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RESEARCH ARTICLE

Expression of multidrug resistance proteins is localized principally to the Malpighian tubules in larvae of the cabbage looper moth, *Trichoplusia ni*

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SUMMARY

The multidrug resistance proteins (MRPs) serve a number of important roles in development, physiological homeostasis and metabolic resistance. In insects, they may also contribute to resistance against xenobiotics including insecticides and plant secondary metabolites. To investigate their contribution to xenobiotic resistance, we have examined the tissue distribution of gene and protein expression of the multidrug resistance proteins TrnMRP1 and TrnMRP4 of the lepidopteran insect, *Trichoplusia ni*. Using quantitative PCR and immunohistochemistry, we have identified high expression levels of both transporters in the Malpighian tubules relative to levels in other major tissues of the body, where they probably contribute to excretion of metabolic wastes or ingested xenobiotics. We have specifically located TrnMRP protein expression in a subpopulation of Malpighian tubule secondary cells. Expression of TrnMRP1 was also detected both at a high level in specific cortical neurons of larval ganglia and at a lower level throughout the cortex, where it may act in signaling or protective functions, respectively. In contrast, expression of TrnMRP4 was low to absent in larval ganglia, with the exception of single cells in the central connective. We discuss the potential implications of this TrnMRP activity on insect development and metabolic resistance.

Key words: ABC transporter, multidrug resistance proteins, Malpighian tubule, *Trichoplusia ni*, insect metabolic resistance.

INTRODUCTION

Members of the ATP-binding cassette (ABC) superfamily of transporters are expressed in the tissues of organisms throughout the Metazoa. Proteins of this superfamily are divided into eight subfamilies, annotated as A to H, based on their sequence similarity and domain conservation, which broadly delineate function and substrate specificity. These efflux pumps have numerous functions in development and organogenesis as well as in resistance of tissues and organisms to drugs and xenobiotics (Cole et al., 1992; van de Ven et al., 2006; Huls et al., 2009). One of the ABC protein subfamilies that can confer drug resistance is the multidrug resistance proteins (MRPs), which belong to sub-family C (ABCC). This family includes MRP1, which was first identified when it was linked to small cell lung cancer resistance to chemotherapy (Cole et al., 1992). With few exceptions, the MRPs are structurally and functionally related proteins that play important roles in resistance to chemotherapy and antiviral agents in humans, typically rendering multiple drugs ineffective (Schuetz et al., 1999; Leggas et al., 2004; James and Davey, 2009). In addition, these proteins have key functions in metazoan metabolism, development and homeostasis, which are reflected by the substrates they transport (Kruh et al., 2001; Deeley and Cole, 2003). Substrates of MRPs include organic anions and neutral organic conjugates produced in phase II metabolism, as well as cyclic nucleotides, bile acids and hormones in the cases of MRP4, MRP5 and MRP8 (Morrow and Cowan, 1990; Schuetz et al., 1999; Russel et al., 2008).

In invertebrates, membrane transporters that include the homologs of human MRPs are believed to contribute to insecticide and antiparasitic resistance by effluxing toxin from cells and tissues (Dow and Davies, 2006; Lespine et al., 2006). Furthermore, the inducible nature of *MRP* gene expression (Chen et al., 2008; Lombardo et al., 2008) may also contribute to the development of xenobiotic-resistant populations. In *D. melanogaster* for instance, Chahine and O'Donnell found that exposure to sufficiently high doses of the cytotoxic MRP substrate methotrexate could induce a broad upregulation of transporter expression including that of the fly *MRP1* ortholog, *dMRP* (Chahine and O'Donnell, 2009). In mammalian cells, insecticide molecules such as organochlorines, organophosphates and pyrethroids induce the expression of genes for several metabolic proteins including the cytochrome P450 enzymes, which render insecticides inactive (Lemaire et al., 2004). This mechanism may also induce the expression of MRPs and their homologs following exposure to substrate molecules, such as in the case of organophosphate exposure in the brown planthopper, *Nilaparvata lugens* (Bao et al., 2010).

In mammals, recent evidence suggests that several ABC transporters play important roles in development and organogenesis. Their expression is often enriched in pluripotent stem cells and metastatic or drug resistant tumors, where they can serve as markers in detecting resistance or pluripotency (Lin et al., 2006; Oevermann et al., 2009). The ABC transporters, including the breast cancer resistance protein (BCRP) and P-glycoprotein, serve to protect pluripotent cell populations from toxic accumulation of metabolic wastes and products of oxidative stress (Huls et al., 2009). In developing dendrites from pluripotent cells, MRP1 is also required to efflux ligands responsible for inducing dendritic differentiation (van de Ven et al., 2006; Jin et al., 2008).

In this study, we have examined the expression of two recently cloned MRP genes of the cabbage looper moth *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae), *TrnMRP1* and *TrnMRP4*, that are orthologs of human *MRP1* and *MRP4*. We hypothesized that expression of these genes would be localized predominantly to barrier tissues of the digestive–excretory system and the nervous system where they may serve in the maintenance of physiological homeostasis. Using quantitative polymerase chain reaction (qPCR) we determined the expression of these genes in fifth instar larvae, and subsequently analyzed the distribution of the TrnMRP1 and TrnMRP4 proteins in those tissues having the highest gene expression. We also hypothesized that exposure to toxic xenobiotics such as a sodium-channel-disrupting pyrethroid insecticide or plant allelochemicals, toxins produced to deter feeding by herbivores, could alter *TrnMRP* expression. Having here confirmed the enrichment and location of the TrnMRP transporters, we discuss the potential implications of these results for insect detoxification mechanisms.

MATERIALS AND METHODS

Insects

Trichoplusia ni were derived from a continuously cultured colony maintained at the Southern Crop Protection and Food Research Centre (SCPFRC), London, Ontario, Canada. The colony was reared using standard procedures (16 h:8 h light:dark photoperiod at 25°C) (Guy et al., 1985) on one of three dietary regimes: cabbage leaves, a wheat-germ-based artificial diet or an artificial diet containing a sublethal dose of 0.025 mg l^{-1} of the pyrethroid insecticide Deltamethrin.

Tissue and protein isolation

RNA and protein were isolated from the tissues of late fifth instar *T. ni* larvae, using the following numbers of caterpillars to obtain sufficient material for each tissue extraction: midgut (3), Malpighian tubules (10), integument (3), brain (30), nerve cord (30), muscle (3) and fat body (3). The tissues were collected by quickly dissecting larvae and immediately placing the isolated tissues in ice-cold RNAlater (Ambion, Streetsville, ON, Canada) for RNA extraction, or into 10 volumes of homogenization buffer [0.1 mol l^{-1} NaH_2PO_4, 1 mmol l^{-1} PTU, 1 mmol l^{-1} dithiothreitol (DTT), 1 mmol l^{-1} EDTA, 1× protease inhibitor cocktail (PIC); Bio-Rad, Mississauga, ON, Canada] at 4°C for protein isolation. Tissue isolated for protein extraction was then quickly homogenized using a Polytron homogenizer (Brinkmann, Rexdale, ON, Canada) yielding a whole cell lysate that was then centrifuged at 2940 ***g*** for 10 min at 4°C to pull down the membrane fraction. The resulting pellet was resuspended in solubilization buffer (0.1 mol l^{-1} NaH_2PO_4, 0.1 mmol l^{-1} PTU, 0.1 mmol l^{-1} DTT, 0.1 mmol l^{-1} EGTA, 20% glycerol, 1× PIC). Protein concentrations were measured in solubilized crude membrane protein samples suspended in homogenization or solubilization buffer by Bradford's assay, using 8 µl of lysate in 792 µl water and 200 µl of Bradford's reagent (Bio-Rad).

RNA extraction and cDNA synthesis

Tissue samples were transferred from RNAlater and homogenized with a glass rod in microtubes using RLT buffer (Qiagen, Mississauga, ON, Canada) containing 1% β-mercaptoethanol. Total RNA was extracted from homogenized tissues according to RNeasy mini kit guidelines for animal cells (Qiagen). Total RNA was quantified using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Ottawa, ON, Canada) with sample purity checked by measuring the 260:280 nm absorbance ratio. The synthesis of cDNA from 1 µg of total RNA was performed using the SuperScript® III Reverse Transcriptase kit (Invitrogen, Burlington, ON, Canada).

qPCR

qPCR was used to determine expression levels of *TrnMRP1* and *TrnMRP4* mRNAs in the larval tissues specified previously. Gene expression was quantified using a Roche LC480 system (Roche, Mississauga, ON, Canada) as follows. Into each experimental reaction well on a 96-well plate, either 2.5 µl of 1:2 diluted cDNA, or water for negative control, was added, plus 5 µl of 2× LightCycler480 Master Mix and the appropriate primer and probe combination at final concentrations of 0.5 and 0.1 µmol l^{-1}, respectively (Table 1). Cycling conditions were: 10 min preincubation at 95°C; 45 amplification cycles of 10 s denaturation at 95°C, a 30 s annealing step at 60°C and a 1 s extension step at 72°C. The final step cooling period was applied with an endpoint of 40°C. Fluorescence readings were acquired at the end of the extension step of each amplification cycle, at an excitation wavelength of 465 nm and an emission wavelength of 510 nm. To generate standard curves for each target and reference gene, cDNA pooled from all tissue sources was serially diluted ten times at a

Table 1. *TrnMRP1*, *TrnMRP4* target gene as well as *eIF4α* and *S5* ribosomal RNA reference gene primer and hydrolysis probe sequences used in qPCR analyses

Name	Type	Sequence (5′ to 3′)
For *TrnMRP1*		
MRP1rt F	Primer	ATCGTATATTGGCGTCTGGTC
MRP1rt R	Primer	AAGCACTTTGTCGTCATTGG
MRP1rt probe	Probe	[DFAM] ACTCCACGCGGCTTCCTGCTTTGT [DTAM]
For *TrnMRP4*		
MRP4rt F	Primer	AGTTACTCAGTTCACATCGTT
MRP4rt R	Primer	TGTAGGATCAACGTTAGCAGTA
MRP4rt probe	Probe	[DFAM] ACTACAGTCGCCGGTCGCTTCA [DTAM]
For *eIF4α*		
eIF4α F	Primer	CTGGATACGCTGTGTGAC
eIF4α R	Primer	ACCTTGCGGCGAGTGTT
eIF4α probe	Probe	[DFAM] CTCTCTCCATCGCTCAGGCTGTCATC [DTAM]
For *S5*		
S5 F	Primer	GTCGACAGCATGCCTTTA
S5 R	Primer	CACATCCACAGCCTGAC
S5 probe	Probe	[DFAM] CATCATCAGAGAGTTTGTCAAGCGCT [DTAM]

rate of 2.5-fold and measured in triplicate. From the resulting standard curves, a correction for gene amplification efficiency was applied by importing standard curve data into each subsequent dataset generated. Experimental expression levels of the target genes *TrnMRP1* and *TrnMRP4* and of reference genes *S5* ribosomal protein and the eukaryotic initiation factor, *eIF4α*, were determined using cDNA samples synthesized from RNA derived from each of the seven *T. ni* tissues analyzed and of larvae reared on each of the three dietary regimes. Data were obtained from three biological replicates, and for each, three technical replicates were sampled. Technical replicates are defined as replicates of identical qPCR reactions. An advanced relative quantification analysis was performed using LC480 system software, resulting in a quantification of target gene mRNA expression relative to reference gene expression. Expression of *TrnMRP1* and *TrnMRP4* were compared across dietary regimes and tissue type using a two-way analysis of variance (ANOVA). Gene expression data were log transformed to correct for heterogeneity of error variance, and Tukey's HSD was used to separate means.

Antibodies

A custom antibody raised in rabbit to recognize TrnMRP4 was generated (EzBiolabs, Carmel, IN, USA) using the antigenic peptide sequence QSLRDLASKAYSEHN, which is residues 1368–1382 of the protein (accession no. ACZ64280). A TrnMRP1-specific antibody was also generated (Invitrogen Custom Antibody Service, Camarillo, CA, USA) using the antigenic peptide sequence VAESQQKVDNYQSI, which is residues 1164–1178 of the TrnMRP1 protein (accession no. ADB45217). A C-terminal cysteine was added to the peptide for KLH site-directed conjugation. A commercial antibody, T24, was also used for detection of TrnMRP protein expression in *T. ni* (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Based on a BLAST (basic local alignment search tool) alignment, the antigen used for T24 antibody synthesis shared 95% and 70% identity with the TrnMRP1 and TrnMRP4 peptide sequences, respectively (Santa Cruz Biotechnology).

Immunocytochemistry

Tissues of the Malpighian tubule and nervous system were fixed in ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS) with 0.1% Triton X-100 (PBST) at 4°C for 4 h, on a shaker at 30 r.p.m. Samples were washed in PBST for 1 h and then blocked by incubation in PBST containing 1% bovine serum albumin (BSA) at 4°C. After overnight blocking, tissues were washed in PBST for 4 h at 4°C and subsequently exposed to 1/400 dilutions of either T24 or TrnMRP-specific antibody in PBST-BSA overnight at 4°C. Specificity control assays were performed by preincubating tissues in either T24 (sc-28287 P; Santa Cruz Biotechnology)- or TrnMRP (Sigma-Aldrich, Oakville, ON, Canada)-blocking peptides at a 1/20 dilution in PBST-BSA for 2 h at 4°C. Tissues pretreated with peptide were then incubated overnight at 4°C in a PBST-BSA solution containing a 1/400 dilution of either the T24 or TrnMRP primary antibodies and 1/20 of the original blocking peptides. After all treatment and control primary antibody incubations, tissues were washed in PBST for 4 h at 4°C and incubated in a 1/200 dilution of anti-rabbit IgG Alexa-Fluor-555-conjugated secondary antibody (Sigma-Aldrich) in PBST-BSA overnight at 4°C. Tissues were then washed in PBST for 4 h at room temperature. Cell nuclei were stained by incubating tissues in a solution of DAPI (4′,6-diamidino-2-phenylindole; 200 ng ml^{-1}) in PBS for 1 h and rinsed three times for 15 min each. After rinsing, tissues were mounted in Geltol Mounting Medium (Immunon, Thermo Shandon, Pittsburgh, PA, USA).

Confocal microscopy

Fluorescence immunolabeling of tissue samples was visualized using a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). A ×63 water immersion objective and a ×10 dry objective were used to examine the subcellular localization and tissue of Alexa Fluor 555 fluorescence, respectively. For imaging, a 488 nm argon laser was used for excitation, and emission was recorded at 565 nm. DAPI nuclear staining was visualized at an excitation wavelength of 385 nm and an emission wavelength of 461 nm. Control experiments were also conducted to determine background fluorescence. These included prepared tissues incubated in secondary antibody but not primary antibody, as well as preimmune-serum-treated tissues.

Western blot analysis

To each 10 µg protein sample, 15 µl of loading buffer (10 mmol l^{-1} DTT, 6.7% sodium dodecyl sulphate, 4.6 mol l^{-1} β-mercaptoethanol, 8 mol l^{-1} urea) and 5 µl of loading dye were added. The samples were then heated to 90°C for 70 s, chilled on ice for 2 min and separated on a 7% polyacrylamide gel for 120 min at 100 V. Gels were then equilibrated in electroblotting buffer (120 mmol l^{-1} glycine, 15.6 mmol l^{-1} Tris) for 5 min. Polyvinylidene fluoride (PVDF) membranes were pre-wet in 100% methanol and equilibrated in electroblotting buffer for 5 min. Proteins were transferred to membranes with a Trans Blot SD, semi-dry transfer cell (Bio-Rad) for 60 min at 24 V. Membranes were blocked overnight in blocking buffer [150 mmol l^{-1} NaCl, 20 mmol l^{-1} Tris, 5% (w/v) skimmed milk powder, 1% (w/v) BSA, pH 7.5] with shaking. Membranes were then incubated for 1 h 45 min with either T24- or TrnMRP4-specific primary antibody diluted 1:200 in blocking buffer. Next, blots were washed at room temperature in blocking buffer for 3× 20 min, with shaking. Membranes were then incubated, with shaking, for 1 h 30 min in secondary goat anti-rabbit horseradish-peroxidase-conjugated antibody (Sigma-Aldrich) diluted 1:3000 in blocking buffer. Washes were then performed as previously described for the primary antibody. The membrane was treated with enhanced chemiluminescence (ECL) Plus western blotting detection reagents (Amersham, Oakville, ON, USA) for 2 min and chemiluminescence detected with a Chemidoc Imaging System and the data processed with Quantity One software (Bio-Rad).

RESULTS

TrnMRP gene expression

TrnMRP1 and *TrnMRP4* mRNA expression levels were compared between different tissues of *T. ni* larvae fed a diet either containing or free of the insecticide Deltamethrin or plant allelochemicals. Although no effect was seen on the expression levels of these genes as a result of changes in the diet, significant differences were observed between the mRNA levels in the different tissues examined (Fig. 1). The relative expression of both *TrnMRP1* and *TrnMRP4* mRNA was highest in the Malpighian tubules, with expression levels up to an order of magnitude higher than in other tissues (Fig. 1A,B; $P<0.0001$). The expression of *TrnMRP4* in the midgut was also greater than in the brain, integument and muscle (Fig. 1B).

TrnMRP protein expression analysis

In this analysis, two antibodies were used to identify protein in extracts of caterpillar Malpighian tubule tissue; a commercial antibody (T24), developed against a mammalian MRP peptide, having similarity to both TrnMRP proteins, and a TrnMRP4-specific antibody raised against a peptide unique to this insect ortholog. In

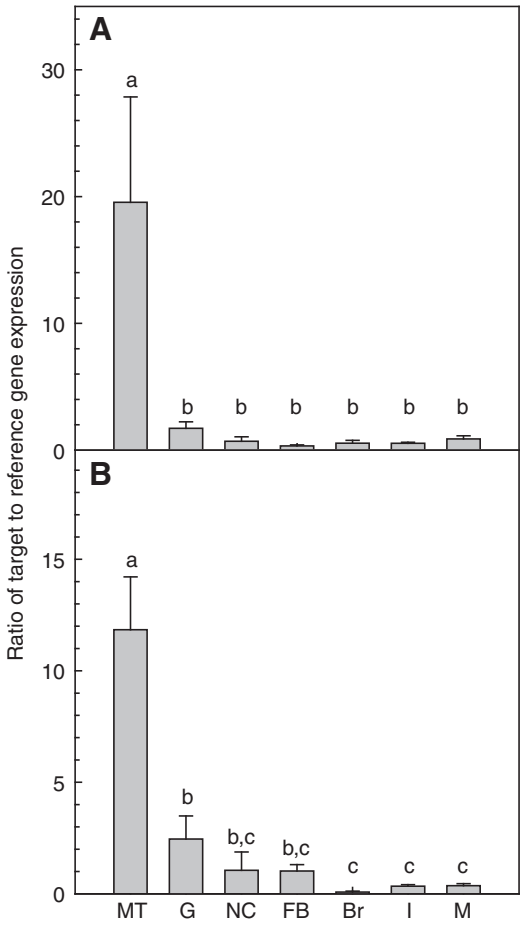

Fig. 1. Transcript expression of *TrnMRP1* (A) and *TrnMRP4* (B) relative to *S5* and *eIF4α* reference genes in tissues from *Trichoplusia ni*. Tissues analyzed were: brain (Br), fat body (FB), integument (I), Malpighian tubules (MT), midgut (G), muscle (M) and nerve cord (NC). Different letters above the columns denote statistically significant differences in gene expression levels between tissues. Values are means + s.e.m. (*N*=3).

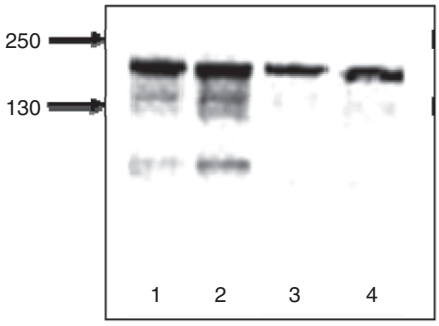

Fig. 2. Detection of TrnMRP expression in Malpighian tubule tissue extracts from fifth instar larval *T. ni*. Protein detected using the MRP-specific T24 antibody (lanes 1 and 2) and TrnMRP4-specific antibody (lanes 3 and 4). Lanes 1 and 3 are the purified membrane fractions and lanes 2 and 4 are whole cell lysates. Molecular masses (kDa) are indicated by arrows to the left of the lanes.

western blots, both antibodies interacted with a protein of an apparent size of ~190 kDa in Malpighian tubule whole-cell lysates (Fig. 2, lanes 2 and 4) as well as in Malpighian tubule membrane fractions (Fig. 2, lanes 1 and 3). Because there is 20 kDa difference in the calculated sizes of TrnMRP1 and TrnMRP4, this suggests that both antibodies were binding to just one protein, TrnMRP4. Protein bands of lower molecular mass were also detected in blots of Malpighian tubule membrane fractions and are considered to be non-specific because of their small size relative to that of a functional transporter protein (Fig. 2, lanes 1 and 2). TrnMRP1 protein expression could not be detected in Malpighian tubule protein blots using a TrnMRP1-specific antibody (data not shown).

Immunolocalization of TrnMRP4 in the Malpighian tubule

TrnMRP4 protein expression was then examined in intact Malpighian tubules using the T24 antibody, and found to be confined to a specific population of secondary cells (Fig. 3A–C). In these secondary cells, TrnMRP4 occurs on both the basolateral and apical cell surfaces, as determined from the strong and intermediate fluorescence signal observed on the basolateral and apical surfaces, respectively, as defined relative to the location of the cell nucleus (Fig. 3E,F). In each of the six Malpighian tubules typically present in a *T. ni* larva, the secondary cells are interspersed among larger principal cells in the distal segment of this organ (Fig. 3C). Each tubule appears to contain between 25 and 30 of these secondary cells. Based on western blot analyses (above) and immunolocalization shown here, these secondary cells appear to be an important location of TrnMRP4 protein expression in the *T. ni* larval Malpighian tubules. Control Malpighian tubule tissue pre- and co-treated with the immunizing peptide along with the T24 antibody confirmed the specificity of protein immunolocalization in the secondary cells (Fig. 3D). In these tissues, background fluorescence in the secondary and primary cells was uniform and low (Fig. 3D).

Immunolocalization of TrnMRPs in the nervous system

qPCR did not identify significant enrichment of *TrnMRP1* or *TrnMRP4* mRNA in nervous system tissues relative to the high levels observed for the Malpighian tubule. However, immunolocalization of the proteins within the nervous system revealed that a very select subset of cells may be expressing these proteins at high levels. Following labeling with a TrnMRP1-specific antibody, weak TrnMRP1 protein expression was revealed throughout the cortex of *T. ni* ganglia, with strong expression present in distinct neuronal cells in this cortical layer (Fig. 4). In comparison, immunohistochemical analyses of ganglia using the MRP-specific T24 antibody produced different results, which are more likely to represent TrnMRP4 protein as was found in western blots (Fig. 2). This antibody typically labeled a cell body situated in the central interneuron surrounded by the ganglial neuropile (Fig. 4D).

DISCUSSION

TrnMRP expression in the Malpighian tubule

The predominant localization of TrnMRPs to the Malpighian tubules suggests that these proteins probably contribute to the efflux and excretory function of this organ. Furthermore, a strong and moderate immunolocalization of these transporters to the basolateral and apical surfaces of secondary cells suggests their possible contribution to either primary or secondary active transport of substrates during osmoregulation. As a transporter of cyclic nucleotides (cAMP and cGMP), urate and other organic anions (Russel et al., 2008), TrnMRPs may have diverse functions, such as mediating cellular cyclic nucleotide concentrations or the excretion of metabolic wastes and toxins. It is therefore possible

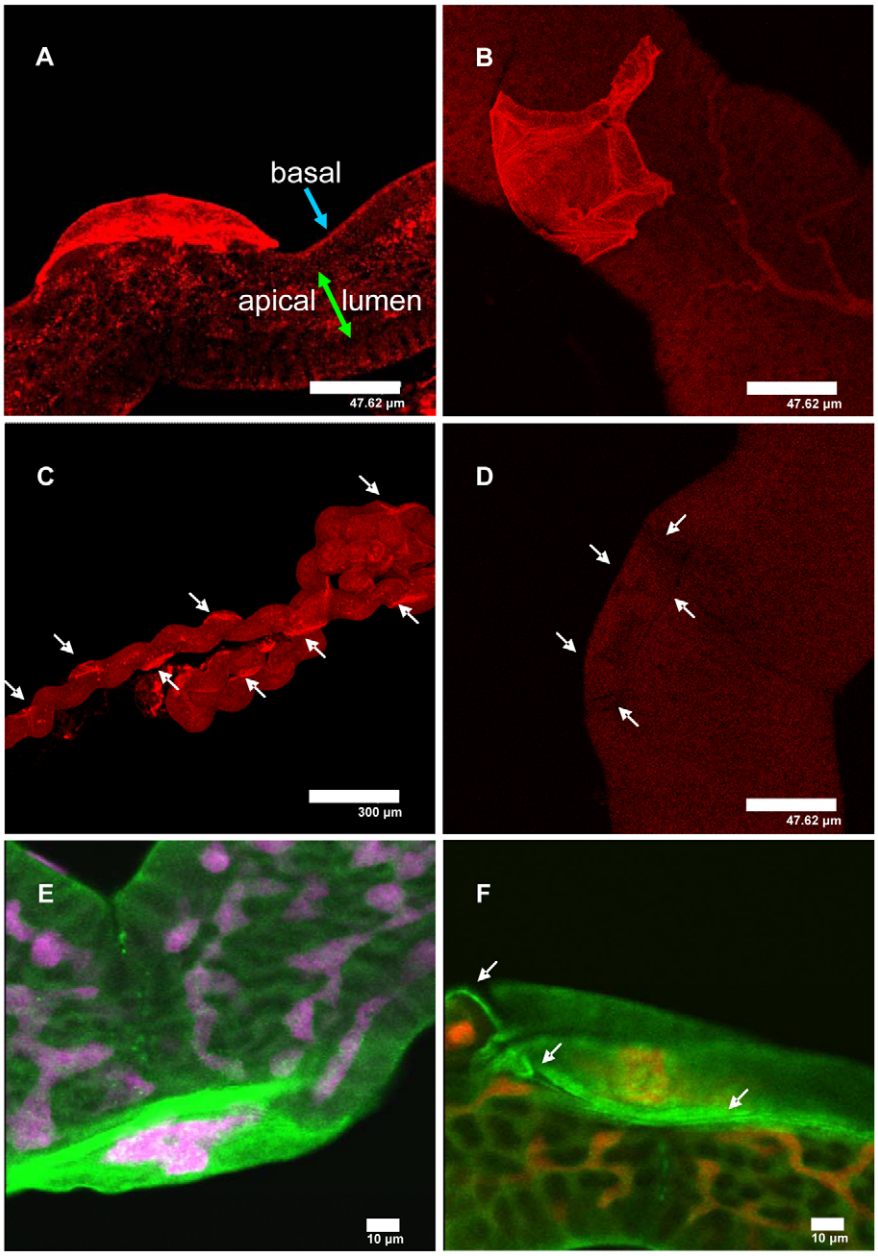

Fig. 3. Immunocytochemical localization of TrnMRP expression in the Malpighian tubule secondary cells of *T. ni*. Heavily labeled secondary cells in red (A,B) and green (E,F) are adjacent to less strongly stained polynucleate primary cells. The apical–lumenal (green arrow) and basal (blue arrow) surfaces of a tubule are indicated in A. (C) Portion of distal Malpighian tubule shown at ×10 magnification with white arrows indicating the location of secondary cells interspersed between primary cells. (D) As C, but using T24 antibody preadsorbed with peptide as a control. A secondary cell is delineated by arrows. (E,F) Tubule cell nuclei labeled with DAPI (shown in magenta in E and orange in F), to show the relative apical–lumenal (E) and basolateral (F) localizations of TrnMRP in secondary cells. Arrows in F indicate observed lateral TrnMRP expression. Antibodies used in localization experiments were the T24 anti-MRP antibody followed by Alexa-Fluor-555-conjugated anti-rabbit IgG secondary antibody.

that TrnMRPs on the apical surface maintain a barrier against toxin entry, whereas those on the basolateral surface may be associated with cyclic-nucleotide-mediated osmoregulation.

Despite the fact that *TrnMRP1* gene expression was highest in the Malpighian tubules, the expression of TrnMRP1 protein was not detectable, possibly because of non-canonical alternative splicing, which we have previously documented for its transcript (Labbé et al., 2011). Low to absent TrnMRP1 protein levels may therefore reflect either the nonsense-mediated decay of transcript or the alternative synthesis of smaller protein products with functions other than in transport, as is the case for human *ABCC12* (MRP9) which has both full-length, 4.5 kb, and short, 1.3 kb, mRNA isoforms (Bera et al., 2002).

Based on its deduced amino acid sequence, TrnMRP4 has a predicted molecular mass of 153 kDa, but it encodes eight *N*-linked glycosylation sites in the large extracellular loop of membrane spanning domain 2 (Labbé et al., 2011), which may result in a substantially increased apparent mass after glycosylation. This could account for the observed molecular mass of TrnMRP4 being nearer to 190 kDa in western blot analyses (Fig. 2). Although TrnMRP4 protein expression was detected in the Malpighian tubules, whole body and midgut protein extracts had minimal to no detectable TrnMRP4 protein (data not shown). This is consistent with our qPCR analyses and suggests the role of this protein is predominantly linked to excretory function.

In the mammalian kidney, expression of MRP proteins is highest in the nephron, where MRP1 is localized to the basolateral surface of the loop of Henle, as well as to the cortical collecting duct (Pei et al., 2002). In comparison, MRP4 is located on the apical surface of the proximal tubule (Van Aubel et al., 2005). In this region, MRP4 mediates the secretion of urate, a major end product of human purine metabolism (Van Aubel et al., 2005). MRP4 also functions in maintaining physiological homeostasis by adjusting the intracellular concentration of cyclic AMP (Nedvetsky et al., 2009). This occurs

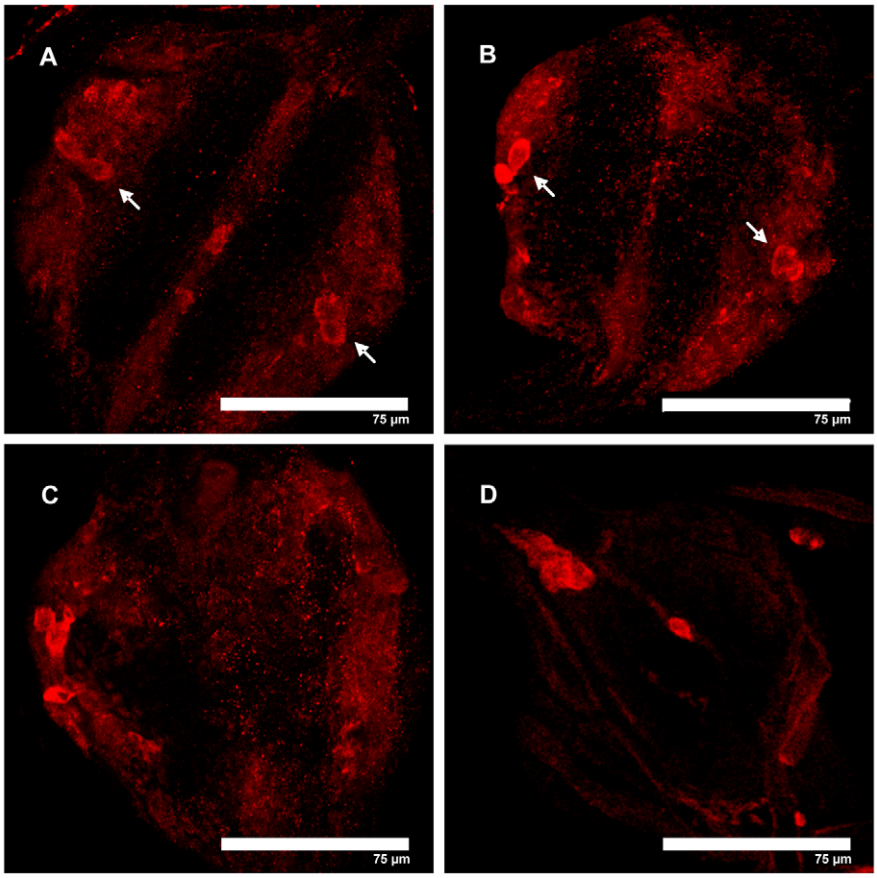

Fig. 4. Immunocytochemical localization of TrnMRP expression in the nerve cord of *Trichoplusia ni* larvae. (A–C) TrnMRP1 protein expression was present in the cortex with particularly strong levels in a specific subset of cell bodies (arrows in A and B), which were detected using a custom synthesized TrnMRP1-specific primary antibody. (D) T24 anti-MRP primary antibody instead labeled a centrally located cell in each ganglion, indicating TrnMRP4 expression in this cell. The secondary antibody used in both immunolocalizations was Alexa-Fluor-555-conjugated anti-rabbit IgG. No signal was observed in the central neuropile with either primary antibody.

by activation of protein kinase A to phosphorylate aquaporin water channels, which are then translocated to principal cells of the renal collecting duct where they may transport water (Nedvetsky et al., 2009).

In insects, Malpighian tubules are suspended in hemolymph and are tightly bound to the midgut by trachea. Until recently, expression of MRP orthologs in *Drosophila* was thought to occur uniformly throughout the body of the developing blastoderm and in the cephalic region of adult flies (Tarnay et al., 2004). However, subsequent microarray studies have shown that Malpighian tubules of *D. melanogaster* are particularly enriched for at least six ABC transporters, including members of the MRP family (Dow and Davies, 2006). In the tobacco hornworm, *Manduca sexta*, ABC transporter substrates nicotine and vinblastine are actively pumped into the tubule lumen, an activity that is inhibited by verapamil (Gaertner et al., 1998). Karnaky et al. have also shown a strong ATP-dependent transport of MRP substrates sulforhodamine 101 and Texas Red, into the lumen of tubules from the cricket *Acheta domesticus* (Karnaky et al., 2001). These studies suggest that MRP ortholog expression is just as relevant among the Insecta as in the mammalian kidney where they serve to efflux metabolic wastes and xenobiotics.

Potential role of TrnMRPs in organogenesis

The distal segment of the *T. ni* Malpighian tubule is made up of two cell types; the predominant principal cells, and a smaller number of secondary cells distributed between every four to seven principal cells. This distal portion of the tubule is bound by trachea to the iliac region of the gut. In *D. melanogaster*, the Malpighian tubules are also made up of at least two cell types, including the principal cells and secondary or 'stellate' cells, named for their irregular, star-like shape. These two cell types are derived from distinct cell lineages and have different roles in tubule function (Denholm et al., 2003). Stellate cells are known to express a number of proteins and receptors that contribute to modulating hormonally controlled osmoregulation (Dow and Davies, 2006). A number of these proteins are stellate-cell-specific and are absent from principal cells, including aquaporins, chloride channels and receptors for the diuretic peptide leucokinin.

An interesting parallel can be drawn between the TrnMRP4 expression in secondary cells of *T. ni* Malpighian tubules, observed in this study, and the development of cells known to have similar embryogenic origins in mammals. In kidney development, progenitor cells of the mesenchyme migrate to, and integrate among, specific epithelial cells that together form the nephron, the functional unit of the kidney (Jung et al., 2005). Budding of mesenchyme precursors and invasion of epithelial tissues are crucial developmental processes in the genesis of numerous tubular organs (Barasch, 2001). In *D. melanogaster*, formation of its nephritic organs, the Malpighian tubules, involves the migration of mesenchyme cells of caudal origin, to form the stellate cells, which intercalate throughout the Malpighian tubule epithelium and then develop an apicobasal polarity. The migratory nature of these secondary cells is considered an important character shared by metastatic cancer cells as well as pluripotent stem cells. That all of these cells are also often enriched in MRP and other ABC transporter proteins suggests that they may share a common developmental status.

It is also interesting to note that the TrnMRP4 ortholog expressed in *Drosophila* is one of the three mature transcripts encoded by the

gene *wunen* (Zhang et al., 1997). Other *wunen* transcripts include a zinc-finger transcription factor GFI1 and a phosphatidic acid phosphatase PAP2, which plays a role in lipid metabolism. In *Drosophila* embryogenesis, *wunen* PAP2 guides germ cells during their migration from the developing gut to the mesoderm. Whether these MRP4 orthologs hold a function in the epithelial transition of mesenchymal cells during organogenesis is still unclear. In support of this possibility, however, is the known function of the *C. elegans* MRP4 ortholog, which functions in organogenesis to prevent the accumulation of metabolic products that would otherwise halt development (Currie et al., 2007).

TrnMRP expression in the nervous system

TrnMRP1 expression was found to be low throughout the cortex of *T. ni* ganglia and high in a subpopulation of neuronal cells in this cortical layer. The diffuse expression in the cortex may explain why only moderate levels of mRNA expression were documented in this tissue. Here, TrnMRP1 may contribute to blood brain barrier function, protecting the neuronal cells of the neuropile in a way similar to P-glycoprotein ABC transporters of the tobacco hornworm, *Manduca sexta* (Murray et al., 1994). In the cortex TrnMRP1 may function to mitigate the toxicity of known MRP1 substrates, including xenobiotics and ingested allelochemicals conjugated to glutathione such as glucosinolates and isothiocyanates (Francis et al., 2005). In addition to this function, the much stronger expression of TrnMRP1 observed in specific neuronal cells of the cortex are likely to have a different role, such as in the protection of signaling function. In the human nervous system, MRP1 is localized to many brain compartments, including capillary endothelial cells (Miller et al., 2000), choroid plexus epithelial cells (Wijnholds et al., 2000) and glial cells (Dallas et al., 2003; Benyahia et al., 2004; Jin et al., 2008; Ronaldson et al., 2010).

This study suggests that TrnMRP4 also localizes to the nervous system of *T. ni* based on T24 antibody immunoreactivity. Although expression was low to absent throughout each ganglion, a single cell of the central connective displayed protein expression. The significance of this result is so far unknown, but the role of MRP4 in mammalian nerve cells is better understood. In humans, MRP4 localizes to the brain capillary endothelium and to astrocytes (Nies et al., 2004). There, MRP4 controls the penetration of xenobiotics into the central nervous system (Leggas et al., 2004; Ose et al., 2008; Yu et al., 2007). It is thus possible that TrnMRP4 has a similar function in the insect nervous system.

Role of TrnMRP expression in xenobiotic metabolism

Expression of MRPs may confer on cells and whole organisms the ability to minimize toxicity. The expression of MRP1 in the soil nematode (*Caenorhabditis elegans*) results in resistance to heavy metals through efflux of their glutathione conjugates (Broeks et al., 1996). These and other efflux pumps may also be involved in resistance to some insecticides. In their microarray analysis of *Drosophila* mRNA expression, Dow and Davies showed that organic solute transporters dominate the Malpighian tubule transcriptome, providing the capacity to actively excrete a broad range of organic solutes and xenobiotics (Dow and Davies, 2006). In this study we have examined the distribution and enrichment of the multidrug resistance proteins TrnMRP1 and TrnMRP4 expressed in the lepidopteran insect, *T. ni*. We have demonstrated that *TrnMRP1* and *TrnMRP4* are highly enriched in the caterpillar Malpighian tubules, where they probably contribute to excretion of metabolic wastes and orally consumed chemicals. Our finding that TrnMRP4 appears to be highly localized to the secondary cells of the insect Malpighian tubule is of particular interest. We have also shown that these transporters are present in small populations of cells of *T. ni* ganglia, where it is suggested that they serve functions in the blood brain barrier, which protects neural integrity, or may themselves contribute to signaling functions similar to those observed for the *D. melanogaster* ABC white protein (Borycz et al., 2008). Although our study did not show that *TrnMRP* expression is modulated by exposure of *T. ni* to the insecticide Deltamethrin or to plant allelochemicals, some studies have documented the upregulation of transporter expression in the Malpighian tubule following exposure to certain substances (Chahine and O'Donnell, 2009).

In summary, we have shown here that the TrnMRPs are highly enriched in the insect Malpighian tubule and particularly in secondary cells. These results therefore link the TrnMRPs, orthologs of transporters with significant pharmacological capabilities, to the function of the insect excretory system. We have also documented the novel finding of TrnMRP1 expression in the *T. ni* nervous system. Together these findings suggest that the TrnMRPs play a diversity of unexpected roles beyond mediating xenobiotic toxicity, including osmoregulation and neural function, which certainly merit further examination.

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