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IMMUNOLOCALIZATION OF AN ANTIGEN ASSOCIATED WITH THE INVERTEBRATE ELECTROGENIC $2Na^+/1H^+$ ANTIPORTER

CLAUDINE KIMURA, GREGORY A. AHEARN, LIZ BUSQUETS-TURNER, S. R. HALEY, CHERIE NAGAO AND H. GERT DE COUET

Department of Zoology, 2538 The Mall, University of Hawaii, Honolulu, HI 96822, USA

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Summary

Epithelial plasma membranes from crustacean gut, kidney and gills have been shown recently to display an electrogenic $2Na^+/1H^+$ antiporter that differs considerably in its physiological properties from the vertebrate electroneutral $1Na^+/1H^+$ exchange paradigm. In this study, we describe the histological and cytological localization of an antigen associated with invertebrate electrogenic $2Na^+/1H^+$ antiport in lobster (*Homarus americanus*) tissues using a monoclonal antibody (MAb 11) raised in mice against purified brush border membranes of the hepatopancreatic epithelium. Previous work showed that MAb 11 inhibited electrogenic $2Na^+/1H^+$ and Ca^{2+}/H^+ exchange by hepatopancreatic brush border membrane vesicles, but was without effect on Na^+-dependent D-glucose transport, suggesting a restricted inhibitory specificity to the cation exchanger. MAb 11 binding occurred at hepatopancreatic epithelial R-cell brush border membranes, at plasma membranes of the antennal gland and gill podocytes, and at vacuolar membranes of hepatopancreatic B- and R-cells, gill nephrocytes and epithelial cells of the antennal gland labyrinth and gill lamellae, as assessed by FITC-labelled secondary antibodies. Control FITC-labelled antibodies raised in mice against vertebrate keratin proteins displayed only weak non-specific binding to the tissues and cells responding intensely to MAb 11, supporting the specific nature of MAb 11 binding to its cognate antigen. The broad histological and cytological distribution of MAb 11 binding to plasma membranes and vacuolar membranes from several lobster organ systems suggests that the physiological activities regulated by its antigen, possibly an element of the invertebrate electrogenic cation exchanger, may be diverse.

Introduction

Na^+/H^+ exchange has been studied extensively in vertebrates over several decades, as it is believed to be involved in the regulation of intracellular pH and cell volume and in the transcellular transport of Na^+ and HCO_3^- (Murer *et al.* 1976; Weinman and Reuss, 1982; Knickelbein *et al.* 1983; Rothstein, 1989; Biemesderfer *et al.* 1992). This antiporter

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system has been characterized as a plasma membrane transport protein found in a number of epithelial and non-epithelial cell types, for which the net uptake of extracellular sodium in exchange for the net extrusion of cytoplasmic protons involves a one-for-one transport stoichiometry. This electroneutral Na^+/H^+ antiporter has been found to exhibit remarkable similarity in kinetics and cation specificities across a wide range of vertebrate species and, as such, has largely been accepted as a paradigm for sodium/proton exchange in all animals (Grinstein, 1988). Further molecular characterization of this transport system has shown that different isoforms of the protein, found in different tissues or cellular locations, display a relatively high degree of amino acid homology and a molecular mass between 80 and 100 kDa (Wakabayashi *et al.* 1992).

This antiporter system has only recently been studied in detail in invertebrate cells. Results of these studies suggest that the Na^+/H^+ antiporter protein in crustacean and echinoderm brush border membrane vesicles of gut, kidney and gill epithelia is electrogenic and displays a transport stoichiometry of $2Na^+/1H^+$ (Ahearn and Clay, 1989; Shetlar and Towle, 1989; Ahearn and Franco, 1990, 1991; Ahearn *et al.* 1990). Using crustacean gastrointestinal cells, the presence of two external cation binding sites with dissimilar binding properties was verified kinetically through external proton and amiloride inhibition of Na^+ transport (Ahearn and Clay, 1989; Ahearn and Franco, 1990, 1991). It has also been reported that this electrogenic system transports calcium, since calcium and sodium were competitive inhibitors of one another and the transport of each was blocked by amiloride (Ahearn and Franco, 1990). In contrast to the broad cation specificity of the invertebrate antiporter, the vertebrate Na^+/H^+ exchanger shows no specificity towards divalent cations (Aronson, 1985).

We have recently isolated a monoclonal antibody (MAb 11) against brush border membrane vesicles of lobster (*Homarus americanus*) hepatopancreatic epithelial cells that significantly inhibited the electrogenic exchange of cations by these membranes and recognized a single protein band on Western blots of hepatopancreas, antennal gland and gill epithelia corresponding to a molecular mass of 185 kDa (de Couet *et al.* 1993). In the present investigation, MAb 11 was used in immunohistological and immunocytological studies to localize its cognate antigen, which may prove to be a protein element of the invertebrate electrogenic $2Na^+/1H^+$ antiporter, in crustacean tissues. Results indicate that the protein was present in brush border membranes of hepatopancreatic epithelial R-cells, in plasma membranes of kidney and gill podocytes and in vacuolar membranes of hepatopancreatic B- and R-cells, gill nephrocytes and epithelial cells of the antennal gland labyrinth and gill lamellae.

Materials and methods

Animals and preparation of tissue samples

Lobsters (*Homarus americanus*) were purchased from commercial dealers in Hawaii and maintained unfed at 10 °C for a few days prior to use in filtered sea water. Tissue samples were prepared for embedding and sectioning according to the paraffin wax methods developed for immunofluorescence by Sainte-Marie (1962). Each tissue sample (5 mm) was removed from a dead animal and quickly put into a tube containing 50 ml of

95 % ethanol pre-cooled to 4 ˚C. The tubes were then placed in a refrigerator (4 ˚C) for 1 h.

After an initial hardening, the samples were trimmed to pieces 2 mm thick and returned to 95 % ethanol for fixation at 4 ˚C for 15 h. The tissue samples were then dehydrated in four changes of pre-cooled absolute ethanol at 4 ˚C for 1.5 h each. Clearing was accomplished by passing the samples through three consecutive baths of pre-cooled xylene for 1.5 h each at 4 ˚C. Tissues were infiltrated through four consecutive changes of filtered, aged paraplast at 56 ˚C for 2 h each, then embedded in paraplast blocks. After cooling, the blocks were stored at 4 ˚C. A Reichert–Jung 820 microtome with Jung disposable blades was used to section the blocks. Tissue sections 7 μm thick were floated on water at 37 ˚C and transferred to clean glass slides, without application of any adhesive. The slides were allowed to dry for 15 min at 37 ˚C, then stored in a desiccator at 4 ˚C for up to 2 weeks.

For immunological testing, the slides were deparaffinized in two consecutive baths of pre-cooled (4 ˚C) xylene for 15 s each. The xylene was removed by three consecutive baths of cold 95 % ethanol for 15 s each, and the alcohol was removed in three consecutive baths of fresh ice-cold (5 ˚C) phosphate buffer solution (PBS) for 1 min each.

Hepatopancreatic cell suspension methods

Lobster hepatopancreas was finely minced in Ca^{2+}- and Mg^{2+}-free saline with 2 mmol l^{-1} EDTA and gently rocked for 30 min on ice to separate individual cells. The resulting cell suspension and debris were filtered through cheesecloth and the filtrate was pelleted in a Sorvall centrifuge at 450 ***g*** for 10 min at 4 ˚C. Cells were resuspended and incubated for 45 min at 5 ˚C in 5 ml of 3 % formaldehyde/lobster saline (Ahearn *et al.* 1983). The pellet was washed three times with lobster saline using 10 min centrifugations at 450 ***g***. The washed pellet was resuspended in 2 ml of MAb 11 in lobster saline (1:100 dilution) and allowed to stand for 1.5 h. This incubation was followed by three additional washes in lobster saline as above and resuspension of the final pellet in 2 ml of FITC-labelled goat anti-mouse IgG in lobster saline (1:250 dilution). The suspension was allowed to stand for 1 h. Three additional 10 min washes were performed on the cell suspension, followed by a final pellet resuspension in 500 μl of lobster saline containing either 40 % glycerol or 40 % Vectashield (Vector Laboratories), the latter being used to reduce fluorescence bleaching during photography after mounting on glass slides.

Preparation of the antibody

Lobster hepatopancreatic brush border membrane vesicles were prepared and purified as described previously (Ahearn *et al.* 1985; de Couet *et al.* 1993) and injected into balb/c mice. The mice were subsequently killed and their spleen was removed. Hybridoma cell lines secreting monoclonal antibodies were produced by fusion of NS-2 myeloma cells and spleen cells and were cloned by limiting dilution. Antibodies were initially selected on the basis of their specific binding to hepatopancreatic brush border membranes and were subsequently assayed by immunohistochemistry, by Western blotting and by functional assays of their ability to interfere with the electrogenic $2Na^+/1H^+$ antiporter of hepatopancreatic brush border membrane vesicles (de Couet *et al.* 1993). Antibody 11

(MAb 11) significantly inhibited electrogenic exchange of cations by these membrane preparations, recognized a target molecule with a molecular mass of 185 kDa on Western blots and was used in all subsequent localization studies reported here.

Immunological techniques

A dilution of MAb 11 (initial concentration approx. 20 mg protein ml^{-1}; Bio Rad protein assay) was prepared with buffer (phosphate-buffered saline) at a ratio of 1:100 to give a total volume of 2 ml. Deparaffinized sections of tissue were removed from the PBS bath and 100 μl of the MAb 11 solution was applied to the tissue. The slides were stored for 1.5 h at 4 °C. As a control, monoclonal anti-cytokeratin CK5 mouse ascites fluid antibody (approx. 20 mg protein ml^{-1}; Bio Rad protein assay) was purchased from Sigma. A dilution of 1:40 to give a total volume of 2 ml was applied to a set of slides as the primary antibody. This antibody was designed to react with keratin in vertebrate tissue and, as such, should not have reacted with any of the invertebrate tissues being tested. This assumption was tested by exposure of the antibody to a wide variety of crustacean epithelial and non-epithelial tissues in the present investigation and all results proved negative (C. Kimura, G. A. Ahearn, L. Busquets-Turner, S. R. Haley, C. Nagao and H. G. de Couet, unpublished observations). The slides were stored for 1.5 h at 4 °C.

FITC-labelled goat anti-mouse IgG (Gibco BRL) was used as the secondary antibody to detect binding of MAb 11 as well as binding of the vertebrate keratin antibody. A dilution of 1:100 was prepared using PBS. All the slides were washed in three consecutive baths of PBS for 1 min each to remove the primary antibody, and 100 μl of the FITC-IgG solution was applied to each sample of tissue. After 1 h at 4 °C, the slides were removed, put through three consecutive baths of PBS, and mounted in a solution of 70 % glycerol in PBS. The slides were observed with a Zeiss Universal compound microscope and pictures were taken with Kodak Pan-X 400 speed film and a Zeiss 35 mm camera under epifluorescence phase contrast or bright-field optics.

Results

In order to localize the antigen for MAb 11 in lobster tissues, histological sections stained with haematoxylin/eosin and photographed under bright-field conditions were compared with similar preparations exposed to both FITC-labelled MAb 11 and FITC-labelled vertebrate keratin antibody (control) and photographed under epifluorescense microscopy.

Hepatopancreas

The hepatopancreas in the lobster is a compact organ composed of numerous epithelial tubules possessing a luminal brush border membrane and four distinct cell types (Fig. 1A). Within a given tubule, B-cells are distinguished by the presence of single large supranuclear vacuoles of various dimensions reported to contain digestive enzymes, at acidic pH, which are synthesized by the cell and secreted into the hepatopancreatic lumen during food digestion (Loizzi, 1971; Gibson and Barker, 1979; Johnson, 1980). The most

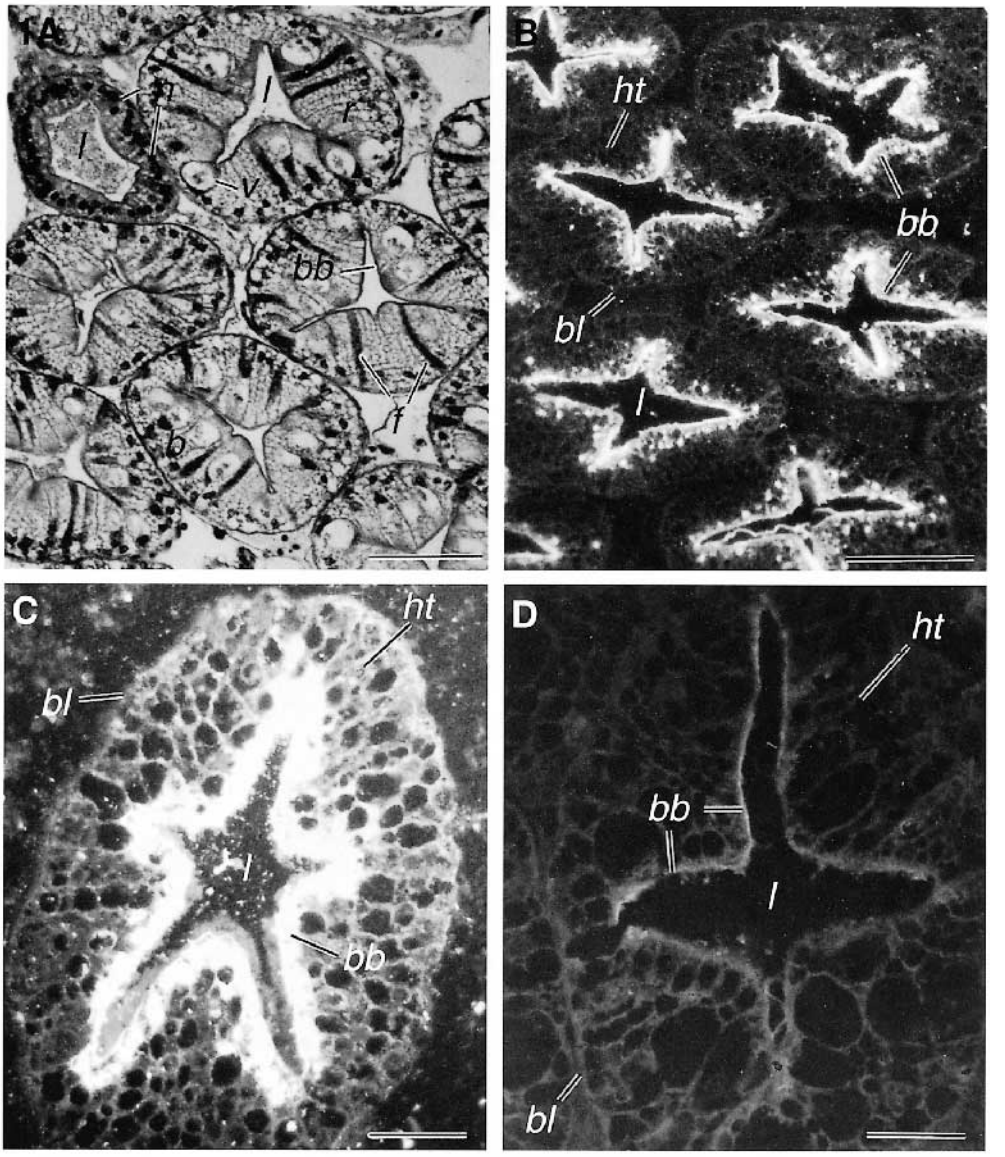
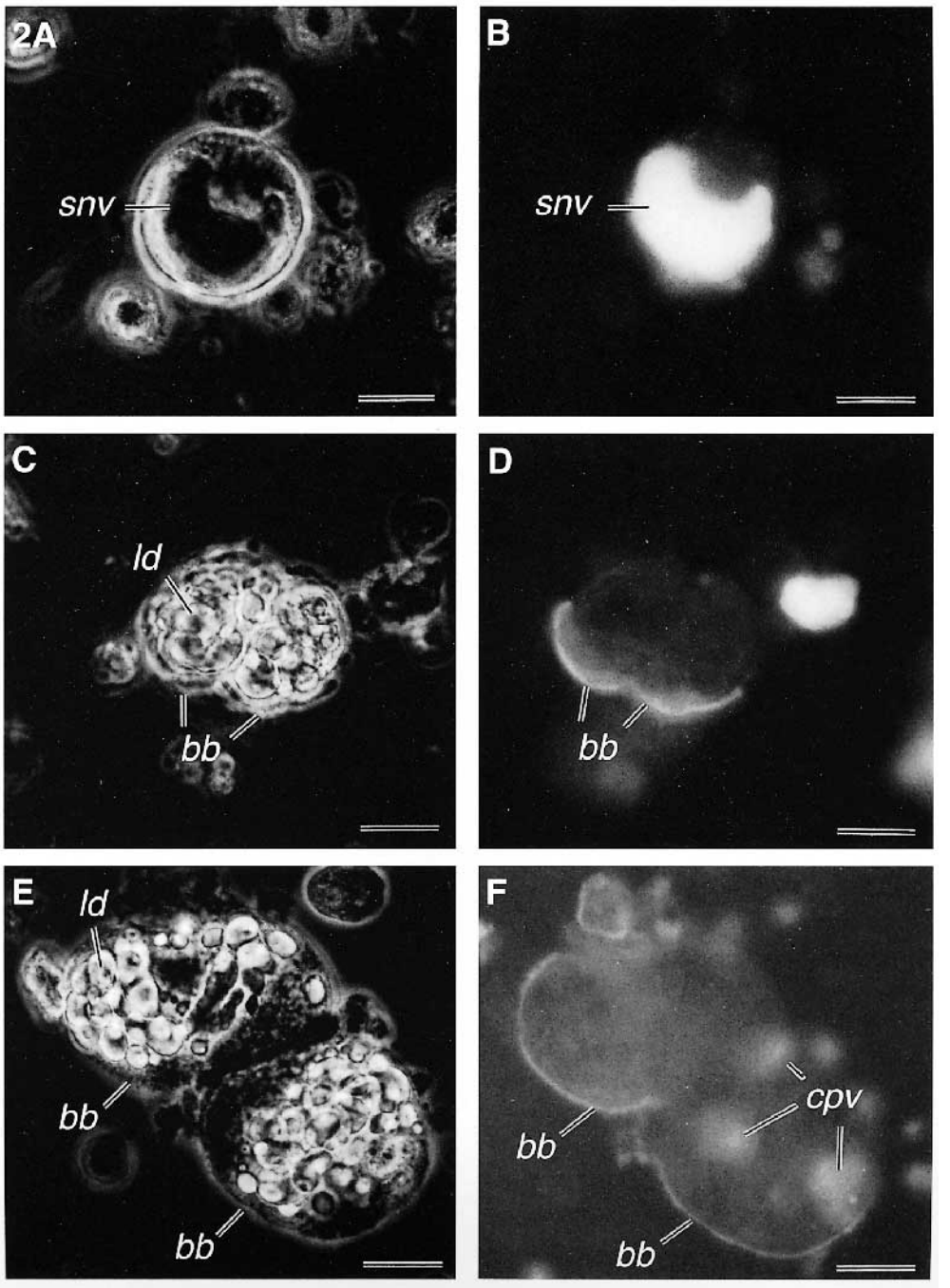


Fig. 1. Transverse sections of lobster hepatopancreas and immunofluorescent localization of mouse monoclonal antibody (MAb 11) to epithelial brush border membrane. (A) Transverse section illustrates the tubular nature of the organ, the luminal membrane of epithelium and the epithelial cell types revealed using haematoxylin/eosin staining. (B,C) Brush border localization of antigen visualized by indirect immunofluorescense using a FITC-labelled goat anti-mouse IgG. (D) Control antibody was produced against mammal keratin and did not react with hepatopancreatic tissues. *l*, tubular lumen; *bb*, epithelial brush border membrane; *b*, B-cells; *v*, hydrolytic supranuclear B-cell vacuole; *r*, absorptive R-cells; *f*, F-cells; *n*, nucleus, *ht*, hepatopancreatic tubule; *bl*, basolateral membrane. Scale bars, in A, B, 50 μm; in C, D, 20 μm.

numerous cells in the tubules are the R-cells, which contain large numbers of lipid droplets stored during absorptive activities following extracellular digestion of dietary components. Dark-staining cells with granular cytoplasm are F-cells, which are believed to mature into terminal B-cells as a result of the elaboration and expansion of



supranuclear vacuoles (Loizzi, 1971). Mitotic embryonic cells, called E-cells, are the smallest cells of the epithelium (not shown in Fig. 1), are located at the distal blind endings of each tubule and possess few cytoplasmic inclusions before they differentiate into either the F- or R-cell lines during maturation.

Exposure of histological sections of lobster hepatopancreas to either MAb 11 or the control vertebrate keratin antibody produced the results shown in Fig. 1B–D. As seen in these photographs, the entire brush border membrane lining each tubule reacted strongly with FITC-labelled MAb 11 (Fig. 1B,C), suggesting localization of its cognate antigen to this site. Small intracellular inclusions in some cells also appeared to react with the antibody in later cell suspension studies. This intracellular labelling appeared to be associated with calcium phosphate storage vacuoles of R-cells (see Fig. 2F). None of the basolateral membranes of the hepatopancreatic epithelial cells showed a response to the antibody, ruling out a serosal location for the antigen. Fig. 1D shows that the hepatopancreatic epithelium did not react with the control vertebrate antibody, eliminating the possibility that the brush border fluorescence seen with MAb 11 was of a non-specific nature.

In order to gain insight into the subcellular localization of the MAb 11 antigen in lobster hepatopancreatic epithelium, the tissue was dissociated into a cell suspension by incubation in a medium free of divalent cations, so that individual epithelial cells could be observed. The histological and ultrastructural morphology of crustacean hepatopancreatic epithelia has been well studied (Loizzi, 1971; Johnson, 1980), and specific intracellular vacuoles performing digestive and storage functions in the intact organ retain sufficient structural characteristics for their identification in suspension. Cells in suspension were first exposed to MAb 11 for 1.5 h followed by incubation for an additional 1 h with FITC-labelled goat anti-mouse IgG to localize the antigen in each hepatopancreatic cell type. Fig. 2A,B shows the fluorescent subcellular localization of MAb 11 antigen in B-cells to the continually expanding supranuclear vacuolar membrane that encloses an acidic hydrolytic enzyme mixture used in extracellular digestive activities of the stomach after it receives secretion products of the hepatopancreas. That the fluorescence of B-cells lies with the vacuolar membrane and not with its contents is apparent because when the cells extrude the vacuolar contents on microscope slides, the secreted vacuolar droplet does not fluoresce.

Fig. 2C,D shows the subcellular localization of MAb 11 antigen to the brush border membrane of hepatopancreatic R-cells in suspension. Restriction of the antigen to this

Fig. 2. Immunocytological localization of mouse monoclonal antibody (MAb 11) to lobster hepatopancreatic epithelial cells in suspension after dissociation from intact tissue. The secondary antibody was a FITC-labelled goat anti-mouse IgG. Control antibody was produced against mammal keratin and was non-reactive to all labelled structures in this plate. (A,B) Hepatopancreatic B-cell (digestive enzyme producing cells) with a large supranuclear vacuole (*snv*) in phase contrast (A) that is immunogenically reactive in epifluorescence (B). (C) Phase contrast images of R-cells (absorptive cells) with abundant cytoplasmic lipid droplets (*ld*). (D) An epifluorescent image of the same cells showing immunoreactivity to MAB 11 localized to the brush border (*bb*). (E) A phase contrast and (F) an epifluorescent picture of two R-cells showing fluorescent labelling of the brush border membranes and of intracellular vacuoles containing putative calcium phosphate concretions (*cpv*). Scale bars, 10 μm.

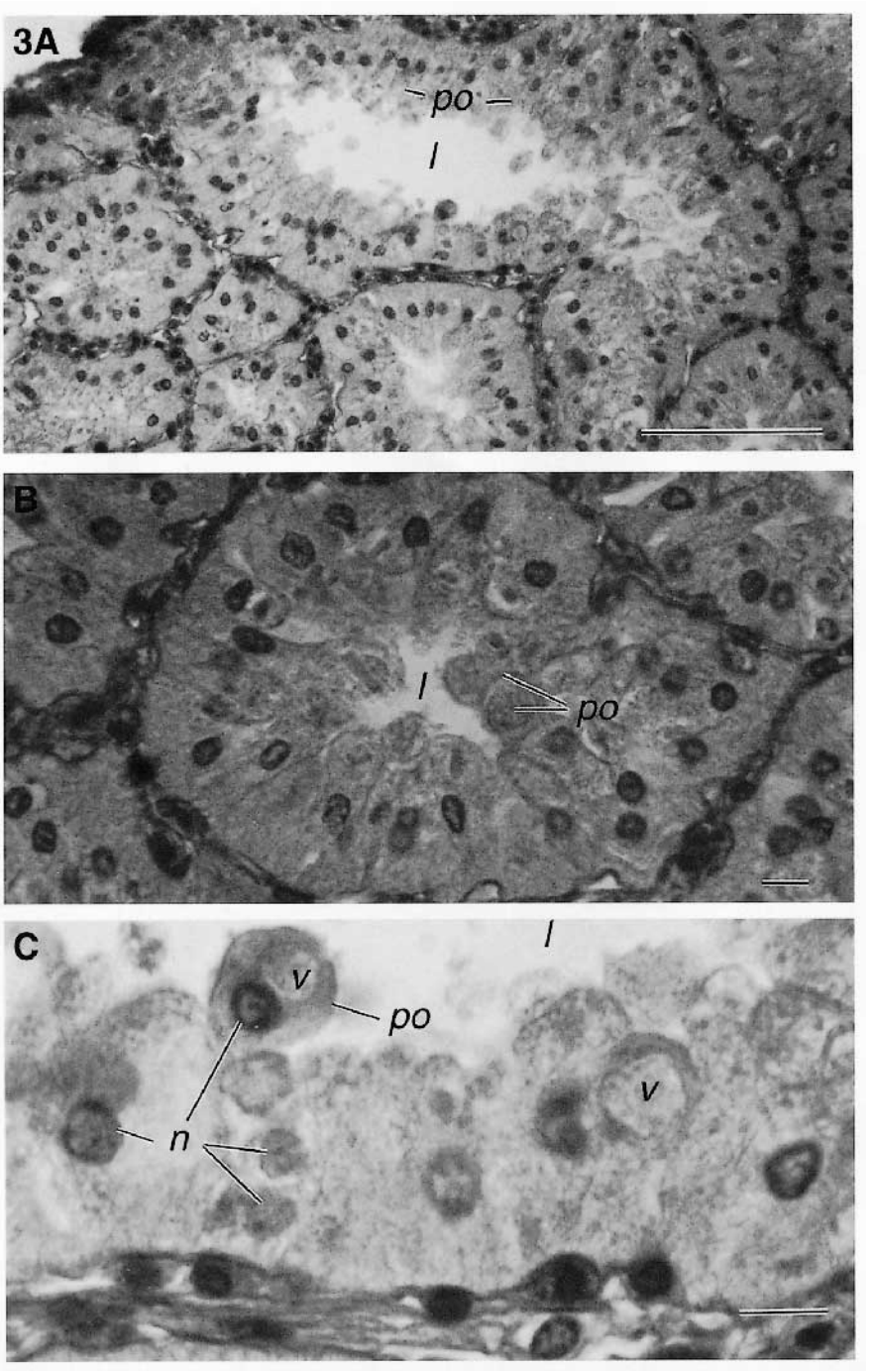


Fig. 3. Histological transverse sections of lobster antennal gland coelomosac at three magnifications stained with haematoxylin/eosin, illustrating the lobule-like nature of the organ with a lumen surrounded by vacuolate podocytes. *l*, lumen; *po*, podocyte; *v*, vacuole; *n*, nucleus. Scale bars, in A, 100 μm; in B, C, 10 μm.

cell pole is clearly apparent in this figure as the basolateral membrane shows no fluorescence under these conditions, supporting the finding that histological localization of MAb 11 binding is to the brush border membrane in hepatopancreatic tubule cross

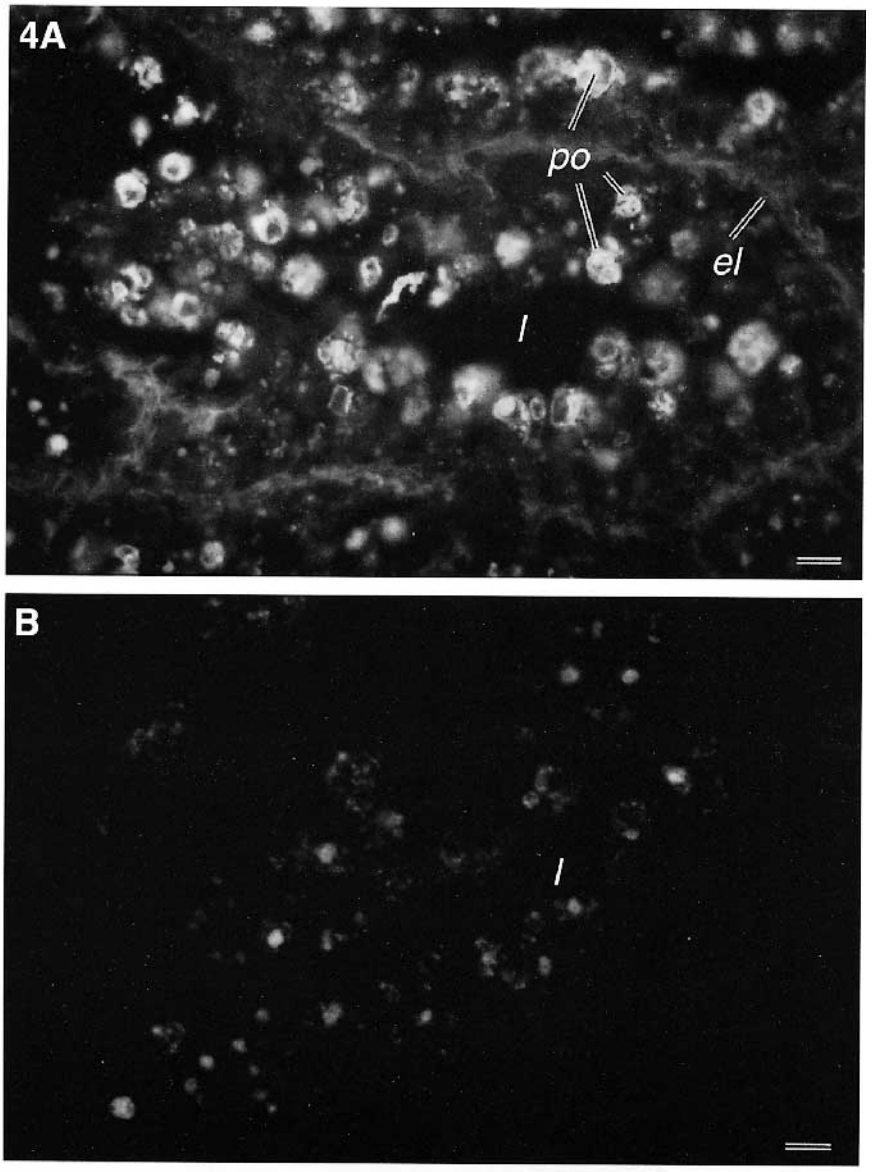
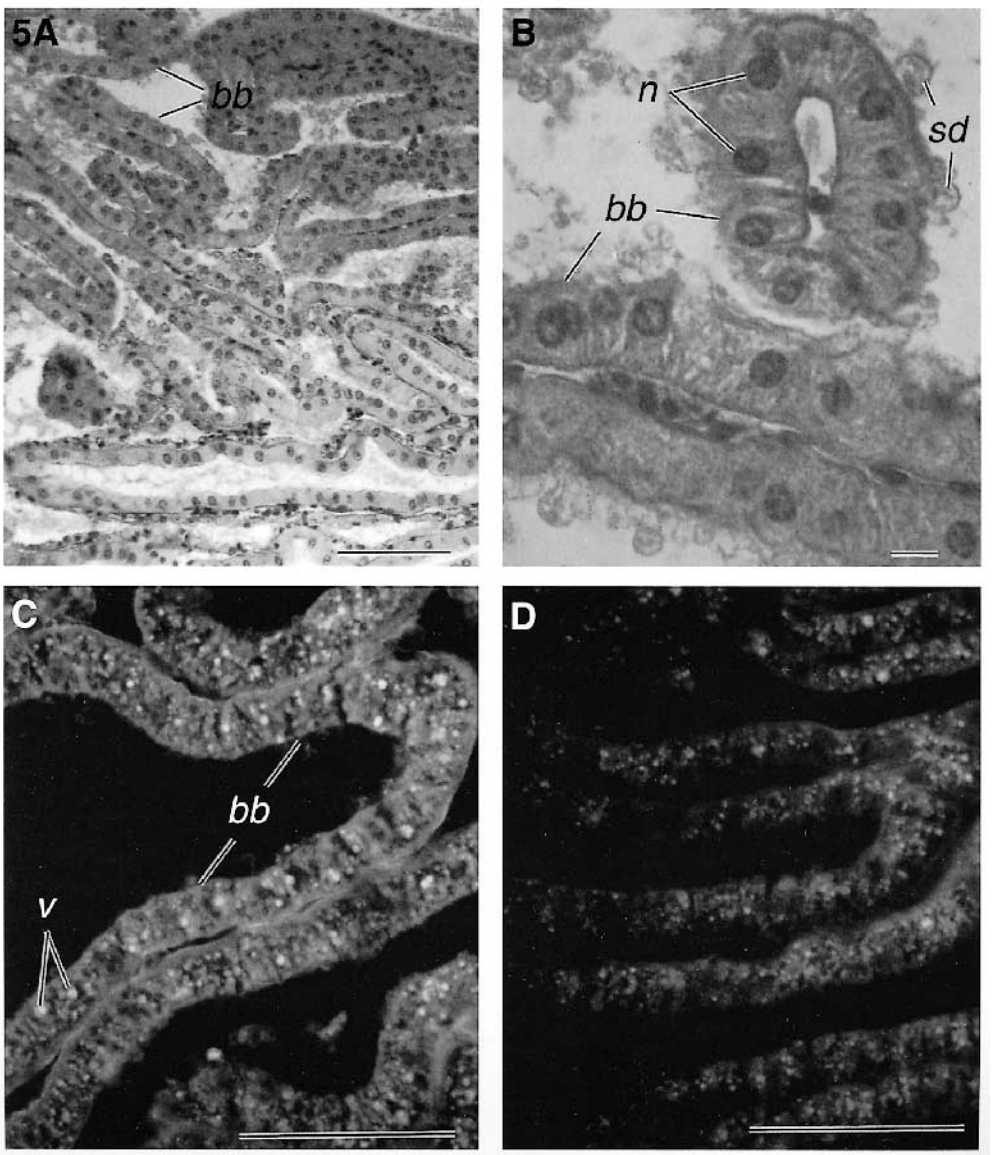


Fig. 4. Immunolocalization of mouse monoclonal antibody (MAb 11) to lobster antennal gland coelomosac podocytes in histological transverse section, illustrating general plasma membrane epifluorescence (A). Secondary antibody was a FITC-labelled goat anti-mouse IgG. Control was treated with an antibody produced against mammal keratin and labelled with a similar FITC marker. Non-specific binding of control keratin antibody was considerably less intense in similar transverse sections than was MAb 11 binding (B), supporting the specific nature of the lobster antibody. *l*, lumen; *po*, podocyte; *el*, edge of lobule. Scale bars, 10 μm.

sections (Fig. 1B,C). None of the other cell types of the hepatopancreas showed a brush border localization of the antigen in suspension, suggesting that it is the R-cells alone that express the antigen on the luminal membrane and that these are responsible for all reported brush border vesicle kinetic properties of the tissue as a whole (Ahearn and Clay, 1989; Ahearn *et al.* 1990; De Couet *et al.* 1993).

Fig. 2E,F indicates that, besides the brush border localization of MAb 11 antigen to R-cell luminal membranes, subcellular localization of the antigen also appears to occur in intracellular vacuolar membranes of these cells associated with calcium phosphate concretion formation, a process that is linked to the moult cycle of the animals in which



periodic hepatopancreatic storage and mobilization of exoskeletal calcium occurs (Johnson, 1980).

Neither hepatopancreatic F-cells nor E-cells, immature cell types giving rise to B- and R-cells, exhibited any reactivity with MAb 11 in suspension (data not shown), suggesting that expression of the antigen only occurred in fully differentiated cell types in this epithelium, but that it may be involved in a variety of physiological operations in those cells where it is found.

Antennal glands

Lobsters possess paired antennal glands (kidneys) with three major anatomical divisions: coelomosac, labyrinth and bladder. The coelomosac is composed of a series of lobule-like structures, each of which contains a lumen surrounded by epithelial cells called podocytes. The coelomosac podocytes are analogous to their vertebrate counterparts in the renal glomerulus, where they perform ultrafiltration to produce primary urine as a result of selective filtration through slit diaphragms between the cellular foot processes (Kummel, 1964; Riegel, 1971; Peterson and Loizzi, 1974; Johnson, 1980). Crustacean podocytes are believed to have both secretory and reabsorptive roles in addition to their ultrafiltration activities (Peterson and Loizzi, 1974). Fig. 3 presents low- and high-magnification histological images of the lobster coelomosac, illustrating the radial arrangement of podocytes around the lumina of coelomosac lobules. At high magnification, several of the podocytes display large intracellular vacuoles. Exposure of coelomosac histological sections to MAb 11 resulted in significant reactivity of the antibody with the podocyte plasma membranes (Fig. 4A). This epifluorescent image suggests that the entire plasma membrane of these cells expresses the appropriate antigen rather than localizing the protein to intracellular vacuolar membranes, as shown by hepatopancreatic epithelial B-cells (Fig. 2A,B). Fig. 4B indicates that the control keratin antibody showed some non-specific fluorescence with some of the coelomosac histological elements, but this was of a considerably lower relative intensity than that displayed by the crustacean antibody, indicating the specific nature of the reaction of MAb 11 with these renal structures.

The antennal gland labyrinth is an epithelial tubular system that has been compared with the vertebrate proximal tubule in structure and function (Peterson and Loizzi, 1973, 1974). Although this structure has a discrete tubular shape in some crustacean species, such as the freshwater crayfish, for marine forms, like the blue crab *Callinectes sapidus* (Johnson, 1980) and the lobster *Homarus americanus*, this organ bears more resemblance to a sponge with numerous cavities and channels. While the coelomosac is divided into

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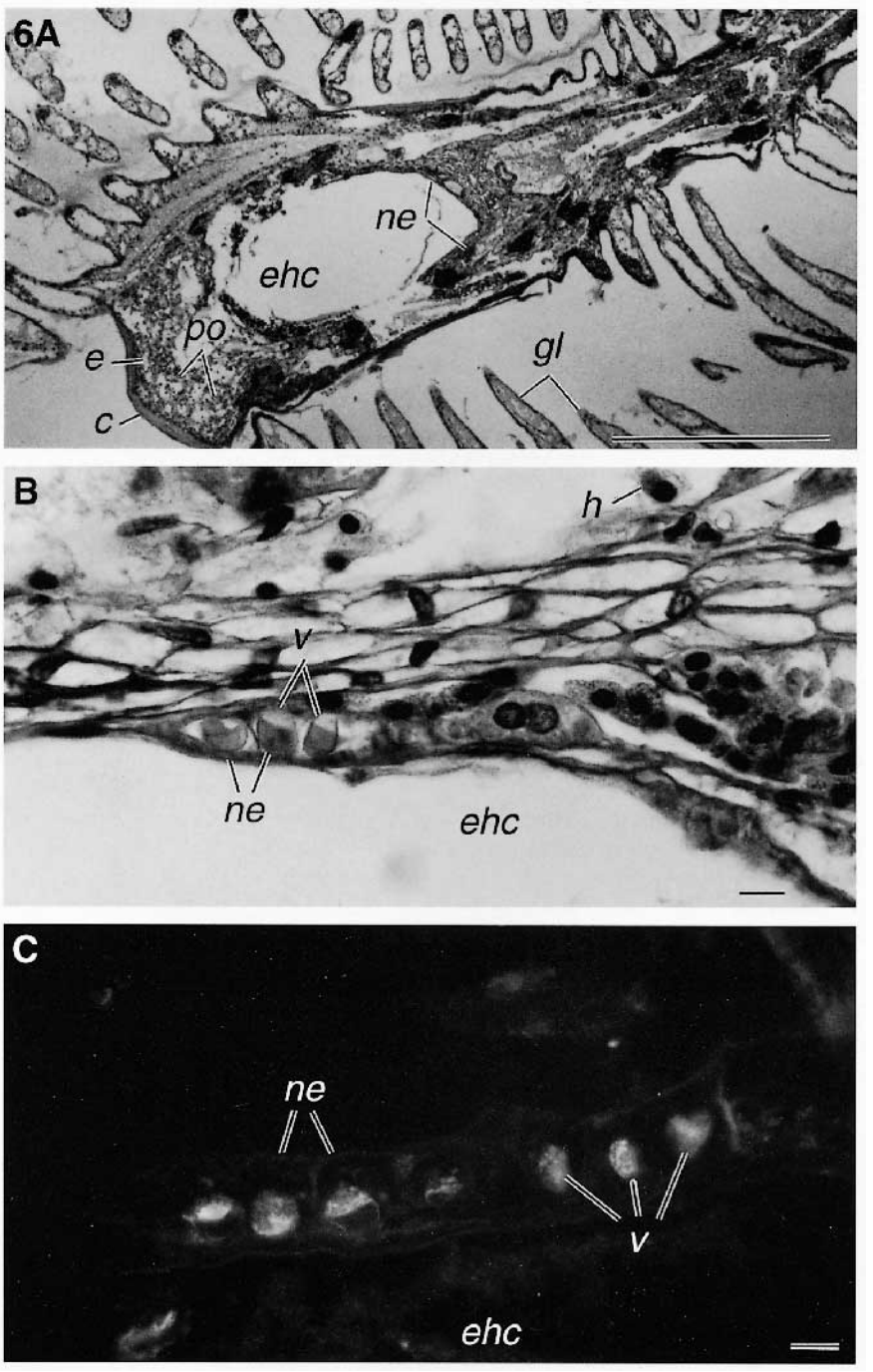
Fig. 5. Transverse sections of lobster antennal gland labyrinth stained with haematoxylin/eosin, illustrating the complex system of cavities and channels comprising the organ and the secretory nature of the epithelial cells lining the cavities (A,B). The figure also displays immunocytological localization of mouse monoclonal antibody (MAb 11) binding to lobster antennal gland labyrinth cell vacuoles and the apparent absence of antibody binding to either the brush border or the basolateral plasma membranes (C). The control keratin antibody showed little reactivity with the tissue (D). *bb*, brush border membrane; *v*, vacuole; *sd*, apparent secretory droplet; *n*, nucleus. Scale bars, in A, C, D, 100 μm; in B, 10 μm.

individual lobules with reduced lumina (Fig. 3), the labyrinth is a complex network of tubules with a voluminous luminal space into which the epithelial apical membrane is extended (Fig. 5A,B). Epifluorescence photographs of the labyrinth epithelium after



incubation of histological sections with MAb 11 showed the lack of brush border reactivity with the antibody (Fig. 5C), suggesting that the antigen is absent in this location. In contrast, numerous intracellular vacuoles appeared to fluoresce strongly with the antibody, perhaps showing localization of its antigen to the membranes surrounding these organelles (Fig. 5C). The control keratin antibody, while exhibiting some non-specific fluorescence with the labyrinth epithelium, showed a much less intense reactivity than that of the crustacean antibody (Fig. 5D), again indicating the probable specificity of the vacuolar reaction produced by MAb 11.

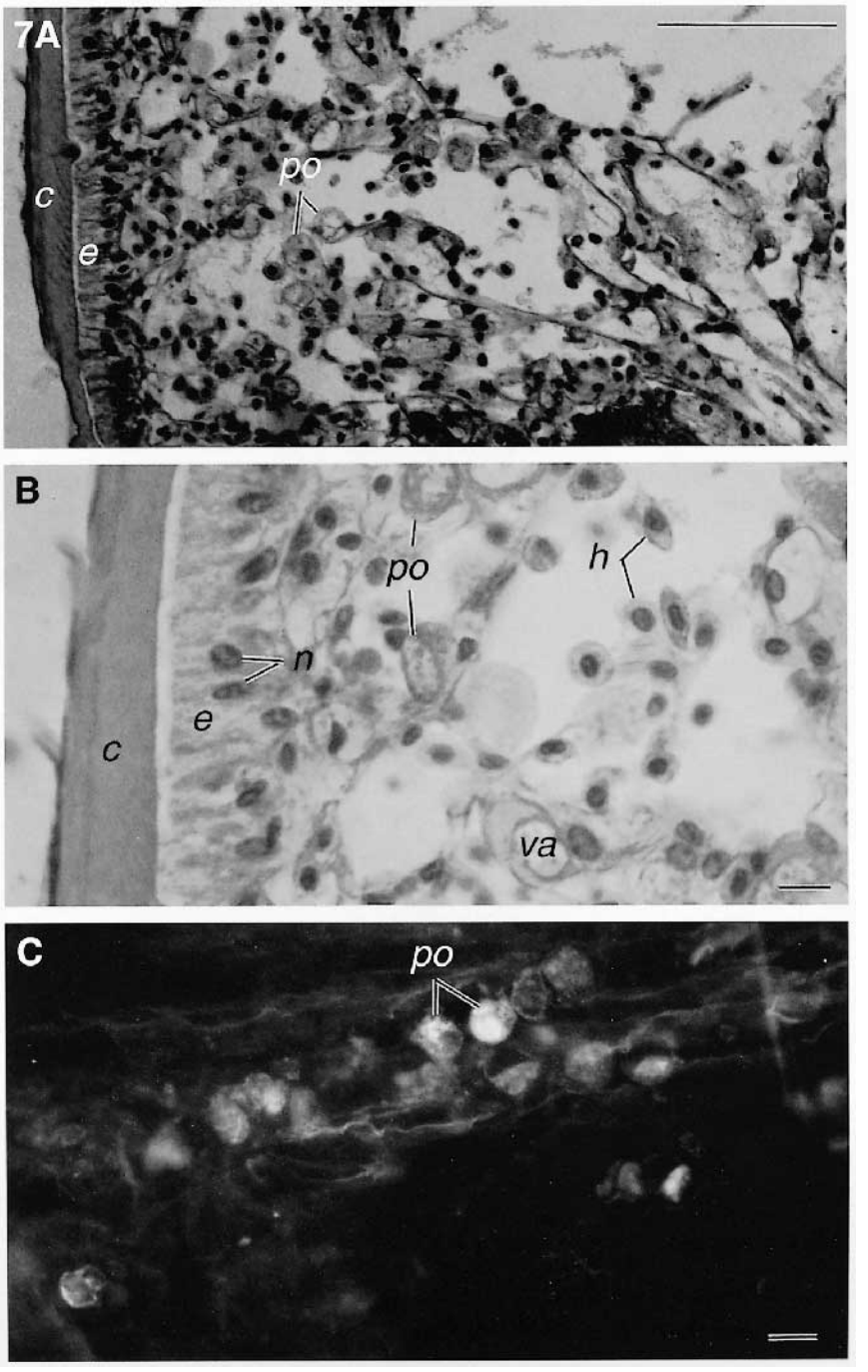
Gills

The gills in the lobster are complex structures with a variety of physiological roles, including osmotic control, respiration, excretion and storage (Drach, 1930; Johnson, 1980). Each gill consists of a central stem that bears leaf-like lamellae extending away from the central core at approximately right angles. Within the central stem, the afferent haemal channel carries deoxygenated blood from the body into the gill and, following gas and solute exchange with the external medium, the efferent haemal channel returns oxygenated blood to the animal by way of the efferent channel (Fig. 6A). Lining the efferent channels containing oxygenated blood are nephrocytes, which are common to many crustacean species (Wright, 1964; Strangways-Dixon and Smith, 1970; Foster and Howse, 1978). Nephrocytes in the lobster are characterized by cytoplasm containing a single large vacuole that, in many cells, occupies more than 50 % of the cell volume (Fig. 6B). Exposure of histological cross sections of the posterior lobster gills to MAb 11 resulted in a strong fluorescence signal from nephrocyte vacuoles (Fig. 6C), probably due to the occurrence of the antigen in the membranes of these organelles. The plasma membranes of these cells did not appear to respond to the FITC-labelled antibody, thereby showing a difference in antigen localization compared with the hepatopancreatic R-cells (Fig. 2D) and coelomosac podocytes (Fig. 4A).

Cross sections of the lobster gill stem shows that it possesses an outer cuticle covering a single epithelial layer (Fig. 7A). The gill stem contains numerous haemocytes and podocytes, the latter strongly resembling similar cells in the antennal gland coelomosac. As with the renal podocytes, the podocytes of the gill stem are large, with a voluminous cytoplasmic vacuole (Fig. 7B). Drach (1930), using a variety of vital dyes accumulated by gill podocytes, suggested that these cells may perform a storage or excretory function in crustaceans, removing substances from the blood throughout the moult cycle. Lobster gill podocytes fluoresced strongly in the presence of MAb 11 (Fig. 7C), but it is unclear from these studies whether podocyte vacuoles or plasma membrane were labelled by the antibody.

Fig. 6. Transverse sections of posterior lobster gill stained with haematoxylin/eosin, illustrating the efferent haemal channel (*ehc*), nephrocytes (*ne*) lining this structure, gill lamellae (*gl*), integumentary epithelium (*e*), stem podocytes (*po*), vacuoles (*v*) and haemocytes (*h*) (A,B). Also shown is the immunocytological localization of mouse monoclonal antibody (MAb 11) binding to nephrocyte vacuolar membranes coupled with a general lack of antibody reactivity to the plasma membranes of these cells (C). Scale bars, in A, 1 mm; in B, C, 10 μm.

Each gill lamella from the lobster has a central haemal channel surrounded by a single-layer epithelium and cuticle (Fig. 8A,B). Haemocytes often fill the haemal channel. Incubation of gill lamellae histological sections with MAb 11 resulted in a significant



FITC-labelling of epithelial structures along the entire length of each lamella (Fig. 8C) and a general lack of reactivity of the keratin antibody to the same tissues (Fig. 8D). Structures labelled with the crustacean antibody appear to be vacuoles, although an electron microscopic investigation would have to substantiate this preliminary observation.

Discussion

The present study indicates that a monoclonal antibody, directed against protein elements of the lobster hepatopancreatic brush border membrane, reacts strongly with cellular components of at least three absorptive and secretory organ systems in these animals, suggesting the possible broad tissue distribution of the antigen to which the antibody is specific. In addition, within a given epithelial organ, multiple subcellular organelle locations of the antigen were found. These results suggest that the protein antigen against which the antibody is reactive may be responsible for a variety of cellular processes that are specific to the organelles exhibiting the protein.

Although an exact identification of the antigen against which the MAb 11 antibody is directed is currently uncertain, a probable candidate for this protein is the electrogenic $2Na^+/1H^+$ exchanger first characterized in kinetic detail in the crustacean hepatopancreatic brush border membrane (Ahearn and Clay, 1989; Ahearn *et al.* 1990). In a recent study, MAb 11 significantly reduced electrogenic amiloride-sensitive $2Na^+/1H^+$ exchange and electrogenic amiloride-sensitive Ca^{2+}/H^+ exchange by brush border membrane vesicles of lobster hepatopancreatic epithelium, but was without effect on Na^+-dependent D-glucose influx in the same preparation, suggesting a relatively specific inhibitory effect on the cation antiporter (de Couet *et al.* 1993). The antibody was used in Western blots of brush border proteins to assess the molecular mass of the antigen. This analysis indicated that the antigen responding to MAb 11 exhibited a molecular mass of 185 kDa, approximately twice that of reported mammalian Na^+/H^+ exchangers (Orlowski *et al.* 1992; Wakabayashi *et al.* 1992; Yun *et al.* 1993). Therefore, although inhibition experiments strongly suggest that the antigen recognized by MAb 11 is the electrogenic cation exchanger previously characterized physiologically in crustacean hepatopancreas, antennal glands and gills, its molecular mass is considerably larger than that of the analogous vertebrate transporter and it may be involved in a number of physiological activities that are not displayed by its mammalian counterpart.

Vertebrate epithelial Na^+/H^+ exchanger isoforms are putatively localized at the brush border membrane (NHE-3; Yun *et al.* 1993), and are involved in transcellular ion transport, or reside at the basolateral membrane (NHE-1; Biemesderfer *et al.* 1992; Wakabayashi *et al.* 1992) and perform a variety of 'housekeeping' roles, such as maintenance of cell volume and pH. None of the Na^+/H^+ isoforms that have currently

Fig. 7. Transverse section of posterior lobster gill stained with haematoxylin/eosin, illustrating the cuticular lining (*c*) of the gill stem, the underlying epithelium (*e*), internal podocytes (*po*) with vacuoles (*va*) and haemocytes (*h*) (A,B). Also shown is the immunocytological localization of mouse monoclonal antibody (MAb 11) binding to stem podocytes (C). It is unclear whether the plasma membrane or vacuolar membranes of these cells are reacting with the antibody. Scale bars, in A, 100 μm; in B, C, 10 μm.

been cloned and sequenced from vertebrate cell types is found on organelle membranes, although there is an indication that an amiloride-insensitive isoform of this exchanger may be localized in vertebrate vacuoles, but its characteristics remain to be disclosed for comparison with NHE-1–4 isoforms (Gurich and Warnock, 1986). The present

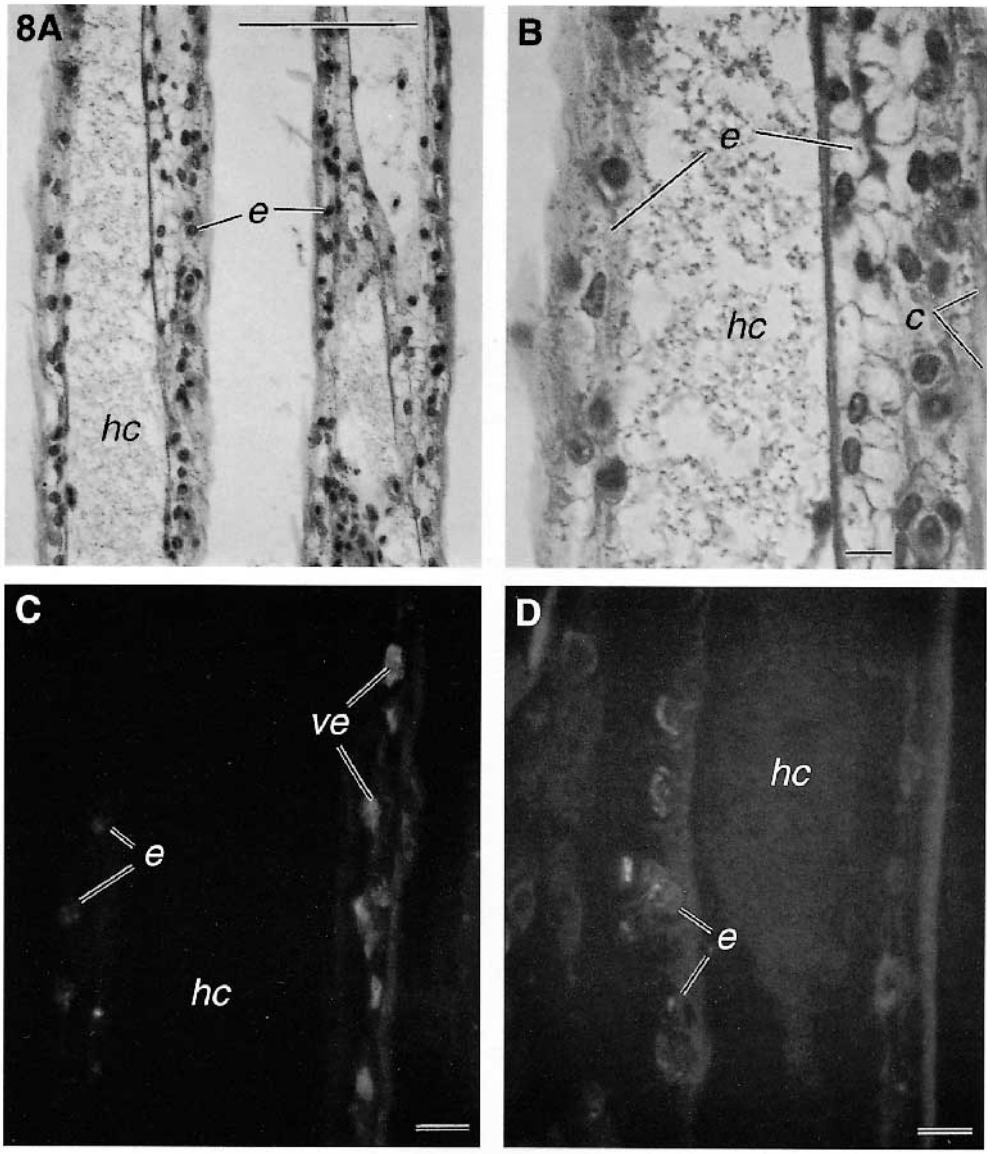


Fig. 8. Transverse section of posterior lobster gill lamellae stained with haematoxylin/eosin, illustrating the epithelial layer (*e*), associated cuticle (*c*) and central haemal channel (*hc*) (A,B). Also shown is the immunocytological localization of mouse monoclonal antibody (MAb 11) binding to epithelial vacuoles (*ve*) coupled with an apparent lack of plasma membrane reactivity with the antibody (C). Control keratin antibody did not bind to vacuolar membranes (D). Scale bars, in A, 100 μm; in B, C, D, 10 μm.

investigation suggests that the antigen binding MAb 11 is localized in at least three subcellular sites in the lobster hepatopancreas. As Figs 1 and 2 indicate, the hepatopancreatic epithelial brush border membrane fluoresces strongly with MAb 11 (associated with FITC-labelled secondary antibody), suggesting a subcellular localization of the antigen at this portion of the tissue. Fig. 2 shows that this luminal fluorescence appears largely, if not entirely, to be due to an R-cell brush border specificity as E-, F- and B-cell apical membranes were without antibody reactivity. R-cells are the most numerous hepatopancreatic epithelial cell, so the bright fluorescence shown in Fig. 1 probably relates to the fact that most of the luminal membrane is derived from fluorescing apical poles of R-cells. Should future studies confirm the identity of the MAb 11 antigen as the electrogenic cation exchanger, these localization studies presented here suggest that previous brush border physiological studies characterizing the transport properties of this exchanger (Ahearn and Clay, 1989; Ahearn *et al.* 1990) were probably investigating R-cell activities alone. R-cells are believed to be largely responsible for most absorptive processes of the crustacean hepatopancreas, transferring nutrients, ions and other substances from the lumen to the blood as a result of transcellular transport (Gibson and Barker, 1979; Johnson, 1980).

In addition to the hepatopancreatic localization of the MAb 11 antigen to the R-cell brush border, the antigen was also localized to vacuolar membranes of B- and R-cells of this organ (Fig. 2). Subcellular localization of a putative electrogenic $2Na^+(1Ca^{2+})/1H^+$ exchanger to the vacuolar membranes of B- and R-cells suggests that this antiporter may have significant roles in luminal acidification during digestion, following apocrine or holocrine secretion of the vacuole contents into the gut lumen, and may also be involved in the cyclic sequestration and release of calcium phosphate during the different stages of the moult cycle. At present, the mechanisms by which the exchanger might operate to bring about these organellar processes is unclear, but synergistic interactions with a vacuolar V-ATPase (Harvey and Nelson, 1992), providing the primary energy input to coupled membrane transport processes, is a strong possibility.

The elements of the lobster kidney showing most intense binding to MAb 11 were the coelomosac podocytes (Figs 3 and 4). Fig. 4 suggests that the antigen was localized to the podocyte plasma membrane rather than being restricted to intracellular vacuolar membranes. Johnson (1980) indicates that these cells perform filtration, secretion and absorption in the crustacean renal organ and it was therefore not surprising that the antigen should be localized to these cells if it is indeed the electrogenic cation exchanger previously characterized from a physiological standpoint. Ahearn and Franco (1990, 1993) studied the sodium and calcium transport activities of lobster antennal gland brush border membrane vesicles after removing bladder tissues from the organs. Their findings indicated that brush border vesicles produced by the combination of coelomosac and labyrinth exhibited an amiloride-sensitive electrogenic cation antiporter activity that exchanged external sodium or calcium for intravesicular protons. It was concluded in these studies that the transporting tissue responsible for these physiological properties was the tubular labyrinth. However, as Fig. 5 of the present study suggests, there was no fluorescent reactivity of MAb 11 to the labyrinth brush border, fluorescence in this organ being restricted to small intracellular vacuoles. Therefore, the predominant reactive cell

type from the lobster antennal glands was the coelomosac podocytes, suggesting that, should the antigen be the electrogenic cation exchanger, its role in kidney function may largely be defined by podocyte physiology.

Figs 6–8 indicate that MAb 11 fluorescence in lobster gills occurs in three separate locations. First, the nephrocytes lining the efferent haemal channel show intense antigen reactivity in their large cytoplasmic vacuoles. Neither the brush border nor the basolateral membranes of these cells demonstrated antigen reactivity. Second, podocytes of the gill stem were also strongly fluorescent, suggestive of either a plasma membrane and/or an intracellular vacuole location of the antigen in these cells (Fig. 7). Lastly, the epithelium of the gill lamellae demonstrated some vacuolar fluorescence activity. Since the crustacean gill performs a large number of physiological activities, including respiration, ion and osmotic regulation, excretion and storage (detoxification), the presence of the antigen in this organ could relate to any of these processes. Of significance in these gill studies, however, was the finding that no fluorescence appeared to be associated with the integumentary epithelial plasma membranes of the lobster gill, which have a suggested ion-regulatory role (Johnson, 1980). Recent work on vesicles from crab gill suggests the presence of the electrogenic cation exchanger in this organ, but its precise cellular or organelle localization remained unclear (Shetlar and Towle, 1989; Towle, 1992). If crab gill is similar to lobster gill in the localization of its ion transport system, and if the antigen localized by the MAb 11 antibody is the electrogenic antiporter, then this carrier process appears to be largely, if not entirely, restricted to vacuolar membranes and possibly to podocyte plasma membranes in this organ and may not be involved in regulating transepithelial ion transport.

In summary, an antigen binding the antibody MAb 11 has been localized to the brush border membrane of hepatopancreatic R-cells, to plasma membranes of kidney and gill podocytes, and to vacuolar membranes of hepatopancreatic B- and R-cells, gill nephrocytes and epithelial cells of antennal gland labyrinth and gill lamellae. Should the antigen identified by this antibody be the previously characterized electrogenic $2Na^+(1Ca^{2+})/1H^+$ antiporter, the localization of this carrier process, or multiple isoforms of a single protein possessing conserved epitopes, to all of these locations implies that a broad range of physiological activities may be regulated by this unique invertebrate exchanger.

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