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ANTIBODIES TO MAMMALIAN AND PLANT V-ATPases CROSS REACT WITH THE V-ATPase OF INSECT CATION-TRANSPORTING PLASMA MEMBRANES

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Summary

In immunobiochemical blots, polyclonal antibodies against subunits of plant and mammalian vacuolar-type ATPases (V-ATPases) cross-react strongly with corresponding subunits of larval *Manduca sexta* midgut plasma membrane V-ATPase. Thus, rabbit antiserum against *Kalanchoe daigremontiana* tonoplast V-ATPase holoenzyme cross-reacts with the 67, 56, 40, 28 and 20 kDa subunits of midgut V-ATPase separated by SDS–PAGE. Antisera against bovine chromaffin granule 72 and 39 kDa V-ATPase subunits cross-react with the corresponding 67 and 43 kDa subunits of midgut V-ATPase. Antisera against the 57 kDa subunit of both beet root and oat root V-ATPase cross-react strongly with the midgut 56 kDa V-ATPase subunit.

In immunocytochemical light micrographs, antiserum against the beet root 57 kDa V-ATPase subunit labels the goblet cell apical membrane of both posterior and anterior midgut in freeze-substituted and fixed sections. The plant antiserum also labels the apical brush-border plasma membrane of Malpighian tubules. The ability of antibodies against plant V-ATPase to label these insect membranes suggests a high sequence homology between V-ATPases from plants and insects.

Both of the antibody-labelled insect membranes transport K^+ and both membranes possess F_1-like particles, portasomes, on their cytoplasmic surfaces. This immunolabelling by xenic V-ATPase antisera of two insect cation-transporting membranes suggests that the portasomes on these membranes may be V-ATPase particles, similar to those reported on V-ATPase-containing vacuolar membranes from various sources.

Introduction

An alkali metal ion pump is present on the apical plasma membrane of many

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insect K^+- and Na^+-transporting epithelial cells. Its localization on gastrointestinal epithelia such as the midgut, Malpighian tubules, salivary glands and rectum was deduced from fluid analysis and electrical voltage measurements *in vivo* (e.g. Ramsay, 1953; Maddrell, 1971) and flux measurements *in vitro* (e.g. Harvey and Nedergaard, 1964). Further localization to ion-transporting apical membranes was based on microelectrode impalements (e.g. Moffett and Koch, 1988) and X-ray microanalysis of tissues (Dow *et al.* 1984). Electron microscopy reveals the presence of F_1-like particles, portasomes, on all of these membranes (see Harvey, 1980; Thurm and Küppers, 1980).

In the insect normally used as a model, *Manduca sexta,* the suspected K^+-transporting membrane, the goblet cell apical membrane (GCAM), was isolated using ultrastructural features, especially the presence of portasomes, as guides (Cioffi and Wolfersberger, 1983). Analysis of purified midgut membrane fractions demonstrated that K^+-stimulated ATPase activity is restricted to GCAM (Wieczorek *et al.* 1986). GCAM ATPase was solubilized and shown to be a vacuolar-type ATPase (V-ATPase) on the basis of its subunit composition and inhibitor sensitivities (Schweikl *et al.* 1989). The GCAM V-ATPase pumps protons towards the goblet cavity (Wieczorek *et al.* 1989), creating a large electrical potential difference (PD) but no proton gradient (Chao *et al.* 1991). The PD drives electrogenic potassium/proton antiport, resulting in secondary active potassium transport towards the cavity. Thus, the GCAM K^+ pump consists of a proton-motive V-ATPase in parallel with an electrogenic K^+/nH^+ antiporter (Wieczorek *et al.* 1991).

The F_1-like particles are thought to couple ATP hydrolysis to the creation of cationic electrochemical gradients in these insect epithelia (see Harvey *et al.* 1981, 1983*b*). Antibodies to the midgut GCAM V-ATPase label the portasome-studded apical membranes in light and electron micrographs of midgut and Malpighian tubules of *M. sexta* larvae (Klein *et al.* 1991) and of sensory sensilla of *Antheraea pernyi* adults (Klein and Zimmermann, 1991).

The amino acid sequences of V-ATPase subunit polypeptides are more than 70% conserved between archaebacteria, higher plants and mammals (Nelson, 1989). If this high sequence-homology extends to insects then antibodies raised against plant and mammalian V-ATPases should cross react with insect V-ATPases. We studied cross reactions of antisera against several xenic V-ATPases and used antiserum against a plant tonoplast V-ATPase to probe sections of *M. sexta* midgut and Malpighian tubule cells. The results support the working hypothesis that GCAM and Malpighian tubule portasomes are V-ATPase particles.

Materials and methods

Antibodies

Rabbit antiserum against the holoenzyme of *Kalanchoe daigremontiana* tonoplast was a gift from Dr Hans Peter Haschke, Technical University of Darmstadt,

Germany. Monospecific antisera against the 72 and 39 kDa subunits of V-ATPase from bovine chromaffin granules were gifts from Dr Nathan Nelson, Roche Institute of Molecular Biology, Nutley, New Jersey. Monospecific antisera to beet root 57 and 70 kDa subunits were gifts from Dr A. B. Bennett, Department of Vegetable Crops, University of California, Davis. Colloidal gold particles (5 nm) were conjugated to goat anti-rabbit antibody and used as a secondary probe for immunocytochemical investigations.

ATPase

Partially purified goblet cell apical membranes were prepared from *M. sexta* midguts (Cioffi and Wolfersberger, 1983). The V-ATPase was solubilized and purified from the partially purified membranes (Schweikl *et al.* 1989; Wieczorek *et al.* 1990).

Light microscopy

Tissue was dissected from the midgut and Malpighian tubules of feeding, fifth-instar larvae, 3–4 days after ecdysis from the fourth instar. It was fixed by inflation with cold paraformaldehyde/lysine/periodate fixative (PLP; McLean and Nakane, 1974) for 1 h or by rapid freezing in liquid propane at −196 °C (Howard and O'Donnell, 1987). Dehydration of PLP-fixed material was carried out in a graded alcohol series. Frozen tissue was transferred to acetone at −80 °C for freeze-substitution. Tissue was embedded in Lowicryl HM-20 resin (Polysciences) at 4 °C and polymerized by ultraviolet irradiation (Altman *et al.* 1984).

Biochemical immunoanalysis

SDS extracts of purified GCAM V-ATPase were separated into subunits by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). The proteins were then blotted onto nitrocellulose or Immobilon (Millipore) membranes (Wieczorek *et al.* 1991). The blots were blocked in 3 % gelatin in Tris-buffered saline (TBS: 20 mmol l^{-1} Tris, 0.5 mol l^{-1} NaCl, pH 7.5) for 1 h. The blots were probed for between 1 h and overnight with antibodies to subunits of xenic V-ATPases and washed (twice) for 10 min in TBS–Tween 20 (0.3 % w/v). A secondary probe of goat anti-rabbit IgG, conjugated with alkaline phosphatase (Sigma), was diluted 1:1000 with TBS–1 % gelatin and used to identify reactive bands. After 1–3 h of incubation with secondary antibody, the blots were washed (twice) for 10 min in TBS–Tween. The antibody complex was visualized using Bio-Rad kit no. 1706460. For comparison with the immunoprobed blots, a duplicate gel was run concurrently and stained conventionally with Coomassie Blue or the blot was stained with Amido Black.

Immunocytochemistry

For immunocytochemical localization of tissue V-ATPase, sections 1 μm thick were dried onto gelatin-covered slides and blocked in a buffer containing bovine serum albumin (BSA) solution (1 % w/v) in TBS for 30 min. These sections were

probed with antibodies to beet root V-ATPase using pre-immune serum as control. The immunolocalization procedure was carried out at 24 °C in a humid chamber. Pre-immune serum or serum containing the antibody was diluted 1:100 with TBS–BSA. After a 12 h incubation period the slides were rinsed twice, with stirring, in TBS–Tween. Tissues were incubated for 1 h with a 1:50 dilution of secondary antibody in TBS–BSA after which the slides were rinsed twice for 10 min with TBS–Tween and then briefly rinsed in deionized water. Light microscopic visualization of the colloidal gold IgG conjugate was augmented by silver enhancement (Intense II kit; Janssen Life Science Products, Piscataway, NJ). Control sections were stained lightly with Toluidine Blue for tissue differentiation.

Results

Immunobiochemistry

Polyclonal antibodies directed against V-ATPases purified from *K. diagremontiana* tonoplast and bovine chromaffin granules cross-reacted with lepidopteran

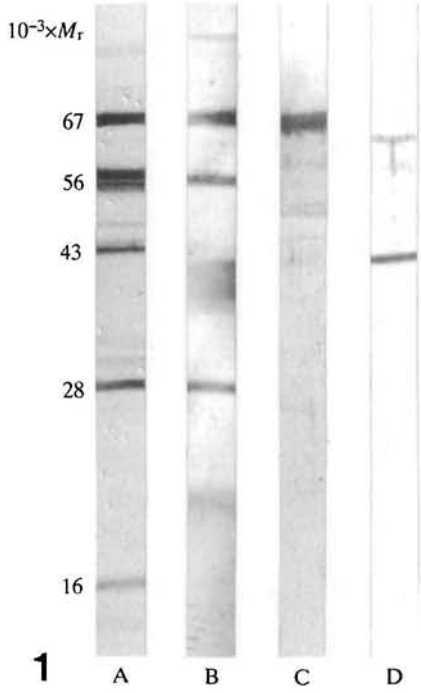


Fig. 1. Immunoblot of *Manduca sexta* V-ATPase. (A) Staining of total protein by Amido Black. (B–D) Immunostaining by polyclonal antibodies. (B) Rabbit serum, dilution 1:100, directed to the V-ATPase holoenzyme, purified from tonoplasts of *Kalanchoe diagremontiana*. (C,D) Monospecific rabbit serum, dilution 1:100 directed to the 72 kDa subunit (C) and directed to the 39 kDa subunit (D) of the V-ATPase from bovine chromaffin granules.

midgut GCAM ATPase in immunoblots (Fig. 1). Antibodies against the holoenzyme from tonoplasts reacted with the 67, 56, 40, 28 and 20 kDa subunits of midgut ATPase (Fig. 1, lane B). An antiserum against the 72 kDa subunit of chromaffin granule ATPase, thought to contain the catalytic site, reacted with the corresponding 67 kDa subunit of midgut ATPase (Fig. 1, lane C). An antiserum against the 39 kDa subunit of chromaffin granule ATPase reacted with the 43 kDa subunit of midgut ATPase (Fig. 1, lane D). Polyclonal antibodies to the 57 kDa subunit of beet root V-ATPase cross-reacted with the *M. sexta* V-ATPase 56 kDa subunit (Fig. 2, lane C). The 70 kDa beet root antibody failed to cross-react with GCAM 67 kDa subunit whereas the 72 kDa chromaffin granule antibody did react with this subunit; this difference may be due to technical problems. Finally,

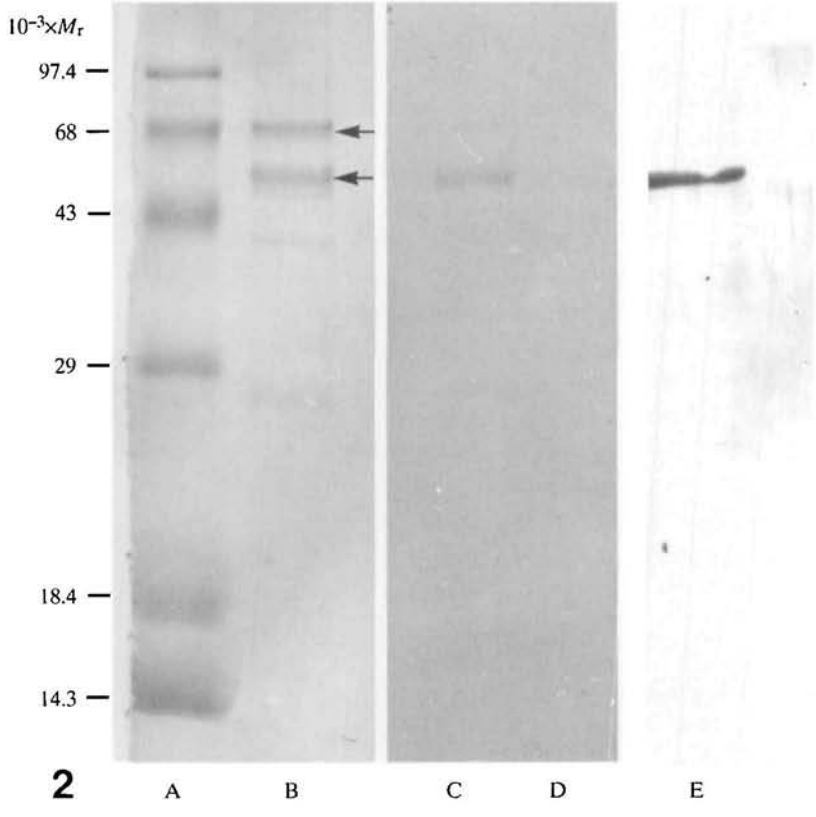


Fig. 2. SDS–PAGE and immunoblot of *Manduca sexta* V-ATPase. (A) Relative molecular mass standards ($M_r \times 10^{-3}$): phosphorylase, 97.4; bovine serum albumin, 68; ovalbumin, 43; carbonic anhydrase, 29; lysozyme, 14.3. (B) GCAM V-ATPase stained with Coomassie Blue. The two most prominently stained bands are from the 56 kDa and 67 kDa subunits (marked with arrows). (C,E) Immunoprobed blot of SDS–PAGE-separated GCAM V-ATPase, showing cross reactivity of the 56 kDa subunit to antibodies to the 57 kDa subunit of beet root tonoplast V-ATPase (C) and oat root tonoplast V-ATPase (E). (D) Blot of SDS–PAGE-separated GCAM V-ATPase probed with antibodies to the 70 kDa subunit of beet root tonoplast V-ATPase.

antibodies to oat root tonoplast V-ATPase cross-reacted with the *M. sexta* V-ATPase 56 kDa subunit (Fig. 2, lane E).

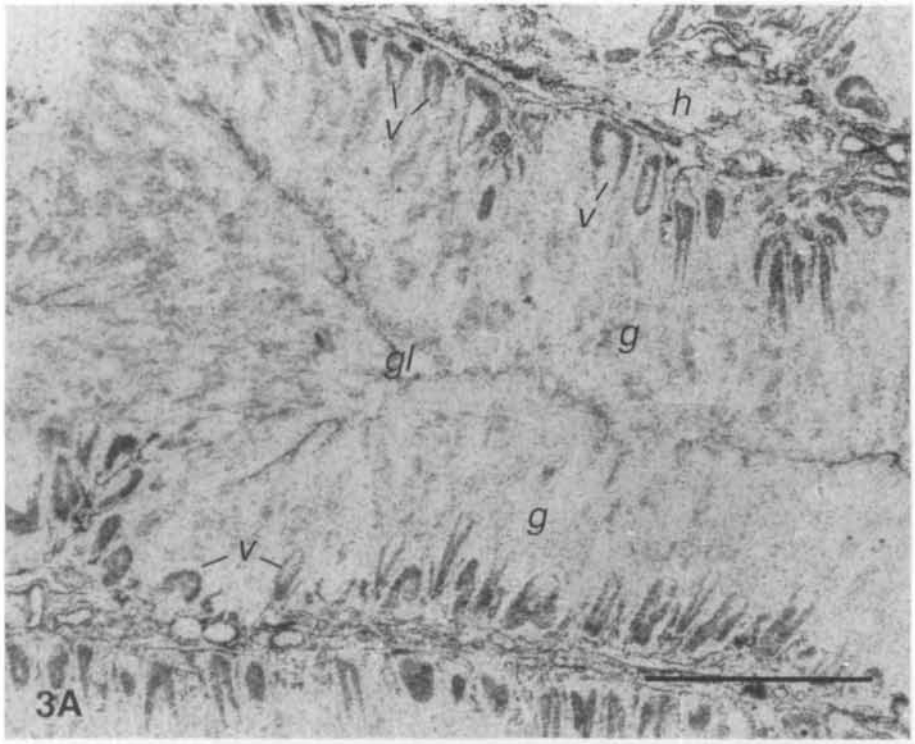
Immunocytochemistry

Sections of midgut probed with antibodies to the beet root 57 kDa V-ATPase subunit showed clear immunolabelling of the apical membrane of goblet cells from both anterior and posterior midgut in light micrographs (*v*, Figs 3A and 4A). The labelling was similar in both freeze-substituted tissue (Fig. 3) and fixed tissue (Fig. 4). The cells are so large (up to 100 μm tall) that there can be little doubt that the localization of the immunolabelling in light micrographs corresponds to the region of the portasome-studded apical membranes. The intensity of this immunocytochemical labelling suggests that V-ATPase must be highly concentrated in GCAM. By comparison, the GCAM of control tissues, which were exposed to pre-immune serum, was unlabelled (Figs 3B and 4B). Strong immunolabelling by antibody to the 57 kDa subunit was also seen on the apical plasma membrane of the Malpighian tubule cells (arrowhead Fig. 4A). Nonspecific staining, characteristic of a rabbit polyclonal serum, when used for probing insect tissue, was also apparent in basal lamellae (*l*), connective tissue (*ct*) and the glycocalyx of the midgut brush border (*bb*), especially in the apical regions. A slight staining could be seen in nuclear material (*n*) (Fig. 3B). Tangential sections through the brush border of PLP-fixed material revealed dense staining of the glycocalyx and moderate nonspecific staining of the brush border itself (Fig. 4B). It is likely that most of the nonspecific labelling involves glycoproteins. Moreover, since background staining was not seen to such an extent in the freeze-substituted tissue, it may be attributable to the PLP fixation method, which is especially effective in preserving carbohydrate side chains (McLean and Nakane, 1974). Thus, the background labelling may be due, in part, to antibody reactions induced by the fixation method.

Discussion

The immunobiochemical cross reactions between polyclonal antibodies raised against the 57 kDa subunit of beet root tonoplast and oat root tonoplast and the

Fig. 3. A section of the anterior region of the midgut of *Manduca sexta*. The tissue was prepared by freeze-substitution. Sections 1 μm thick were probed with either (A) immune serum containing antibodies to the 57 kDa subunit of beet root tonoplast V-ATPase or (B) pre-immune rabbit serum. Colloidal-gold-conjugated goat anti-rabbit antibody was used to localize the rabbit antibody. (A) Tissue probed with specific antibodies to the 57 kDa subunit of the V-ATPase; specific staining can be seen on the apical projections (*v*) of the goblet cell [midgut (*g*), haemocoel (*h*), midgut lumen (*gl*)]. (B) Control section treated with pre-immune rabbit serum. Nonspecific staining is characteristic in rabbit IgG probing of insect tissues. A very high affinity can be deduced for basal glycoproteins (*l*, basal lamellae; *ct*, connective tissue). A slight affinity can be deduced for nuclear material (*n*). No staining is evident in the region of the goblet cell cavity. Scale bars, 50 μm.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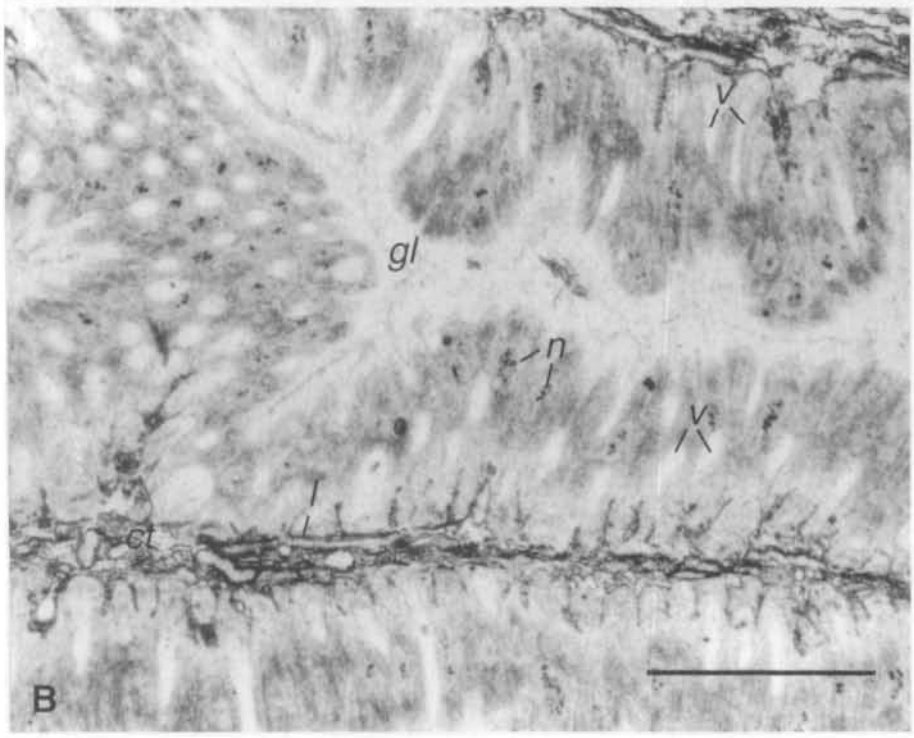
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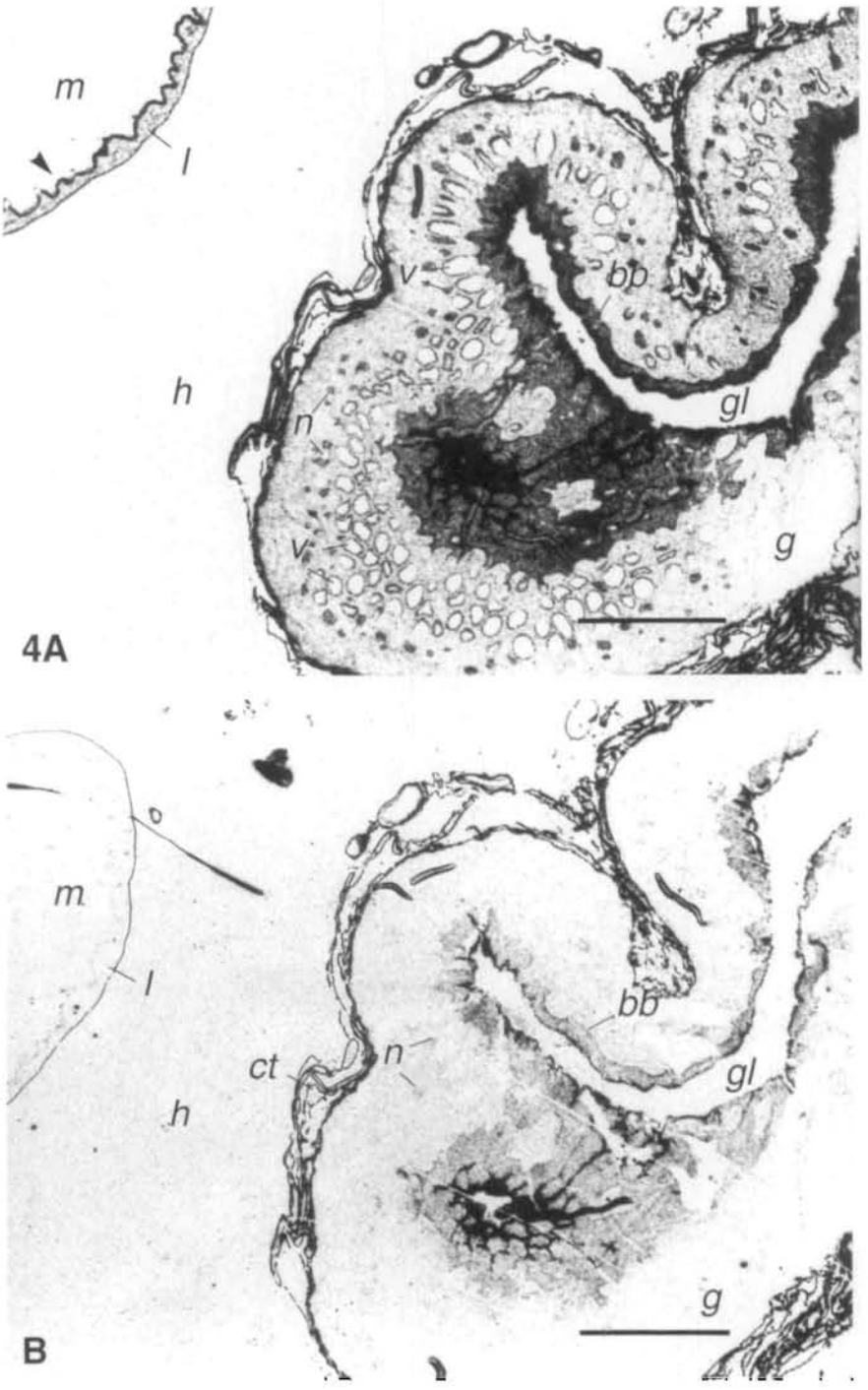

72 kDa subunit of chromaffin granule V-ATPase and the corresponding 56 kDa and 67 kDa subunits from GCAM V-ATPase (Figs 1 and 2), as well as the other cross reactions reported here, support the hypothesis that GCAM ATPase shares

Fig. 4. A section of the posterior region of the midgut of *Manduca sexta*. The tissue was fixed with paraformaldehyde/lysine/periodate. Sections 1 μm thick were probed with either (A) immune serum containing antibodies to the 57 kDa subunit of beet root tonoplast V-ATPase or (B) pre-immune rabbit serum. Colloidal-gold-conjugated goat anti-rabbit antibody was used to localize the rabbit antibody. (A) Tissue probed with specific antibodies for the 57 kDa subunit of the V-ATPase. Specific staining can be seen on the apical projections (*v*) of the goblet cell and on the brush border of the Malpighian tubule (*m*, arrowhead). An intensification of staining of the midgut brush border (*bb*) is also apparent. (B) Control section treated with pre-immune rabbit serum. As in Fig. 3B, there is strong positive nonspecific binding of rabbit serum to glycoproteins of connective tissue elements. In the lower part of the picture the section is tangential through the brush border, revealing dense nonspecific staining of the glycocalyx and moderate staining of the brush border (*bb*) itself, especially in the apical regions. Other labels as in Fig. 3. Scale bars, 100 μm.

many epitopes with distantly related, xenic V-ATPases. The beet root antibodies preferentially labelled *M. sexta* goblet cell apical membrane from both anterior (Fig. 3) and posterior (Fig. 4) midgut. This immunocytochemical evidence supports the immunobiochemical evidence in demonstrating the kinship between plant and insect V-ATPases. Klein *et al.* (1991) have already shown, by light microscopy, that monoclonal antibodies against several subunits of the *M. sexta* midgut V-ATPase specifically label the goblet cell apical membrane of *M. sexta* posterior midgut cells and, by electron microscopy, that monoclonal antibodies to the 67 kDa subunit of the midgut V-ATPase also label the same membrane. The labelling of this membrane with a xenic antibody suggests that the plant V-ATPase contains the epitope responsible for the labelling. The present results show, for the first time, specific labelling of anterior midgut GCAM. Like posterior midgut, anterior midgut transports K^+ (Cioffi and Harvey, 1981) and has portasomes on its GCAM (Cioffi, 1979). The beet root antibodies also label the apical membrane of *M. sexta* Malpighian tubule cells (Fig. 4), supporting the hypothesis (Klein *et al.* 1991) that a V-ATPase energizes the cation transport that underlies fluid secretion by these organs.

The high sequence homology between distantly related V-ATPases leads one to expect that the *M. sexta* antibodies of Klein *et al.* (1991) and the beet root antibody used in this study would label the V-ATPase-containing endomembranes of midgut cells as well as they label the portasome-studded plasma membranes. Since the *M. sexta* goblet cell apical membrane develops from an intracellular vacuole (Hakim *et al.* 1988), the V-ATPase of *M. sexta* endomembranes could be similar to that of goblet cell apical membrane. The slight intensification of cytoplasmic stain in this study is consistent with labelling of V-ATPases in endomembranes. Klein *et al.* (1991) suggest that the faint labelling of goblet cell cytoplasm by monoclonal antibodies against midgut V-ATPase might be explained by a reaction with specific epitopes of prospective GCAM ATPase while the enzyme was *en route* from its site of synthesis to GCAM. The intense labelling of portasome-studded apical membranes by antibodies against V-ATPases in both studies suggests that the

concentration of the enzyme in these plasma membranes is much higher than that in endomembranes. This conclusion is supported by biochemical evidence. SDS–PAGE gels of purified goblet cell apical membranes (Harvey *et al.* 1983*a*; Wieczorek *et al.*, 1991) contain major components corresponding to the subunits of solubilized V-ATPase (Schweikl *et al.* 1989; Wieczorek *et al.* 1990), implying that the V-ATPase is a major constituent of GCAM.

This paper, like that of Klein *et al.* (1991), supports the hypothesis that certain insect apical plasma membranes are energized by a primary, electrogenic, proton-motive V-ATPase (Wieczorek *et al.* 1991). The proton-motive V-ATPase, by itself, can only impose an electrical PD across the membrane (Al-Awqati, 1986). The presence of carriers, ion channels and water movements determines the physiological utilization of the generated PD. In lepidopteran midgut a large PD alkalizes the lumen (Dow, 1984; Dow and Harvey, 1988; Dow and O'Donnell, 1990; Chamberlin, 1990; Chao *et al.* 1991) and drives amino acid/cation symport from lumen to cells (Hanozet *et al.* 1989; Hennigan and Wolfersberger, 1989). In Malpighian tubules, net K^+ transport drives fluid secretion (Maddrell, 1971) by a process that is inhibited by bafilomycin A_1 (Bertram *et al.* 1991). Midgut V-ATPase antibodies label the apical membrane of auxiliary cells in *A. pernyi* sensory sensilla (Klein and Zimmermann, 1991). The pump PD across this membrane modulates the receptor current (Thurm and Küppers, 1980).

The presence of F_1-like particles (Gupta and Berridge, 1966; Smith, 1969) first suggested that goblet cell apical membrane might be responsible for active K^+ transport across the lepidopteran midgut epithelium (Anderson and Harvey, 1966) and guided the isolation of this membrane (Cioffi and Wolfersberger, 1983). Similar particles are present on the apical membrane of Malpighian tubule and sensory sensilla auxiliary cell apical membrane (see references in Harvey, 1980; Thurm and Küppers, 1980), *Neurospora crassa* and plant vacuoles (Bowman *et al.* 1989; Klink and Lüttge, 1991; Morré *et al.* 1991; Taiz and Taiz, 1991), chromaffin granules (Schmidt *et al.* 1982), guinea pig brain synaptic vesicles (Stadler and Tsukita, 1984) and kidney acidifying membranes (Brown *et al.* 1987). In many of these cases the particle-containing membrane has V-ATPase activity and labels with antibodies to the V-ATPase. Considering the homology of the B and A subunits of V-ATPase with the α and β subunits of F-ATPase and the proposed similarity of V-ATPase and F-ATPase structure (e.g. Nelson, 1991), it is not surprising that portasomes appear to be V_1 ATPase particles just as 'lollipops' are F_1 ATPase particles.

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