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ENHANCED XENOBIOTIC TRANSPORTER EXPRESSION IN NORMAL TELEOST HEPATOCYTES: RESPONSE TO ENVIRONMENTAL AND CHEMOTHERAPEUTIC TOXINS

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Summary

Many aquatic organisms are resistant to environmental pollutants, probably because their inherent multi-drug-resistant protein extrusion pump (pgp) can be co-opted to handle man-made pollutants. This mechanism of multixenobiotic resistance is similar to the mechanism of multidrug resistance exhibited in chemotherapy-resistant human tumor cells. In the present study, a variety of techniques were used to characterize this toxin defense system in killifish (*Fundulus heteroclitus*) hepatocytes. The cellular localization and activity of the putative drug efflux system were evaluated. In addition, *in vitro* and *in vivo* studies were used to examine the range of expression of this putative drug transporter in the presence of environmental and chemotherapeutic toxins. The broad range of pgp expression generally observed in transformed mammalian cells was found in normal cells of our teleost model. Our findings suggest that the expression of the pgp gene in the killifish could be an excellent indicator of toxin levels or stressors in the environment.

Key words: xenobiotic resistance, *Fundulus heteroclitus*, hepatocyte, biomarker, multidrug transporter.

Introduction

The multidrug-resistance (MDR) phenotype is the ability of cells to acquire resistance to many structurally unrelated cytotoxic compound. A major biochemical characteristic of MDR cells is the overproduction of the p-glycoprotein (pgp) (Juliano and Ling, 1976). In humans, the MDR1 gene encodes this p-glycoprotein, a high-molecular-mass membrane protein, overexpression of which is associated with the development of resistance to several antineoplastic agents (Gottesman and Pastan, 1993). These include anthracyclines, vinca alkaloids and epipodophyllotoxins as well as environmental contaminants such as polycyclic aromatic hydrocarbons (Yeh et al., 1992). Many known substrates for pgp are natural products that are used in cancer chemotherapy. The expression of the MDR1 gene in human neoplasms correlates with the poor chemotherapeutic efficacy of the disease (Chan et al., 1993).

The human p-glycoprotein belongs to the large superfamily of integral membrane transporters, the ATP binding cassette (ABC) superfamily, or traffic ATPases, which have been identified in a variety of normal tissue and cell types including the apical surfaces of epithelial cells in the intestine, kidney and liver and the endothelium of the blood–brain barrier (Gottesman and Pastan, 1993; Childs and Ling, 1994; Klein et al., 1999). The role of the p-glycoprotein in conferring drug resistance suggests that this protein may be involved in defense against toxic compounds. The p-glycoprotein acts as an energy-dependent drug efflux pump, and overexpression of this MDR1 gene product is, therefore, associated with a decreased intracellular accumulation of drugs in multidrug-resistant cell lines. The role of this gene was established by gene transfer experiments, which demonstrated that the expression of the mouse and human MDR1 genes results in the development of the MDR phenotype in their respective cells (Choi et al., 1988; Fairchild et al., 1990).

High levels of MDR1 gene expression (following drug treatment) have been found in several human tumors including ovarian, colon, renal, adrenal, lung and breast cancers as well as in leukemia, lymphoma, myeloma, sarcoma and neuroblastoma (Bell et al., 1985; Gerlach et al., 1987; Kakehi et al., 1988; Bourhis et al., 1989; Goldstein et al., 1989; Chan et al., 1990). Thus, specific factors are important in the regulation of pgp expression in malignant tissues, but the mechanisms involved in the regulation of MDR1 gene expression are still unclear (Hu et al., 2000). Furthermore, there appear to be multiple levels of regulation including transcriptional regulation, possibly post-transcriptional regulation and, as a consequence of transcription, MDR1 gene amplification (Scotto et al., 1986; Roninson, 1992; Hu et al.,

2000). In tumors, the occurrence of differential expression of pgp without gene amplification indicates that specific transcription factors are involved in its regulation (Gottesman et al., 1991), while in cells subjected to chemical stress, increased MDR1 mRNA levels have been observed (Uchiumi et al., 1993), suggesting that *trans*-activation of MDR1 by nuclear factors may be another mechanism leading to gene overexpression.

Marine organisms that inhabit estuaries and coastal zones are continuously exposed to a variety of naturally occurring toxins such as plant alkaloids and mycotoxins, and it has been postulated they must be equipped with a functioning defense system against such natural environmental chemicals (Kurelec, 1992; Bard, 2000). Many of the same organisms, particularly bottom-dwelling species such as flounder, mussels, sponges and the killifish (*Fundulus heteroclitus*), are regularly exposed to urban and industrial pollutants that accumulate in the sediment. These marine animals are typically exposed to complex mixtures of different chemical pollutants such as heavy metals, polycholorinated biphenyls, pesticides and polycyclic aromatic hydrocarbons. Most of these are potential carcinogens or mutagens and tend to accumulate in aquatic organisms if they are taken up from water, food and sediment. Interestingly, the levels of such pollutants in the tissues of these organisms are often lower than the concentrations in the surrounding environment (Chan et al., 1992; Kurelec et al., 1992). Thus, the tolerance of these organisms is likely to be based on a common mechanism of clearance, rather than biochemical or metabolic modifications of the individual toxins (Galgani et al., 1992; Burgeot et al., 1994). In this respect, their responses are the same as those of multidrug-resistant (MDR) tumor cells (Ling, 1995), in which pgp membrane glycoprotein overexpression serves to reduce the accumulation of diverse drugs (i.e. pollutants) (Morrow and Cowan, 1988; Bain et al., 1997; Barrand et al., 1997; Michiele et al., 1997). In addition, the accumulation of xenobiotics increased in all aquatic organisms tested when pgp function was hampered by verapamil, a known inhibitor of mammalian pgp activity (Yusa and Tsuruo, 1989; Kurelec, 1992; Waldmann et al., 1995).

P-glycoprotein-like activity has been detected in the tissues of some toxin-resistant aquatic invertebrates, and several observations indicate that this activity may be elevated by exposure to toxins (Kurelec et al., 1992; Minier et al., 1993; Galgani et al., 1996; Muller et al., 1996). Using antibodies generated against mammalian pgp proteins, expression of the putative pgp protein has been detected in various tissues in the guppy *Poecilia reticulata* and more recently in the liver of the killifish *Fundulus heteroclitus* (Hemmer et al., 1995; Cooper et al., 1999). The study in killifish was the first to demonstrating expression of the pgp homolog in a teleost from a polluted environment (Cooper et al., 1999). P. S. Cooper, P. A. Van Veld and K. S. Reece (unpublished results) have submitted a 2752 bp partial sequence, accession no. AF099732, of *Fundulus heteroclitus* multidrug-resistance transporter homolog that contains homologous regions to human MDR1 p-glycoprotein (Chen et al., 1986) and to the partial clones evaluated from the Atlantic winter flounder *Pleuronectes americanus* (Chan et al., 1992).

In the present study, a variety of techniques were used to characterize this toxin defense system in *Fundulus heteroclitus*. We evaluated how the putative drug efflux system in the liver of *Fundulus heteroclitus* complements the mammalian p-glycoprotein system in both cellular localization and activity. Additional studies evaluated the range of expression of this putative drug transporter with respect to toxin concentration with both *in vitro* and *in vivo* assays. What follows are arguments in support of the idea that the natural defense system of marine animals, particularly the killifish, is structurally and functionally similar to the MDR in mammals. In general, the vast range of pgp expression observed in mammalian cells is found in transformed cells, whereas this similar range of expression is seen in normal hepatocytes of *Fundulus heteroclitus*. These characteristics strongly suggest that the expression of the pgp gene in the killifish could be an excellent indicator of toxin or stressor levels in the environment.

Materials and methods

Animals

Adult killifish (*Fundulus heteroclitus* L.), males and females, were collected at Pellicer Creek in the Guana Tolomato Matanzas National Estuarine Research Reserve (GTM NERR) near Marineland, Florida. Pellicer Creek is considered a pristine area in this NERR and is located adjacent to the University of Florida, Whitney Laboratory Marine Research Facility. After collection, animals were housed in The Whitney Laboratory aquarium facilities prior to *in vitro* and *in vivo* experiments.

Isolation and culture of Fundulus heteroclitus *primary hepatocytes*

Hepatocytes were isolated using a modification of the collagenase disassociation procedure described previously (Smith et al., 1987). Animals were anesthetized with 3-aminobenzoic acid ethyl ester (tricaine) before excision of the liver. The liver(s) were minced and held in a traditional perfusion buffer (25 mmol l^{-1} Na_2HPO_4, 3 mmol l^{-1} KCl, 120 mmol l^{-1} NaCl, 10 mmol l^{-1} glucose and 0.1 % bovine serum albumin, pH 7.4) containing 1 mg ml^{-1} collagenase for approximately 20 min prior to filtration through eight-ply gauze and isolation by centrifugation at 800 ***g***. The cells were resuspended and washed in L-15 culture medium (Life Technologies) before layering over a Percoll (Sigma) step gradient (20 %, 30 % and 40 %) to enable separation of red blood cells from hepatocytes. Following gradient centrifugation (800 ***g*** for 15 min), the hepatocytes were removed by pipette and washed in L-15 medium, and the yield was determined. Viability was evaluated by the lactate dehydrogenase (LDH) and Trypan Blue exclusion assays. Monolayers of cells were plated onto six-well polystyrene dishes precoated with poly-L-lysine (approximately 10^7 cells

per well in L-15 medium containing 10 % fetal bovine serum) and maintained at 15 °C for 16–24 h prior to experimental manipulation of the culture medium.

Membrane protein isolation and preparation

An enriched plasma membrane fraction was isolated using a slightly modified procedure as described previously (Laine et al., 1991). Briefly, *Fundulus heteroclitus* hepatocytes previously cultured in six-well dishes were washed with phosphate-buffered saline (PBS), and the cells in each well were isolated (scraped) into 1 ml of harvesting buffer (in mmol l^{-1}): 10 Pipes, 100 KCl, 3 NaCl, 3.5 $MgCl_2$, 1 EGTA, and manufacturer-recommended amounts of protease inhibitor cocktail (Roche Biochemicals), pH 7.2, collected, and then processed through one freeze/thaw cycle in liquid nitrogen. A parallel procedure was followed for killifish liver tissue in which six livers (approximately 50 mg per liver) were added to 1 ml of harvesting buffer prior to a freeze/thaw cycle in liquid nitrogen. Complete lysis of cells was achieved by processing all cells through a progression of a 20.5-gauge needle, a 21.5-gauge needle and then 10 times through a 25-gauge needle. The cell suspension was centrifuged at 800 ***g*** to pellet nuclei, and the resulting supernatant was then centrifuged at 38 000 ***g*** for 30 min to obtain the desired membrane fraction (pellet). Membrane fractions were resuspended in harvesting buffer containing 5 % sodium dodecyl sulfate (SDS), incubated for 2 h on ice and centrifuged at 10 000 ***g*** for 20 min at 4 °C to remove insoluble material. Protein concentrations were determined for each sample using the BCA protein assay kit (Pierce).

Polyacrylamide gel electrophoresis and immunoblotting

One-dimensional sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS–PAGE) of cellular components was carried out using 3 % to 8 % gradient gels in a Tris-acetate buffer system under reducing conditions (Laemmli, 1970). Cross-reactivity of antibody C219 (see below) with marine organism pgps tended to show a rather broad band with potential smearing. We attempted to generate a somewhat 'tighter' band by resolving membrane preparations on 3 % to 8 % Tris-acetate gels (Novex) *versus* the more traditional Tris-glycine. The intention was to try to show a trend of expression without the need for densitometry or statistical evaluation. Resolved proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore) in transfer buffer (25 mmol l^{-1} Bicine, 25 mmol l^{-1} Bis-Tris, 1 mmol l^{-1} EDTA, 10 % methanol) for 2 h at 400 mA (Laine et al., 1997), and the resulting blots were blocked in Tris-buffered saline (TBS) containing 5 % nonfat dried milk for 4 h. Monoclonal mouse antibody prepared against human p-glycoprotein C219 (Signet Laboratories) was applied at 5 μg ml^{-1} for 1 h and, after washing in TBS containing 0.3 % Tween-20, anti-mouse IgG horseradish-peroxidase-conjugated secondary antibody (New England Biolabs) was added. The blots were visualized with enhanced chemiluminescence using a FluorS scanning Imager (Bio-Rad).

Cellular drug incorporation and analysis

Doxorubicin accumulation into killifish hepatocytes was evaluated in the presence and absence of verapamil following previously described procedure (Schuurhuis et al., 1989; Baldini et al., 1995; Lai et al., 1998). Briefly, a monolayer of cells was plated in 3.5 cm glass-bottomed plastic dishes (MatTek Corporation), and the cells were then incubated in medium containing 50 μg ml^{-1} doxorubicin (Sigma) in the presence or absence of 10 μmol l^{-1} verapamil (Sigma). After a designated time, the dishes were washed twice with PBS solution before microscopic evaluation and measurement of doxorubicin autofluorescence. Images were captured on a Leica fluorescence microscope equipped with fluorescein optics (excitation filter, 450–490 nm; dichroic mirror, 510 nm; barrier filter, 520 nm) using a Hamamatsu 3CCD camera with HPS-SCI C5810 Firmware (Version 1.72). The photometric measurement field areas were identical in all captured images, and all images were obtained systematically from replicate dishes in the same experiment. Results were replicated in multiple independent experiments. The fluorescence images were digitized on a 255-level gray scale, which was linear with respect to fluorescence intensity and, therefore, was proportional to the quantity of doxorubicin. The relative fluorescence for each image was calculated by subtracting the mean gray level for a background area containing no cell images from the mean gray level per image measured for 180±6 cells (color added). In all experiments, cell viability was 90 % or more, as shown by Trypan Blue dye exclusion and LDH assays.

We followed a published procedure (Schuurhuis et al., 1989) to identify the fluorescence quenching that results from doxorubicin intercalating into DNA (nuclear quenching). Essentially, we isolated nuclei from cells cultured with doxorubicin in the absence and presence of verapamil and, using a spectrofluorimeter, measured the nuclear doxorubicin fluorescence signal over time as a percentage of the total amount of doxorubicin in the nuclei (Schuurhuis et al., 1993). Generally, for these hepatocytes, 10^7 cells will very rapidly quench approximately 2 μmol l^{-1} doxorubicin fluorescence; since we were 40 times above this concentration of drug, we expected, and observed, an approximately 5 % loss of fluorescence. This was within the standard deviations calculated for the signal readings. We assumed that no quenching of the doxorubicin occurred in non-nuclear cellular compartments.

Tissue staining and immunohistochemistry

Freshly isolated livers were fixed in 4 % paraformaldehyde followed by immersion in 30 % sucrose in TBS for 16 h. The livers were then embedded in OCT compound (Tissue-Tek) and quick-frozen, and cryostat sectioning was performed to give 10 μm sections mounted on glass slides precoated with gelatin (Zhuang et al., 1999). Slides were air-dried and, if desired, stored at −20 °C. Cellular arrangement and tissue details of frozen sections were evaluated by standard staining of selected slides with Harris Hematoxylin and Eosin Y. Slides

were incubated with mouse monoclonal antibody C219 (10 μg ml^{-1}) (Signet Laboratories) raised against human MDR1 p-glycoprotein to localize the mammalian p-glycoprotein homolog (Kartner et al., 1985; Okochi et al., 1997; van den Elsen et al., 1999). Antibody specificity was visualized using rabbit anti-mouse IgG–Alexa Fluor 594 conjugate (Molecular Probes) at 15 μg ml^{-1} incubated for 1 h at room temperature (22 °C). Slides were also counterstained with 4′,6-diamidino-2-phenylindol (DAPI) to visualize cell nuclei.

Immunoelectron microscopy

Immunoelectron microscopy was performed with the assistance of the University of Florida Biotechnology Research Center Electron Microscopy Core Laboratory, Gainesville, FL. Specimens of *Fundulus heteroclitus* liver were immersed in 4 % paraformaldehyde and 1 % glutaraldehyde in PBS, pH 7.2, at room temperature for 30 min. After fixation, the tissue was washed three times in PBS, dehydrated in a graded ethanol series, embedded in Unicryl (SPI Supplies, West Chamber, PA, USA) and then ultraviolet-polymerized at 4 °C for 2 days. Ultrathin sections (70 nm) of embedded liver were cut using a Reichert Ultracut R ultramicrotome (Leica, Deerfield, IL, USA). Tissue sections were collected on Butvar-coated 100 mesh nickel grids, blocked for 10 min with 1 % non-fat dried milk in 1× high-salt Tween 20 (HST), and washed in HST for 5 min. The preparations were then incubated overnight at 4 °C in a moist chamber containing monoclonal C219 primary antibody (Signet Laboratories) (20 μg ml^{-1}) diluted with HST (1:1). A non-associated monoclonal antibody (Mab 83.5; generated against a slime mold glycoprotein) in HST (1:1000) was used as a control. The sections were then washed in HST followed by several washes in PBS and incubated in 10 μl droplets of 18 nm colloidal gold-affinipure goat anti-mouse IgG secondary antibody in PBS (1:30) for 1 h at room temperature. After incubation, the sections were washed three times in PBS for 10 min each and post-fixed with McDowell and Trump's fixative for 10 min. Sections were then jet-washed with distilled water and post-stained with 2 % aqueous uranyl acetate for 5 min followed by 5 min with Reynold's lead citrate and a final jet wash. Liver sections were examined using a Hitachi H-7000 electron microscope (Hitachi Instruments Inc., San Jose, CA, USA), and micrographs were taken on a Gatan BioScan/Digital Micrograph 2.5 (Gatan Inc., Pleasanton, CA, USA).

Results

Doxorubicin efflux

The effectiveness of many cytotoxic drugs used in human cancer chemotherapy is reduced by expression of the 170 kDa p-glycoprotein that confers multidrug resistance (MDR) (Saves and Masson, 1998). This transporter in human cancer cells has broad specificity for various unrelated substrates, including anthracyclines such as doxorubicin (DXR), and numerous studies on mammalian cells expressing multidrug transporter activity indicate a reduced accumulation of these substrates (Sharom, 1997; Shapiro and Ling, 1998). We wanted to utilize the natural fluorescence of DXR to evaluate uptake of the human chemotherapy drug in normal killifish hepatocytes. To evaluate DXR accumulation, monolayers of adherent cells in six-well dishes were incubated with the same concentration of DXR and analyzed at different times (20–80 min) by image analysis. This demonstrated a limited uptake of DXR by the cells but no significant increase in drug accumulation over the period measured (Fig. 1). To determine whether verapamil, a competitive substrate or inhibitor of multidrug transport activity in mammalian systems (Slater et al., 1982), affects the uptake of DXR in killifish hepatocytes, DXR in the presence of 10 μmol l^{-1} verapamil was incubated with cells as described previously. Analysis of the DXR fluorescence in these experiments indicated a significant time-dependent increase in DXR accumulation (Fig. 1), which was markedly greater than that found following DXR treatment alone (Figs 1, 2).

Protein expression in response to toxins

We now wanted to expand on the observation that the chemotherapy drug doxorubicin is expelled from the killifish hepatocytes in a verapamil-sensitive manner. Interestingly, a drug-resistance activity similar to characteristics of the multidrug-resistance activity demonstrated by the human MDR-pgp in cancer cells is found in killifish liver cells. In the mammalian situation, the pgp is present on the secretory domain of the plasma membrane of polarized cells, such as hepatocytes, renal proximal tubule cells, pancreatic ductule cells and small intestinal mucosal cells (Thiebaut et al., 1987). We therefore isolated and purified plasma membranes from hepatocytes cultured in the laboratory in the presence of

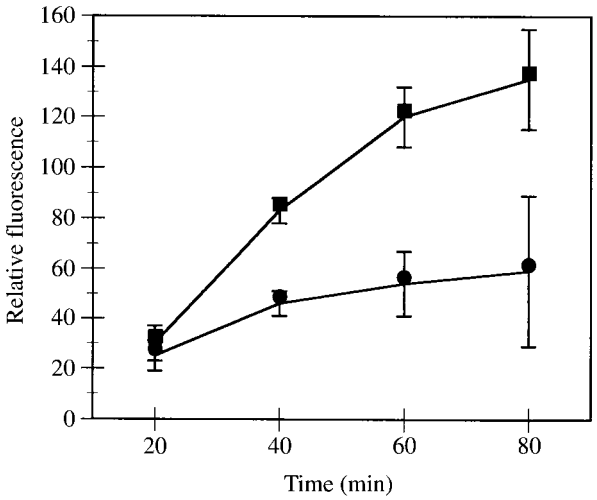

Fig. 1. Relative fluorescence in cultured *Fundulus heteroclitus* primary hepatocytes following exposure to doxorubicin (DXR) (50 μg ml^{-1}) (filled circles) or to DXR plus 10 μmol l^{-1} verapamil (filled squares) for 20–80 min. The results shown are the means ± standard deviations of one representative experiment with triplicates for each data point.

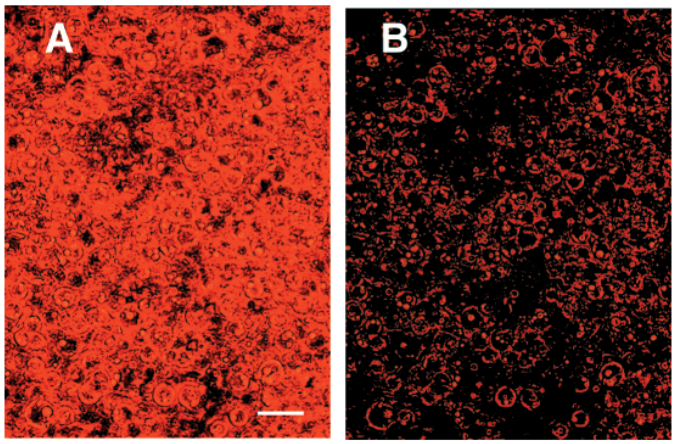

Fig. 2. Representative cellular doxorubicin (DXR) fluorescence in cultured *Fundulus heteroclitus* primary hepatocytes after exposure to 50 μg ml^{-1} DXR for 80 min in the presence (A) and in the absence (B) of 10 μmol l^{-1} verapamil. Scale bar, 25 μm. In all experiments, cell viability was always greater than 90 %, as determined by Trypan Blue dye exclusion or lactate dehydrogenase assays.

environmental contaminants and a human chemotherapeutic drug. Immunoblots were performed using protein isolated from hepatocytes incubated in varying concentrations of the human chemotherapy drug doxorubicin (Baldini et al., 1995), the first metabolite of the organophosphate insecticide Dursban (chlorpyrifos oxon) (Lanning et al., 1996) and the known hepatocarcinogen *N*-nitrosodiethylamine (Hendricks et al., 1994). The concentration of these toxins was significantly lower than reported in previous tests (Hendricks et al., 1994; Baldini et al., 1995; Lanning et al., 1996). SDS–PAGE followed by western blotting was performed on membrane preparations from all three chemical treatments of killifish primary hepatocytes and probed with monoclonal antibody, Mab C219 (Fig. 3). These unenhanced immunoblots demonstrate a concentration-dependent increase in a polypeptide of approximately 170 kDa typically identified as

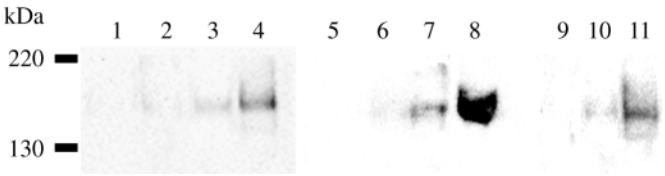

Fig. 3. Immunoblots of human antibody C219 against plasma membrane fractions isolated from cultured *Fundulus heteroclitus* primary hepatocytes. Hepatocytes were incubated for 16 h either in the presence of toxins, doxorubicin (DXR) (lanes 2–4), chlorpyrifos oxon (lanes 6–8) and *N*-nitrosodiethylamine (DEN) (lanes 10 and 11), or in their absence (lanes 1, 5 and 9). The concentrations of DXR were 1 nmol l^{-1} (lane 2), 10 nmol l^{-1} (lane 3) and 100 nmol l^{-1} (lane 4). The concentrations of chlorpyrifos oxon were 0.5 nmol l^{-1} (lane 6), 5 nmol l^{-1} (lane 7) and 50 nmol l^{-1} (lane 8). The concentrations of DEN were 0.1 μmol l^{-1} (lane 10) and 1 μmol l^{-1} (lane 11). Each lane contains 20 μg of sample.

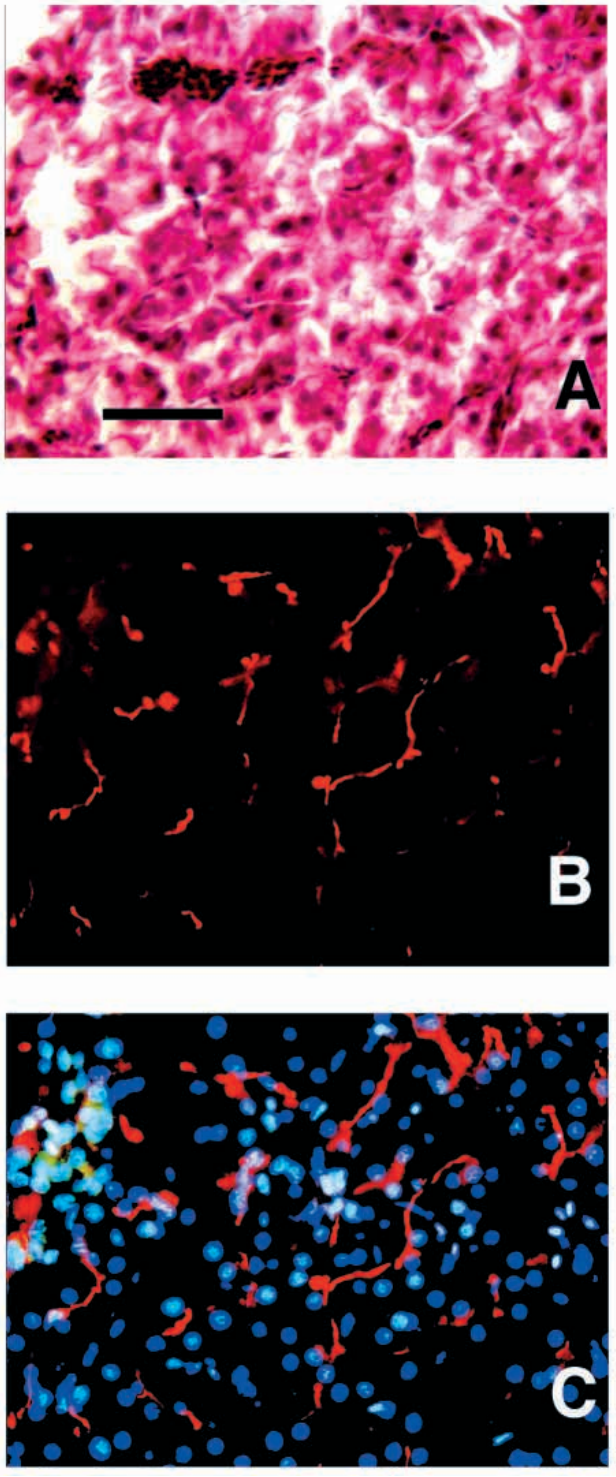

Fig. 4. Immunolocalization of the putative *Fundulus heteroclitus* xenobiotic transporter in frozen sections of liver. (A) Hematoxylin and Eosin Y staining of a liver section representative of an animal incubated either in the presence or in the absence of the toxin chlorpyrifos oxon. (B) Immunofluorescence labeling of a frozen section of liver probed with C219 primary antibody and visualized with Alexa Fluor 594 IgG, representing an animal incubated in 50 pmol l^{-1} chlorpyrifos oxon for 16 h. (C) Same section as shown in B except double-labeled with DAPI to visualize DAPI labeling of nuclei (blue). Scale bar, 50 μm.

pgp when observed in mammalian preparations and also as detected in a previous study of *Fundulus heteroclitus* liver (Cooper et al., 1999).

Histochemical localization of putative transporter

To evaluate the localization and distribution of this putative efflux protein within the cell, histochemical analysis was performed on liver tissue from treated animals. Male and female *Fundulus heteroclitus* were maintained in 2 l aquaria for 16 h either in the presence or in the absence of 50 pmol l^{-1} chlorpyrifos oxon (DowElanco), prior to excision of the liver and cryostat sectioning. Frozen sections 10 μm thick were stained with DAPI to enable nuclei to be identified and then probed with monoclonal antibody C219 specific for the

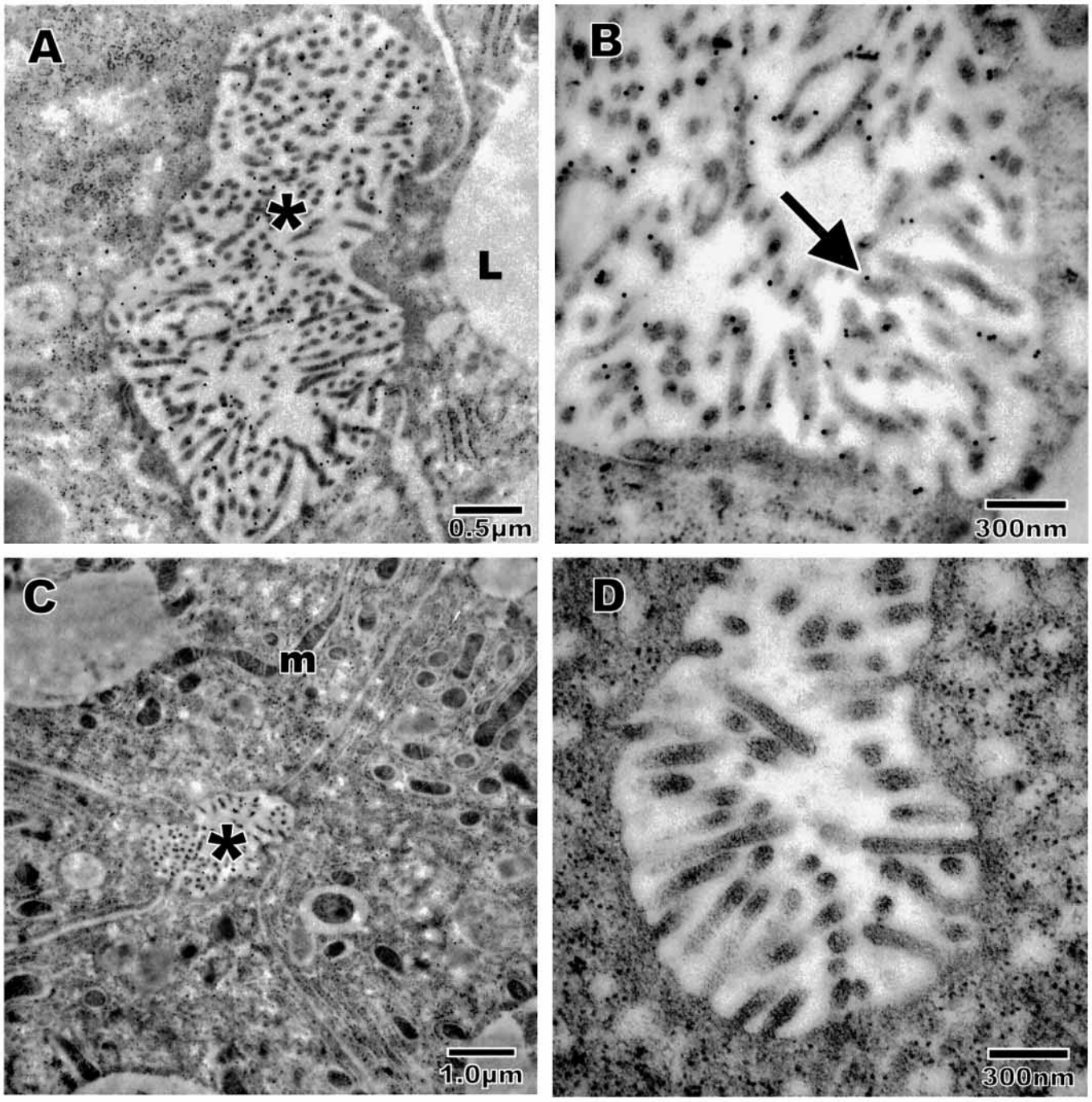

Fig. 5. Electron microscope immunolabeling using monoclonal antibody (Mab) C219 in *Fundulus heteroclitus* hepatocytes. (A) Mab C219 immunoreactivity can be seen over the bile canaliculus. (B) A higher-power micrograph showing that the 18 nm gold labeling is specifically associated with the micovilli of the bile canaliculus. (C) Control Mab 83.5 had no specific distribution of gold labeling within the cell. (D) At a higher magnification, there is no association between gold particles and microvilli in the control. The non-uniform dark specks observed throughout the cell are ribosomes. *, canaliculus; m, mitochondria; L, lipid droplets; a gold particle in B is identified by an arrow.

human p-glycoprotein drug pump (Kartner et al., 1985; Okochi et al., 1997). Other sections representing both control fish and animals incubated with toxin were selectively stained with Hematoxylin and Eosin (H&E) for standard morphological evaluation (Fig. 4A). Both control and experimental hepatocytes appeared morphologically normal, and both were characterized by flat, anastomosing plates of cells as revealed by H&E stain (Fig. 4). Alexa Fluor 594 Red (Molecular Probes) was used to detect mouse primary antibodies using immunofluorescence (Fig. 4B,C). As shown in Fig. 4C, a combination of antibody C219 fluorescence and contrasting DAPI staining of nuclei (not present in Fig. 4B) showed intense staining of the plasma membranes forming the walls of the canaliculi. Similar structures have been observed when histochemical staining for the enzyme ATPase was performed in mammalian hepatocytes to identify the bile canaliculi since the secretion of bile is considered to be an energy-dependent process (Wheater et al., 1985). Bile is synthesized by all hepatocytes and is secreted into a system of minute canaliculi that form a network between the hepatocytes. At this level of resolution, the canaliculi show no discrete structure of their own but consist of fine channels running between adjacent hepatocytes in which the walls of the canaliculi are formed by the plasma membranes of adjacent cells. Control immunofluorescence experiments using only secondary Alexa Fluor 594 antibody showed no labeling of any cellular structures (results not shown), and additional testing using a monoclonal primary antibody prepared against human beta actin protein labeled none of the cellular areas identified using monoclonal C219 (results not included). The association of staining with the plasma membrane of cells adjacent to bile canaliculi shown in Fig. 4 suggests that the putative *Fundulus heteroclitus* p-glycoprotein activity may be involved in the excretion of xenobiotics into the bile.

Electron microscopy localization of putative transporter

The distribution and localization of the putative drug pump in different cellular compartments of *Fundulus heteroclitus* hepatocytes were studied using electron microscope immunolabeling of the putative p-glycoprotein in liver tissue excised from animals incubated in 100 pmol l^{-1} chlorpyrifos oxon for 16 h. P-glycoprotein labeling was determined using antibody against human pgp, C219, and controls for labeling specificity used a monoclonal antibody Mab 83.5, generated against a slime mold glycoprotein. In the killifish hepatocyte, gold labeling was detected exclusively in the bile canaliculi associated with the finger-like projections (microvilli) of the heptocyte surface that extend into the lumen of the canaliculus (Fig. 5A,B). This finding agrees with our histochemical labeling observations and mimics results obtained for the localization of the p-glycoprotein in human hepatocytes. Controls for labeling specificity using slime mold Mab 83.5 showed little or no labeling within the hepatocyte and no particular pattern of distribution (Fig. 5C,D).

In vivo *toxin studies and the effects of nutrient deprivation on transporter expression*

Further evidence of pgp expression in response to toxins was obtained from the liver tissue of animals treated in the aquarium. Immunoblot analysis using Mab C219 was performed on hepatocyte membrane protein fractions obtained from whole animals incubated in the presence and absence of 100 pmol l^{-1} doxorubicin or chlorpyrifos oxon. Both the doxorubicin- and oxon-treated animals showed significant expression, in comparison with the control animals, of the human pgp homolog when exposed to very low concentrations of toxin (Fig. 6A). We were also interested in whether increased expression of the killifish liver pgp could be observed in response to other stressors such as nutrient deprivation in the form of amino acid starvation. Previous studies had detected enhanced human MDR1 and rat mdr1a expression in response to environmental stressors such as heat shock (Chin et al., 1990), hepatectomy (Thorgeirsson et al., 1987) and alterations in nutrient levels (Tanimura et al., 1992). We therefore incubated cultured primary hepatocytes for 9 h in either Hanks' balanced salt solution (HBSS) plus vitamins (amino-acid-starved conditions) or L-15 lacking serum (amino-acid-fed conditions). Immunoblots using Mab C219 as probes demonstrated that expression of the putative killifish pgp was sensitive to nutrient deprivation in the form of amino acid starvation (Fig. 6B). Interestingly, these observations again demonstrate the many similarities of expression between the mammalian and the putative killifish pgp.

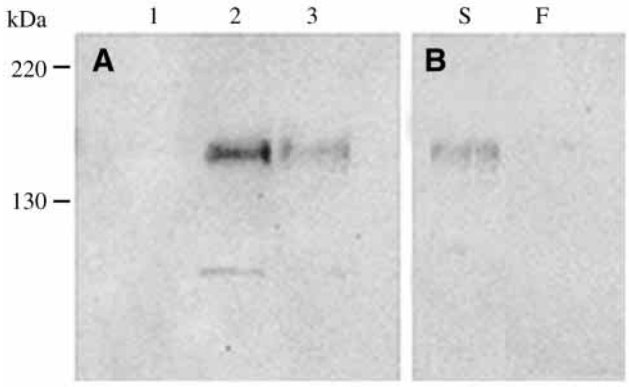

Fig. 6. Immunoblots of plasma membrane protein fractions isolated from *Fundulus heteroclitus* liver tissue (A) and from *Fundulus heteroclitus* cultured primary hepatocytes (B) using monoclonal antibody (Mab) C219. (A) Mab C219 staining of plasma membrane protein fractions from excised livers of animals maintained in 21 aquaria in the absence of toxins (lane 1) or in the presence of 100 pmol l^{-1} chlorpyrifos oxon (lane 2) or doxorubicin (lane 3) for 16 h. (B) Mab C219 staining of plasma membrane protein fractions from primary hepatocytes cultured for 9 h in the absence (S) or presence (F) of amino-acid-containing medium. Each lane was loaded with 20 μg of sample.

Discussion

Previous studies have indicated that two species of mussel (bottom-dwelling, sessile filter feeders), *Anodonta cygnea* and *Mytilus galloprovincialis*, thrive in heavily polluted environments and possess a verapamil-sensitive ability to bind the xenobiotic 2-acetylaminofluorene, a model carcinogen, to membrane vesicles (Kurelec and Pivcevic, 1989; Galgani et al., 1996; Kurelec et al., 1996). Also, a 170 kDa plasma membrane pump has been implicated in the responses of aquatic organism, including mussels and sponges, to high levels of pollutants (Kurelec et al., 1992; Galgani et al., 1996). Fish studies from other investigators have shown active extrusion of drugs from killifish renal proximal tubules using fluorescent substrates and confocal microscopy (Fricker et al., 1999; Gutmann et al., 1999). In addition, a recent study showed expression of a putative p-glycoprotein in killifish liver from a polluted environment together with expression in killifish hepatic neoplasms (Cooper et al., 1999). Thus, the natural defense system of marine animals seems to be similar to multidrug resistance in mammals. Little characterization of the efflux systems of marine organisms has, however, been reported. In addition, little is understood about the large increases in pgp expression that can occur without hepatocarcinogenesis. In this study, we wanted to demonstrate similarities between the killifish and the human drug pump, thereby characterizing its activity in this species, and to evaluate its potential usefulness in environmental quality assessment.

Using an antibody prepared against the human p-glycoprotein drug pump, we identified a polypeptide(s) with similar relative mobility in hepatocytes of the teleost *Fundulus heteroclitus*. We demonstrated that this putative mammalian homolog(s) is sensitive to low and varying concentrations of different toxins both *in vitro* and *in vivo*. Interestingly, background expression of this protein(s) both from cultured hepatocyes and from liver tissue collected from animals inhabiting a local pristine environment was very low when evaluated by immunocytochemistry and, although not absolutely proven, the approximately 170 kDa band identified on our blots is probably a killifish homolog of the mammalian p-glycoprotein drug pump.

Efflux experiments using cultured primary killifish hepatocytes demonstrated the ability of these normal cells to eliminate the chemotherapy drug doxorubicin in a manner previously observed by human drug-resistant cancer cells (Baldini et al., 1995). There is, therefore, a parallel in drug efflux activity between normal killifish hepatocytes and multidrug-resistant human cancer cells. This observation hints at the potential substrate diversity and changing level of expression of the drug pump in the normal killifish hepatocyte. Transport of doxorubicin also suggests that we are observing expression of the mammalian p-glycoprotein homolog and not of the liver-specific sister p-glycoprotein (spgp) since it is known that daunomycin, a member of the same family of drugs as doxorubicin, was not transported by spgp (Childs et al., 1995; Lecureur et al., 2000). Our observations were further supported by immunoblots generated from cultured primary killifish hepatocytes incubated with varying concentrations of three very different toxins. Protein expression, as measured by human antibody C219, was sensitive to nanomolar concentrations of chlorpyrifos oxon, doxorubicin and the hepatocarcinogen *N*-nitrosodiethylamine. Again, the basal levels of expression observed in our model were consistently very low, which enables the sensitivity of the system to toxins to be assessed. This sensitivity was also found during *in vivo* tests in which picomolar concentrations of doxorubicin and chlorpyrifos oxon elicited levels of expression discernible from those of control animals that had no toxins included in their aquarium water. Such sensitivity strongly suggests that this efflux system could be the major detoxification system within the animal and that human cancer cells have, by certain regulatory mechanisms, maintained this system.

The expression of mammalian p-glycoprotein has been characterized in hepatocytes and is known to be restricted to the bile canaliculus; a similar location and distribution of the homolog in killifish hepatocyte is to be expected if the killifish p-glycoprotein complements its mammalian counterpart. Frozen section histocytochemistry and electron microscope immunocytochemical studies demonstrated that the localization of the putative killifish homolog was indeed similar to that of its mammalian counterpart, and this polarized location in the canalicular microvilli of the hepatocyte suggests its involvement in the excretion of xenobiotics and other endogenous materials into the bile.

The relationship between p-glycoprotein expression and hepatocarcinogenesis in mammalian systems is not well understood. There is some indication of coordination of regulation between p-glycoprotein and the cytochrome P4503A family members in the mammalian liver (Silverman and Schrenk, 1997) and that, even in killifish, alterations in p-glycoprotein expression are related to tumor progression (Cooper et al., 1999). Our results suggest that p-glycoprotein-like activity in the normal killifish liver is very sensitive to a variety of xenobiotics and that a broad range of expression can occur independent of any tumor progression. This property might be useful in developing more sensitive assays of environmental changes than are currently available. Previous studies have indicated that other stressors, including heat shock stress, affect the expression of p-glycoprotein (Miyazaki et al., 1992). We also show that alterations in nutritional state such as amino acid deprivation, result in increased expression of this p-glycoprotein homolog in cultured killifish hepatocytes, suggesting a complex involvement of this xenobiotic efflux gene in cellular organization.

In conclusion, the present study further characterized the expression and activity of xenobiotic efflux pumps in marine organisms, specifically in the killifish (*Fundulus heteroclitus*). Our results confirm and enlarge on previous observations that the p-glycoprotein homolog in teleosts is an important first line of defense in detoxification. In addition, and most importantly, they show that significant expression in liver tissue can be independent of tumor progression. The killifish xenobiotic

efflux pump resembles human p-glycoprotein in sharing not only the same substrates and expression characteristics exhibited by human cancer cells but also the intracellular location and distribution observed in human hepatocytes. The range of pgp expression and activity observed in normal killifish hepatocytes using the human cancer drug doxorubicin as a substrate paralleled that of p-glycoprotein expression exhibited in human cancer cells. This observation contrasts with pgp expression in normal mammalian cells, in which RNA and protein levels generally remain low. Pgp activity in killifish is sensitive to very low and varying concentrations of toxins, indicating that pgp expression has important potential as an ecological indicator of environmental stressors.

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