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IMMUNOLOCALIZATION OF THE 17 kDa VACUOLAR H^+-ATPase SUBUNIT c IN *HELIOTHIS VIRESCENS* MIDGUT AND MALPIGHIAN TUBULES WITH AN ANTI-PEPTIDE ANTIBODY

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Summary

The transmembrane sector of V-ATPases is involved in proton conduction across the membrane where a 15–17 kDa proteolipid forms a putative proton channel. An affinity-purified rabbit polyclonal antibody was developed to an antigenic and putatively extracellular region of a cloned 17 kDa proteolipid. In larval tissue sections, this antibody labeled the midgut goblet cell apical membrane in *Heliothis virescens* (Lepidoptera: Noctuidae) and the apical membrane in Malpighian tubules from *H. virescens* and *Manduca sexta* (Lepidoptera: Sphingidae). The antibody also recognized the 17 kDa protein in an immunoblot of *H. virescens* Malpighian tubule homogenate. Northern blot analysis revealed the presence of two transcript sizes in the midgut (1.9 and 1.2 kb) and Malpighian tubules (2.2 and 1.9 kb). Our results strongly support the hypothesis that the 17 kDa protein is a component of the V-ATPase, where it is thought to be the proton-conducting subunit. This polyclonal antibody may provide a powerful tool for V-ATPase regulation studies, while the use of the anti-peptide antibody approach may be helpful for the immunolocalization of other ductins.

Key words: plasma membrane, goblet cell, gap junction, 16 kDa proteolipid, Lepidoptera, larvae, insect, V-ATPase, *Heliothis virescens*, confocal microscopy.

Introduction

The lepidopteran larval midgut epithelium is one cell thick and is composed of two main cell types, columnar and goblet cells, although regenerative and endocrine cells are also present (Cioffi, 1979; Baldwin *et al.* 1993). A vacuolar ATPase (V-ATPase), previously thought to be a K^+ pump, energizes the midgut goblet cell plasma membrane as well as the plasma membrane of Malpighian tubules and other transporting epithelia in lepidopterans (Schweikl *et al.* 1989; Wieczorek *et al.* 1991; Klein *et al.* 1991; Klein, 1992). The vacuolar nature of this pump in the midgut goblet cell accords with the origin of the goblet cell cavity, which arises from a closed vesicle during embryonic development of the midgut (Hakim *et al.* 1988).

The V-ATPase proton pump most commonly functions in the acidification of cytoplasmic vesicles and vacuoles and it is present in most eukaryotic cells (Nelson, 1992). First identified in organelles, it is also localized in the plasma membrane of vertebrate transporting epithelia, such as frog skin (Harvey, 1992), in osteoclasts (Chatterjee *et al.* 1992) and in kidney (Brown *et al.* 1992). The V-ATPase is arranged in two structural sectors, a peripheral or V_1 sector and an integral or V_o sector. The peripheral sector is composed of multiple subunits and, among others, it contains the catalytic subunit A and the regulatory subunit B, both of which possess ATP binding sites. Three molecules of both A and B are thought to be present in V_1 in a single ATPase complex (Nelson, 1992; for reviews of V-ATPases, see Harvey and Nelson, 1992). The V_o sector is mainly composed of the putative proton channel formed by six copies of a protein known as subunit c or 16 kDa proteolipid, with molecular masses ranging from 15 to 18 kDa depending on the organism (Meagher *et al.* 1990; Mandel *et al.* 1988; Hanada *et al.* 1991; Hasebe *et al.* 1992; Dow *et al.* 1992; Pietrantonio and Gill, 1993).

This subunit c belongs to a family of highly conserved proteins known as ductins (Holzenburg *et al.* 1993; Finbow and Pitts, 1993). Although these proteins share a very high percentage of amino acid identity, they have been implicated in diverse functions (Finbow *et al.* 1991). These include proton transport when forming the putative proton channel in the V-ATPase (Nelson, 1992), Ca^{2+}-dependent acetylcholine transport in the electric ray (*Torpedo marmorata*) electric organ, where they aggregate to a 200 kDa complex to constitute the mediatophore (Birman *et al.* 1986, 1990; Cavalli *et al.* 1993; Leroy *et al.* 1994), and intercellular communication at gap junctions in the lobster *Nephrops norvegicus*

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hepatopancreas (Leitch and Finbow, 1990; Finbow *et al.* 1992) and bovine brain (Dermietzel *et al.* 1989). Furthermore, even within the same organism, homologous proteins or the same protein may have different roles. For example, in yeast, two different genes that encode for proteins sharing 56.7 % of their identity perform two different functions; the *VMA11* gene (17 kDa product) is required for V-ATPase activity, being involved in the assembly of the subunit c (Umemoto *et al.* 1991), while the *VMA3* gene encodes the 16 kDa V-ATPase proteolipid (Nelson and Nelson, 1989). In *T. marmorata*, the same 15 kDa protein has been implicated in both the mediatophore and the V-ATPase (Brochier and Morel, 1993). In this last study, immunolocalization with a monoclonal antibody was the key step in elucidating these two functions.

We have previously reported the cloning of a 17 kDa protein isolated from a tobacco budworm, *Heliothis virescens*, larval midgut and Malpighian tubule cDNA library (Pietrantonio and Gill, 1993), which is highly homologous to the published sequences of other ductins. On this basis, and considering that the V-ATPase is very abundant in these tissues (Klein *et al.* 1991; Klein, 1992), we speculated that this cDNA encoded the proton-transporting integral subunit of the V-ATPase (Pietrantonio and Gill, 1993). We now present studies on the immunolocalization of the 17 kDa protein in *H. virescens* and *M. sexta*, obtained using an anti-peptide affinity-purified polyclonal antibody. Our results support the role of the 17 kDa protein in the plasma membrane V-ATPase of *H. virescens* larval midgut and Malpighian tubules and in *M. sexta* larval Malpighian tubules. Confocal laser microscopy, however, did not provide consistent evidence for the presence of this protein in gap junctions in the intermolt larval midgut in *H. virescens*.

Materials and methods

Insects

Heliothis virescens larvae were reared on a modified pinto bean diet (Shorey and Hale, 1965) and *Manduca sexta* larvae on a diet described by Bell and Joachim (1976). Eggs and larvae of both insect species were kept at 27 °C on a 16 h:8 h L:D cycle. Only actively feeding animals were used for experiments and they were reared individually to stage them precisely. Larvae were dissected to obtain midgut and Malpighian tubules in ice-cold saline containing (mmol l^{-1}) K^+, 39; Na^+, 15; Ca^{2+}, 6; Mg^{2+} 20; HCO_3^-, 10; Hepes, 10; sucrose, 145; and Cl^-, 96 (Bindokas and Adams, 1988).

Northern blotting

Total RNA from fifth-instar *H. virescens* midgut and Malpighian tubules was obtained by the method of Chomczynski and Sacchi (1987). Poly(A+) RNA was isolated through an oligo (dT)-cellulose type 3 column (Collaborative Biomedical Products, Becton Dickinson, Bedford, MA) equilibrated with 20 mmol l^{-1} Tris–HCl, 0.5 mmol l^{-1} NaCl and 1 mmol l^{-1} EDTA. Poly(A+) RNA was eluted with 10 mmol l^{-1} Tris–HCl and 1 mmol l^{-1} EDTA and analyzed in a 0.87 % agarose, 0.66 mol l^{-1} formaldehyde gel as described in Davis *et al.* (1986). RNA was transferred onto a Magnagraph nylon membrane (Micron Separations Inc., Westboro, MA), which was then baked for 1 h at 80 °C. Pre-hybridization, probe synthesis and hybridization conditions as well as development were performed using the non-radioactive detection Genius System (Boehringer Mannheim, Indianapolis, IN), as indicated by the manufacturer. The 1.9 kb cDNA fragment that encodes the 17.2 kDa protein (Pietrantonio and Gill, 1993) was used as a template for digoxigenin-11-dUTP-labeled probe synthesis by the random primed DNA labeling method. Probe concentration in the hybridization solution was 25 ng ml^{-1}. Pre-hybridization and hybridization steps were performed with 50 % formamide at 42 °C. Washes were 2×15 min with 2× sodium chloride/sodium citrate buffer (SSC) containing 0.1 % SDS at room temperature and 2×15 min with 0.1× SSC containing 0.1 % SDS at 65 °C. Detection was carried out using a 1:10 000 dilution of the anti-DIG alkaline-phosphatase-labeled secondary antibody and the chemiluminescent reagent Lumi-phos 530 (Boehringer Mannheim).

Hydropathy plot and antigenicity index

The deduced amino acid sequence of the putative 17 kDa vacuolar ATPase subunit was analyzed in a hydropathy plot (Kyte and Doolittle, 1982) with a selected window size of 11 residues using the DNA Strider software. Antigenicity index values were obtained using the sequence analysis software package from the Computer Genetics Group, University of Wisconsin. The hydropathy plot, the antigenicity index values and current models of the 16–17 kDa protein structure (Finbow *et al.* 1992, 1993; Jones *et al.* 1994) were used to select a region of the protein for the design of a peptide used for antibody production.

Antisera

Anti-17 kDa anti-peptide antiserum production

Polyclonal antisera were developed by Research Genetics (Huntsville, AL) in female New Zealand white rabbits, using multiple antigenic peptides (MAPs) technology. The antigen was an eight-lysine peptide core to which a 12-residue synthetic peptide was coupled through the C terminus (about 8 kDa MAP-peptide). An alanine residue also formed part of the structural lysine core. Three immunizations scheduled to occur every 2 weeks were performed by injecting the rabbits with 0.5 mg of MAP-peptide with an equal volume of Freund's adjuvant into 3–4 subcutaneous dorsal sites. The specificity of the antisera was checked by ELISA using the MAP-peptide, resulting in a titer of 1:80 000.

Antibody purification

An affinity gel was synthesized using 4 mg of peptide (synthetic peptide + lysine peptide core) coupled through amino groups to 10 ml of a carboxyl-activated support Affigel 10 (Bio-Rad Laboratories, Richmond, CA). Briefly, the peptide dissolved in 20 ml of dry dimethylsulfoxide (DMSO), and 100 μl of dry triethylamine (Sigma Chemical Co., St Louis, MO) was added to Affigel that had been rinsed with dry

DMSO. This mixture was incubated overnight at room temperature on a slow rotator. Unreacted peptide and DMSO were removed by vacuum filtration and the column was washed three times with 50 ml of DMSO. The column was resuspended and washed several times in 1 mol l^{-1} acetic acid (500 ml), then washed with sterile distilled water until a pH of 6.5 had been achieved in the flow-through. The column was then stored in sterile water containing 0.05 % sodium azide at 4 ˚C. All antibody purification steps were carried out at 4 ˚C. Affinity gel corresponding to 1 ml bed volume was equilibrated with 5× phosphate-buffered saline (PBS). 10 ml of antiserum was diluted with 10 ml of 10× PBS and added to the gel. After overnight incubation on a slow rotator, the suspension was poured into a disposable Bio-Rad column. The column was then washed with 5× PBS (60–80 ml) until no protein could be detected spectrophotometrically in the flow-through. Antibodies were eluted with 10 ml of 100 mmol l^{-1} sodium citrate (pH 2.5) in 1 ml fractions collected in tubes containing 200 μl of 1 mol l^{-1} Tris (pH 8.5). Fractions were pooled and concentrated under nitrogen by periodic addition of 1× PBS using an Amicon ultrafiltration cell system and a Diaflo PM30 filter (Amicon, Inc., Beverly, MA). Concentration was stopped when the pH reached 7.5. Protein concentration was determined and affinity-purified antibodies were stored in PBS with 10 % glycerol at 4 ˚C. From 10 ml of serum, about 600 μg of specific antibodies was obtained in different purifications.

Western blotting

Malpighian tubules of fifth-instar *H. virescens* were dissected and collected in ice-cold buffer consisting of 10 mmol l^{-1} Tris–HCl at pH 9, 0.1 mmol l^{-1} EDTA, 5 % SDS, 10 mmol l^{-1} NaCl and 1 mmol l^{-1} phenol methyl sulfoxide (PMSF), modified from Brochier and Morel (1993). Tissue was vortexed for 1 min, further homogenized with a mini pestle in an Eppendorf tube and left for 10 min on ice. Protein concentration was determined using the BCA protein assay reagent (Pierce, Rockford, IL). SDS–PAGE was run with precast gradient gels of 4 % to 20 % (Bio-Rad Laboratories), as indicated by the manufacturer. Samples containing 300 μg of protein were boiled for 3 min in sample treatment buffer before loading. Proteins were transferred to an Immobilon-P (PDVF) membrane (Millipore, Bedford, MA) in transfer buffer containing 15 % methanol using a Bio-Rad mini blot system (92 V, 90 min) at 4 ˚C. The blot was blocked for 45 min with 5 % normal goat serum (NGS) (Vector Laboratories, Inc., Burlingame, CA) in PBS containing 0.05 % Tween (PBST), exposed overnight to anti-peptide affinity-purified antibodies (30 μg ml^{-1} in PBST), washed for 3×10 min with PBST and developed using goat anti-rabbit alkaline-phosphatase-labeled secondary antibodies at a concentration of 1:2000 (HyClone Lab. Inc., Logan, UT) and Western Blue substrate (Promega Corp., Madison, WI).

Immunohistochemistry

Paraplast sections

Midgut contents and peritrophic membranes of *H. virescens* and *M. sexta* larvae were discarded during dissection, and tissues were flushed with buffer and immediately fixed overnight in Bouin's solution (water-saturated picric acid 72 % v/v, glacial acetic acid 5 % and 23 % of 37 % formaldehyde solution) at 4 ˚C. After fixation, tissues were serially dehydrated for 2×30 min in 70 %, 96 % and 100 % ethanol followed by 2×1 h in chloroform. Tissues were penetrated in Paraplast-Xtra (Fisher Scientific, Pittsburgh, PA) at 60 ˚C for 1 h and blocks were stored at 4 ˚C. Using a rotatory microtome, sections 8–15 μm in thickness were cut, placed on polylysine-coated slides and dried for 2 days at 39 ˚C.

Immunocytochemistry

Sections were dewaxed and rehydrated by soaking them in two changes of toluene for 15 min and then in two 10 min changes of 100 %, 96 % and 70 % ethanol and 30 min in water. After a 5 min incubation with 0.02 % proteinase K (20 mAnson units mg^{-1}, Gibco BRL, Grand Island, NY; 1 mAnson unit is the amount of enzyme that liberates 1 μmol of Folin-positive amino acid in 1 min at 37 ˚C using hemoglobin as a substrate) in 50 mmol l^{-1} Tris–HCl (pH 7.5) at room temperature, the sections were washed for 3×5 min with PBS containing 0.05 % Triton X-100 (PBSTx). Sections were blocked with 5 % NGS in PBSTx for 1 h, incubated overnight with either 5 % NGS in PBSTx or 1:200 preimmune serum in PBSTx as negative controls, or with anti-peptide affinity-purified antibodies (30 μg ml^{-1} protein in PBSTx containing 2 % NGS). Sections were washed for 3×15 min with PBSTx and incubated for 1 h with goat anti-rabbit biotin-labeled secondary antibodies at 1:200 dilution (Hyclone), washed for 4×3 min with PBSTx and incubated for 1 h with streptavidin biotinylated horseradish peroxidase complex (Amersham Corporation, Arlington Heights, IL) at 1:100 dilution in PBSTx. After washing for 4×3 min with PBSTx, 3,3′-diaminobenzidine (DAB) peroxidase tablet sets (one-third dilution in water) (Sigma Fast, Sigma Chemical Co.) were used for detection. Slides were mounted in glycerol–gelatin. Photographs were taken on Kodak Gold 100 ASA film with a Zeiss Axiophot microscope using Nomarski optics.

Tissue sections for immunofluorescence were processed as above using as secondary antibody a Texas-Red-labeled goat anti-rabbit IgG (H+L) (Vector). Tissue sections used as positive controls for V-ATPase immunodetection were probed with rabbit antiserum produced against the expressed product of a V-ATPase B subunit cDNA clone obtained from larval midgut and Malpighian tubules of the mosquito *Culex quinquefasciatus* (L. S. Ross and S. S. Gill, unpublished results). Preimmune and immune sera from female rabbits were used at 1:100 dilution. Sections were examined by conventional epifluorescence. Tissue sections probed with the anti-peptide antibody were also analyzed by confocal microscopy, which was performed with a Bio-Rad MRC-600 LSCM equipped with a krypton/argon mixed-gas laser.

Results

Northern blot

The 1.9 kb cDNA clone encoding the 17.2 kDa protein had

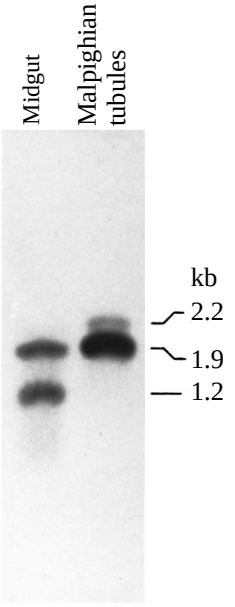

Fig. 1. Northern blot of *Heliothis virescens* midgut (8.5 μg) and Malpighian tubule (2 μg) poly(A)+ RNA. Transcript sizes were estimated using a 0.24–9.5 kb RNA as a standard.

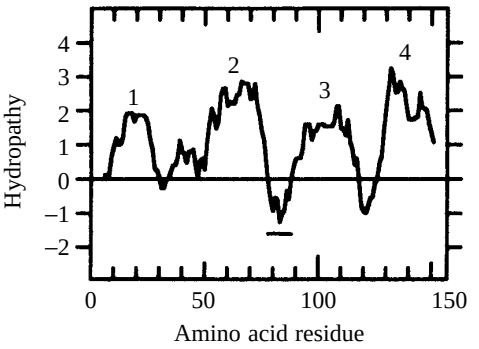

Fig. 2. Hydropathy plot of the 17 kDa protein predicted amino acid sequence. Sequences contained within the upper panel are hydrophobic, whereas sequences in the lower panel are hydrophilic. The second hydrophilic loop marked with a black bar, consisting of amino acid residues 78–89 (DAPSNNYTLYKG), was used for production of anti-peptide antibodies. Antigenicity indexes for these amino acid residues were 0.9, 0.9, 1.3, 1.7, 1.7, 1.7, 1.15, 0.75, 0.75, 0.3, −0.6 and −0.3, respectively.

been isolated from an *H. virescens* midgut and Malpighian tubule cDNA library; consequently, a northern blot was performed to detect the presence of the transcript in these tissues. Two different transcript sizes were observed in each tissue, 2.2 and 1.9 kb in Malpighian tubules and 1.9 and 1.2 kb in midgut (Fig. 1).

Antibody characterization

As shown in Fig. 2, the predicted sequence of the *H. virescens* 17 kDa protein reveals a highly hydrophobic protein with four putative intramembrane regions. According to this prediction and other studies on the yeast and lobster proteins, most of the proteolipid is embedded in the membrane (Finbow *et al.* 1992, 1993; Jones *et al.* 1994). Antigenicity index values for most of the protein sequence are coincidentally low (not shown). The anti-peptide strategy was chosen to produce a specific antibody against the 17 kDa protein. The sequence selected for anti-peptide antibody production was DAPSNNYTLYKG, encompassing residues 78–89 in the *H. virescens* deduced amino acid sequence. This region has higher antigenicity indexes ranging from 1.7 to −0.6 and putatively corresponds to the extracellular loop marked in Fig. 2. The development of a high titer of antibodies in the sera of immunized rabbits shows that this peptide is antigenic. More importantly, the amino acid sequence of this region is variable among members of the ductin family, as shown in Table 1, possibly increasing the chances of antibody recognition of a specific ductin. Affinity-purified antibodies identified a protein of the expected size on a western blot of Malpighian tubule homogenate (Fig. 3). Two other protein bands at about 86 kDa and 100 kDa showed a reaction with the antibody. These immunoreactive bands were not observed when the homogenate was incubated in sample treatment buffer for 30 min at 37 °C instead of being boiled prior to SDS–PAGE analysis. However, a new band at 28 kDa was detected under these conditions (data not shown).

Microscopy

Fig. 4 shows highly specific staining of the *H. virescens* midgut goblet cell apical membrane (Fig. 4A) and Malpighian tubule apical membrane (Fig. 4C), as well as the staining of *M. sexta* Malpighian tubule apical membrane (Fig. 4E) obtained with the anti-peptide affinity-purified antibodies. The extent of this labeling for all tissues was similar to that obtained with the anti-B subunit antiserum (data not shown), strongly supporting the involvement of the 17 kDa protein in the V-ATPase. Furthermore, similar staining patterns were observed with both antibodies using midgut sections obtained from a single insect (data not shown). No non-specific labeling was observed in the negative controls (Fig. 4B,D,F). The antibodies also label the apical membrane of *M. sexta* Malpighian tubules, which is consistent with the fact that the peptide antigen is similar to the corresponding *M. sexta* sequence (Table 1).

In order to investigate the presence of the 17 kDa protein in the gap junctional area, confocal microscopy was performed, since this technique has been successfully used to study vertebrate gap junctions (Severs *et al.* 1993). Confocal analysis confirmed the results obtained with light microscopy in *H. virescens* midgut, showing that the anti-peptide antibody specifically labeled the goblet cell cavities (Fig. 5A,B,C). However, consistent labeling of intercellular membranes in the midgut epithelium was not achieved. The typical puncta normally present in gap junction fluorescent labeling (Severs *et al.* 1993) was observed in only four optical sections in one insect (results not shown). The labeling of the columnar cell apical brush border in Fig. 5A, which is also observed in the negative control with the preimmune serum (Fig. 5D), is due

Table 1. *Sequence alignment of various homologous proteins in the region selected for anti-peptide antibody production*

		Reference
Heliothis virescens	D A P S N N Y . . . . . T L Y K G	1
Manduca sexta	D S P S N N Y . . . . . T L Y R G	2
Drosophila melanogaster	E E P S . K Y . . . . . S L Y R G	3
Nephrops norvegicus	D E A P T . Y . . . . . T L Y Q G	4
Ascaris lumbricoides	T S A S A G Y . . . . . T L ***D*** K G	5
Torpedo marmorata	T E . . . D I . . . . . . L F K S	6
Bos taurus	N D . . . G I . . . . . S L Y R S	7
Human	N D . . . D E . . . . . S L Y K S	8
Human kidney	N D . . . D I . . . . . S L Y K S	9
Mouse	T D . . . G I . . . . . T L Y R S	10
Avena sativa	N P K A K P Y . . . . . ***F*** L F D G	11
Neurospora crassa	T Q D H . . Y . . . . . ***A*** L Y T G	12
Schizosaccharomyces pombe	K Q . . . . I L . . . . S L Y S G	13
Saccharomyces cerevisiae VMA3	G Q K Q A . . . . . L Y T G	14
Saccharomyces cerevisiae VMA11	. S P T E D Y . . . . . T L F N G	15
Saccharomyces cerevisiae FP3	. S P T E D Y . . . . . T L F N G	16
Saccharomyces cerevisiae PPA1	T V A T A E N M Y S K S ***N*** L Y T G	17
Consensus	- - - - - - - - - - - - - **T L Y - G**	
	S F S	

References: 1, Pietrantonio and Gill (1993); 2, Dow *et al.* (1992); 3, Meagher *et al.* (1990); 4, Finbow *et al*. (1992); 5, Hannon *et al.* (1990); 6, Birman *et al.* (1990); 7, Mandel *et al.* (1988); 8, Hasebe *et al.* (1992); 9, Gillespie *et al.* (1991); 10, Hanada *et al.* (1991); 11, Lai *et al.* (1991); 12, Sista *et al.* (1994); 13, Toyama *et al.* (1991); 14, Nelson and Nelson (1989); 15, Umemoto *et al.* (1991); 16, Shih *et al.* (1990); 17, Apperson *et al.* (1990).

Amino acids in bold and italic type do not fit the consensus.

to the non-specific binding of the Texas-Red-labeled secondary antibody as observed in negative controls performed without primary antibodies (results not shown).

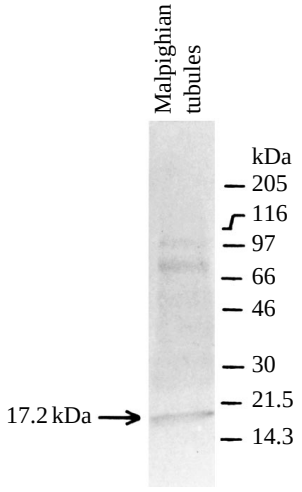

Fig. 3. Western blot of *Heliothis virescens* Malpighian tubule homogenate probed with affinity-purified antibodies to the DAPSNNYTLYKG sequence. Protein standards were Amersham Rainbow, low and high molecular mass range. The predominant band is at 17.2 kDa.

Discussion

Immunohistochemical studies on the ductin family of proteins are scarce in the literature. There are three other reports on immunolocalization with antibodies generated against these 15–18 kDa proteins. The first study was performed with anti-*N. norvegicus* protein polyclonal antibodies (Buultjens *et al.* 1988), the second with a monoclonal antibody against *T. marmorata* 15 kDa protein (Brochier and Morel, 1993) and the third with polyclonal antibodies against *T. marmorata* mediatophore (Brochier *et al.* 1993).

Immunohistochemical studies of ductins present a number of problems. First, the extensive sequence homology makes it difficult to identify specific ductins. Second, there is no abundant source of proteolipid for purification other than possibly from *N. norvegicus* and *T. marmorata*, and this, coupled with the fact that expression of these proteins in *Escherichia coli* results in cell death, makes it difficult to obtain sufficient protein for immunizations. Third, few antigenic sites may be present in this highly hydrophobic protein, making unpurified polyclonal antisera unspecific or unable to label the protein in a particular tissue (Brochier *et al.* 1993). We have previously attempted to express the 17 kDa protein for antibody production in *E. coli* using the expression vector pQE9 (Qiagen Inc., Chatsworth, CA), which is designed

to produce a fusion protein with six histidine residues to facilitate purification using a nickel column. However, induction of expression of the cloned fragment resulted in a significant decrease in cell growth in a time-dependent manner, which could be observed using SDS–PAGE (data not shown), and attempts to isolate the protein through the column from these cultures were unsuccessful. It appears that expression of this protein in bacterial cultures is toxic to the host cells. It is possible that, owing to its high hydrophobicity, the protein interacts with the host cell membranes, disrupting their function.

As an alternative strategy, a synthetic peptide corresponding to a putative extracellular loop with high antigenicity was used to develop anti-17 kDa antibodies. This anti-peptide antibody strategy has been successfully used to map another group of transmembrane channel proteins, the connexins, which are the

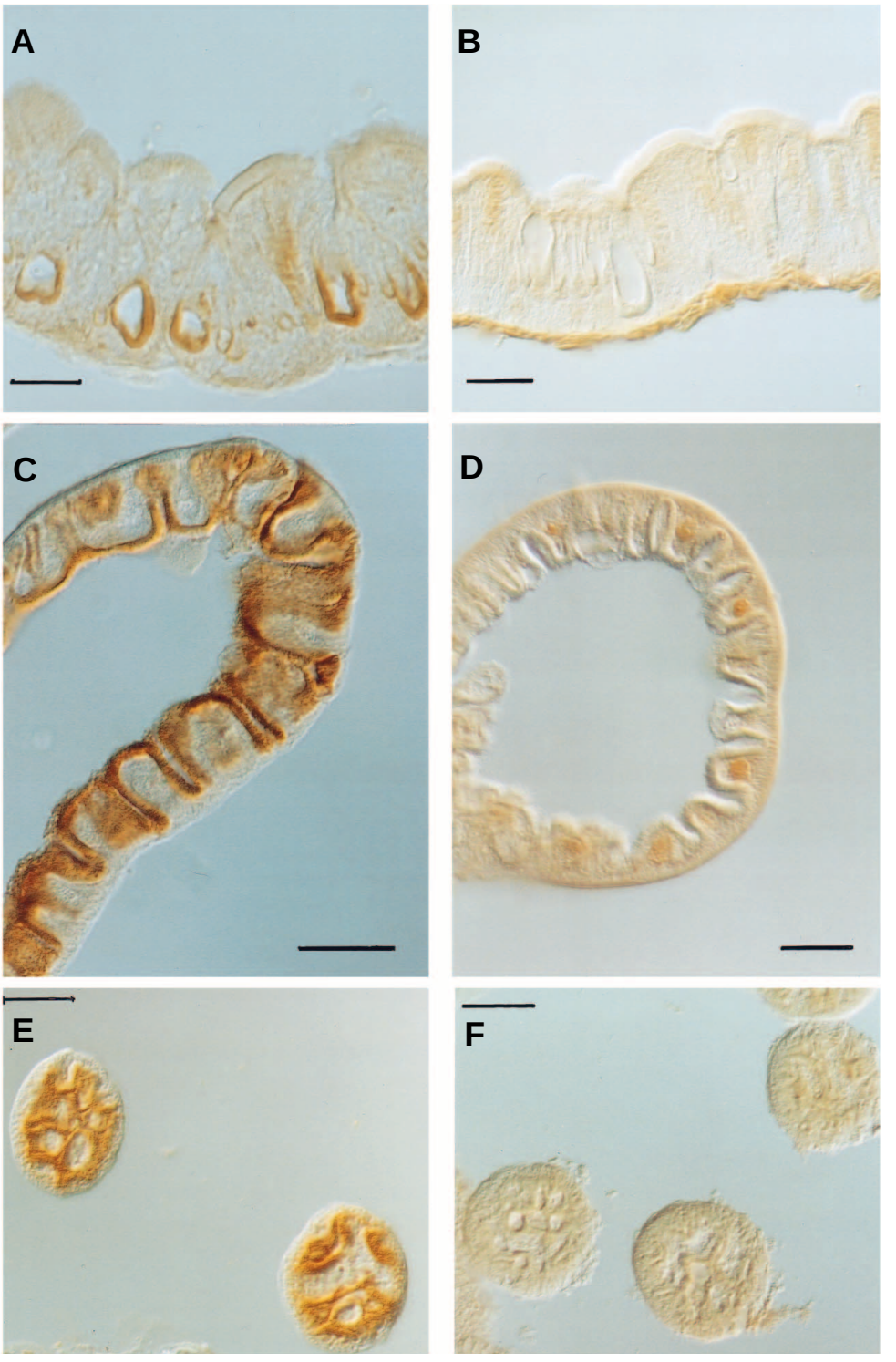

Fig. 4. Light microscopy. *Heliothis virescens* middle midgut, fifth instar, day 0 (4–6 h after ecdysis) (A) and Malpighian tubule, fifth instar, day 1 (24–48 h after ecdysis) (C) labeled with the anti-peptide affinity-purified antibody showing staining in the goblet cell apical membranes (A) and Malpighian tubule apical membrane (C). Negative controls of the same tissues incubated with preimmune serum: midgut (B) and Malpighian tubule (D). *Manduca sexta*, fifth instar, day 0 (0–24 h after ecdysis) Malpighian tubule apical membrane labeled with the anti-peptide antibody (E) or incubated with preimmune serum (F). Scale bars, A, B, 20 μm, C–F, 30 μm.

gap junction proteins of vertebrates (Evans *et al.* 1992). The anti-peptide antibody used in this study was able to detect the 17 kDa protein in western blots of *H. virescens* Malpighian tubules, and immunocytochemical studies showed that it bound to the apical membranes of *H. virescens* midgut goblet cells, and to apical membrane in Malpighian tubules of both *H. virescens* and *M. sexta*, providing strong evidence that the 17 kDa protein is a V-ATPase subunit. Malpighian tubules were the tissue of choice for western blotting because of their high specific V-ATPase content, which made the preparation of membranes or proteolipid isolation unnecessary for its detection. The two bands with molecular masses of 86 kDa and 100 kDa that also showed faint antibody binding are consistent with the presence of an aggregate the size of five or six protein

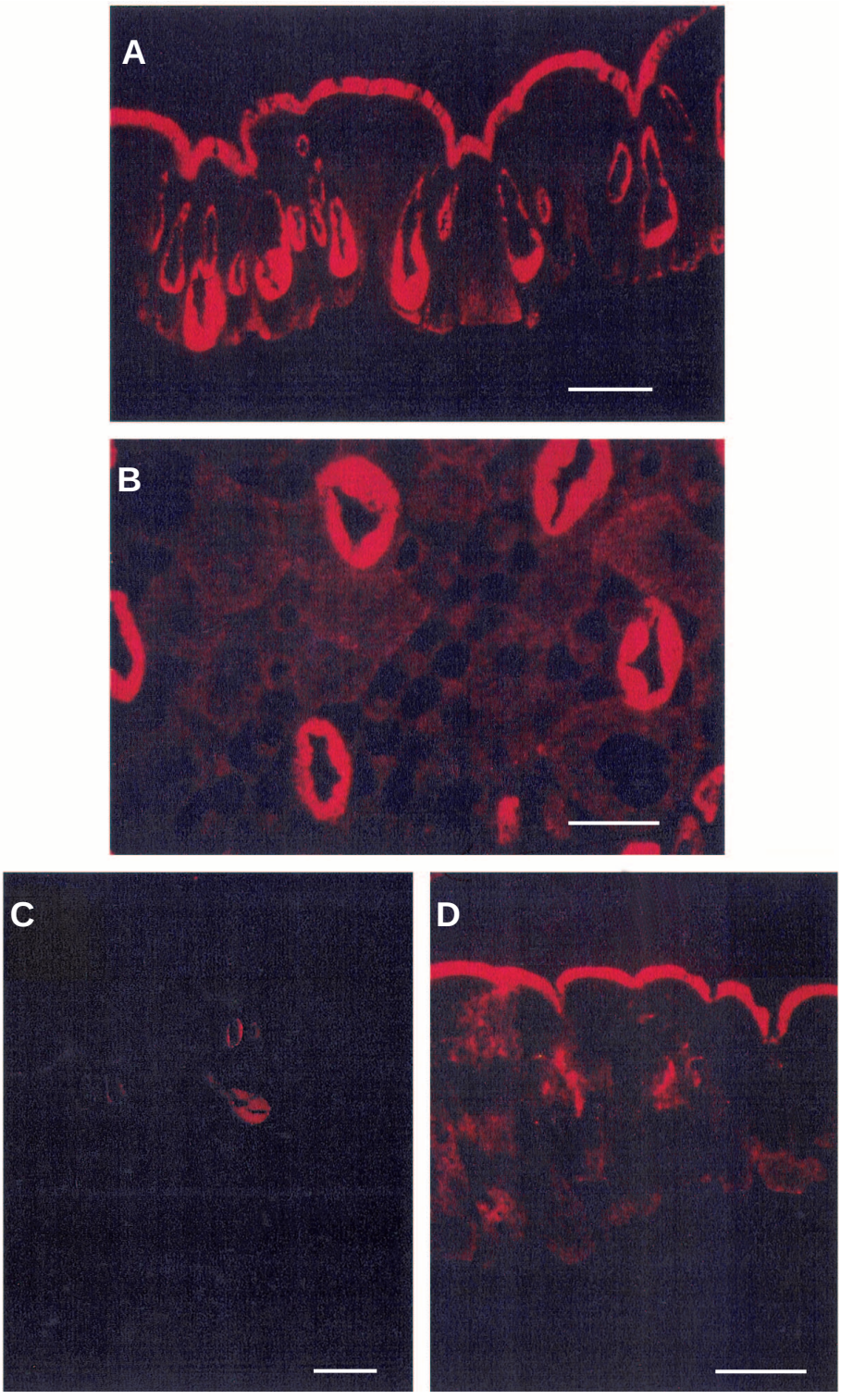

Fig. 5. Confocal microscopy of *Heliothis virescens* midgut, fifth instar, day 0 (4–6 h after ecdysis). A, B and C, anti-peptide affinity-purified antibody midgut labeling showing staining in the goblet cell cavities. (A) Longitudinal section through the middle midgut. (B) Horizontal longitudinal section of the same tissue. (C) Posterior-middle midgut. Notice the two different goblet cell shapes, also present in *Manduca sexta* midgut (Cioffi, 1979). (D) Preimmune control of a middle midgut longitudinal section to show the absence of staining in goblet cavities and the non-specific labeling of the brush border. Scale bars, A, D, 20 μm; B, 10 μm; C, 30 μm.

molecules, possibly present at lower concentration than the 17 kDa monomer. Similar aggregation patterns have been reported in another study. SDS–PAGE of mouse gap junction preparations shows a 16 kDa band and also two fainter bands at about 90 kDa and a dimer band at about 28 kDa (Finbow *et al.* 1994). The proteolipid is known to aggregate in SDS-containing buffers, especially after it is heated (Finbow and Pitts, 1993). In our experiments when the Malpighian tubule homogenate was not boiled, only the 28 kDa band, consistent with the formation of a dimer, was observed in addition to the 17 kDa band (not shown). The staining in tissue sections was highly specific, was consistent with the involvement of the 17 kDa protein in the V-ATPase, and was seen using both light and confocal microscopy. These results are strong evidence that the antibody only recognizes the 17 kDa protein as a monomer or multimer.

Northern blot analysis is not only in agreement with the expression of the 17 kDa protein in both tissues but also indicates that there are tissue-specific differences in the transcription of the gene(s) encoding the proteolipid. Southern blot analysis appears to indicate the presence of only one gene (data not shown), although the existence of other related genes cannot be excluded. The *H. virescens* midgut transcript sizes of 1.9 and 1.2 kb are comparable with those of 1.9 and 1.4 kb present in *M. sexta* midgut (Dow *et al.* 1992). In Malpighian tubules, two transcript sizes are also present, one of 2.2 kb and another of 1.9 kb, the latter transcript appearing to be the most abundant (Fig. 1). It is not known whether these various transcripts encode protein variants or whether the multiplicity of transcript sizes (2.2, 1.9 and 1.2 kb) may have regulatory significance (Fig. 1). As suggested by Dow *et al.* (1992), these various transcript sizes could have arisen from differential splicing and/or may correlate with different functions or localizations of the 17 kDa protein, for example its association with the V-ATPase in organelles and plasma membrane or, controversially, gap junctional areas.

This study shows for the first time the immunolocalization of the V_o sector component or the 17 kDa subunit c in the midgut goblet cell apical membrane and in the Malpighian tubule apical membrane of lepidopteran larvae. These labeling patterns in tissue sections of *H. virescens* and *M. sexta* obtained with the anti-peptide antibody and with the anti-B subunit antiserum are similar to those shown with monoclonal antibodies against the midgut V-ATPase V_1 sector in *M. sexta* midgut and Malpighian tubules by Klein *et al.* (1991). Our results are also similar to those obtained for the same tissues from *M. sexta* with the anti-B subunit antibody from beetroot by Russell *et al.* (1992). The cross reactivity of the anti-mosquito B subunit antiserum with the 57 kDa lepidopteran protein is not surprising since this subunit sequence is highly conserved in different organisms (Gill and Ross, 1991). Similarly, the binding of the anti-peptide affinity-purified antibodies to *M. sexta* Malpighian tubule apical membranes was expected since the peptide-antigen sequence differs in only two positions from the corresponding *M. sexta* sequence, and these are conservative changes, S instead of A, and R instead of K (Table 1).

In the intermolt midgut, the anti-17 kDa protein affinity-purified antibody did not consistently label areas of cell–cell contact. If the 17 kDa protein is a constituent of gap junctions in the midgut, it is possible that the majority of *H. virescens* intermolt midgut gap junctions may be too small (<0.2 μm) to be detected by laser confocal microscopy (Green *et al.* 1993).

In summary, we have localized the subunit c or proton-transporting subunit from the V-ATPase in *H. virescens* midgut goblet cell apical membranes and Malpighian tubule apical membranes. The generation of anti-peptide antibodies is, therefore, a powerful tool for understanding V-ATPase regulation in lepidopteran tissues, and studies are being conducted to achieve this goal. Finally, the region selected for antibody production is probably exposed in the V-ATPase so that the production of anti-peptide antibodies against this region of homologous ductins could be useful in similar localization studies.

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