ESCUELA DE PREGRADO
FACULTAD DE CIENCIAS
UNIVERSIDAD DE CHILE

"Caracterización molecular de una nueva proteína Patched-related en *Apis mellifera* y *Drosophila melanogaster*"

Seminario de Título entregado a la Universidad de Chile en cumplimiento parcial de los requisitos para optar al Título de Ingeniero en Biotecnología Molecular.

Por

Luis Eduardo Pastenes Opazo

Junio, 2011
Santiago - Chile

Directora de Seminario de Título: **Dra. Verónica Cambiazo Ayala**

ESCUELA DE PREGRADO – FACULTAD DE CIENCIAS – UNIVERSIDAD DE CHILE

INFORME DE APROBACIÓN
SEMINARIO DE TÍTULO

"Caracterización molecular de una nueva proteína Patched-related en *Apis mellifera* y *Drosophila melanogaster*"

Se informa a la Escuela de Pregrado de la Facultad de Ciencias que el Seminario de Título presentado por el candidato

LUIS EDUARDO PASTENES OPAZO

Ha sido aprobado por la Comisión de Evaluación, en cumplimiento parcial de los requisitos para optar al Título de Ingeniero en Biotecnología Molecular.

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Santiago de Chile, junio 2011

BIOGRAFÍA

Mi nacimiento tuvo lugar el 31 de mayo de 1974 en la ciudad de Santiago, Chile. Mis padres son Inés Del Carmen Opazo Quezada y Luis Francisco Pastenes Donaire. Tengo tres hermanos, Claudio Aníbal (35), Felipe Andrés (27) y Gemita Inés (25, *"la regalona"*). Mi infancia la viví en Batuco, una localidad situada 25 km al norponiente de Santiago. A los seis años de edad, mis padres deciden trasladarse a la capital. Cursé mi educación básica en un establecimiento de la comuna de Recoleta (Colegio "San Benildo"). Completé mi educación media en el emblemático Liceo A-17, "Internado Nacional Barros Arana". El gran cariño por las Ciencias Naturales, sobre otras disciplinas, me condujo a desarrollar la idea de estudiar una carrera universitaria en el área científica del saber. En el año 1999 ingresé a la Facultad de Ciencias de la Universidad de Chile. ¡Nunca pensé en otra universidad! A fines del 2001 me adherí a un laboratorio de investigación (Lab. de Bioinformática y Expresión Génica) para aprender la formación y disciplina científica. Gracias a la Prof. y Dra. Verónica Cambiazo tuve la oportunidad de participar en dos proyectos de investigación, en los que apliqué herramientas de biología molecular para el estudio de las bases moleculares del desarrollo embrionario temprano de *Drosophila melanogaster*. En este largo andar logré participar, como autor principal o co-autor, de varios congresos nacionales y publicaciones científicas de corriente principal. Actualmente, estoy cursando un programa de postgrado en la Universida de Chile.

DEDICATORIA

"Enseñar no es una función vital, porque no tiene el fin en sí misma; la función vital es aprender".

Aristóteles (384 a. C. – 322 a. C.)
Su logro más absoluto fue la Biología. Su segunda conquista fue la Lógica.

Dedico este trabajo por completo
a mi familia.

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Al culminar este largo sendero, no exento de dificultades en su trayecto, quisiera agradecer el apoyo de todas aquellas personas (en un orden cronológico de eventos) que, de una u otra manera, colaboraron en mi egreso:

A Patricia Grandón y Margarita Olivares (oficina de Bienestar), por asistir mi ingreso, permanencia y término en la carrera. Gracias por su invaluable ayuda.

A mis abuelos maternos, Ana y Aníbal, por otorgarme abrigo y respaldo durante el inicio de esta travesía.

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Y por cierto, a Verónica y MG. Gracias por compartir su laboratorio y sus investigaciones con este humilde alumno.

ÍNDICE DE CONTENIDOS

LISTA DE ABREVIATURAS

[α-^{32}P]dCTP	Radio-isótopo de 2'-desoxicitidina 5'-trifosfato
A/P	Antero-posterior
aa	Aminoácidos
*Am*Ptr	Ptr de *Apis mellifera*
Blast	Herramienta de búsqueda de alineamientos locales básicos
bp	Pares de bases
BSA	Albúmina sérica de bovino
$CaCl_2$	Cloruro de calcio
cDNA	DNA complementario
Cu^{2+}	Cobre
$CuSO_4$	Sulfato cúprico
dap	Diaminopimelato
Disp	Dispatched
*Dm*Ptr	Ptr de *Drosophila melanogaster*
DNA	Ácido desoxirribonucleico
dsRNA	RNA de doble hebra
dT	desoxitimina
FBS	Suero fetal bovino
FITC	Fluoresceína-5-isotiocianato
HCl	Ácido clorhídrico
Hh	Hedgehog
HMGCR	HMG-CoA reductasa
KCl	Cloruro de potasio

kDa	KiloDalton
LDL	Lipoproteína de baja densidad
M-MLV RT	Transcriptasa reversa del virus de la Leucemia Murina de Moloney
mRNA	RNA mensajero
Na_2CO_3	Carbonato de sodio
NaCl	Cloruro de sodio
NCBI	Centro Nacional para la Información Biotecnológica
Ni-NTA	Níquel-nitrilotriacetato
NP-40	2-[2-[4-(2,4,4-trimetilpentan-2-il)fenoxi]etoxi]etanol
NPC1	Niemann-Pick tipo C1
OD	Densidad óptica (absorbancia)
ORF	Marco de lectura abierto
PAGE	Electroforesis en gel de poli-acrilamida
PAUP*	Análisis filogenético usando Parsimonia (y otros métodos)
PBS	Búfer fosfato salino
PCR	Reacción en cadena de la polimerasa
Ptc	Patched
Ptr	Patched-related
qPCR	PCR cuantitativo en tiempo real
RACE	Amplificación rápida de los extremos del cDNA
RLM-RACE	RACE mediado por RNA ligasa
RNA	Ácido ribonucleico
RND	Resistencia-Nodulación-División celular
rp49	Proteína ribosomal 49
RT-PCR	PCR en transcripción reversa

SCAP	Proteína activadora a través del clivaje de SREBP
SDS	Dodecil-sulfato sódico
Smo	Smoothened
SSD	Dominio sensor de esteroles
TM	Transmembrana
Tris	2-amino-2-hidroximetil-propano-1,3-diol
Triton X-100	2-[4-(2,4,4-trimetilpentan-2-il)fenoxi]etanol
Tween-20	2-[2-[3,4-bis(2-hidroxietoxi)oxolan-2-il]-2-(2-hidroxietoxi)etoxi]etil dodecanoato
v/v	Volumen/volumen
w/v	Peso/volumen

RESUMEN

En el presente trabajo de investigación se realizó la identificación y caracterización molecular del gen *patched-related* (*ptr*) y su proteína, en *Apis mellifera* y *Drosophila melanogaster*. Las proteínas Ptr están estrechamente relacionadas en topología predicha y organización de dominios a la proteína codificada por el gen de polaridad de segmento de *Drosophila* conocida como Patched. Las proteínas Ptrs tienen potencialmente 12 dominios de transmembrana dispuestos en dos series de 1+5 segmentos de membrana, conteniendo un dominio sensor de esteroles (SSD) conservado y motivos funcionales GxxxD y PPXY. El análisis filogenético mostró que las Ptrs corresponden a una clase de proteínas de insectos que comparten un alto nivel de identidad de secuencia. El análisis mediante PCR cuantitativo en tiempo real reveló que el gen *ptr* se expresa preferentemente en las etapas embrionarias del desarrollo de *A. mellifera*; interesantemente, este patrón de expresión temporal también se observó para el homólogo de *D. melanogaster*, sugiriendo que estas proteínas podrían estar involucradas en la morfogénesis del embrión. Para comprender la función de Ptr a nivel molecular, se investigó la distribución subcelular de *Dm*Ptr. Se evidenció, mediante pruebas bioquímicas, que la proteína *Dm*Ptr está estrechamente asociada con las membranas. Consecuentemente, la inmunoreactividad de Ptr parece estar localizada en los sitios de formación de surcos de membrana durante la celularización de los embriones de *D. melanogaster*. Estos estudios indican que las Ptrs pertenecen a una clase de proteínas de transmembrana de insectos, no caracterizadas previamente, que comparten un alto porcentaje de homología. Los análisis de expresión génica de *ptr* y de localización de la proteína sugieren que Ptr podría cumplir un papel en el desarrollo, participando en procesos que requieren crecimiento y estabilización de la membrana plasmática.

MANUSCRITO

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

Luis Pastenes,[1] Freddy Ibáñez,[1] Carmen Bolatto,[2] Leonardo Pavéz,[1] and Verónica Cambiazo[1*]

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 *Dm*Ptr. We have shown by biochemical analysis that *Dm*Ptr 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. Arch. Insect Biochem. Physiol. 68:156–170, 2008.

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 humans, 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 *Dm*Ptr 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* (*Am*Ptr). 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 $CaCl_2$, 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.

Amptr and *Dmptr* cDNA Cloning

Putative *A. mellifera ptr* cDNAs were identified by Blast analysis, using *D. melanogaster* Ptr (*Dm*Ptr, GenBank accession number NM_136365) as the protein input. Two putative cDNAs (GenBank accession numbers NW_055478 and NW_048484), initially named *Amptr1* and *Amptr2*, with amino acid sequence similarity to the N- and the C-terminal of *Dm*Ptr, were found. PCR primers (s1: 5′-CGTCAAGATGCGATAGGAGC-3′, and a1: 5′-TATTCTCCGCTTACTACCACCTGT-3′) were designed to test whether *Amptr1* and *Amptr2* 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 (*Amptr*, 2,620 bp) was assembled from the *Amptr1*, the *Amptr1-Amptr2* amplicon, and *Amptr2* sequences and used to design gene-specific primers for 5′- and 3′-RACE PCR.

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

5′- and 3′-RACE PCR

The 5′- and 3′-ends of the *Amptr* 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′-GACGCAAACCTGGCAGTGGTAATG-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′-AGGCGTCCTCTATGGAATGTCTC-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 *Amptr* (s3: 5′-TTACAATCCTACCGACAACC-3′, and a3: 5′-TTCTCCGCTTACTACCACCT-3′) and *dap* (ds1: 5′-TTGCATTAGAGCACGGAGTC-3′ and da1: 5′-GCGTATCTGAAGCGTTTGG-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/μl) 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 μg) 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 *Dm*ptr 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 generate 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 *Dm*Ptr 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 *Dm*Ptr 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 Cu^{2+}-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 μg of vector DNA by using Cellfectin Reagent according to standard techniques (Invitrogen). Expression of *Dm*Ptr construct was induced 48 h post-transfection by adding $CuSO_4$ 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×/1.4 Oil at 1,024-pixel resolution.

*Dm*Ptr 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 *Dm*Ptr 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 preimmune 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 frozen embryos using equilibrium sedimentation in a sucrose density step gradient as described by Zhang and Hsieh (2000). To examine whether *Dm*Ptr 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 30,000*g* 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-*Dm*Ptr 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* (*Dm*Ptr, 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 *Am*Ptr1 and *Am*Ptr2 amino acid sequences were compared to that of *Dm*Ptr, we observed that *Am*Ptr1 showed a 58% of identity with 479 aa from the C-terminal end of *Dm*Ptr, while *Am*Ptr2 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 *Am*Ptr and *Dm*Ptr 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 *Am*Ptr and *Dm*Ptr 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 *Am*Ptr and *Dm*Ptr (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.

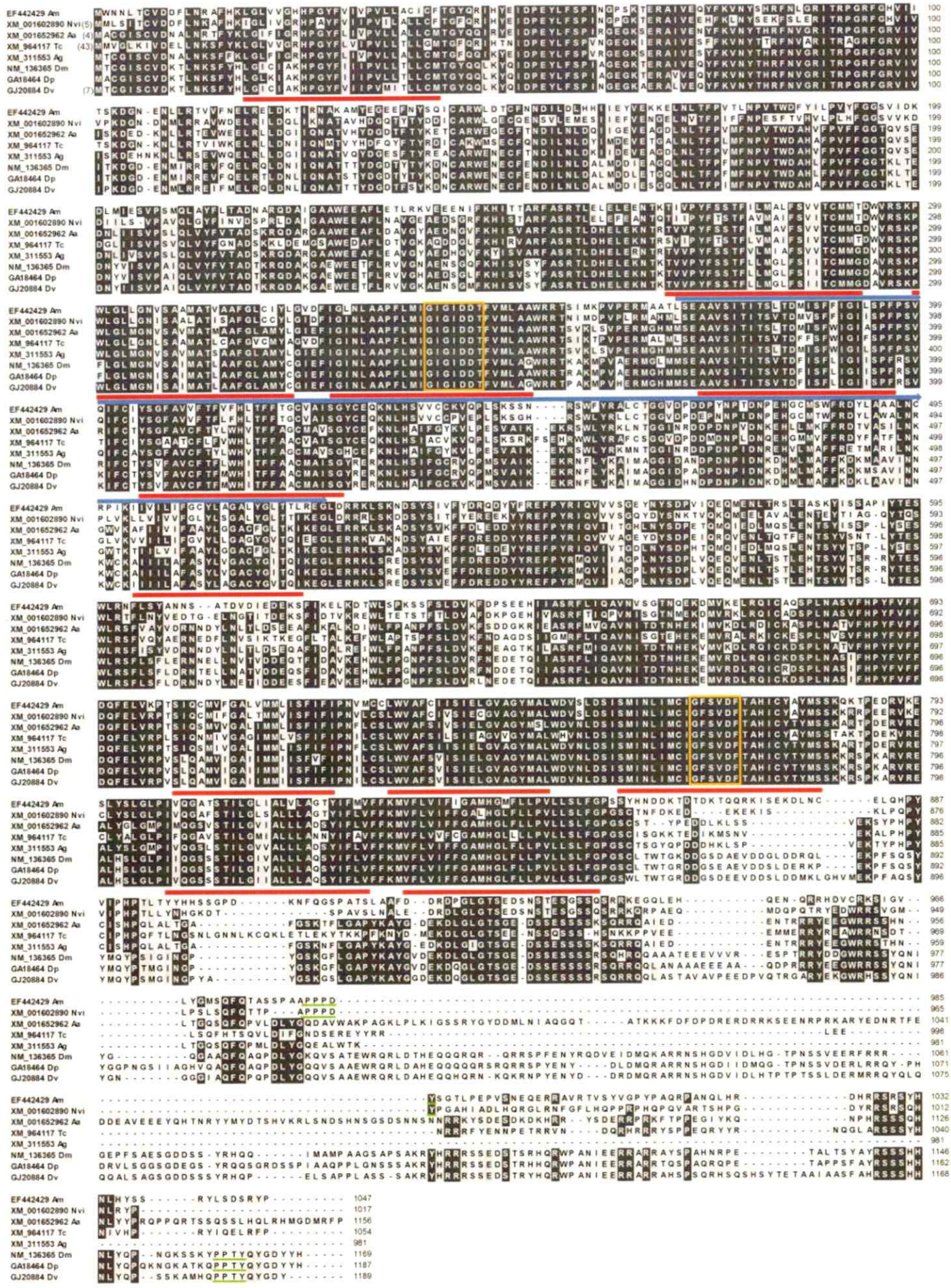
EF442429 Am
XM_001602890 Nvi (5)
XM_001652962 Aa (4)
XM_964117 Tc (43)
XM_311553 Ag
NM_136365 Dm
GA18464 Dp
GJ20884 Dv (7)

Figure 1

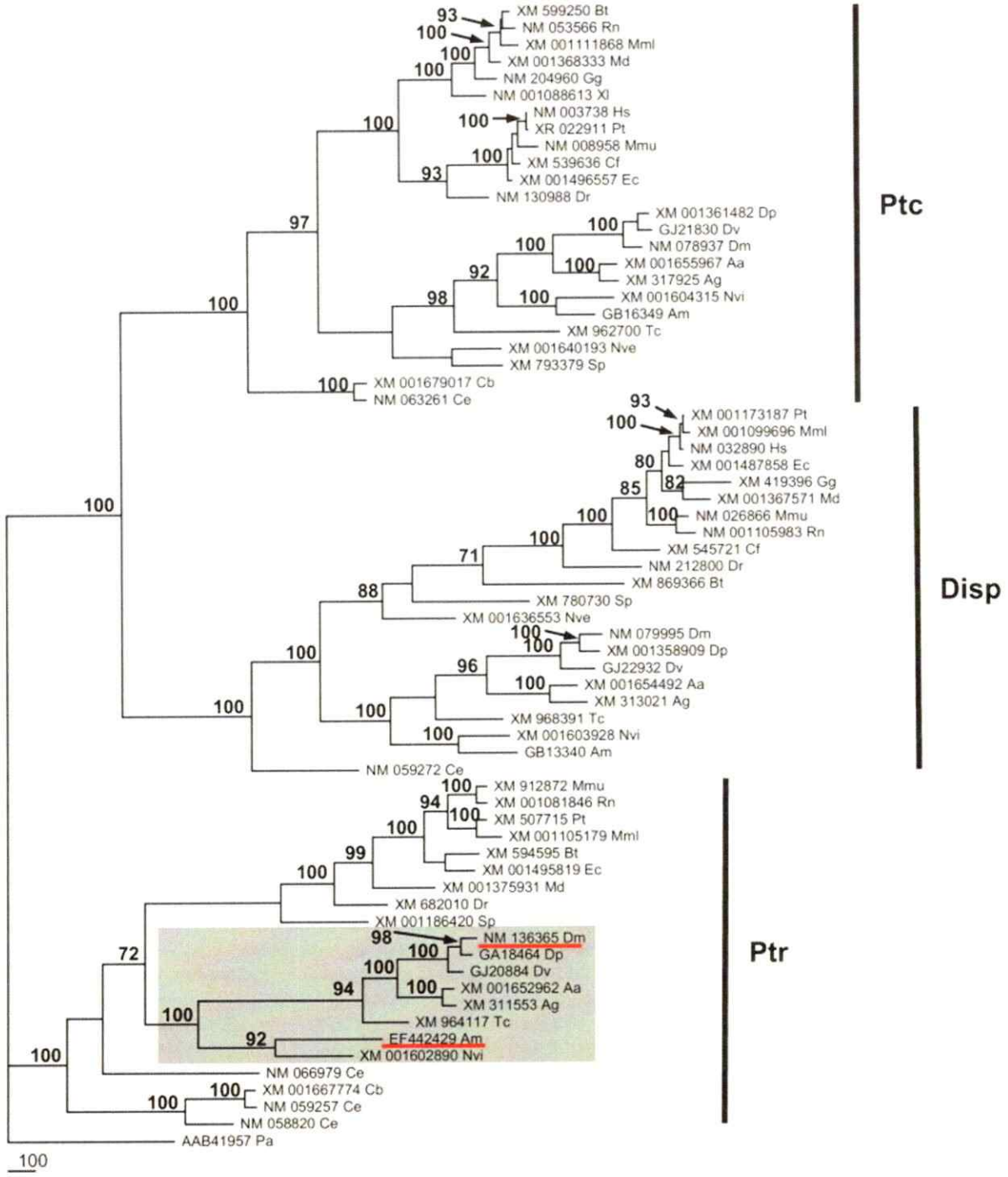

Fig. 2. Phylogenetic tree comparing the sequences of Ptr, Ptc, and Disp proteins Analysis was performed with multiple alignments from amino acid sequences using ClustalW. Bootstrap values (1,000 pseudo-replicates) are shown above branches. The tree is displayed rooted by *Pseudomonas aeruginosa* RND 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*; Xl, *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]

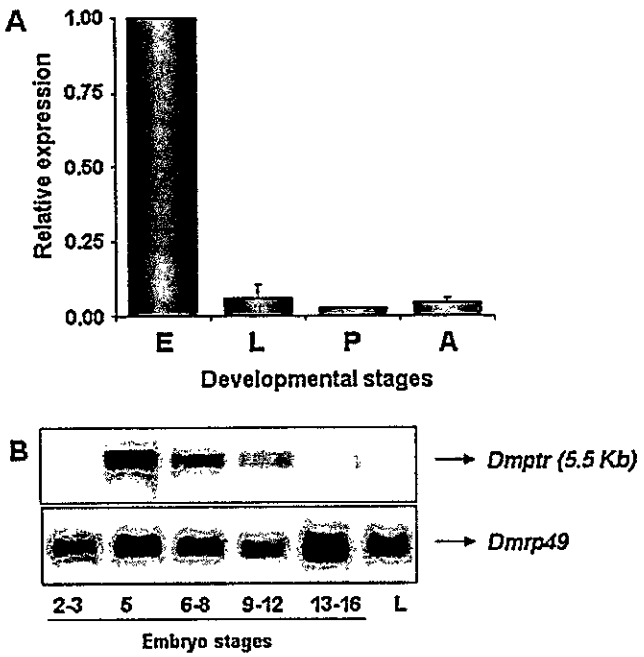

Fig. 3. Analysis of *Amptr* and *Dmptr* gene expression. A: Total RNA was isolated from 2-day embryos (E), 4-day larvae (L), unpigmented pupae (P), and adults (A). For each developmental stage, the relative expression of *Amptr* was normalized using *dap* spike mRNA (see Materials and Methods). The results are presented as a fraction of the highest value of relative expression. B: *D. melanogaster* total RNA (10 μg) from stages 2–3, 5, 6–8, 9–12, and 13–16 embryosand 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 *Dm*Ptr association with membranes (Fig. 5A, lane 1). *Dm*Ptr remains associated with the membranes after PBS and alkaline treatment at pH 10, indicating a tight association between *Dm*Ptr 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 *Dm*Ptr could be released by the detergent (Fig. 5, lanes 6–7), we noted that a minor fraction of *Dm*Ptr 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 *Dm*Ptr is a transmembrane protein. In addition, we examined the subcellular localization of *Dm*Ptr using an epitope-tagged version of the protein. A construct expressing *Dm*Ptr-V5 fusion protein was transfected into a *Drosophila* culture cell line (S2R+) and the subcellular localization of *Dm*Ptr was determined by immunofluorescence using anti-V5 antibodies (Fig. 5B, panels a and c). We observed that *Dm*Ptr is localized to the cell surface and dispersed throughout the cytoplasm in a punctate pattern. In a higher-magnification view, *Dm*Ptr-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 *Am*Ptr and *Dm*Ptr 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

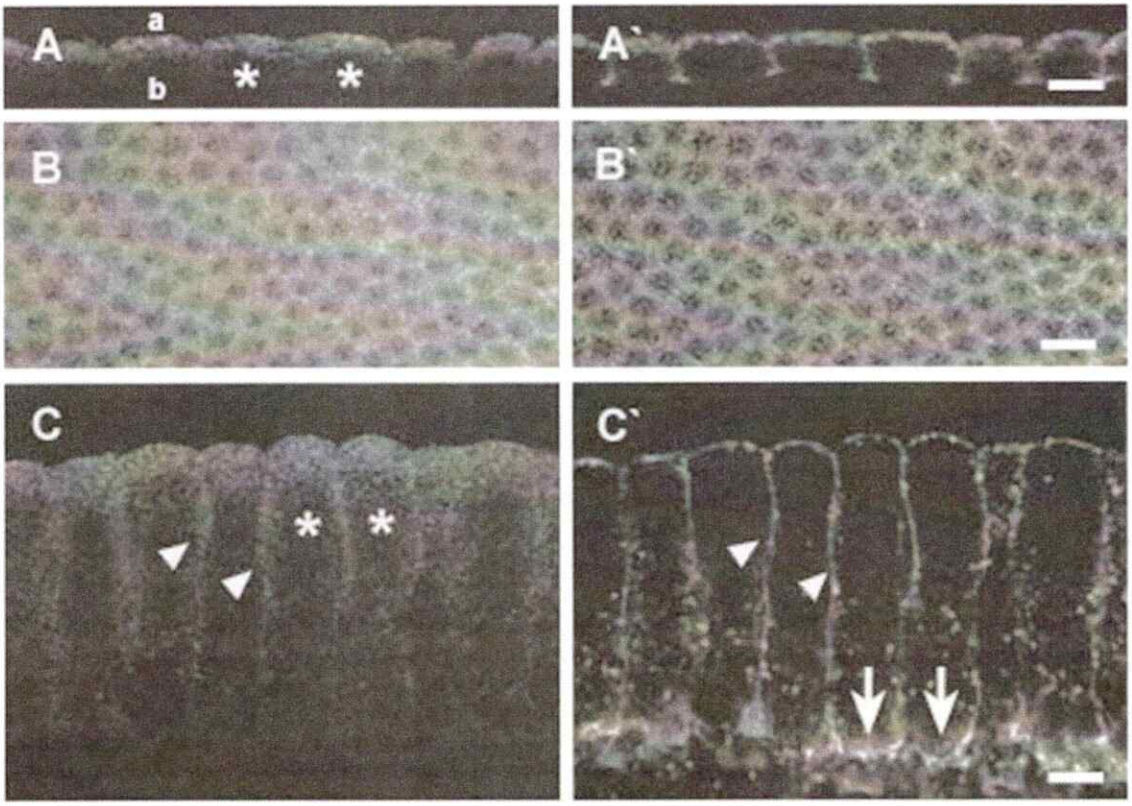

Fig. 4. *Dm*Ptr localization by antibody staining and confocal microscopy in whole-mount stage 5 embryos. Embryos were double-stained for: *Dm*Ptr (**A**,**B**,**C**); and F-actin; (**A′**,**B′**,**C′**) a, apical; b, basal; *, nuclei. (A and B) Sections from an embryo during the slow phase of cellularization. *Dm*Ptr 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 μm in A; 15 μm in B; 5 μm 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 *Am*Ptr and *Dm*Ptr 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. casteneum* seem to lack

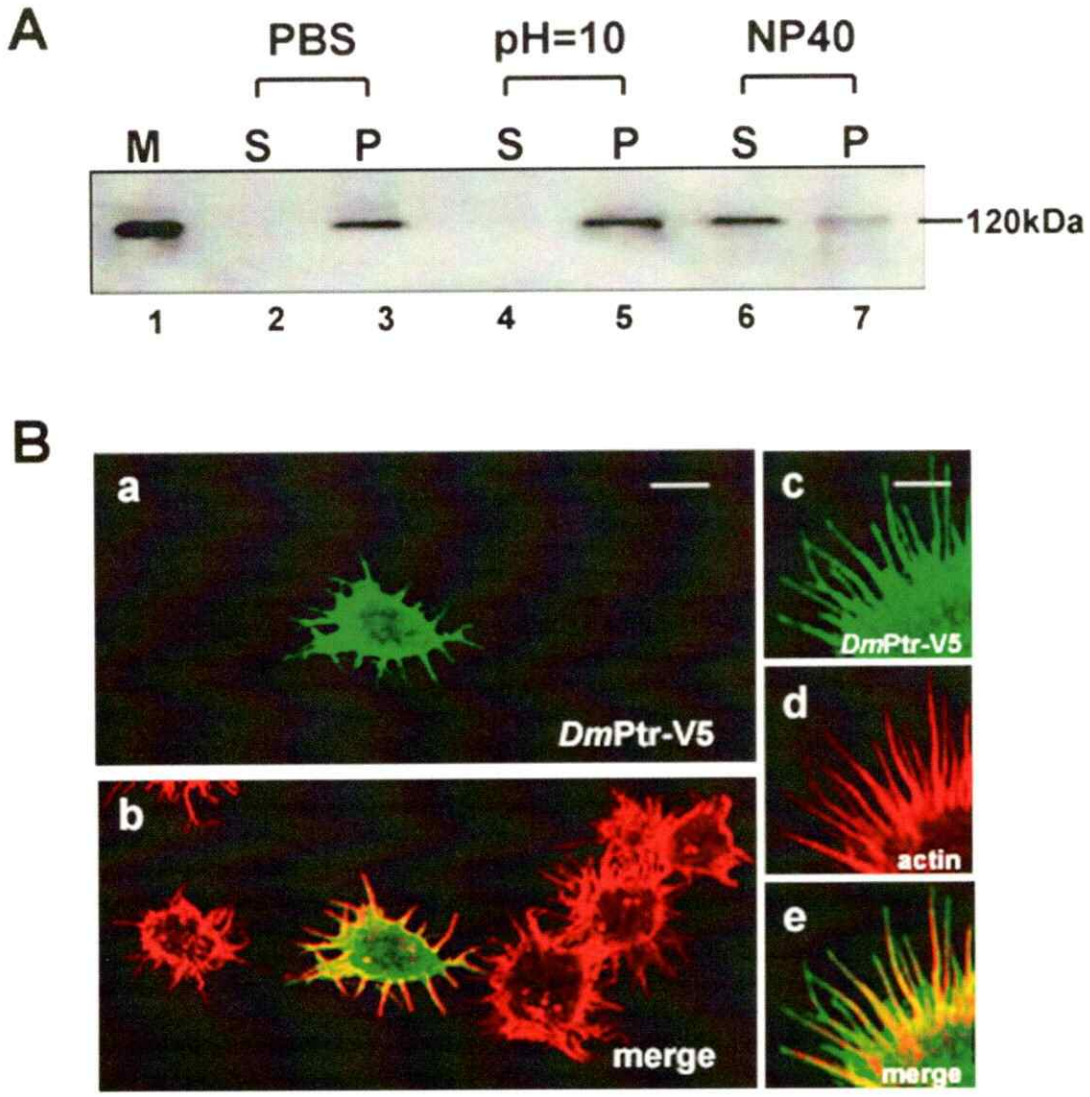

Fig. 5. Membrane association of *Dm*Ptr. **A:** Embryos (0–4 h) membranes were prepared by cellular fractionation on a sucrose step gradient (M). Three solubilizing reagents were used to wash the membrane fraction. These reagents were PBS, 100 mM Na_2CO_3 (pH 10), and 1% NP-40. After washing, the same volume of supernatant (S) and pellet (P) was loaded on the gel and subjected to Western blot analysis using an anti-*Dm*Ptr polyclonal antibody (1:1000 dilution). Estimated molecular weight of *Dm*Ptr, 120 kDa. **B:** Subcellular localization of the *Dm*Ptr-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 *Am*Ptr and *Dm*Ptr 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 *Dm*Ptr, which shares a high level of sequence identity with *Am*Ptr. 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-*Dm*Ptr antibody, we observed that during the slow phase of cellularization *Dm*Ptr 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 *Dm*Ptr was consistent with a peripheral localization of the *Dm*Ptr-V5 fusion protein in S2R+ cells and with our biochemical analysis showing that *Dm*Ptr was tightly associated with embryo membranes. The spatial expression pattern of *Dm*Ptr 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 enrichement on the newly forming lateral cell membranes and is required for a normal embryo cellularization, suggesting that *Dm*Ptr 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 *Dm*Ptr 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.

ACKNOWLEDGMENTS

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CONCLUSIONES

En relación con los resultados experimentales obtenidos en el presente trabajo de investigación, se puede concluir lo siguiente:

1. Los cDNAs de largo completo de los genes *ptr* de *Apis mellifera* (*Amptr*) y *Drosophila melanogaster* (*Dmptr*) codifican para una proteína de transmembrana de múltiple-paso (12 posibles hélices alfa). Las topologías predichas de las proteínas *Am*Ptr y *Dm*Ptr comparten características comunes en cuanto a dominios estructurales (dos casetes de 1+5 segmentos de transmembrana) y dominios y motivos funcionales (SSD, GxxxD y PPXY). Además, las secuencias proteicas predichas de *Am*Ptr y *Dm*Ptr son altamente conservadas con las Ptrs de otras especies de insectos. En consecuencia, se propone que las Ptrs de insectos pertenecerían a una nueva subfamilia conservada de proteínas de transmembrana, previamente no caracterizadas, que contienen un dominio sensor de esteroles.

2. El patrón de expresión temporal de *Amptr* insinúa que este gen puede estar jugando un papel durante la embriogénesis de *A. mellifera*. Además, los resultados en los patrones de expresión temporal del gen *Dmptr*, junto con la localización de la proteína Ptr en los sitios donde ocurre adición de membrana durante la celularización en embriones de *D. melanogaster*, y consistente con nuestra predicción de la secuencia proteica, son coherentes con el papel potencial de *DmPtr* en el crecimiento del surco de la membrana. Por lo tanto, la evidencia sugiere que la expresión de los genes *ptr* de ambas especies de insectos estaría regulada durante el desarrollo, siendo expresados preferentemente en etapas embrionarias.

PRESENTACIONES

2004. **Pastenes L**, Zúñiga A, González-Agüero M, Méndez MA, Cambiazo V. Caracterización molecular del gen *patched-related* (*ptr*), un posible nuevo receptor de Hedgehog durante la gastrulación de *Drosophila*. Presentación en panel en la XVIII Reunión Anual de la Sociedad de Biología Celular de Chile. Pucón, Chile (13-17 de octubre).

2005. **Pastenes L**, Zúñiga A, Méndez MA, Cambiazo V. El gen CG11212 de *Drosophila melanogaster* codifica una nueva proteína de transmembrana altamente conservada en los insectos. Presentación en panel en la XIX Reunión Anual de la Sociedad de Biología Celular de Chile. Pucón, Chile (16-20 de octubre).