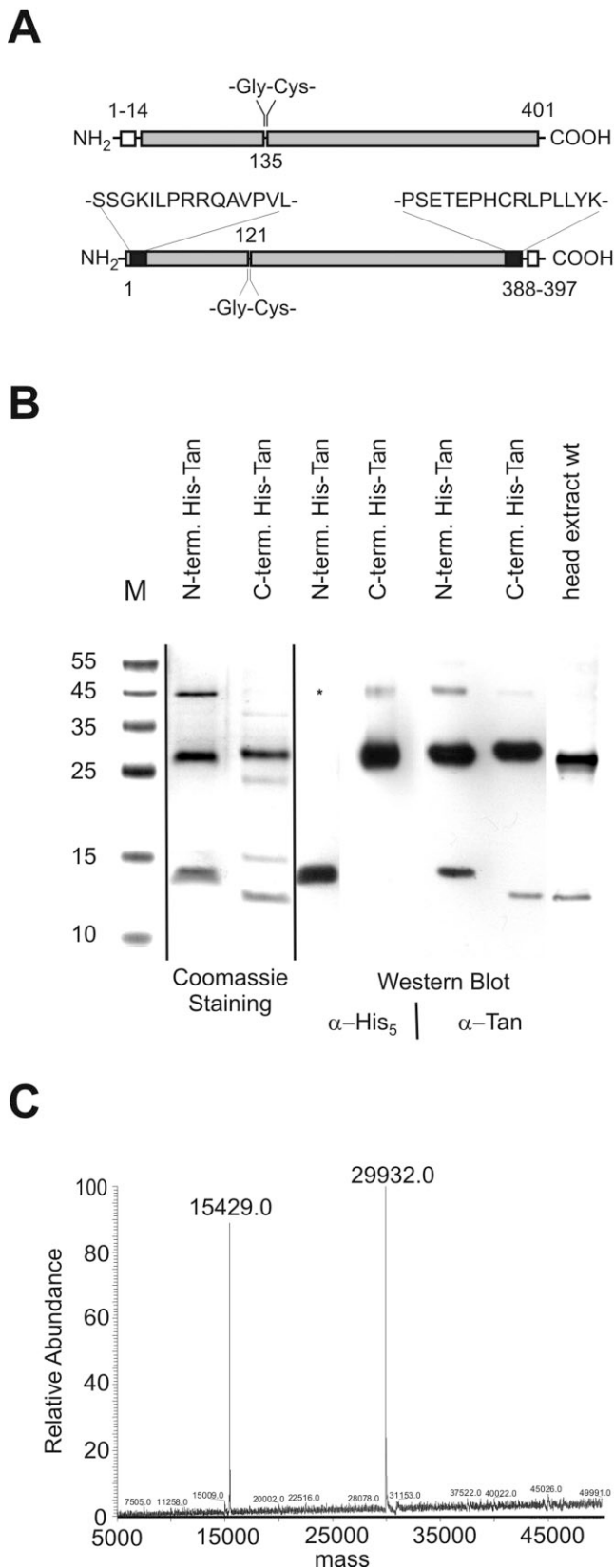
Fig. 1.   Tan preprotein is cleaved into two subunits. **A:** The structures of the His_6-Tan fusion protein constructs are depicted. Boxes represent the encoded amino acid sequences. Numbers refer to protein length and positions of His_6-tags (1–14 and 308–397) and the —Gly—Cys— motif. Black squares represent the position of the peptide sequences, which were used for antiserum production. **B:** SDS-PAGE of amino-terminal and carboxy-terminal His_6-Tan expressed in *E. coli*, affinity-purified on Protino columns and stained with Coomassie brilliant blue is shown in lane 1 and 2, respectively, from left to right. The carboxy-terminal His_6-Tan affinity column preparation reveals a few minor bands of contaminating protein. Lanes 3–6 show a Western blot of amino-terminal (lane 3 and 5) and carboxy-terminal (lane 4 and 6) His_6-Tan fusion protein. Lanes 3 and 4 were probed with anti-His_5 antiserum. Only His_6-tag-containing fragments are labeled, indicating processing of the fusion protein. The amount of unprocessed Tan preprotein varies depending on expression and preparation time. Lane 5 and 6 were probed with a 1:1 mixture of antiserum ap61 and ap63 against the amino-terminal and the carboxy-terminal Tan peptides. Lane 7 shows a Western blot of the soluble fraction of a *Drosophila* head extract labeled with the same anti-Tan peptides antiserum mixture as in lanes 5 and 6. Note that the amount of loaded protein varied. M, size marker. **C:** Deconvoluted mass spectrum of an HPLC-MS run of Tan averaged for run times 11.0–12.3 minutes. The original spectrum was obtained between m/z 700 and m/z 2,000. Deconvolution was performed with the proprietary software Bioworks Browser and selected masses of 15,429 and 29,932.

mained unlabeled, which is again a strong indication for precursor processing. The degree of processing was dependent on temperature and time (data not shown). Three-hour expression at 28°C as used for Figure 1 gave rise predominantly to the cleaved subunits with small amounts of unprocessed precursor.

Antisera with high selectivity for Tan

We have previously shown by RNA in situ hybridization to 10-μm head cryosections that *tan* is transcribed in the retina of 1–3-day-old flies (True et al., 2005). Still, putative Tan protein expression in photoreceptor cells R1–R8 must be verified directly. Moreover, determination of Tan's subcellular distribution is of particular importance for understanding its function in synaptic transmission of photoreceptor cells. In addition, our aim was to verify the existence of the two subunits' composition in *Drosophila*. To enhance the probability of antiserum production and to detect both cleavage products with high specificity, we raised rabbit antisera against a mixture of the two Tan peptides corresponding to aa 2–16 and to aa 373–387 of the *tan* reading frame (Fig. 1A). Both peptides were linked to a carrier and were used for affinity purification of two of the produced antipeptide antisera, ap61 and ap63. A 1:1 mixture of affinity-purified ap61 and ap63 was then employed in a 1:10,000 dilution on Western blots of *E. coli* expressed amino-terminal (Fig. 1B, lane 5) and carboxy-terminal His$_6$-Tan fusion protein (Fig. 1B, lane 6). The observed staining pattern of unprocessed precursor and of the two putative subunit bands confirmed that His$_6$-Tan in vitro indeed underwent the expected maturation process. We next asked whether the observed cleavage of Tan protein in vitro occurs in vivo as well. We therefore probed a Western blot of a soluble supernatant fraction of head extracts of wild-type flies with the antiserum. Labeling of a 13.8-kDa and a 29.9-kDa fragment (Fig. 1B, lane 7) and the absence of any precursor-sized band unequivocally demonstrate Tan processing in *Drosophila* in vivo.

The —Gly$_{121}$—Cys$_{122}$— motif is the cleavage site for Tan precursor processing

The —Gly—Cys— motif as site for autocatalytic cleavage is conserved among IAT genes in fungi (Tobin et al., 1995). Because heterologous expression of *Drosophila* Tan in *E. coli* gave rise to processed enzyme subunits, we used the aminoterminal His$_6$-Tan fusion protein for mass determination of its subunits to establish the site of cleavage. Electrospray ionisation mass spectrometry (ESI-MS) revealed two mass peaks closely coinciding with the calculated molecular masses for the putative subunits (Fig. 1C). One prominent peak indicated a molecular weight of 15,429 Da, compared with a calculated molecular weight of 15,416 Da for the amino-terminal fragment. A deviation of 13–15 Da from the calculated molecular weight was obtained repeatedly and cannot be explained at present. A second prominent mass peak appeared at 29,932 Da. This value is within the standard deviation range of 2/10,000 Da and matches very closely the calculated molecular weight of 29,934 Da for the carboxy-terminal fragment starting with Cys$_{122}$. We therefore conclude that Tan is indeed cleaved between Gly$_{121}$ and Cys$_{122}$, which emphasizes again a close relation to the large group of fungal IATs.

Immunocytochemistry reveals photoreceptor-cell-specific expression of Tan

To determine the cellular and subcellular localization of Tan in the eye and the optic lobe of the fly, we initially incubated head cryosections with the affinity-purified antiserum ap63 against the Tan peptides. Strong immunoreactivity was detected in photoreceptor cells (Fig. 2A). The label extended into the narrow axons and expanded terminals of R1–R6 in the lamina and of R7 and R8 in the medulla. Immunolabeling of processes in the medulla was restricted to the distal half and did not penetrate the serpentine layer separating its distal and proximal halves. To confirm that Tan expression in eye and optic lobe is confined to photoreceptor cells, we crossed the *GMR-gal4* driver line with the *UAS-n-Syb-GFP* reporter line. The *GMR* enhancer activates expression of *gal4* in all photoreceptor cells (Moses and Rubin, 1991). The *n-Syb-GFP* reporter was employed because the n-Syb portion facilitates transport of the GFP fusion protein to axonal endings, which enhances the neuronal cell labeling with anti-GFP antiserum. Figure 4A shows a *GMR-gal4/UAS-n-Syb-GFP* fly brain horizontal section labeled with anti-GFP antiserum. It revealed GFP expression in all photoreceptor cells, with particularly strong label of the axonal terminals in lamina and medulla. A double labeling with anti-GFP and anti-Tan peptides antiserum ap63 is shown in Figure 4B. The complete overlap of Tan and GFP expression clearly revealed that Tan expression is confined to photoreceptor cells.

Replenishment of the histamine pool through hydrolysis of carcinine is the putative function of Tan. De novo, however, histamine is synthesized in photoreceptor cells by decarboxylation of histidine (Melzig et al., 1996). To identify additional cells that might replenish their histamine pool by cleavage of carcinine by Tan, we searched for overlapping anti-Tan and antihistamine immunoreactivity in head cryosections. As shown previously (Borycz et al., 2002; Buchner et al., 1993), immunolabeling of histamine was found in photoreceptors and in neurons of the lobula and the central brain (Fig. 2C,D). Anti-Tan labeling on formaldehyde-fixed preparations showed, in addition to the photoreceptor cells, labeling neither in the optic lobe nor in the central brain (Fig. 2A,B). Because the antihistamine immunolabeling required fixation with EDAC (Panula et al., 1988), subsequent formaldehyde fixation and double labeling with anti-Tan antiserum gave rise to images of poor resolution, reduced labeling intensity, and high background (data not shown). This technical problem excluded a colocalization on the same preparation. From comparison of the separate imunolabeling images, we could not detect a colocalization between histamine and Tan outside of photoreceptor cells.

Double labeling of wild-type fly head freeze sections with antichaoptin mab 24B10, which is specific to photoreceptor cell membranes (Fujita et al., 1982; Zipursky et al., 1984), and with anti-Tan serum ap63 was performed to investigate the subcellular localization of Tan in the retina. Saggital sections through the retina revealed labeling of Tan in photoreceptor somata, excluding the rhabdomere membrane structures, which were labeled by the antichaoptin antibody (Fig. 2F). Although double labeling of Tan and chaoptin revealed the cytosolic localization of Tan in the retina, it did not disclose the subcellular
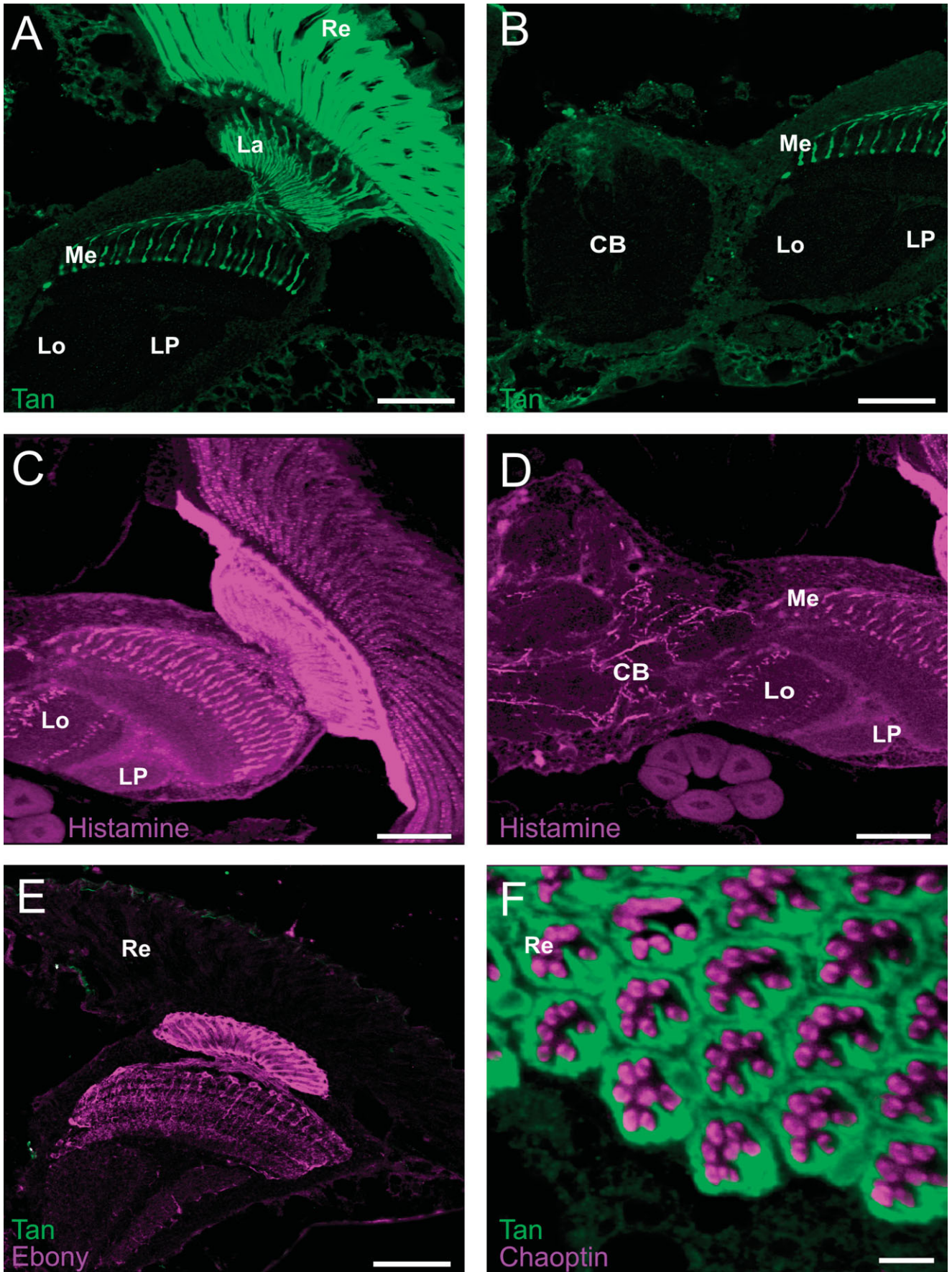
Fig. 2. Tan expression in eyes and optic lobes. Confocal images of 10-μm fly head cryosections were prepared. **A:** Labeling with anti-Tan peptides antiserum ap63 reveals expression in photoreceptor cells and their axonal extensions in lamina and medulla. **B:** Ap63 anti-Tan labeling in optic lobe and central brain. **C:** Histamine labeling in eye and optic lobe. **D:** Histamine labeling in optic lobe and central brain. **E:** Double labeling of the *P{g1557} tan* mutant with anti-Tan antiserum SA2 and anti-Ebony antiserum. **F:** Saggital section of the retina labeled with anti-Tan peptides antiserum ap63 and antichaoptin mab 24B10. Re, retina; La, lamina; Me, medulla; Lo, lobula; LP, lobula plate; CB, central brain. Scale bars = 40 μm in A–E, 4 μm in F.

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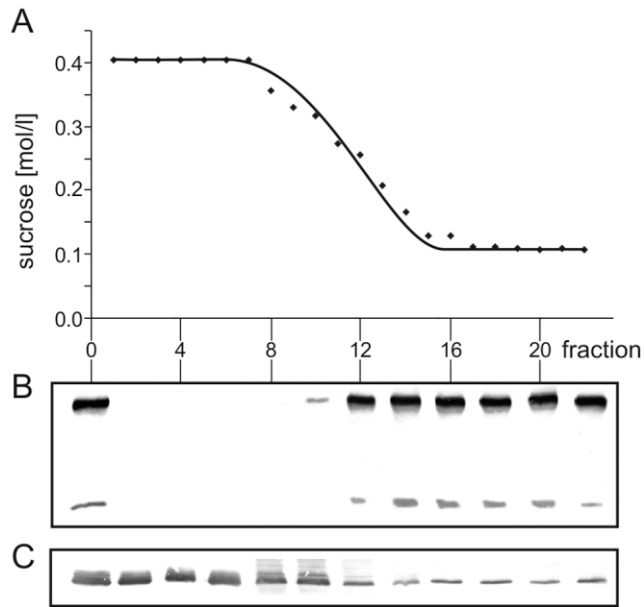HISTAMINE NEUROTRANSMITTER IN *DROSOPHILA* VISION 607

Fig. 3. Subcellular distribution of Tan in head extracts. **A:** Density profile of a sucrose gradient for separation of subcellular fractions of fly head extract. Every second fraction was resolved on a denaturing polyacrylamide gel and transferred to nitrocellulose. **B:** A Western blot with a 1:1 mixture of anti-Tan peptides antiserum ap61 and ap63 reveals Tan containing fractions. **C:** A separate blot with anti-CSP mab ab49 shows the distribution of the synaptic vesicle marker protein. At zero position, the input material (clear head extract) was analyzed.

distribution in the axonal terminals under our experimental conditions.

Tan is not firmly associated with subcellular structures

To investigate whether Tan is firmly bound to vesicle membranes, we separated the soluble fraction of *Drosophila* head extracts from membranes and vesicles. A head extract was loaded on top of a 0.2 M and 0.4 M sucrose step gradient (Fig. 3A), and the vesicle fraction was spun down into the high-density region of the gradient. Aliquots of the gradient fractions were resolved either directly (Fig. 3B) or after tenfold concentration in a 15% SDS-PAGE (Fig. 3C), and proteins were subsequently transferred to a nitrocellulose membrane. Immunochemical detection of Tan was clearly restricted to the low-density fraction of soluble proteins (Fig. 3B). Synaptic vesicles were detected with mab ab49 against CSP. Strong CSP labeling was obtained in the fractions of 0.2–0.4 M sucrose. A small amount of CSP detected in the low-density gradient fractions must have been released from synaptic vesicle membranes during extract preparation (Fig. 3C). Because not even traces of Tan immunoreactivity appeared in the high-density fraction of the gradient, a firm association of Tan with synaptic vesicles can be excluded. Still, subcellular accumulation of Tan as the result of weak interactions with membranous structures, which disintegrated under the employed experimental conditions, might have been missed and must await an immunocytochemical EM analysis.

Neuronal Tan expression as opposed to glial Ebony expression

Potential colocalization of Ebony and Tan was of particular interest at sites of neurotransmitter release in lamina and medulla, insofar as both proteins have been proposed to be involved in histamine neurotransmitter inactivation and recycling (Borycz et al., 2002; Richardt et al., 2002, 2003). This putative recycling pathway would involve inactivation of histamine by Ebony through β-alanyl conjugation and subsequent liberation of histamine by Tan through hydrolysis of carcinine. These mechanisms are conceivable only if Tan- and Ebony-containing cell structures are in close proximity and if a specific transport system is available.

Ebony expression had been localized to epithelial glia of the lamina and distal glia of the medulla (Richardt et al., 2002). To investigate the possible colocalization of Tan and Ebony, we raised the rat antiserum SA2 against affinity-purified full-length Tan expressed as His$_6$-tag fusion protein in rat. We used the *tan* mutant *P{g1557}*, which, according to missing "on" and "off" transients (True et al., 2005), has no Tan function in the eye, as negative control for the anti-Tan immunoreactivity of the employed serum SA2. The observed loss of labeling (Fig. 2E) revealed the specificity of the anti-Tan serum. Concurrent anti-Ebony labeling in lamina and medulla glia (Richardt et al., 2002) served as positive internal control. After verification of SA2 specificity through the lack of labeling in the absence of Tan expression, we performed double-labeling experiments with anti-Tan SA2 and the previously characterized anti-Ebony peptide antiserum (Richardt et al., 2003). In the lamina, horizontal sections showed Tan labeling of photoreceptor axonal terminals surrounded by Ebony-labeled epithelial glia (Fig. 4C,D). This arrangement of immunoreactivity was corroborated in sagittal sections. Photoreceptor terminals with Tan immunolabeling were framed by Ebony immunoreactivity in the three glia cells surrounding each cartridge and extended into its inner margins, around the terminals (Fig. 4E). In the medulla, Tan and Ebony labeling revealed a group of closely aligned extensions that reach but do not cross the serpentine layer (Fig. 4C,F). Tan-positive axonal terminals of photoreceptors R7 and R8 alternate with adjacent extensions of Ebony-positive glia revealing sites of close association between the two structures (Fig. 4F, arrows).

DISCUSSION

Tan belongs to the family of cysteine peptidases

The Tan protein exhibits about 50% similarity to fungal IATs. Similar amino acids are scattered over the entire sequence length (True et al., 2005), suggesting that the encoded enzyme function might also have been conserved. This would be surprising, in that Tan and IAT operate in divergent biochemical pathways. IATs possess three enzymatic activities: an endopeptidase activity for precursor maturation (Tobin et al., 1995), a hydrolase activity for cleavage of the carboxyamide bond in isopenicillin-N, and an acyl-group transfer activity to form penicillin-G and its derivatives (Alvarez et al., 1993). Previous investigations on Tan function have revealed a hydrolase activity for
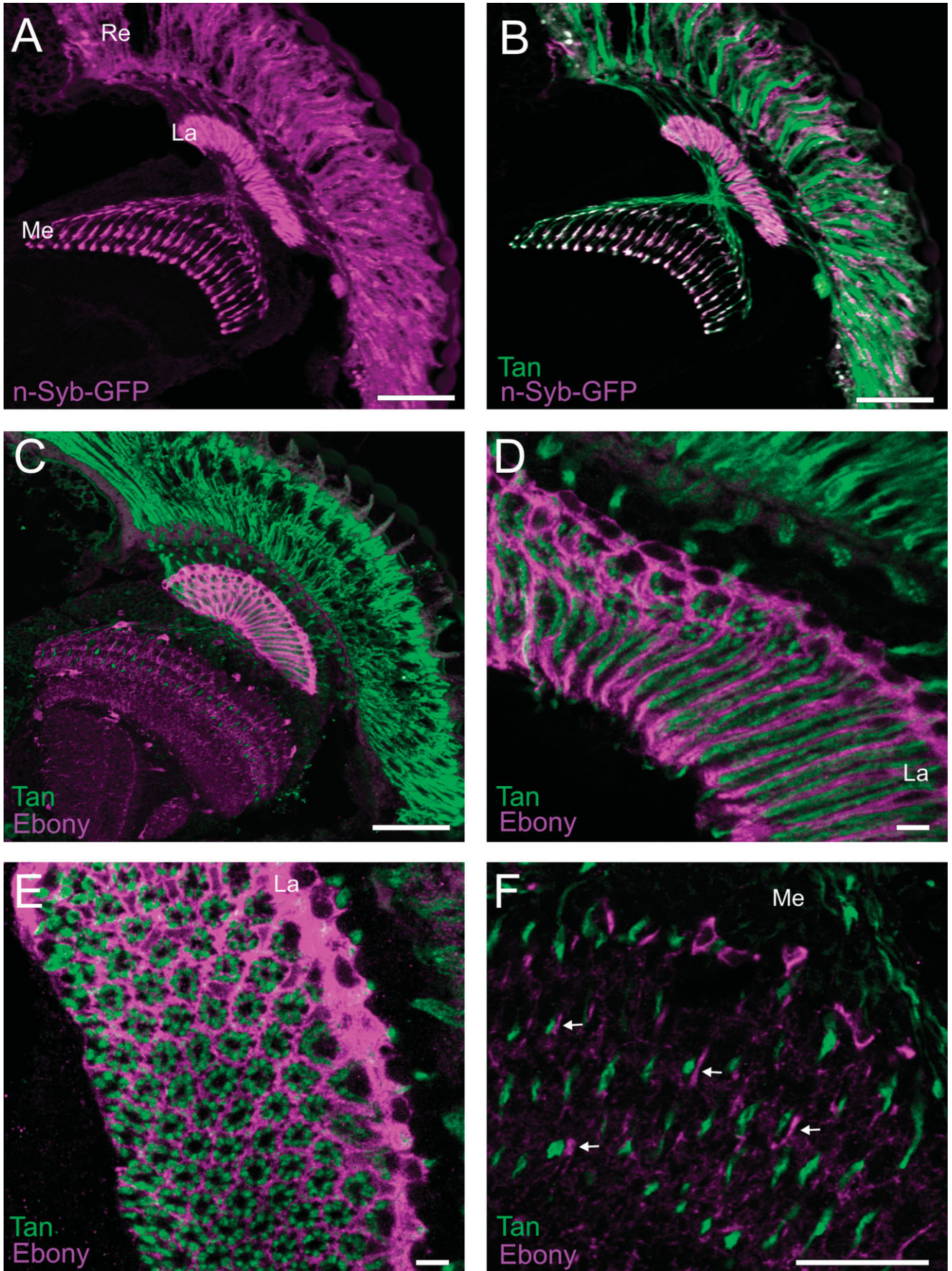
Figure 4

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β-alanyl conjugates of histamine and dopamine, which mechanistically might correspond to isopenicillin-N cleavage of IATs (True et al., 2005). Provided that the acyl-group transfer activity of IATs would also be a function of Tan, the histamine metabolite N-acetyl histamine (Elias and Evans, 1983; Sarthy, 1991) would be a candidate product. Recent investigations on *Sacrophaga bullata* have indeed supported the evidence for an accumulation of histamine metabolites in the eye, which could unfortunately not be identified by HPLC (Borycz et al., 2002). However, to date, formation of N-acetyl histamine has been reported only from specific N-acylases, enzymes distinct from Tan (Wright, 1987).

In this report, we have asked whether Tan also shares the endopeptidase activity with IATs, as suggested by the sequence conservation of the —Gly—Cys— motif apparent in all heterodimeric IATs. This would give rise to a heterodimeric subunit structure of Tan. We have shown by Western blotting that Tan is cleaved into two subunits in *Drosophila* head extracts as well as after expression as His_6-tag fusion protein in *E. coli*. The determination of the subunits' molecular weights through ESI-MS confirmed cleavage between Gly_{121} and Cys_{122}. This finding led us to ask whether Tan precursor maturation is also dependent on an intrinsic autocatalytic cleavage activity. Previous investigations of IAT processing, including a number of *E. coli*-expressed mutant proteins, had shown that processing was strictly dependent on the —Gly—Cys— motif (Tobin et al., 1993). This motif has so far not been observed to be involved in the processing of native *E. coli* proteins. Therefore, it is the identity of this processing site and the accumulated sequence homology surrounding the —Gly—Cys— motif in Tan that led us to exclude an *E. coli*-dependent cleavage reaction on Tan. Instead, we predict that Tan processing is also an autocatalytic process. Definitive proof, however, must await future experimental evidence.

The reason for the consistent 13–15-Da mass deviation from the calculated molecular weight of the aminoterminal subunit remains to be elucidated. It could be the result of an internal amino acid side chain reaction that takes place during autocatalytic cleavage (Perler et al., 1997). We speculate, in light of the overall sequence similarity between Tan and IATs, particularly around the conserved —Gly—Cys— motif, that the reaction mechanism underlying the putative intrinsic endopeptidase activity, which leads to cleavage of the Tan precursor, might be similar to the one operating in IATs.

According to sequence conservation and maturation from a precursor, the protein Tan can be classified to clan PB of the large family of cysteine peptidases (Barrett and Rawlings, 2001). Cysteine peptidases were described as "self-processing precursor proteins that use a cysteine residue to cleave a single internal peptide bond in the precursor, although the mature protein might express other kinds of enzymatic activity, commonly hydrolysis or transfer of amide bonds." Tan clearly fulfills this requirement. The number of enzymes known to be formed from preproteins independent of an additional endopeptidase is still growing. Self-catalyzed protein rearrangements are commonly initiated by an N-O or N-S acyl rearrangement at a specific serine, threonine, or cysteine residue (Perler et al., 1997). Our forthcoming experiments are aimed at substituting the —Gly—Cys— motif by in vitro mutagenesis to elucidate the reaction mechanism leading to mature Tan.

Tan expression localizes to photoreceptors R1–R8

Our previous *tan*-RNA in situ hybridization experiments had revealed transcriptional activity in the retina, most likely in photoreceptor cells (True et al., 2005). However, RNA in situ hybridization signals are sometimes difficult to assign to a specific cell type because the signal is usually restricted to the cell body, preventing cell identification based on the characteristic shape and distribution of marker proteins obtained from antibody labeling, particularly in the nervous system. We have therefore raised specific antisera against Tan peptides and the His_6-Tan fusion protein to determine its expression pattern. By concurrent labeling of GFP expressed as n-synaptobrevin fusion protein in photoreceptor cells, we show that Tan expression in the eye and the optic lobe is confined to photoreceptor cells R1–R8. These results corroborate previous predictions of retinal *tan* expression based on genetic evidence (Hotta and Benzer, 1970).

Antihistamine immunoreactivity confirmed previous results showing label in photoreceptors and in a number of lobula and CNS intrinsic neurons (Melzig et al., 1998). The lobula and CNS intrinsic neurons had been shown to fail in taking up histamine after deprivation in the histidine decarboxylase null mutant hdc^{JK910}, whereas photoreceptor cells revealed a fast uptake mechanism. From this difference, Melzig et al. concluded that the histamine-containing photoreceptor neurons, which showed uptake, use histamine as fast neurotransmitter, whereas neurons that revealed no uptake, such as the lobula and central brain intrinsic neurons, would rather use histamine as neuromodulator. Although we could not obtain a reasonable double labeling with antihistamine and anti-Tan antiserum, the comparison of separate preparations indicated that colocalization most likely is limited to photoreceptors and thus to cells that use histamine as fast neurotransmitter.

Double labeling with anti-Tan and antichaoptin antiserum revealed a cytosolic distribution of Tan in the retinal portion of the photoreceptor cells opposed to a rhabdomeric accumulation of the membrane-bound chaoptin immunoreactivity. The localization of Tan in photoreceptors requires that, according to the proposed model, its substrate carcinine must travel from Ebony-expressing epithelial glia and medulla neuropil glia to photoreceptor axonal terminals. Histamine, on the other hand, must first be transported from the synaptic cleft into the glia before it can be transformed into carcinine by Ebony. Transporters have not been identified for the histamine

Fig. 4. Tan and Ebony expression in eye and optic lobe. Confocal images of 10-μm fly head cryosections were prepared. **A:** Labeling of a *GMR-gal4/UAS-n-Syb-GFP* head section with anti-GFP antiserum. **B:** Double labeling of the *GMR-gal4/UAS-n-Syb-GFP* preparation with anti-GFP and anti-Tan peptides antiserum ap63. **C–F:** Immunolabeling with anti-Tan antiserum SA2 and anti-Ebony antiserum C: Horizontal section with colocalization of Tan and Ebony in lamina and medulla. An enlargement of a horizontal section through the lamina is shown in D. A sagittal section through lamina cartridges in E reveals Tan expression in photoreceptor terminals surrounded by epithelial glia expression of Ebony. F: Tan labeling in medulla terminals of R7 and R8 axons, with Ebony expression in glia extensions. Scale bars = 40 μm in A–C; 4 μm in D,E; 20 μm in F.

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transport into the glia or for the carcinine transfer out of the glia and into the photoreceptor axonal terminals, and the mechanistic details of the translocation are unknown to date. Because the established Tan function is cleavage of histamine from carcinine (Borycz et al., 2002; True et al., 2005), one could expect that Tan activity is concentrated at axonal terminals, the putative site of carcinine uptake and cleavage as well as of histamine loading into vesicles. Capitate projections (Stark and Carlson, 1986), which are glial invaginations in photoreceptor axons, have been discussed as candidate sites for uptake of carcinine into photoreceptor axonal endings (Fabian-Fine et al., 2003). It was also suggested that the axonal membrane opposite capitate projections is the site of synaptic vesicle recycling. Provided that Tan hydrolyzes carcinine entering the photoreceptor axon opposite to capitate projections, the resulting carcinine depletion would eventually build up a local concentration gradient between glial and axonal carcinine and pull more carcinine through the cell membranes by an as yet unknown mechanism. Alternatively, Tan activity could be coupled to or depend on association with a vesicular histamine transporter at the synaptic vesicle membrane. However, our density gradient fractionation experiments excluded a firm association of Tan with vesicles and therefore call into question a synaptic vesicle membrane-associated function of Tan. A weak interaction with a membrane-bound protein complex, however, could have been missed under our experimental conditions. The final answer to this question has to await electron microscopic immunolabeling experiments.

The anti-Tan immunoreactivity revealed a broad distribution of Tan in photoreceptor somata and axons. In considering this distribution, we have to notice that only a very small fraction of Tan is present at its putative site of carcinine hydrolysis activity, the axonal terminals. As another function, Tan might be capable of catalyzing an acyl-group transfer, an activity that is observed in its IAT homologues. This activity could convert histamine into the inactive N-acetyl-histamine (Sarthy, 1991), a reaction that might not necessarily be defined for the axonal terminals. However, no such Tan activity has been determined, nor has an N-acetyl-histamine derivative been identified in the photoreceptors to date.

Local proximity of Tan and Ebony immunoreactivity in optic lobes

To verify the involvement of Tan and Ebony in a putative histamine neurotransmitter cycle, we examined whether adjoining sites of Tan and Ebony expression actually exist. The observed proximity of Tan and Ebony immunoreactivity was indeed the most striking result of our colocalization experiments. In the lamina, Tan-containing axonal terminals are surrounded by Ebony-positive epithelial glia. Also, in the columns of the distal medulla, Ebony-containing extensions of medulla neuropil glia and Tan-positive photoreceptor axonal terminals appear lined up side by side. This close association is one prerequisite for a transport of carcinine from glia to photoreceptor axons. The other prerequisite for fast replenishment of the histamine pool in the photoreceptor axon is a transport system for histamine and carcinine to cross the cell membranes of glia and photoreceptor, respectively. This remains to be identified.

We observed, in addition to the Ebony expression in the optic lobe, anti-Ebony labeling at the proximal and distal

border of the lobula and lobula plate (Fig. 4C) and in the central brain (data not shown). This label is not associated with an adjoining site of Tan expression. Therefore, we expect this Ebony to play a role in biogenic amine inactivation by β-alanyl conjugation, which is unrelated to the putative histamine/carcinine cycle in photoreceptor cells.

In summary, localization of Tan in photoreceptor cells and evidence for the close proximity of Tan- and Ebony-expressing cellular structures in the photoreceptor neurons and the associated glia, respectively, substantiate the predicted model of a histamine/carcinine cycle that operates at the photoreceptor synapse.

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