

Molecular Characterization of a Novel Patched-Related Protein in *Apis mellifera* and *Drosophila melanogaster*

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The molecular identification and characterization of the *patched-related (ptr)* gene and protein in *Apis mellifera* and *Drosophila melanogaster* are reported. Ptr proteins are closely related in predicted topology and domain organization to the protein encoded by the *Drosophila* segment polarity gene *patched*. Ptrs have 12 potential transmembrane domains arranged in two sets of 1+5 membrane-spanning segments containing a conserved sterol-sensing domain (SSD) and functional GxxxD and PPXY motifs. Phylogenetic analysis showed that Ptrs belong to a previously uncharacterized class of insect proteins that share a high level of sequence identity. Analysis using quantitative real-time polymerase chain reaction (qPCR) indicates that *ptr* gene is preferentially expressed during embryo stages of *A. mellifera* development; interestingly, this pattern of temporal expression was also observed for the *D. melanogaster* homologue, suggesting that these proteins might be involved in embryo morphogenesis. To understand Ptr function at the molecular level, we investigated the subcellular distribution of *DmPtr*. We have shown by biochemical analysis that *DmPtr* protein is tightly associated with membranes. Consistently, Ptr immunoreactivity appears to be localized at the sites of membrane furrow formation during cellularization of *D. melanogaster* embryos. These studies indicated that Ptrs belong to a previously uncharacterized class of insect transmembrane proteins that share a high level of sequence identity. Our analysis of *ptr* gene expression and protein localization suggest that Ptr might fulfil a developmental role by participating in processes that require growth and stabilization of plasma membrane.

KEYWORDS: *Apis mellifera*; sterol-sensing domain; patched-related; membrane protein; *D. melanogaster*

INTRODUCTION

The membrane protein Patched (Ptc) and its ligand, the cholesterol-modified morphogen Hedgehog (Hh), control numerous processes during embryonic development. In *Drosophila*, Ptc and Hh specify cell fate in the developing embryo and along the anteroposterior (A/P) axis of the adult wing. During vertebrate development, Ptc is involved in neural tube differentiation and axon guidance (Ingham and McMahon, 2001). In hu-

mans, dysfunction of Hh–Ptc signaling is associated with a variety of malignancies (Ingham, 1998). Biochemical and genetic studies indicate that upon binding to Hh, Ptc relieves the inhibition of the activity of another membrane protein, Smoothened (Smo), which transduces the Hh signal to downstream effectors (Hooper and Scott, 1989). Ptc proteins have been identified in a number of species, and their roles in Hh signal transduction appear to be conserved.

Ptc is proposed to contain 12 potential trans-

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membrane domains (Hooper and Scott, 1989), five of which comprise a putative 180-amino acid sterol-sensing domain (SSD) (Carstea et al., 1997; Loftus et al., 1997). A common feature shared by many SSD-containing proteins is their participation in processes that involve the transport of lipids, sterols, or sterol-modified proteins (Kuwabara and Labouesse, 2002). This domain was initially identified in HMG-CoA reductase (HMGCR) and SREBP cleavage activating protein (SCAP), two proteins involved in sterol synthesis and sterol-dependent transcription, respectively (Hampton, 2002). Additional SSD-containing proteins are Niemann-Pick disease type C1 (NPC1) with functions in cholesterol trafficking (Carstea et al., 1997) and Dispatched (Disp), a protein that shares sequence similarity to Ptc and facilitates the release of Hh from Hh-producing cells (Burke et al., 1999).

Recently, a group of membrane proteins, the Patched-related (Ptr), were characterized in *Caenorhabditis elegans* (Kuwabara and Labouesse, 2002). They are closely related in predicted topology to Ptc and Disp and share the greatest sequence similarity within their putative SSDs. Studies using dsRNA-mediated interference suggested that several of the *C. elegans* Ptrs are involved in development processes, including cell growth, patterning, and molting (Zugasti et al., 2005). Functional analysis of *C. elegans* Ptc-1 and Ptrs has revealed that they play essential roles in germline cytokinesis, as well as in different morphogenetic processes that involve vesicle trafficking (Kuwabara et al., 2000; Michaux et al., 2000; Zugasti et al., 2005; Perens and Shaham, 2005). Considering the fact that *C. elegans* lacks the components of the Hh signal transduction pathway, it has been proposed that Ptc-1 and Ptrs perform functions that differ from those previously assigned to Ptc and Disp, among them, the regulation of lipid transport and vesicle trafficking during development (Zugasti et al., 2005; Perens and Shaham, 2005).

Since Ptrs appear to be conserved through evolution (Zugasti et al., 2005), studies can be performed with a variety of model organisms to expand or unify their possible action models. In this regard, we have isolated a *D. melanogaster ptr*

gene (*Dmptr*) in a subtractive hybridization screening designed to identify genes that are differentially expressed at early stages of embryogenesis (González-Agüero et al., 2005). We have cloned the full-length cDNA of *Dmptr* and deduced that the product is a 1,169-amino acid protein with 12 putative transmembrane alpha helices and a SSD conserved domain. When we compared the amino acid identity of the whole protein and the SSD domain among SSD-containing proteins from different species of insects and vertebrates, we found that *DmPtr* belongs to a divergent, previously uncharacterized, class of insect Ptr proteins. In the present study, we extend the analyses of these novel SSD-containing proteins by cloning and characterizing a full-length cDNA encoding a Ptr protein from *A. mellifera* (*AmPtr*). We show that the expression of *ptr* genes from both species is developmentally regulated, being preferentially expressed in embryo stages. Moreover, biochemical analysis revealed that, consistent with our protein sequence prediction, Ptr was found to be associated with embryo membranes, whereas immunohistochemistry analyses allowed us to localize this protein to the growing plasma membranes of newly forming cells in early *D. melanogaster* embryos. Additional studies that are currently in progress will help us to understand Ptr function during development in *D. melanogaster* and to increase our understanding of the mechanisms that regulate cellular activities of insect Ptrs.

MATERIALS AND METHODS

Experimental Insects

Embryos, larvae, pupae, and adult workers of Africanized *A. mellifera* were collected from hives of the experimental apiary of the National Institute of Agropecuary Research, Santiago, Chile. Developmental stages were determined according to criteria established by Jean-Prost (1987). Specimens were washed with 0.1% Ringer-Triton solution (182 mM KCl, 46 mM NaCl, 3 mM CaCl₂, and 10 mM Tris-HCl, pH 7.2), frozen in liquid nitrogen, and stored at -80°C. *D. melanogaster* embryos

and larvae were handled and staged as described in González-Agüero et al. (2005).

RNA Extraction and cDNA Synthesis

Total RNA was extracted using the TRI Reagent Kit (Ambion, Austin, TX) according to the manufacturer's instructions. The quantity and quality of the RNA were assessed by spectrophotometry (OD 260/280) and by electrophoresis on 1.2% (w/v) formaldehyde-agarose gels. Single-strand cDNA was synthesized in a standard reverse transcription reaction, using 200U M-MLV reverse transcriptase (Promega, Madison, WI) and 0.5 µg oligo(dT)₁₅ (Promega). This cDNA was used as template for both standard and quantitative polymerase chain reaction (qPCR) assays.

*Ampt*r and *Dmpt*r cDNA Cloning

Putative *A. mellifera ptr* cDNAs were identified by Blast analysis, using *D. melanogaster Ptr* (*DmPtr*, GenBank accession number NM_136365) as the protein input. Two putative cDNAs (GenBank accession numbers NW_055478 and NW_048484), initially named *Ampt*r1 and *Ampt*r2, with amino acid sequence similarity to the N- and the C-terminal of *DmPtr*, were found. PCR primers (s1: 5'-CGTCAA-GATGCGATAGGAGC-3', and a1: 5'-TATTCTCCG-CTTACTACCACCTGT-3') were designed to test whether *Ampt*r1 and *Ampt*r2 were part of a single transcript. RT-PCR reactions using total RNA from *A. mellifera* embryos as template produced a single PCR product (1,025 bp) that was isolated, cloned into pGEM-T Easy vector (Promega), and sequenced. The resulting composite cDNA sequence (*Ampt*r, 2,620 bp) was assembled from the *Ampt*r1, the *Ampt*r1-*Ampt*r2 amplicon, and *Ampt*r2 sequences and used to design gene-specific primers for 5'- and 3'-RACE PCR.

*Dmpt*r was amplified by long-distance RT-PCR with the gene-specific primers, using Advantage 2 PCR mix (Clontech, Mountain View, CA). As template total RNA isolated from stage 6–7 embryos was used. A single band of ~3,500 bp, that matched the size of the predicted coding sequence, was iso-

lated from the gel, subcloned into pGEM-T Easy vector (Promega), and sequenced.

5'- and 3'-RACE PCR

The 5'- and 3'-ends of the *Ampt*r transcript were amplified using the RLM-RACE GeneRacer™ kit, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Briefly, 5 µg of total RNA from *A. mellifera* embryos was used as the starter material. For 5'-end amplification, the adaptor-ligated cDNA was PCR amplified using Advantage-2 Polymerase Mix (Clontech), GeneRacer™ 5' Primer and 10 µM of Gene-Specific Primer a2 (5'-GACGCA-AACCTGGCAGTGGTAATG-3') under the following conditions: 2 min at 94°C, 5 cycles of 30 s at 94°C and 40 s at 72°C, 5 cycles of 30 s at 94°C and 40 s at 70°C and 25 cycles of 30 s at 94°C, 30 s at 65°C and 40 s at 68°C. The resulting PCR products were used in a Nested PCR reaction with GeneRacer™ 5' Nested Primer and the Gene-Specific Primer a2. The Nested PCR program comprises 2 min at 94°C and 25 cycles of 30 s at 94°C, 30 s at 65°C and 2 min at 68°C. The PCR product (1,100 bp) was cloned into pGEM-T Easy vector and sequenced. For 3'-end amplification, the same adaptor-ligated cDNA was amplified using GeneRacer™ 3' Primer, the Gene-Specific Primer s2 (5'-AGGCGTCCT-CTATGGAATGTCTC-3') and the same PCR enzyme and programs. The resulting PCR product (650 bp) was cloned into pGEM-T Easy vector and sequenced.

qPCR Assays

Samples of cDNA from *A. mellifera* at different stages of development were produced as described later, in the section on RNA Extraction and cDNA Synthesis. To normalize *A. mellifera* data, a spike RNA was in vitro transcribed from the vector pGIBS-*dap* (ATCC 87486) and added to the total RNA prior to cDNA synthesis in a 1:1000 ratio. Specific primers for *Ampt*r (s3: 5'-TTACAATCC-TACCGACAACC-3', and a3: 5'-TTCTCCGCTTAC-TACCACCT-3') and *dap* (ds1: 5'-TTGCATTAGAGC-ACGGAGTC-3' and da1: 5'-GCGTATCTGAAGCGT-TTGG-3') were designed using Primer Premier 5.0

software (BioSoft International, Palo Alto, CA). Reactions were monitored by using LightCycler 1.5 Instrument (Roche, Basel, Switzerland) and LightCycler FastStart DNA Master SYBR Green I (Roche). A standard curve was generated for *Amptr* and *dap* based on serial dilutions (10^1 – 10^{-2} pg/ μ l) of plasmid templates. The thermal cycle conditions were: Pre-incubation at 95°C for 10 min, 40 cycles of 95°C for 3 s, 57°C for 10 s, and 72°C for 25 s. The purity of amplified products was verified by melting curves analysis. Control reactions included a subset of PCR components lacking the cDNA template. The initial amount of *Amptr* in each sample was calculated from the standard curve, using the default (fit point/arithmetic) method of LightCycler Software Version 3.5 (Roche) and normalized to the values of *dap*. Data represent the averaged of two experimental replicates from two independent sets of samples.

Northern Blot Analysis

Total RNA (10 μ g) from *D. melanogaster* staged embryos and third-instar larvae was fractionated in 1% (w/v) agarose gels containing 6% (v/v) formaldehyde. Northern transfer, membrane hybridization, and washing were carried out as described by González-Agüero et al. (2005). A *Dmptr* probe (650 bp) was labeled with [α - 32 P]dCTP, using the random primer labeling method (Invitrogen). Blots were stripped and re-hybridized with a 540-bp-labeled fragment of *ribosomal protein 49 (rp49)* cDNA. Blots were exposed to phosphor screens and scanned with a phosphorimaging instrument (Molecular Imager FX; Bio-Rad, Hercules, CA). Relative hybridization levels were determined by densitometry using the Quantity One software (Bio-Rad).

Antibody Production

An 825-bp fragment of *Dmptr* cDNA of the deduced amino acid sequence was PCR amplified and cloned into pTrcHis2Topo vector (Invitrogen). The resulting histidine-tagged protein was purified using Ni-NTA agarose (Invitrogen) according to the manufacturer's instructions and employed to gen-

erate an antiserum in rabbits following the protocol described by Knudsen (1985). Freund's complete and incomplete adjuvants were used for the primary immunization and for subsequent boosts, respectively. Pre-immune and immune sera were affinity purified against the *DmPtr* fusion protein, previously subjected to SDS-PAGE, and transferred to nitrocellulose. After staining the nitrocellulose with Ponceau S to visualize the protein, the appropriate band was cut out and incubated with the antiserum overnight at 4°C. The nitrocellulose strip was washed four times with 20 mM Tris pH 7.4, 0.1 M NaCl, 0.1% Tween-20, and bound antibodies were eluted from the strips with 100 mM glycine (pH 3.0); the eluted fraction was neutralized with one-tenth volume of Tris 1 M (pH 8.0). On Western blots of embryo extracts, a single band corresponding to the calculated size of *DmPtr* was detected at a dilution of 1:1000.

Transfection and Immunofluorescent Staining of S2R⁺ Cells

Primers specific for the *Dmptr* transcript were used to create a PCR product encompassing the entire open reading frame (ORF). The product was cloned into the pMT/V5-His-TOPO vector (Invitrogen) and sequenced to confirm its identity. The plasmid was then used for the transient transfection of S2R⁺ cells under conditions of Cu²⁺-inducible expression. Cells were cultured in Schneider's *Drosophila* Medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. For transient transfections, 3×10^6 cells were cultured in 35-mm culture dishes for 24 h and then transfected with 3 μ g of vector DNA by using Cellfectin Reagent according to standard techniques (Invitrogen). Expression of *DmPtr* construct was induced 48 h post-transfection by adding CuSO₄ to a final concentration of 0.5 mM to the cell medium. At 24 h later, induced and uninduced (control) cells were harvested and transferred onto coverslips for 4 h before fixing with 4% paraformaldehyde. Cells were permeabilized with phosphate-buffered saline (PBS), 0.1% saponin for 15 min and then blocked with PBS, 5%

BSA, 0.1% saponin for 45 min before incubation with mouse monoclonal anti-V5 antibody (1:500, Sigma). Cells were washed three times in PBS, 0.1% saponin, and incubated with the secondary antibodies: anti-mouse Alexa Fluor 488 (1/500, Molecular Probes, Eugene, OR). Alexa Fluor 546 phalloidin (1:200, Molecular Probes) was used to stain F-actin. Confocal images were collected using a Confocal Laser Scanning Microscope-510 META (Zeiss) and processed using LSM Image Browser software (Zeiss) and Adobe Photoshop 7.0. The pinhole diameters for each fluorescence channel were set between 1.3–1.4 μm . All images were taken using objective Plan-Apochromat 63 \times /1.4 Oil at 1,024-pixel resolution.

***DmPtr* Immunolocalization in Embryos**

D. melanogaster (Canton S) embryos at cellular blastoderm stage were collected as described in González-Aguero et al. (2005). Embryos were dechorionated using 50% commercial bleach solution in PBS, 0.03% Triton X-100. Embryos were fixed in a 1:1 mixture of heptane and 4% formaldehyde for 90 min, vitelline membranes were removed by the hand-peeling procedure (Rothwell and Sullivan, 2000), and then embryos were postfixed in 4% paraformaldehyde for 30 min. Embryos were incubated at 4°C for 16–18 h in blocking solution containing 3% BSA, 0.1% Triton X-100, 50 mM glycine in PBS, and then incubated in *DmPtr* affinity-purified antibody, diluted 1:25. After overnight incubation at 4°C with primary antibody, the samples were incubated for 1 h with Alexa Fluor 546 α -rabbit (1:200, Molecular Probes) antibodies. FITC-conjugated phalloidin (1:200, Sigma) was used to stain F-actin. All the confocal images were obtained and processed as described above. Affinity-purified pre-immune serum does not generate a visible signal (data not shown).

Membrane Association Analysis

In this analysis, 4-h collections of embryos were dechorionated, rinsed, and stored at -80°C . A membrane fraction was prepared from 1.5 g of fro-

zen embryos using equilibrium sedimentation in a sucrose density step gradient as described by Zhang and Hsieh (2000). To examine whether *DmPtr* was an integral or peripheral membrane protein, the membrane fraction was treated with PBS, 0.1 M Na_2CO_3 , pH 10.0 or 1% (v/v) NP-40 in 50 mM Tris-HCl, pH 7.5 for 30 min, and then centrifuged at 30,000g at 4°C for 30 min to generate supernatant and pellet fractions. The pellets were solubilized in SDS sample buffer, and the supernatants were lyophilized, followed by solubilization in SDS sample buffer. Both fractions were analyzed on SDS-PAGE and Western blot (Harlow and Lane, 1999), using the anti-*DmPtr* antibody, diluted 1:1000. As a control of membrane fraction identity we checked for the presence of *D. melanogaster* Ptc in our membrane preparations using a monoclonal anti-Ptc antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA).

Bioinformatics

Initial sequence homology searches were done against honey bee genome (all assemblies) sequence database, using the Blastn and Blastx programs at NCBI. Homologous protein sequences from various invertebrate and vertebrate species, derived as best matches to *D. melanogaster* Ptr, Ptc and Disp protein sequences, were acquired by using NCBI, FlyBase, and BeeBase databases and Blast tools. Domain-based analyses using SMART and InterPro, protein topology, and hydropathy were predicted by using HMMTOP v.2.0. BioEdit v.7.0 software was used to perform a multiple sequence alignment, using ClustalW. A parsimony analysis of the protein sequence alignment was performed using PAUP* v.4.0b10. Bootstrap analyses were conducted using 1,000 resampling. Accession numbers for all genes used for phylogenetic analysis are shown in Figure 2.

RESULTS

Molecular Characterization of *AmPtr* and *DmPtr*

We began a characterization of the *AmPtr* gene by obtaining a full-length cDNA sequence. In do-

ing so, we Blast searched the *A. mellifera* cDNA sequences at NCBI using the deduced amino acid sequence of *Drosophila ptr* (*DmPtr*, 1,169 aa; GenBank accession number NM_136365) as input. The analysis revealed a high sequence identity with two putative cDNAs, named as *AmPtr1* and *AmPtr2*. When the deduced *AmPtr1* and *AmPtr2* amino acid sequences were compared to that of *DmPtr*, we observed that *AmPtr1* showed a 58% of identity with 479 aa from the C-terminal end of *DmPtr*, while *AmPtr2* showed 60% of identity with 317 aa from the N-terminal end of *D. melanogaster* protein, suggesting that *A. mellifera* cDNAs might be part of a single transcript. Following amplification of *A. mellifera* embryo cDNA with primers designed from the nucleotide sequences of *AmPtr1* and *AmPtr2*, we obtained a single PCR product of 1,025 bp. After cloning and sequencing of the PCR product, a composite cDNA sequence of 2,650 bp was assembled and used to design the specific primers for 5'- and 3'-RACE PCR assays. RACE analysis allowed us to identify a single *AmPtr* transcript of 3,825 bp that was also detected by Northern analysis (data not shown). This full-length cDNA containing an ORF of 3,141 bp, starting at nucleotide 308 and ending at nucleotide 3,448, encodes a predicted protein of 1,047 amino acid residues (GenBank accession number EF442429); the stop codon was located 351 bp upstream of the poly(A) tail, and no usual polyadenylation (AAUAAA) signal was found. The intron/exon organization of the *AmPtr* locus was deduced by comparing the full-length cDNA to the corresponding genomic sequence. A Blast search in the *A. mellifera* genome at NCBI database revealed that our *AmPtr* cDNA sequence had a perfect match to exonic sequences of contig *AmeLG8_WGA353_4* (GenBank accession number NW_001253519), which contains the full-length genomic structure for *AmPtr* (26,692 nucleotides). Comparative analysis of the full-length cDNA and the genomic sequence permitted characterization of 9 exons separated by 8 introns.

Hydropathy analysis estimated that *AmPtr* and *DmPtr* have 12 potential transmembrane (TM) domains and a putative SSD, which consists of 158

amino acids that form five consecutive membrane spanning domains (Fig. 1). The predicted topology of *Ptrs* is similar to that calculated for *Ptc*, *Disp*, and *C. elegans* *Ptr* proteins (Kuwabara and Labouesse, 2002), including a long C-terminal intracellular tail and large extracellular loops between TM segments 1 and 2 and 7 and 8. The deduced amino acid sequence of these proteins contain important conserved motifs common to SSD-containing proteins (Fig. 1), such as GxxxD motifs present in the middle of the TM4 and TM10 (Taipale et al., 2002) and a PPXY found in the C-terminal of *Ptrs* from *A. mellifera*, *N. vitripennis* and three *Drosophila* species, which might be a target for ubiquitin ligases (Hicke and Dunn, 2003).

When we compare the amino acid sequence of *AmPtr* and *DmPtr* with *C. elegans* *Ptr*-10 and *Ptr*-2 low level of sequence identities (averaged 23%) were detected even within the conserved SSD regions (34%). However, a screening for *Ptr* homologues at NCBI, Flybase, and Beebase databases retrieved predicted insect gene products with a high percentage of identity (averaged 56%) to *AmPtr* and *DmPtr* (Fig. 1). *Ptr* homologues share a 12-pass modular topology, the presence of an SSD, and putative functional domains such as GxxxD, suggesting that *Ptr* structures are highly conserved among insect species. Then, we asked whether the sequence of the SSD was conserved between the structurally related insect SSD-containing proteins, *Ptc*, *Disp*, and *Ptrs*. Sequence comparisons between the SSD from insect *Ptrs* with equivalent domains from *Ptc* and *Disp* proteins only reach average values of 20% of identity (data not shown). Moreover, pairwise comparisons between complete *Ptr* proteins and *Ptc* and *Disp* from *A. mellifera* and *D. melanogaster* reveal low identity values (between 10.1% and 13.6%). A phylogenetic analysis based on 68 *Ptc*, *Disp*, and *Ptr* protein sequences from various invertebrates and vertebrates species, resolved three separate clades of proteins and further indicated the closer sequence relationships *Ptr* proteins from insect species (Fig. 2). Thus, this result suggests that *Ptrs* belong to a new class of insect SSD-containing proteins.

EF442429 Am XM_001602890 Nvi(5) WNNLTCVDDFLNR AFH LKGLVGVHHPGYFVLPVLLACIGFTGYQRHVE DPEYLFSPINP SKTERAIVEQYFKVNYSHRFNLGRITRPGRFQHVIT 100
 XM_001652962 Aa (4) MGLSTTCVDDFLNR AFH LKGLVGVHHPGYFVLPVLLACIGFTGYQRHVE DPEYLFSPINP SKTERAIVEQYFKVNYSHRFNLGRITRPGRFQHVIT 100
 XM_964117 Tc (43) MVQLKIVDELNKSFFYKLGIVVGRHPGYFVLPVLLACIGFTGYQRHVE DPEYLFSPINP SKTERAIVEQYFKVNYSHRFNLGRITRPGRFQHVIT 100
 XM_311553 Ag NTGQTSVCDNALNKSFFYKLGIVVGRHPGYFVLPVLLACIGFTGYQRHVE DPEYLFSPINP SKTERAIVEQYFKVNYSHRFNLGRITRPGRFQHVIT 100
 NM_136365 Dm NCGISCVDKT LKNSFFYKLGIVVGRHPGYFVLPVLLACIGFTGYQRHVE DPEYLFSPINP SKTERAIVEQYFKVNYSHRFNLGRITRPGRFQHVIT 100
 GA18464 Dp NCGISCVDKT LKNSFFYKLGIVVGRHPGYFVLPVLLACIGFTGYQRHVE DPEYLFSPINP SKTERAIVEQYFKVNYSHRFNLGRITRPGRFQHVIT 100
 GJ20884 Dv (7) TCGISCVDKT LKNSFFYKLGIVVGRHPGYFVLPVLLACIGFTGYQRHVE DPEYLFSPINP SKTERAIVEQYFKVNYSHRFNLGRITRPGRFQHVIT 100

EF442429 Am TSKDGN-ENLRLRTVVFNLELREEDKTRNAKAMVYEGEENYSQICARWLDTCFNNDLDLHHIEYVENKKEENLTFPVTLPVTFGGVSDIK 199
 XM_001602890 Nvi VPKDGN-DNMLRRAVWDELRLLDGLKNAATVHYDGGDTTYKTECARWEGEGCEQNSVLESMETIEFVENGEENLTFPVTLPVTFGGVSDIK 199
 XM_001652962 Aa TSKDGN-KNLLRTEVWELELRLDGLIQAATVHYDGGDTTYKTECARWEGEGCEQNSVLESMETIEFVENGEENLTFPVTLPVTFGGVSDIK 199
 XM_964117 Tc TSKDGN-KNLLRTEVWELELRLDGLIQAATVHYDGGDTTYKTECARWEGEGCEQNSVLESMETIEFVENGEENLTFPVTLPVTFGGVSDIK 199
 XM_311553 Ag TSKDGN-KNLLRTEVWELELRLDGLIQAATVHYDGGDTTYKTECARWEGEGCEQNSVLESMETIEFVENGEENLTFPVTLPVTFGGVSDIK 199
 NM_136365 Dm TSKDGN-KNLLRTEVWELELRLDGLIQAATVHYDGGDTTYKTECARWEGEGCEQNSVLESMETIEFVENGEENLTFPVTLPVTFGGVSDIK 199
 GA18464 Dp TSKDGN-KNLLRTEVWELELRLDGLIQAATVHYDGGDTTYKTECARWEGEGCEQNSVLESMETIEFVENGEENLTFPVTLPVTFGGVSDIK 199
 GJ20884 Dv TSKDGN-KNLLRTEVWELELRLDGLIQAATVHYDGGDTTYKTECARWEGEGCEQNSVLESMETIEFVENGEENLTFPVTLPVTFGGVSDIK 199

EF442429 Am QLMTEVSPVMSQLAVFLTADNARQDA GAWEAEAFLETLRKEV EENIKFHTIYARFASRTLELEENIKTIIPVYFSSFTLMALFVSVTCMMTDWVRSKP 299
 XM_001602890 Nvi QILLS-VPAVQLGYFVFNVDSPRLDAI GAWEAEAFLETLRKEV EENIKFHTIYARFASRTLELEENIKTIIPVYFSSFTLMALFVSVTCMMTDWVRSKP 299
 XM_001652962 Aa QMLTITVSPVSLQLVYFVADSRKQDAR GAWEAEAFLETLRKEV EENIKFHTIYARFASRTLELEENIKTIIPVYFSSFTLMALFVSVTCMMTDWVRSKP 299
 XM_964117 Tc QDLITVSPVSLQLVYFVADSRKQDAR GAWEAEAFLETLRKEV EENIKFHTIYARFASRTLELEENIKTIIPVYFSSFTLMALFVSVTCMMTDWVRSKP 299
 XM_311553 Ag QDLITVSPVSLQLVYFVADSRKQDAR GAWEAEAFLETLRKEV EENIKFHTIYARFASRTLELEENIKTIIPVYFSSFTLMALFVSVTCMMTDWVRSKP 299
 NM_136365 Dm QDLITVSPVSLQLVYFVADSRKQDAR GAWEAEAFLETLRKEV EENIKFHTIYARFASRTLELEENIKTIIPVYFSSFTLMALFVSVTCMMTDWVRSKP 299
 GA18464 Dp QDLITVSPVSLQLVYFVADSRKQDAR GAWEAEAFLETLRKEV EENIKFHTIYARFASRTLELEENIKTIIPVYFSSFTLMALFVSVTCMMTDWVRSKP 299
 GJ20884 Dv QDLITVSPVSLQLVYFVADSRKQDAR GAWEAEAFLETLRKEV EENIKFHTIYARFASRTLELEENIKTIIPVYFSSFTLMALFVSVTCMMTDWVRSKP 299

EF442429 Am WLGLLGNVSAAMATVAAFGLCIYLVDFIGLNLAAFFLMIGIGIDDTFVMLAAWRRTSIMPVPERMAATLSEAAVSIITSLTDMISFFIHLSPFPSSV 399
 XM_001602890 Nvi WLGLLGNVSAAMATVAAFGLCIYLVDFIGLNLAAFFLMIGIGIDDTFVMLAAWRRTSIMPVPERMAATLSEAAVSIITSLTDMISFFIHLSPFPSSV 399
 XM_001652962 Aa WLGLLGNVSAAMATVAAFGLCIYLVDFIGLNLAAFFLMIGIGIDDTFVMLAAWRRTSIMPVPERMAATLSEAAVSIITSLTDMISFFIHLSPFPSSV 399
 XM_964117 Tc WLGLLGNVSAAMATVAAFGLCIYLVDFIGLNLAAFFLMIGIGIDDTFVMLAAWRRTSIMPVPERMAATLSEAAVSIITSLTDMISFFIHLSPFPSSV 399
 XM_311553 Ag WLGLLGNVSAAMATVAAFGLCIYLVDFIGLNLAAFFLMIGIGIDDTFVMLAAWRRTSIMPVPERMAATLSEAAVSIITSLTDMISFFIHLSPFPSSV 399
 NM_136365 Dm FLGLMGNVSAAMATVAAFGLCIYLVDFIGLNLAAFFLMIGIGIDDTFVMLAAWRRTSIMPVPERMAATLSEAAVSIITSLTDMISFFIHLSPFPSSV 399
 GA18464 Dp FLGLMGNVSAAMATVAAFGLCIYLVDFIGLNLAAFFLMIGIGIDDTFVMLAAWRRTSIMPVPERMAATLSEAAVSIITSLTDMISFFIHLSPFPSSV 399
 GJ20884 Dv FLGLMGNVSAAMATVAAFGLCIYLVDFIGLNLAAFFLMIGIGIDDTFVMLAAWRRTSIMPVPERMAATLSEAAVSIITSLTDMISFFIHLSPFPSSV 399

EF442429 Am QIFCYISGFVAVFTFLVHLLTFFTGCVAISGYCEQKLNLSVVCQKVPVLSKSSN- - -RSWLYRLLCTGGVDPDDPNYPTDNPEHGCMSWFRDYLAAALNR 495
 XM_001602890 Nvi QIFCYISGFVAVFTFLVHLLTFFTGCVAISGYCEQKLNLSVVCQKVPVLSKSSN- - -RSWLYRLLCTGGVDPDDPNYPTDNPEHGCMSWFRDYLAAALNR 495
 XM_001652962 Aa RIFCYISGFVAVFTFLVHLLTFFTGCVAISGYCEQKLNLSVVCQKVPVLSKSSN- - -RSWLYRLLCTGGVDPDDPNYPTDNPEHGCMSWFRDYLAAALNR 495
 XM_964117 Tc TIFCYISGFVAVFTFLVHLLTFFTGCVAISGYCEQKLNLSVVCQKVPVLSKSSN- - -RSWLYRLLCTGGVDPDDPNYPTDNPEHGCMSWFRDYLAAALNR 495
 XM_311553 Ag QIFCYISGFVAVFTFLVHLLTFFTGCVAISGYCEQKLNLSVVCQKVPVLSKSSN- - -RSWLYRLLCTGGVDPDDPNYPTDNPEHGCMSWFRDYLAAALNR 495
 NM_136365 Dm RIFCYISGFVAVFTFLVHLLTFFTGCVAISGYCEQKLNLSVVCQKVPVLSKSSN- - -RSWLYRLLCTGGVDPDDPNYPTDNPEHGCMSWFRDYLAAALNR 495
 GA18464 Dp KIFCYISGFVAVFTFLVHLLTFFTGCVAISGYCEQKLNLSVVCQKVPVLSKSSN- - -RSWLYRLLCTGGVDPDDPNYPTDNPEHGCMSWFRDYLAAALNR 495
 GJ20884 Dv KIFCYISGFVAVFTFLVHLLTFFTGCVAISGYCEQKLNLSVVCQKVPVLSKSSN- - -RSWLYRLLCTGGVDPDDPNYPTDNPEHGCMSWFRDYLAAALNR 495

EF442429 Am RPIKILVILVIFGCVLGAALYGLITLREGLDRRRLSKNDSYSIVYDRQDYVREFFPYRIQVVSQYVNSQVPIQEQMENLRSLEASKYISSAPVITYTES 595
 XM_001602890 Nvi PLVKLLVIVIFGCVLGAALYGLITLREGLDRRRLSKNDSYSIVYDRQDYVREFFPYRIQVVSQYVNSQVPIQEQMENLRSLEASKYISSAPVITYTES 595
 XM_001652962 Aa GWVKAFVIVIFGCVLGAALYGLITLREGLDRRRLSKNDSYSIVYDRQDYVREFFPYRIQVVSQYVNSQVPIQEQMENLRSLEASKYISSAPVITYTES 595
 XM_964117 Tc GLVKVIVIVIFGCVLGAALYGLITLREGLDRRRLSKNDSYSIVYDRQDYVREFFPYRIQVVSQYVNSQVPIQEQMENLRSLEASKYISSAPVITYTES 595
 XM_311553 Ag GWTKVIVIVIFGCVLGAALYGLITLREGLDRRRLSKNDSYSIVYDRQDYVREFFPYRIQVVSQYVNSQVPIQEQMENLRSLEASKYISSAPVITYTES 595
 NM_136365 Dm KWCKAIVIVIFGCVLGAALYGLITLREGLDRRRLSKNDSYSIVYDRQDYVREFFPYRIQVVSQYVNSQVPIQEQMENLRSLEASKYISSAPVITYTES 595
 GA18464 Dp KWCKAIVIVIFGCVLGAALYGLITLREGLDRRRLSKNDSYSIVYDRQDYVREFFPYRIQVVSQYVNSQVPIQEQMENLRSLEASKYISSAPVITYTES 595
 GJ20884 Dv KWCKAIVIVIFGCVLGAALYGLITLREGLDRRRLSKNDSYSIVYDRQDYVREFFPYRIQVVSQYVNSQVPIQEQMENLRSLEASKYISSAPVITYTES 595

EF442429 Am WLRNPLNSYANN- - -ATDVIDEKSFKELKDWLSPKSWLDFVDPKPEEHIASRFLQAVNVSSTNQEIMVVKELRQICADSPNLASVFPYVYVFF 693
 XM_001602890 Nvi WLRNPLNSYANN- - -ATDVIDEKSFKELKDWLSPKSWLDFVDPKPEEHIASRFLQAVNVSSTNQEIMVVKELRQICADSPNLASVFPYVYVFF 693
 XM_001652962 Aa WLRNPLNSYANN- - -ATDVIDEKSFKELKDWLSPKSWLDFVDPKPEEHIASRFLQAVNVSSTNQEIMVVKELRQICADSPNLASVFPYVYVFF 693
 XM_964117 Tc WLRNPLNSYANN- - -ATDVIDEKSFKELKDWLSPKSWLDFVDPKPEEHIASRFLQAVNVSSTNQEIMVVKELRQICADSPNLASVFPYVYVFF 693
 XM_311553 Ag WLRNPLNSYANN- - -ATDVIDEKSFKELKDWLSPKSWLDFVDPKPEEHIASRFLQAVNVSSTNQEIMVVKELRQICADSPNLASVFPYVYVFF 693
 NM_136365 Dm WLRNPLNSYANN- - -ATDVIDEKSFKELKDWLSPKSWLDFVDPKPEEHIASRFLQAVNVSSTNQEIMVVKELRQICADSPNLASVFPYVYVFF 693
 GA18464 Dp WLRNPLNSYANN- - -ATDVIDEKSFKELKDWLSPKSWLDFVDPKPEEHIASRFLQAVNVSSTNQEIMVVKELRQICADSPNLASVFPYVYVFF 693
 GJ20884 Dv WLRNPLNSYANN- - -ATDVIDEKSFKELKDWLSPKSWLDFVDPKPEEHIASRFLQAVNVSSTNQEIMVVKELRQICADSPNLASVFPYVYVFF 693

EF442429 Am DQFELVLRPISIQCMVFGALVMMILSFIPIPNVMSCLWVAFSISIELGVAGYMALWVLDVSDLSMNLIMC GFSVDFTAHICYAMSSKQKTPEDRVKE 793
 XM_001602890 Nvi DQFELVLRPISIQCMVFGALVMMILSFIPIPNVMSCLWVAFSISIELGVAGYMALWVLDVSDLSMNLIMC GFSVDFTAHICYAMSSKQKTPEDRVKE 793
 XM_001652962 Aa DQFELVLRPISIQCMVFGALVMMILSFIPIPNVMSCLWVAFSISIELGVAGYMALWVLDVSDLSMNLIMC GFSVDFTAHICYAMSSKQKTPEDRVKE 793
 XM_964117 Tc DQFELVLRPISIQCMVFGALVMMILSFIPIPNVMSCLWVAFSISIELGVAGYMALWVLDVSDLSMNLIMC GFSVDFTAHICYAMSSKQKTPEDRVKE 793
 XM_311553 Ag DQFELVLRPISIQCMVFGALVMMILSFIPIPNVMSCLWVAFSISIELGVAGYMALWVLDVSDLSMNLIMC GFSVDFTAHICYAMSSKQKTPEDRVKE 793
 NM_136365 Dm DQFELVLRPISIQCMVFGALVMMILSFIPIPNVMSCLWVAFSISIELGVAGYMALWVLDVSDLSMNLIMC GFSVDFTAHICYAMSSKQKTPEDRVKE 793
 GA18464 Dp DQFELVLRPISIQCMVFGALVMMILSFIPIPNVMSCLWVAFSISIELGVAGYMALWVLDVSDLSMNLIMC GFSVDFTAHICYAMSSKQKTPEDRVKE 793
 GJ20884 Dv DQFELVLRPISIQCMVFGALVMMILSFIPIPNVMSCLWVAFSISIELGVAGYMALWVLDVSDLSMNLIMC GFSVDFTAHICYAMSSKQKTPEDRVKE 793

EF442429 Am SLYSGLPVGQASTLGLLALVLAGTYIFWVFFKMVFLVIFFGAMHGLFLLPVLLSLFGPQSCYHNDKKTDTKQQRKISEKDLNC- - -ELQHPY 887
 XM_001602890 Nvi CLYSGLPVGQASTLGLLALVLAGTYIFWVFFKMVFLVIFFGAMHGLFLLPVLLSLFGPQSCYHNDKKTDTKQQRKISEKDLNC- - -ELQHPY 887
 XM_001652962 Aa ALYGLGMPVQGSTLGLVALLAQSYIFLVFFKMVFLVIFFGAMHGLFLLPVLLSLFGPQSCYHNDKKTDTKQQRKISEKDLNC- - -ELQHPY 887
 XM_964117 Tc CLYALGLPVGQASTLGLLALVLAGTYIFWVFFKMVFLVIFFGAMHGLFLLPVLLSLFGPQSCYHNDKKTDTKQQRKISEKDLNC- - -ELQHPY 887
 XM_311553 Ag ALYSGLPVGQASTLGLVALLAQSYIFLVFFKMVFLVIFFGAMHGLFLLPVLLSLFGPQSCYHNDKKTDTKQQRKISEKDLNC- - -ELQHPY 887
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 GA18464 Dp ALYSGLPVGQASTLGLVALLAQSYIFLVFFKMVFLVIFFGAMHGLFLLPVLLSLFGPQSCYHNDKKTDTKQQRKISEKDLNC- - -ELQHPY 887
 GJ20884 Dv ALYSGLPVGQASTLGLVALLAQSYIFLVFFKMVFLVIFFGAMHGLFLLPVLLSLFGPQSCYHNDKKTDTKQQRKISEKDLNC- - -ELQHPY 887

EF442429 Am VPHPTLYYHSSGPD- - -KNFGQSPATSLAAFD- - -DRDPLGLTSEDSNSYEGSSQSRKKEGLEH- - -QEN-QRRHDVCRKSIQV- - 966
 XM_001602890 Nvi VPHPTLLVYHKGDT- - -SPAVSNLALE- - -DRDLGLTSEDSNSYEGSSQSRKKEGLEH- - -QEN-QRRHDVCRKSIQV- - 966
 XM_001652962 Aa CISHPQLALTGA- - -FGSKTFAGAPYKAYG- - -DEKDLGLTSGE- - -DSSESSSSKQRRQAIED- - -EMTRRYEGWRRSHTN- - 969
 XM_964117 Tc CISHPQLALTGA- - -FGSKTFAGAPYKAYG- - -DEKDLGLTSGE- - -DSSESSSSKQRRQAIED- - -EMTRRYEGWRRSHTN- - 969
 XM_311553 Ag CISHPQLALTGA- - -FGSKTFAGAPYKAYG- - -DEKDLGLTSGE- - -DSSESSSSKQRRQAIED- - -EMTRRYEGWRRSHTN- - 969
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EF442429 Am - - - - -LYMSQFQTASPAAPPPD- - - 985
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 NM_136365 Dm YG- - - - -GQAGAFQDLYGQKQVSAEWRQRDLTHEQQQRQ- - -QRRSPFENYRQDVEIDMQARRNSHGDVIDLHG- - -TPNSSVEERFR- - - 1061
 GA18464 Dp YGGPNSIIAGHQAQFQADPDLGQVSAEWRQRDLTHEQQQRQ- - -QRRSPFENYRQDVEIDMQARRNSHGDVIDLHG- - -TPNSSVEERFR- - - 1071
 GJ20884 Dv YGN- - - - -GGQIADQFPQDLYGQVSAEWRQRDLTHEQQQRQ- - -QRRSPFENYRQDVEIDMQARRNSHGDVIDLHG- - -TPNSSVEERFR- - - 1075

EF442429 Am - - - - -YSGTLPEPVNQEQRQAVRVS YGYPY AQRANQLHR- - - - -DHRRSSSHY 1032
 XM_001602890 Nvi - - - - -YSGTLPEPVNQEQRQAVRVS YGYPY AQRANQLHR- - - - -DHRRSSSHY 1032
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 XM_964117 Tc - - - - -NRRRYENPPTTRKVN- - -DQHRRYSPLEQRYK- - -NQGLARSSSH 1040
 XM_311553 Ag - - - - -NRRRYENPPTTRKVN- - -DQHRRYSPLEQRYK- - -NQGLARSSSH 981
 NM_136365 Dm GEPFSAESODDS- - -YRHOQ- - - -IMAMPAGSAPSAKR- - -YHRRSSSDS- - -TSRHRQWPA- - -NIEERRARAYS- - -AHNRPE- - - -TALTSYARSSSH 1146
 GA18464 Dp DRVLSGGSODES- - -YRQSGRSDSP- - -IAAQLPLNGSSAKR- - -YHRRSSSDS- - -TSRHRQWPA- - -NIEERRARAYS- - -AHNRPE- - - -TAPPSAYRSSSH 1162
 GJ20884 Dv QQALSAGSODESSYRHQP- - - -ELSAAPLASS- - -SAKRYHRRSSSDS- - -TRYHRQWPA- - -NIEERRARAYS- - -SQRSHSSHYETA- - -TAIAASFAHRSSSH 1168

EF442429 Am NLYHSS- - - - -RYLSDSRYP- - - 1047
 XM_001602890 Nvi NLYR- - - - - 1017
 XM_001652962 Aa NLYYRQPPQRTSSQSSLHQLRHMGMDFP- - - 1156
 XM_964117 Tc NLYH- - - - -RYIQELRFP- - - 1054
 XM_311553 Ag - - - - - 981
 NM_136365 Dm NLYQR- - - - -NGKSSKPPTYQGDYYH- - - 1169
 GA18464 Dp NLYQR- - - - -QKNKATKPPPTTYQGDYYH- - - 1187
 GJ20884 Dv NLYQR- - - - -SSKAMHPPPTTYQGDYYH- - - 1189

Figure 1

Expression Pattern of *Amptr* and Its Homologue *Dmptr*

To examine the expression of *Amptr*, we performed quantitative PCR analyses by using RNA preparations from different developmental stages of *A. mellifera*. As shown in Figure 3A, the relative expression level of *Amptr* reached its highest value in embryonic stages and markedly decreased in 4-day larvae, remaining at low levels in pupae and adults. This result indicates that *Amptr* is transcriptionally regulated during the life cycle of *A. mellifera* and suggest its possible involvement in *A. mellifera* embryogenesis. Next, we evaluated whether the *D. melanogaster* homologue of *Amptr* exhibited a similar pattern of developmental expression. Northern blot analysis (Fig. 3B) revealed that *Dmptr* transcript was undetectable in preblastoderm embryos (stages 2–3). In 5 and 6–8 embryonic stages, the abundance of the transcript dramatically increases; interestingly, this is a period during which key morphogenetic process take place to cellularize the embryo and initiate gastrulation. *Dmptr* transcript levels declined sharply in 9–12-stage embryos and remain low throughout the rest of embryogenesis and in the third-instar larvae, indicating that embryonic levels of *Dmptr* transcript are subject to strong temporal regulation.

The higher level of *Dmptr* gene expression at cellularization led us to hypothesize that this gene might play a role during early embryo development. For an indication of its function, we examined the distribution of the protein by immunofluorescence and confocal microscopy. The distribution of

DmPtr protein during cellularization of the embryo (stage 5) was analyzed using a polyclonal antibody raised against *DmPtr* (see Materials and Methods). Double immunofluorescence analyses were performed by staining the embryos with the anti-*DmPtr* antibody and FITC-phalloidin to label actin filaments (F-actin), which are located in close apposition to the cell membranes during embryo cellularization. Sagittal views of the embryo during the first or slow phase of cellularization reveal that *DmPtr* immunoreactivity is mainly detected in the apical domain of the newly forming cells and to a moderate extent, is present along the growing plasma membranes (Fig. 4A), giving rise to a honeycomb pattern that can be observed in transversal focal sections of the cellular blastoderm surface (Fig. 4B). F-actin-rich cortical layer covers the whole surface of the embryo and clearly marks the ingrowing membranes (Fig. 4A',B'). During the second or fast phase of cellularization, *DmPtr* immunoreactivity is observed on the growing lateral membrane, which extended downward from the cell surface and enclosed each nucleus within a long columnar cell (Fig. 4C–C', arrowheads), whereas in the apical cellular domain the immunoreactivity appears to be localized to the cytoplasm and/or to cytosolic vesicles of uniform size evenly distributed between the cell apices and the nuclei (Fig. 4C). *DmPtr* staining was not detected in the basal constrictive rings where F-actin concentrates at this phase of cellularization (Fig. 4C', arrows). Before cellularization, we did not detect *Ptr* immunoreactivity at a level that was above background. Thus, our observations of protein distribution are consistent with the idea that *DmPtr* might have a role during embryo cellularization.

Subcellular Distribution of *DmPtr*

As *Ptr* proteins are predicted to contain 12 transmembrane spans, they should be localized either to the cell surface or to a membranous cellular compartment. To test the subcellular distribution of *Ptr*, we performed cellular fractionation experiments with early *D. melanogaster* embryos, since we could detect the *D. melanogaster* protein in the

Fig. 1. Comparison of the structures of insect SSD-containing proteins. Protein sequence alignment of selected insect *Ptrs*, including *A. mellifera* (EF442429 Am), *N. vitripennis* (XM_001602890 Nvi), *A. aegypti* (XM_001652962 Aa), *T. castaneum* (XM_964117 Tc), *A. gambiae* (XM_311553 Ag), *D. melanogaster* (NM_136365 Dm), *D. pseudoobscura* (GA18464 Dp), and *D. virilis* (GJ20884 Dv). Sequence similarities are shown by the intensities of the gray background. Protein features are shown as follows: red bars, transmembrane domains; blue bars, SSD; yellow open box, GxxxD motif; green line, PPXY motifs.

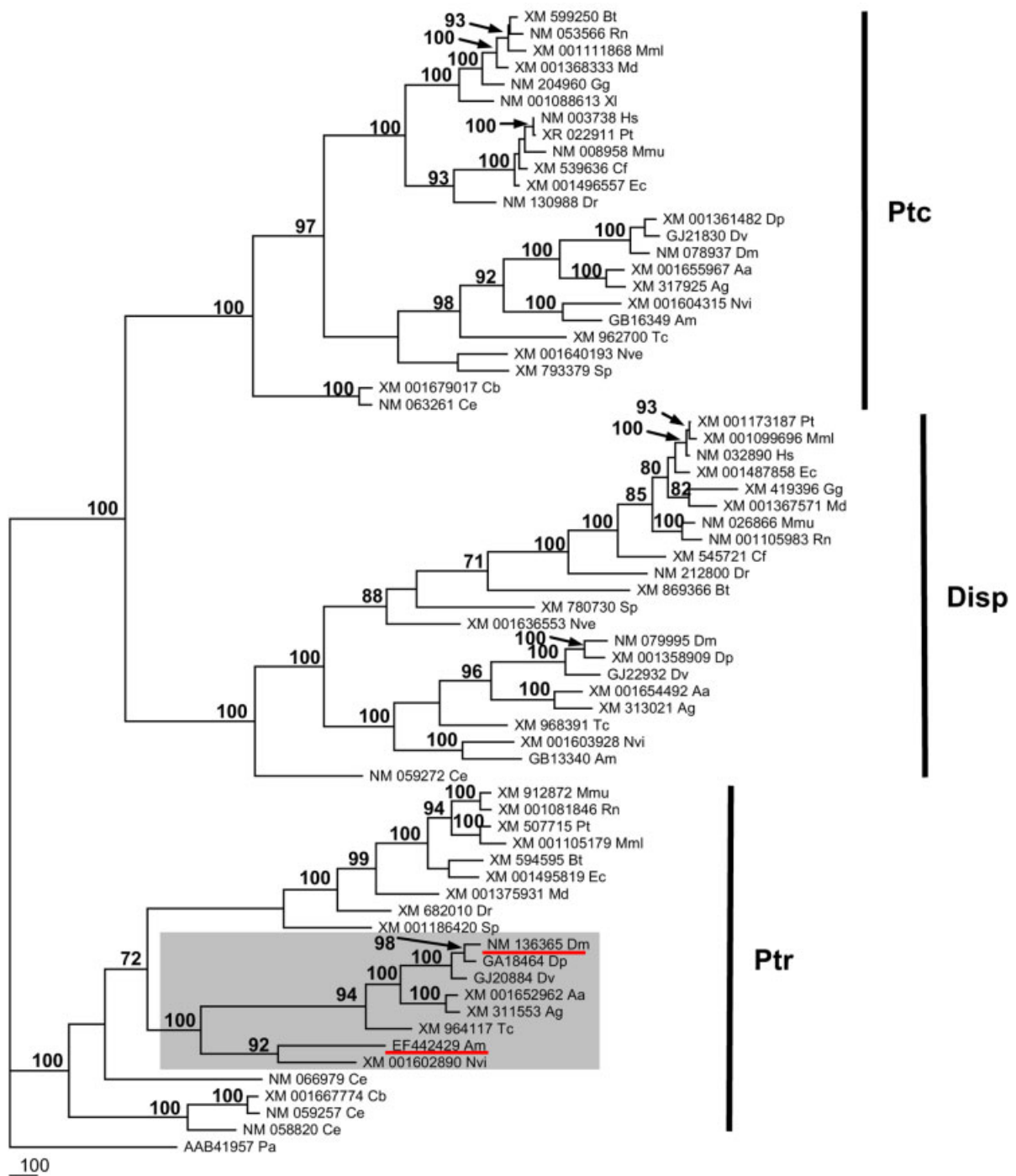


Fig. 2. Phylogenetic tree comparing the sequences of Ptr, Ptc, and Disp proteins. Analysis was performed with multiple alignments from amino acid sequences using ClustalW. Bootstrap values (1,000 pseudo-replicates) are shown above branches. The tree is displayed rooted by *Pseudomonas aeruginosa* RND family exporter MexD (GenBank accession number AAB41957). Protein sequences were from: Aa, *Aedes aegypti*; Ag, *Anopheles gambiae*; Am, *Apis mellifera*; Bt, *Bos taurus*; Cb, *Caenorhabditis briggsae*; Ce, *Caenorhabditis elegans*; Cf, *Canis familiaris*; Dm, *Drosophila melanogaster*; Dp, *Drosophila pseudoobscura*; Dr, *Danio rerio*; Dv, *Drosophila virilis*; Ec, *Equus caballus*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Md, *Monodelphis domestica*; Mml, *Macaca mulatta*; Mmu, *Mus musculus*; Nve, *Nematostella vectensis*; Nvi, *Nasonia vitripennis*; Pt, *Pan troglodytes*; Rn, *Rattus norvegicus*; Sp, *Strongylocentrotus purpuratus*; Tc, *Tribolium castaneum*; XI, *Xenopus laevis*. GenBank accession numbers are indicated. AmPtr and DmPtr are underlined; insect Ptrs are shown inside gray box. [Color figure can be viewed in the online issue which is available at www.interscience.wiley.com]

phila pseudoobscura; Dr, *Danio rerio*; Dv, *Drosophila virilis*; Ec, *Equus caballus*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Md, *Monodelphis domestica*; Mml, *Macaca mulatta*; Mmu, *Mus musculus*; Nve, *Nematostella vectensis*; Nvi, *Nasonia vitripennis*; Pt, *Pan troglodytes*; Rn, *Rattus norvegicus*; Sp, *Strongylocentrotus purpuratus*; Tc, *Tribolium castaneum*; XI, *Xenopus laevis*. GenBank accession numbers are indicated. AmPtr and DmPtr are underlined; insect Ptrs are shown inside gray box. [Color figure can be viewed in the online issue which is available at www.interscience.wiley.com]

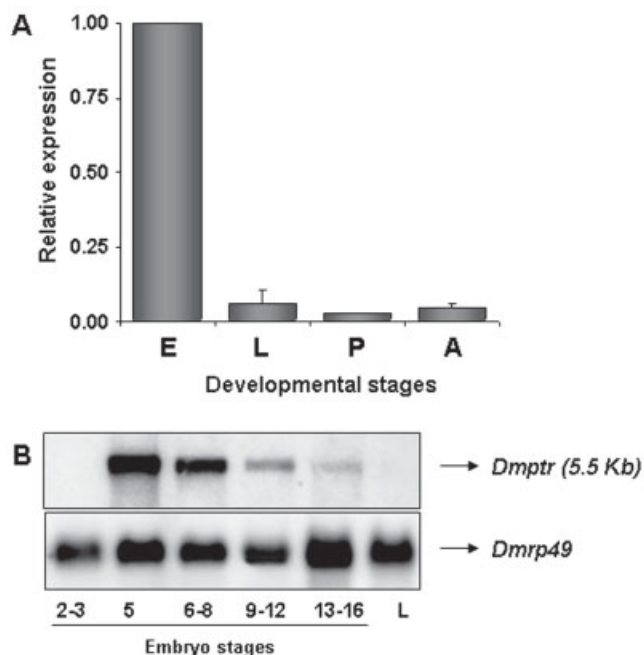


Fig. 3. Analysis of *Amptr* and *Dmptr* gene expression. **A**: Total RNA was isolated from 2-day embryos (E), 4-day larvae (L), unpigmented pupae (P), and adults (A). For each developmental stage, the relative expression of *Amptr* was normalized using *dap* spike mRNA (see Materials and Methods). The results are presented as a fraction of the highest value of relative expression. **B**: *D. melanogaster* total RNA (10 μ g) from stages 2–3, 5, 6–8, 9–12, and 13–16 embryos and third instar larva (L) were hybridized with [α - 32 P]dCTP-labeled probe for *Dmptr* (650 bp, upper panel) or *Dmrp49* (540 bp, lower panel). Estimated molecular weight of *Dmptr* mRNA is shown inside the parenthesis. Relative *Dmptr* transcript levels were estimated by normalization to *rp49* hybridization signals.

different fractions by using the anti-Ptr antibody. In doing so, membrane fractions were isolated according to their buoyant densities in sucrose step gradients (Zhang and Hsieh, 2000) and treated with various solubilizing reagents to examine the nature of *DmPtr* association with membranes (Fig. 5A, lane 1). *DmPtr* remains associated with the membranes after PBS and alkaline treatment at pH 10, indicating a tight association between *DmPtr* and membrane (Fig. 5, lanes 2–3 and 4–5). When the nonionic detergent NP-40 was used to solubilize the membranes, a large fraction of *DmPtr* could be released by the detergent (Fig. 5, lanes 6–7), we noted that a minor fraction of *DmPtr* remained

insoluble, this was probably due to an incomplete solubilization of the membranes, since we did not perform serial NP-40 washes. These results are consistent with the prediction that *DmPtr* is a transmembrane protein. In addition, we examined the subcellular localization of *DmPtr* using an epitope-tagged version of the protein. A construct expressing *DmPtr*-V5 fusion protein was transfected into a *Drosophila* culture cell line (S2R+) and the subcellular localization of *DmPtr* was determined by immunofluorescence using anti-V5 antibodies (Fig. 5B, panels a and c). We observed that *DmPtr* is localized to the cell surface and dispersed throughout the cytoplasm in a punctate pattern. In a higher-magnification view, *DmPtr*-V5 immunostaining can be observed along the cell filopodia (Fig. 5B, panels c–e). No immunostaining was detected in uninduced cells with the anti-V5 antibody (data not shown).

DISCUSSION

Sequence Analysis of *AmPtr* and *DmPtr* Proteins

A. mellifera and *D. melanogaster* Ptrs share characteristics common to all known Ptc, Disp, and *C. elegans* Ptr proteins (Kuwabara and Labouesse, 2002). These proteins are predicted to have 12-membrane spanning domains with cytoplasmic N- and C-terminal ends. The membrane domains can be further subdivided into two cassettes of 1+5, which are separated by a large intracellular loop. Carried within the first set of TM domains is an SSD, a phylogenetically conserved domain that has been identified in several multipass transmembrane proteins involved in the transport of lipids, sterols, or sterol-modified proteins and in cholesterol homeostasis (Kuwabara and Labouesse, 2002). The role of the SSD is still open to debate; questions have arisen as to whether SSD has a function common to all the SSD-containing proteins. Recent studies indicate that in HMG-CoA reductase and SCAP, the SSD modulates sterol-dependent interactions with the resident endoplasmic reticulum proteins, Insig-1 and Insig-2 (Yang et al., 2002; Sever et al., 2003), whereas in NPC1, SSD mediates LDL-cholesterol trafficking to the plasma

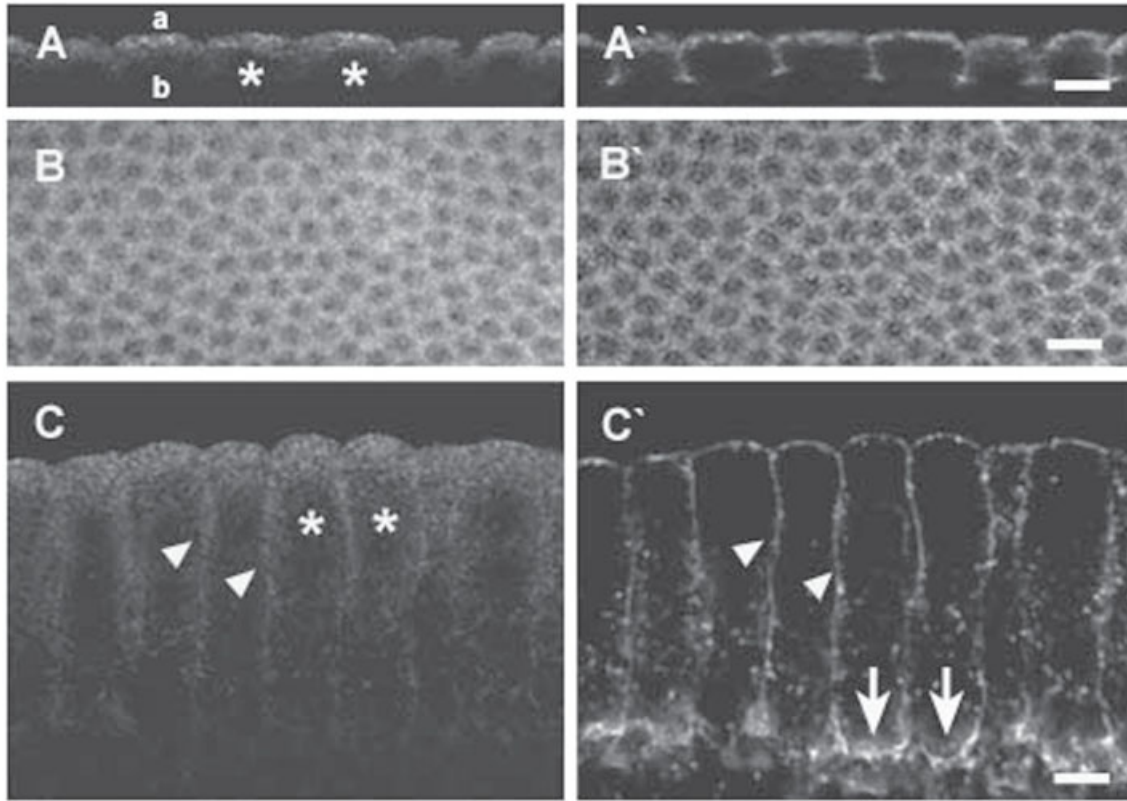


Fig. 4. *DmPtr* localization by antibody staining and confocal microscopy in whole-mount stage 5 embryos. Embryos were double-stained for: *DmPtr* (A,B,C); and F-actin; (A',B',C') a, apical; b, basal; *, nuclei. (A and B) Sections from an embryo during the slow phase of cellularization. *DmPtr* is localized to the apical cellular region and moderately to the newly formed furrows as seen in a sagittal

image of the embryo (A) or in a surface view (B). C: *DmPtr* distribution during the fast phase of cellularization. *Ptr* immunoreactivity is detected along the length of the growing lateral membrane (arrowheads), no immunoreactivity was observed to associate with the basal F-actin constrictive rings (C', arrows). Scale bars = 5 μ m in A; 15 μ m in B; 5 μ m in C.

membrane and endoplasmic reticulum (Millard et al., 2005). In the case of *Ptc*, an obvious suggestion would be that the SSD has a role in binding the cholesterol adducts of *Hh*. However, the data indicate that the absence of the cholesterol moiety does not alter the *in vitro* affinity of *Ptc* for *Hh* (Pepinsky et al., 1998). Moreover, mutations in the SSD of *D. melanogaster Ptc* do not interfere with *Hh* binding *in vivo* (Martin et al., 2001; Strutt et al., 2001). Instead, the SSD seems to regulate the vesicular trafficking of *Ptc* between the plasma membrane and the endosomes (Martin et al., 2001; Strutt et al., 2001). A similar function has been reported for the SSD in *C. elegans Ptr-7*, since mutations in this domain affect the subcellular localization of this protein (Perens and Shaham, 2005).

The predicted topology of *AmPtr* and *DmPtr* includes a long C-terminal intracellular tail showing the most prominent structural differences between insect *Ptrs* (Fig. 1). The C-terminal tail of *D. melanogaster Ptc* is required for both protein internalization and turnover (Lu et al., 2006) and contains a PPXY motif that is predicted to bind HECT and WW domain ubiquitin ligases, such as the *Drosophila Nedd4* protein, which targets transmembrane receptors containing the PPXY motif for endocytosis (Hicke and Dunn, 2003). Interestingly, the C-terminal of *Ptrs* from *A. mellifera*, *N. vitripennis*, and the three *Drosophila* species contain a PPXY sequence that might be a target for such ubiquitin ligases, whereas *Ptr* proteins from *A. aegypti*, *A. gambiae*, and *T. castaneum* seem to lack

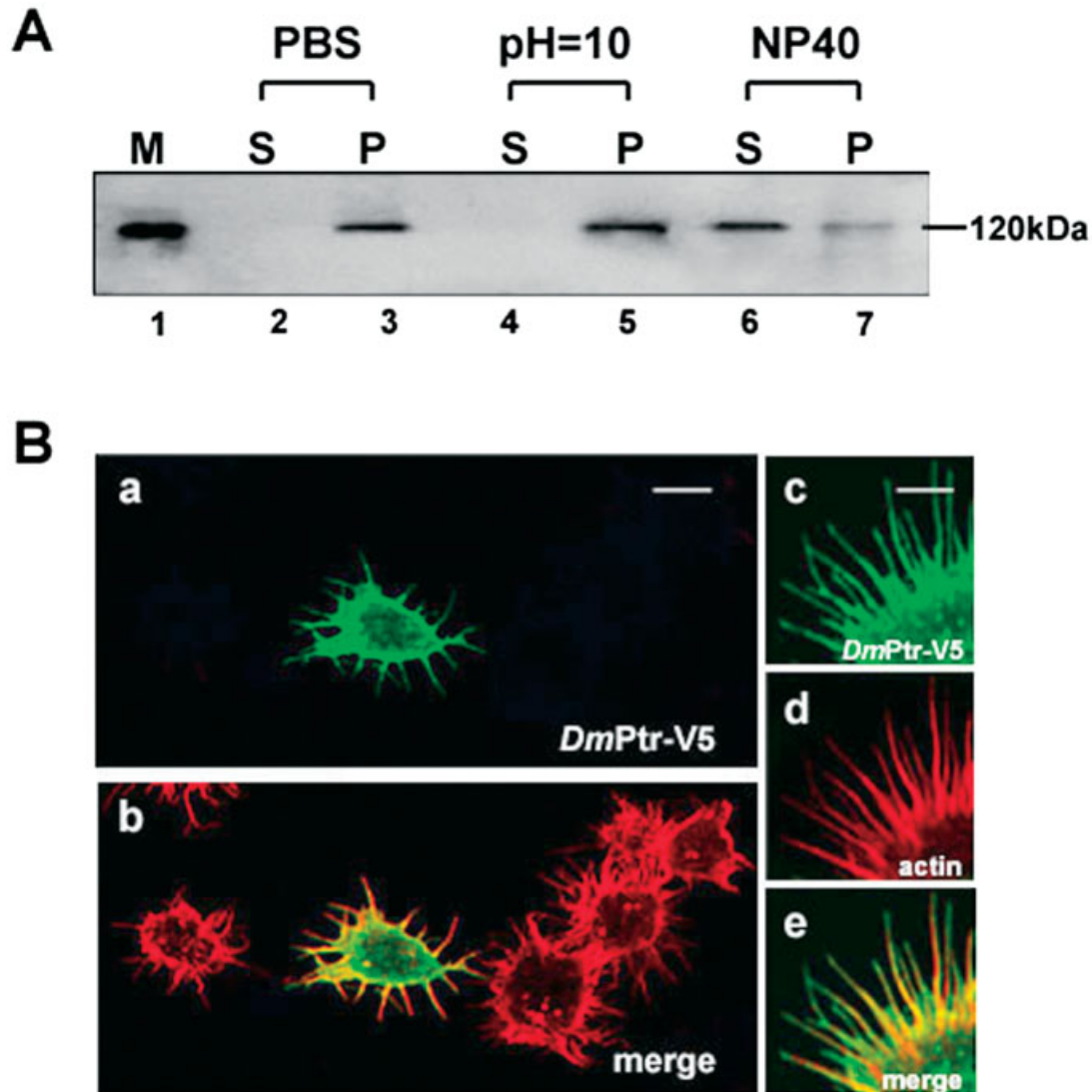


Fig. 5. Membrane association of *DmPtr*. **A**: Embryos (0–4 h) membranes were prepared by cellular fractionation on a sucrose step gradient (M). Three solubilizing reagents were used to wash the membrane fraction. These reagents were PBS, 100 mM Na₂CO₃ (pH 10), and 1% NP-40. After washing, the same volume of supernatant (S) and pellet (P) was loaded on the gel and subjected to Western

blot analysis using an anti-*DmPtr* polyclonal antibody (1:1000 dilution). Estimated molecular weight of *DmPtr*, 120 kDa. **B**: Subcellular localization of the *DmPtr-V5* fusion protein. (a–e) S2R+ cells were transiently transfected with the fusion protein and stained with an anti-V5 monoclonal antibody (green in a, b, c, and e) and phalloidin to visualize actin (red in b, d, and e). Scale bar = 3 μm.

this conserved motif (Fig. 1). Another structural characteristic of SSD-containing proteins is the presence of large extracellular loops between TM segments 1 and 2 and 7 and 8. The extracellular loops appear to be necessary for protein function since deletion of *D. melanogaster* Ptc second loop blocks the ability of cells to bind and transduce Hh signal (Briscoe et al., 2001). Similarly, deletion

of *C. elegans* Ptr-7 first and second loop affects protein function, suggesting that Ptr activity might be regulated by extracellular signals (Perens and Shaham, 2005). Insect Ptrs, as well as Ptc, Disp, and *C. elegans* Ptr proteins, share an overall similarity in membrane topology with members of the RND family of prokaryotic permeases (Tseng et al., 1999). In addition, all of the present GxxxD motifs,

in the middle of the TM4 and the TM10, these residues and their position in the protein are highly conserved between RND permeases, and they are essential for their transporter function (Tseng et al., 1999). Consistently with this structural similarity, it has been reported that Ptc and Disp are able to behave as transmembrane molecular transporter (Ma et al., 2002; Taipale et al., 2002). Therefore, topology and domain analysis indicate a close structural relation between the Ptr proteins described here and SSD-containing proteins from other species.

Our data indicate that the predicted *AmPtr* and *DmPtr* protein sequences are highly conserved among other insect species as *N. vitripennis* (67% and 51% identity), *A. gambiae* (59% and 62% identity), *T. castaneum* (57% and 54% identity), *A. aegypti* (55% and 63% identity), *D. pseudoobscura* (51% and 90% identity), and *D. virilis* (51% and 86% identity). Sequence comparisons of the Ptr insect orthologues indicate that these proteins are more closely related to each other than to other SSD-containing proteins, such as Ptc and Disp. Thus, insect Ptrs appear to belong to a conserved, previously uncharacterized subfamily of SSD-containing proteins.

Expression Analysis of *ptr* Gene and Its Encoded Protein


Our results on the temporal expression pattern of *AmPtr* gene suggest that it might be playing a role during *A. mellifera* embryogenesis. To gain insight into the cellular functions of Ptrs, we took advantage of the well-established *D. melanogaster* model to analyze the expression pattern of the *DmPtr*, which shares a high level of sequence identity with *AmPtr*. The results indicate that *DmPtr* transcripts are highly accumulated during embryo cellularization (stage 5), a developmental stage at which the plasma membrane invaginates to form cleavage furrows between 6,000 nuclei that are localized at the cortex of the embryo. Cellularization proceeds first in a slow phase (40 min) and then in a fast phase (20 min) to form individual cells within a polarized epithelium (Foe et al., 1993).

When we analyzed Ptr protein distribution using an anti-*DmPtr* antibody, we observed that during the slow phase of cellularization *DmPtr* was detected on the apical cellular domain. As cellularization progress Ptr immunoreactivity was localized at the sites where membrane formation is taking place. The distribution of *DmPtr* was consistent with a peripheral localization of the *DmPtr*-V5 fusion protein in S2R+ cells and with our biochemical analysis showing that *DmPtr* was tightly associated with embryo membranes. The spatial expression pattern of *DmPtr* in embryos during cellularization is reminiscent of that described for *D. melanogaster* transmembrane membrane protein Syntaxin 1 (Burgess et al., 1997), which displays a similar enrichment on the newly forming lateral cell membranes and is required for a normal embryo cellularization, suggesting that *DmPtr* is recruited to the invaginating membranes and might play a role in furrow extension.

To our knowledge, Ptr proteins have been only characterized in *C. elegans*, where they seem to regulate vesicular transport during developmental processes that require the growth and stabilization of the plasma membrane (Kuwabara et al., 2000; Perens and Shaham, 2005). Within the *C. elegans* germline syncytium, bipolar cytokinesis involves membrane fusion that requires vesicular transport and stabilization of the incomplete membrane furrows within the syncytium. It has been suggested that in *C. elegans*, *ptc-1* and Ptr proteins contribute to the process of syncytial cytokinesis by helping establish or maintain the incomplete plasma membrane furrows that separate individual nuclei within the syncytium (Kuwabara, 2000). As it has been mentioned, the evidence generated in the *C. elegans* model, which lacks other Hh pathway components, suggests that a novel, or perhaps ancestral, activity for Ptc and Ptrs has been uncovered that is not dependent on Hh or Smo but relies on a fundamental mechanism of cytokinesis that implies the regulation of vesicle trafficking. Interestingly, it has been shown that the regulated mobilization of intracellular pools of vesicles at defined sites of the plasma membrane underlies membrane growth and surface polarization during *D. melanogaster*

cellularization. Moreover, proteins that are specifically induced during cellularization, such as Nullo and Slam (Hunter et al., 2002; Lecuit et al., 2002), represent developmental regulators of membrane growth during cellularization. Whether the insect Ptrs are also involved in such functions constitute the obvious questions which will support our future investigations. Nevertheless, our results on the temporal expression pattern of *Dmptr* gene along with Ptr protein localization at sites where membrane addition occurs during cellularization are consistent with a potential role of *DmPtr* in membrane furrow growth. Certainly, further work will be required to determine the molecular function(s) of insect Ptrs; toward that goal, the high sequence identity between these proteins would allow to perform functional assays in a model system such as *D. melanogaster*, which has proved more amenable to genetic screenings.

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LITERATURE CITED

- Briscoe J, Chen Y, Jessell TM, Struhl G. 2001. A hedgehog-insensitive form of patched provides evidence for direct long-range morphogen activity of sonic hedgehog in the neural tube. *Mol Cell* 7:1279–1291.
- Burgess RW, Deitcher DL, Schwarz TL. 1997. The synaptic protein syntaxin 1 is required for cellularization of *Drosophila* embryos. *J Cell Biol* 138:861–875.
- Burke R, Nellen D, Bellotto M, Hafen E, Senti KA, Dickson BJ, Basler K. 1999. Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified hedgehog from signaling cells. *Cell* 99:803–815.
- Carstea ED, Morris JA, Coleman KG, Loftus SK, Zhang D, Cummings C, Gu J, Rosenfeld MA, Pavan WJ, Krizman DB, Nagle J, Polymeropoulos MH, Sturley SL, Ioannou YA, Higgins ME, Comly M, Cooney A, Brown A, Kaneski CR, Blanchette-Mackie EJ, Dwyer NK, Neufeld EB, Chang T-Y, Liscum L, Strauss III JE, Ohno K, Zeigler M, Carmi R, Sokol J, Markie D, O'Neill RR, van Diggelen OP, Elleder M, Patterson MC, Brady RO, Vanier MT, Pentchev PG, Tagle DA. 1997. Niemann-P C1 disease gene: homology to mediators of cholesterol homeostasis. *Science* 277:228–231.
- Foe VE, Odell GM, Edgar BA. 1993. Mitosis and morphogenesis in the *Drosophila* embryo: point and counterpoint. In: Martinez-Arias A, editor. *The development of Drosophila melanogaster*. Vol 1. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. p 149–300.
- González-Agüero M, Zúñiga A, Pottstock H, Del Pozo T, González M, Cambiazo V. 2005. Identification of genes expressed during *Drosophila melanogaster* gastrulation by using subtractive hybridization. *Gene* 345:213–224.
- Gotoh N, Kusumi T, Tsujimoto H, Wada T, Nishino T. 1999. Topological analysis of an RND family transporter, MexD of *Pseudomonas aeruginosa*. *FEBS Lett* 456:32–36.
- Hampton RY. 2002. Proteolysis and sterol regulation. *Annu Rev Cell Dev Biol* 18:345–378.
- Harlow E, Lane D. 1999. *Using antibodies: a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. 495 p.
- Hicke L, Dunn R. 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu Rev Cell Dev Biol* 19:141–172.
- Hooper JE, Scott MP. 1989. The *Drosophila patched* gene encodes a putative membrane protein required for segmental patterning. *Cell* 59:751–765.
- Hunter C, Sung P, Schejter ED, Wieschaus E. 2002. Conserved domains of the Nullo protein required for cell-surface localization and formation of adherens junctions. *Mol Biol Cell* 13:146–157.
- Ingham PW. 1998. The *patched* gene in development and cancer. *Curr Opin Genet Dev* 8:88–94.
- Ingham P, McMahon A. 2001. Hedgehog signaling in animal development: paradigms and principles. *Genes Dev* 15:3059–3087.
- Jean-Prost P. 1987. *Apicultura: Conocimiento de la Abeja*,

- Manejo de la Colmena. Madrid: Ediciones Mundi-Prensa. 573 p.
- Knudsen KA. 1985. Proteins transferred to nitrocellulose for use as immunogens. *Anal Biochem* 147:285–288.
- Kuwabara PE, Lee MH, Schedl T, Jefferis GS. 2000. A *C. elegans* *patched* gene, *ptc-1*, functions in germ-line cytokinesis. *Genes Dev* 14:1933–1944.
- Kuwabara PE, Labouesse M. 2002. The sterol-sensing domain: multiple families, a unique role? *Trends Genet* 18:193–201.
- Lecuit T, Samanta R, Wieschaus E. 2002. *Slam* encodes a developmental regulator of polarized membrane growth during cleavage of the *Drosophila* embryo. *Dev Cell* 2:425–436.
- Loftus SK, Morris JA, Carstea ED, Gu JZ, Cummings C, Brown A, Ellison J, Ohno K, Rosenfeld MA, Tagle DA, Pentchev PG, Pavan WJ. 1997. Murine model of Niemann–P C disease: mutation in a cholesterol homeostasis gene. *Science* 277:232–235.
- Lu X, Liu S, Kornberg TB. 2006. The C-terminal tail of the Hedgehog receptor Patched regulates both localization and turnover. *Genes Dev* 20:2539–2551.
- Ma Y, Erkner A, Gong R, Yao S, Taipale J, Basler K, Beachy PA. 2002. Hedgehog-mediated patterning of the mammalian embryo requires transporter-like function of *patched*. *Cell* 111:63–75.
- Martín V, Carrillo G, Torroja C, Guerrero I. 2001. The sterol-sensing domain of Patched protein seems to control Smoothed activity through Patched vesicular trafficking. *Curr Biol* 11:601–607.
- Michaux G, Gansmuller A, Hindelang C, Labouesse M. 2000. CHE-14, a protein with a sterol-sensing domain, is required for apical sorting in *C. elegans* ectodermal epithelial cells. *Curr Biol* 10:1098–10107.
- Millard EE, Gale SE, Dudley N, Zhang J, Schaffer JE, Ory DS. 2005. The sterol-sensing domain of the Niemann–P C1 (NPC1) protein regulates trafficking of low density lipoprotein cholesterol. *J Biol Chem* 280:28581–28590.
- Pepinsky RB, Zeng C, Wen D, Rayhorn P, Baker DP, Williams KP, Bixler SA, Ambrose CM, Garber EA, Miatkowski K, Taylor FR, Wang EA, Galdes A. 1998. Identification of a palmitic acid-modified form of human Sonic hedgehog. *J Biol Chem* 273:14037–14045.
- Perens EA, Shaham S. 2005. *C. elegans* *daf-6* encodes a patched-related protein required for lumen formation. *Dev Cell* 8:893–906.
- Rothwell WF, Sullivan W. 2000. Fluorescent analysis of *Drosophila* embryos. In: Sullivan W, Ashburner M, Scott Hawley, R, editors. *Drosophila* protocols. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. p 141–157.
- Sever N, Yang T, Brown MS, Goldstein JL, DeBose-Boyd RA. 2003. Accelerated degradation of HMG CoA reductase mediated by binding of *insig-1* to its sterol-sensing domain. *Mol Cell* 11:25–33.
- Strutt H, Thomas C, Nakano Y, Stark D, Neave B, Taylor AM. 2001. Mutations in the sterol-sensing domain of Patched suggest a role for vesicular trafficking in Smoothed regulation. *Curr Biol* 11:608–613.
- Taipale J, Cooper MK, Maiti T, Beachy PA. 2002. Patched acts catalytically to suppress the activity of Smoothed. *Nature* 418:892–897.
- Tseng TT, Gratwick KS, Kollman J, Park D, Nies DH, Goffeau A, Saier MH Jr. 1999. The RND permease superfamily: an ancient, ubiquitous and diverse family that includes human disease and development proteins. *J Mol Microbiol Biotechnol* 1:107–125.
- Yang T, Espenshade PJ, Wright ME, Yabe D, Gong Y, Aebersold R, Goldstein JL, Brown MS. 2002. Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. *Cell* 110:489–500.
- Zhang CX, Hsieh T. 2000. Preparation of membrane proteins from *Drosophila* embryos. In: Sullivan W, Ashburner M, Hawley RS, editors. *Drosophila* Protocols. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. p. 563–569.
- Zugasti O, Rajan J, Kuwabara PE. 2005. The function and expansion of the Patched- and Hedgehog-related homologs in *C. elegans*. *Genome Res* 15:s1402–1410.