The Journal of Experimental Biology **199**, 1019–1027 (1996)
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JEB0199

TEMPORAL AND SPATIAL DISTRIBUTION OF V-ATPase AND ITS mRNA IN THE MIDGUT OF MOULTING *MANDUCA SEXTA*

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Accepted 30 January 1996

Summary

The spatial and temporal distribution of the plasma membrane V-ATPase and its encoding mRNA in the midgut of *Manduca sexta* were investigated during the moult from the fourth to the fifth larval instar. Digoxigenin-labelled RNA probes were used for *in situ* hybridization of V-ATPase mRNA of both peripheral and integrated subunits; monoclonal antibodies to subunits of the peripheral sector of the purified plasma membrane V-ATPase were used for immunocytochemistry. Extensive mRNA labelling was found in both mature columnar and goblet cells of intermoult and moulting larvae. Hybridization screening in several tissues suggested that only cells with increased V-ATPase biosynthesis were labelled by our hybridization method. Mature goblet cells contain a large amount of V-ATPase in the apical plasma membrane and were therefore expected to contain V-ATPase mRNA. The intense mRNA signal found in mature columnar cells was unexpected. However, after refining the techniques of tissue preparation, immunolabelling in apical blebs of columnar cells was demonstrated. Since this immunoreactivity did not appear to be membrane-associated, it suggested a cytosolic localization of peripheral V_1 subunits. The mRNA encoding subunit A of the peripheral V_1 sector was distributed unevenly in columnar cells with a strong apical preference, whereas the mRNA for the proteolipid of the integral V_0 sector was evenly distributed in the cytosol. This spatial pattern reflected the distribution of free ribosomes and rough endoplasmic reticulum in the cell, supporting the view that V_1 subunits are synthesized at free ribosomes, whereas the V_0 subunits are synthesized at the rough endoplasmic reticulum. All undifferentiated cells exhibited intense mRNA signals for V-ATPase subunits of both holoenzyme sectors from the start of proliferation and thus precursors of columnar and goblet cells could not be distinguished.

Key words: *in situ* hybridization, immunocytochemistry, mRNA localization, V-ATPase, tobacco hornworm, midgut, *Manduca sexta*.

Introduction

The insect plasma membrane V-ATPase is a well-established member of the family of proton-transporting vacuolar-type ATPases. It was first purified from the posterior midgut of *Manduca sexta* larvae (Wieczorek *et al.* 1986; Schweikl *et al.* 1989). Six subunits from the multi-subunit enzyme have been sequenced: subunit A (67 kDa; Gräf *et al.* 1992), subunit B (56 kDa; Novak *et al.* 1992), subunit E (28 kDa; Gräf *et al.* 1994*a*), the 14 kDa subunit (Gräf *et al.* 1994*b*), the proteolipid (17 kDa; Dow *et al.* 1992) and the novel 13 kDa subunit G (Lepier *et al.* 1996). Biochemical and immunocytochemical studies indicated that the enzyme was localized exclusively in the goblet cell apical membrane (Wieczorek *et al.* 1986; Schweikl *et al.* 1989; Klein *et al.* 1991) along three well-defined regions of the midgut (Russell *et al.* 1992). The V-ATPase may, therefore, provide a suitable celltype-specific marker protein for the apical plasma membrane domain in the goblet cells. However, V-ATPases also occur ubiquitously in endomembranes (Harvey and Nelson, 1992). This poses a significant problem: do cells discriminate different target membranes for V-ATPase sorting and, if so, how do they do it? So far, nothing is known about sorting signals for V-ATPases.

In midgut goblet cells, the question of membrane target definition is further complicated since the goblet cell apical membrane develops from an endosomal goblet cavity precursor compartment (GCPC) during embryogenesis and subsequent larval moults. At ecdysis, the GCPC fuses with the apical plasma membrane to form the goblet cavity (Hakim *et al.* 1988; Baldwin and Hakim, 1991). During successive larval moults, the differentiation of stem cells into goblet or

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columnar cells can be easily observed. At every moult, the number of epithelial cells increases approximately fourfold. The new cells intercalate, in a well-organized pattern, between the persisting mature cells (Baldwin and Hakim, 1991). Exploiting current knowledge about the molecular biology of the insect plasma membrane V-ATPase and about midgut morphology during development, we chose the midgut of moulting *Manduca sexta* as a model system in which to investigate the establishment, maintenance and modulation of plasma membrane composition in polarized epithelial cells. Sumner *et al.* (1995) have recently shown that, during the moult, V-ATPase activity in goblet cells is regulated by the loss of the peripheral catalytic V_1 sector of the holoenzyme. As a first step in analysing the biosynthetic pathways of V-ATPases, we describe in this paper the spatial and temporal distribution of V-ATPase mRNA and protein in developing and mature midgut cells, both in feeding intermoult larvae and during the moult from the fourth to fifth instar.

Materials and methods

Insects

Manduca sexta (Johannson) larvae (Lepidoptera, Sphingidae) were reared according to Schweikl *et al.* (1989) under long-day conditions (16 h of light) at 27 °C using a synthetic diet. Posterior midguts were taken from intermoult (feeding) larvae and larvae moulting from the fourth to the fifth instar. Staging of the moulting larvae was based on external morphological features (Baldwin and Hakim, 1991; modified by Sumner *et al.* 1995): briefly, larvae gain mass until they weigh approximately 0.8 g (stage A); upon entry into the moult, they stop feeding (1.15 g, stage B; in our cultures, animals initiate moulting at a slightly lower mass than that given in Baldwin and Hakim, 1991); the head capsule bends down and becomes extensively green (stage C); the base of the head capsule becomes first slightly opaque (stage D) and then entirely opaque with the mandibles visible but unpigmented (stage E); then the mandibles become pigmented (stage F) and finally ecdysis occurs (stage G, freshly moulted larvae). About 2 h after moulting, the larvae resume feeding (stage H).

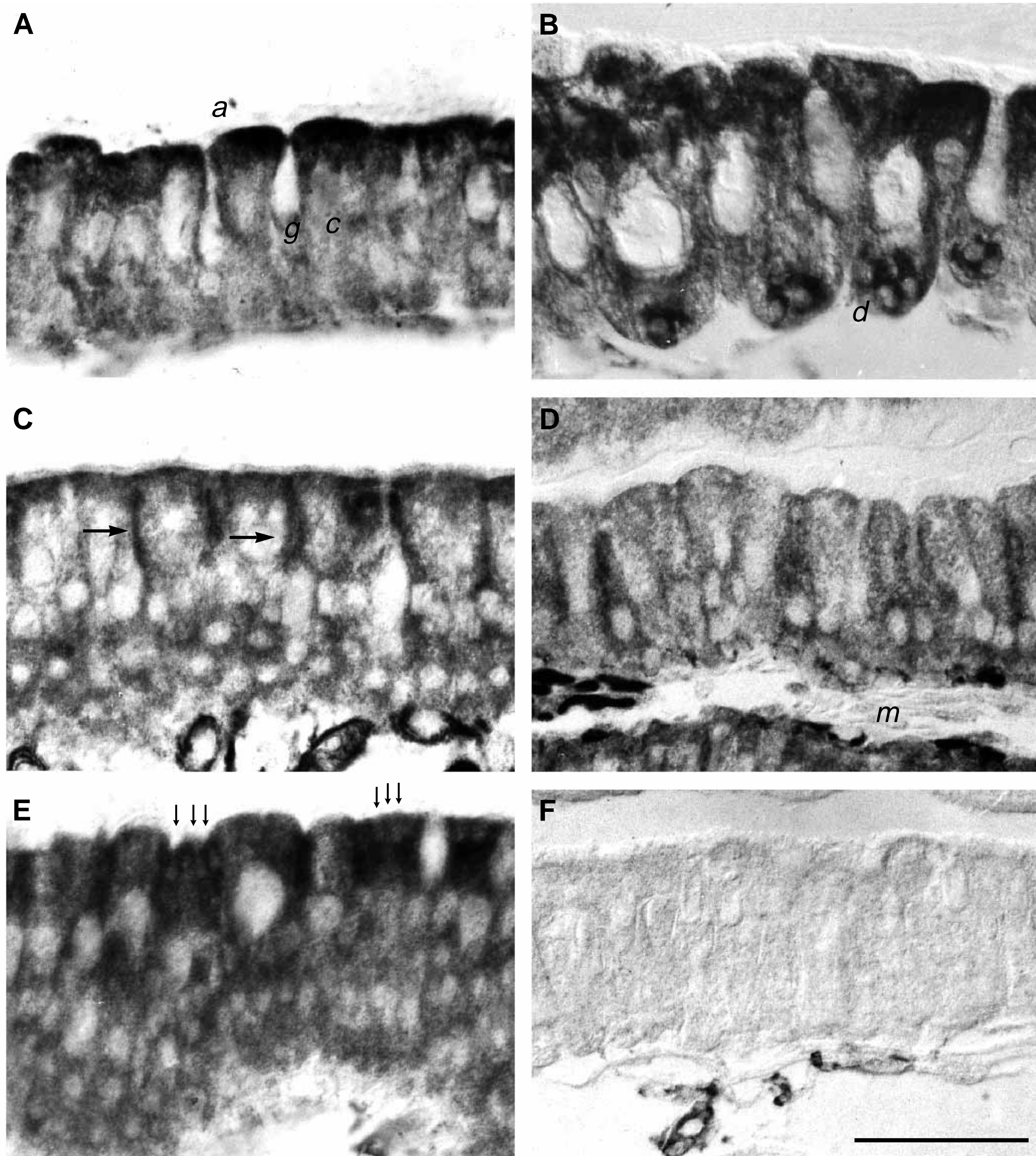
In situ *hybridization*

Digoxigenin-labelled RNA probes for *M. sexta* V-ATPase subunits were produced by *in vitro* transcription from pBluescript phagemids, using T7-RNA polymerase (Boehringer Mannheim, Germany) following the protocol recommended by the manufacturer. To produce a probe for the mRNA encoding peripheral subunit A (67 kDa), the cDNA was isolated by digestion with BssH II from a pBluescript II KS clone, containing bp 1580–1900 of the coding region (Gräf *et al.* 1992). To produce a probe for the mRNA encoding the membrane-integral proteolipid (17 kDa, 1428 bp), the cDNA was isolated by digestion with Pvu II from a pBluescript SK clone (kindly provided by Julian Dow; Dow *et al.* 1992). Hybridization of the RNA probes with the total *M. sexta* midgut polyA+ RNA was confirmed on dot blots (data not shown). Nonsense probes derived from the polylinker of pBluescript II KS (173 bp) served as negative controls for hybridization.

Fig. 1. Distribution of mRNA encoding V-ATPase subunit A in the midgut during the moult to the fifth instar. *In situ* hybridization on longitudinal cryosections with an RNA probe, visualized under differential interference contrast microscopy (DIC). (A) Intermoult fifth larval instar showing an apically enhanced hybridization signal. (B) Moulting stage B, showing small nests of proliferating cells already bearing the hybridization signal. (C) Late moulting stage D with a striped hybridization signal (arrows), which may be due to enhanced levels of mRNA in the apical portion of mature goblet cells. At this stage, the densely labelled differentiating cells in the basal layer of the epithelium start to intercalate between the mature cells and their nuclei become oriented (lower row of nuclei, nuclei of new goblet cells; middle row, nuclei of new columnar cells; upper row, nuclei of mature columnar cells; cf. Baldwin and Hakim, 1991); the heavy staining of the tracheae at the bottom of the picture is due to unspecific silver precipitation. (D) In late moulting stage E, the hybridization signal in the mature columnar cells is now evenly distributed, with differentiating cells showing a slightly stronger signal. (E) Stage G (oblique section), in the freshly moulted larva, the apical polarization of the hybridization signal is re-established; note the small size of the new goblets (arrows). (F) Intermoult fifth instar, control by *in situ* hybridization with nonsense RNA. *a*, apical; *c*, columnar cell; *d*, differentiating cell; *g*, goblet cell; *m*, muscle. Scale bar, 50 μm.

Moulting and intermoult larvae were chilled in crushed ice for 10 min and then their entire midguts were dissected out under ice-cold fixative (Lavdowsky's fixative: ethanol:formalin:acetic acid:water; 50:10:4:40). Slices approximately 2 mm thick, containing all the midgut contents and some adjacent Malpighian tubules, were cut from the posterior portion. This preparation circumvented removal of the peritrophic membrane and led to improved preservation of apical cell borders. Specimens were returned to the same fixative for 1 h and then rinsed three times for 5 min in $0.1\,mol\,l^{-1}$ sodium phosphate, pH 7.3 (PB). The tissue was cryoprotected by immersion in PB containing increasing concentrations of sucrose, 10 %, 20 % (twice for 10 min each) and 30 % sucrose (twice for 10 min and once for 60 min). The tissue was then cryo-embedded as described by Klein *et al.* (1991). In a few cases, entire fourth-instar intermoult larvae were cut into six portions, fixed and embedded. Cryosections (7 μm) were cut on a Microm cryotome at −25 °C, collected on glass slides coated with 0.01 % polylysine and air-dried at room temperature.

After postfixation for 2–5 min in 4 % formaldehyde in PB, the cryosections were prehybridized for 15 min at room temperature in 2×SSC (SSC: $0.03\,mol\,l^{-1}$ trisodium citrate, $0.3\,mol\,l^{-1}$ sodium chloride, pH 7.0) with 30 % formamide for subunit A mRNA, 4×SSC with 40 % formamide for proteolipid mRNA or in 5×SSC with 20 % formamide for the nonsense RNA. Hybridization was accomplished in a moist chamber overnight at 38 °C with droplets of $1\,ng\,\mu l^{-1}$ of digoxigenin-labelled RNA probes in the same solution, containing in



addition 10 % of dextran sulphate and 100 ng μl^{-1} of yeast tRNA. Washes were carried out first using 2×SSC twice for 15 min at room temperature (20–23 °C, low stringency) and then using 0.1×SSC twice for 15 min at 38 °C (high stringency). After a second postfixation for 5 min in 4 % formaldehyde in PB, subsequent rinsing twice for 5 min in phosphate-buffered saline, PBS (8 g l^{-1} sodium chloride, 0.2 g l^{-1} potassium chloride, 1.44 g l^{-1} disodium hydrogen phosphate, 0.24 g l^{-1} potassium dihydrogen phosphate, pH 7.4) and pre-incubation for 15 min in PBA (PBS containing 1 % bovine serum albumin), sections were incubated for 90 min at room temperature with anti-digoxigenin antibodies (0.8 nm colloidal gold-conjugated sheep anti-digoxigenin antibodies; Boehringer Mannheim, Germany), diluted 1:30 in PBA.

Sections were then rinsed six times for 5 min in distilled water before silver enhancement of the gold particle staining (silver enhancement kit; Boehringer Mannheim, Germany). The reaction was stopped by rinsing three times for 5 min in distilled water. Finally, the sections were covered by Mowiol 4-88 (Hoechst, Germany).

Immunocytochemistry

Midguts of moulting and intermoult larvae were dissected as described above except that 2 % formaldehyde and 2.5 % glutardialdehyde in PB (pH 7.3) or 4 % formaldehyde in PB (pH 7.3) were used as fixatives. Immunolabelling was carried out according to Klein *et al.* (1991) and subsequent staining followed the protocol of Sumner *et al.* (1995). Two protein-G-purified monoclonal antibodies were applied, both exhibiting identical patterns in midgut immunolabelling: antibody 221-9 is directed to subunit A, and antibody 224-3 is directed to two polypeptides with molecular masses of approximately 20 kDa and 56 kDa. The latter two polypeptides are not yet characterized, but they co-purify with the midgut plasma membrane V-ATPase (U. Klein, M. Timme, F. J. S. Novak, A. Lepier, W. R. Harvey and H. Wieczorek, in preparation). Visualization was accomplished with 5 nm gold-conjugated secondary antibodies diluted as recommended by the manufacturer (sheep anti-mouse IgG, whole molecule, Sigma, Germany) and by silver enhancement as described above. Control sections were incubated with secondary antibodies only.

Electron microscopy

Larval midguts were prepared under ice-cold fixative (2 % formaldehyde, 2.5 % glutardialdehyde in PB, pH 7.3), fixed in the same solution for 2 h and postfixed with 1 % OsO_4 in PB for 1 h on ice, dehydrated in a graded ethanol series and embedded in Epon 812 (TAAB Laboratories, Germany). Ultrathin sections were cut on a RMC MT6000 ultramicrotome, stained with uranyl acetate and lead citrate and examined in a Philips CM10 electron microscope.

Results

Columnar and goblet cells both contain significant V-ATPase mRNA

The two antisense RNA probes for subunit A and for the proteolipid labelled both columnar and goblet cells of midguts from intermoult larvae (Figs 1A, 2A). No labelling was found in controls created with nonsense mRNA (Fig. 1F). This result was unexpected, since V-ATPase immunoreactivity had previously been detected only in the apical membrane region of goblet cells and no immunolabelling had been found in columnar cells. However, since V-ATPases occur ubiquitously in endosomal membranes, every cell is expected to contain the appropriate mRNA. To screen for the pattern of labelling intensity in different tissues, *in situ* hybridization for subunit A mRNA was carried out on a variety of tissues from different body areas. Malpighian tubules exhibited clear labelling along most of their length, except at their most posterior loop region (Fig. 3A,B). Labelling was also detected in fat body cells (Fig. 3B) and, to differing degrees, in nerve cell somata of the brain (Fig. 3C). The neuropile and all muscles were unlabelled (Figs 1D, 3C). In cross sections of entire fourth-instar larvae, the epidermal epithelium showed clear labelling in most areas, the rectal epithelium was only slightly labelled and the salivary glands were unlabelled (not shown). In summary, V-ATPase mRNA appears to be labelled by our method, but only in those cells that are highly enriched in V-ATPase mRNA.

To test for a significant level of V-ATPase protein in columnar cells, we re-investigated the midgut for V-ATPase immunoreactivity. In addition to the goblet cell apical membrane, immunolabelling was also prominent in apical

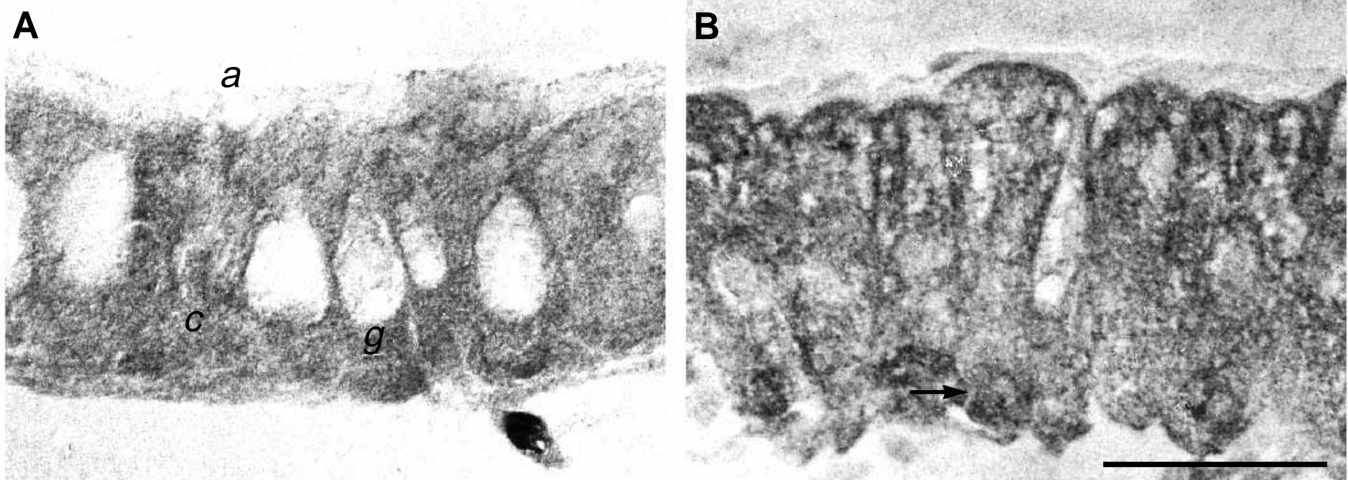


Fig. 2. Distribution of mRNA encoding V-ATPase proteolipid in the midgut during the moult. *In situ* hybridization of cryosections with an RNA probe, viewed under DIC. (A) Intermoult fifth larval instar showing an evenly distributed hybridization signal. (B) Stage C; no change in distribution was found during moult, the differentiating cells (arrow) are faintly visible. *a*, apical; *c*, columnar cell; *g*, goblet cell. Scale bar, 50 μm.

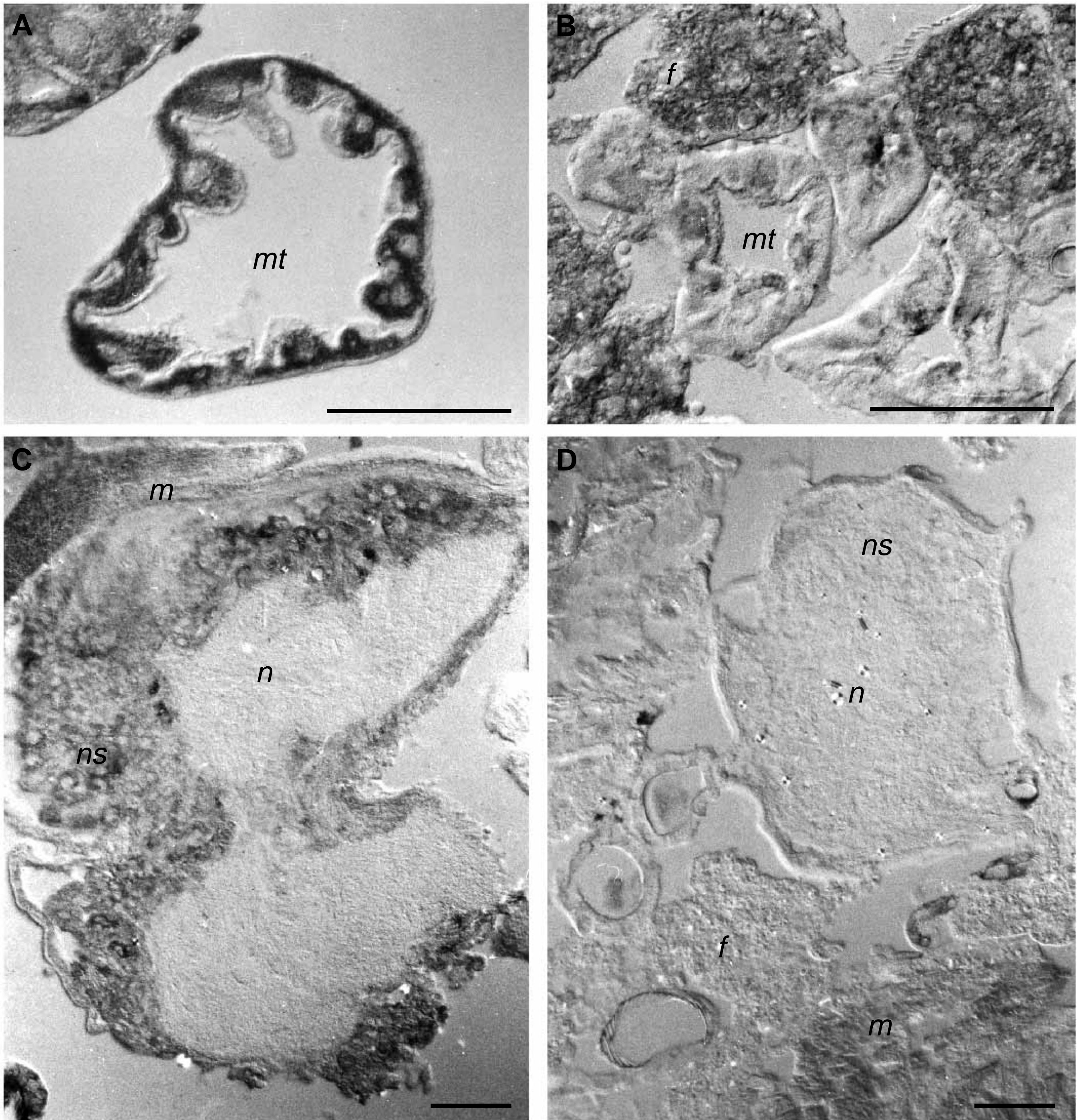


Fig. 3. Hybridization levels of mRNA encoding V-ATPase subunit A in several larval tissues. *In situ* hybridization of cryosections with an RNA probe, viewed under DIC. (A) Malpighian tubule in the midgut region. (B) Malpighian tubule close to its proximal loop near the rectum. (C) Brain tissue, divided into neuropile and nerve cell somata, surrounded by fat body; some muscles are visible. (D) Thoracic segment, control hybridization with plasmid RNA. *f*, fat body; *m*, muscle; *mt*, Malpighian tubules; *n*, neuropile; *ns*, nerve cell somata. Scale bars, 50 μm.

protrusions of the columnar cells that had been preserved by a refined tissue preparation technique in which apical cell borders were better preserved (Fig. 4A,B). Such apical blebs are common during moulting and serve for apocrine secretion of cellular material (Baldwin and Hakim, 1991). In rare cases, we also found them in midguts of intermoult larvae.

Local distribution of subunit A mRNA and proteolipid mRNA differs

In situ hybridization showed an even distribution of proteolipid mRNA throughout the cytosol in both cell types of the midgut (Fig. 2A), whereas subunit A mRNA was concentrated in the apical regions (Fig. 1A). Proteolipid mRNA

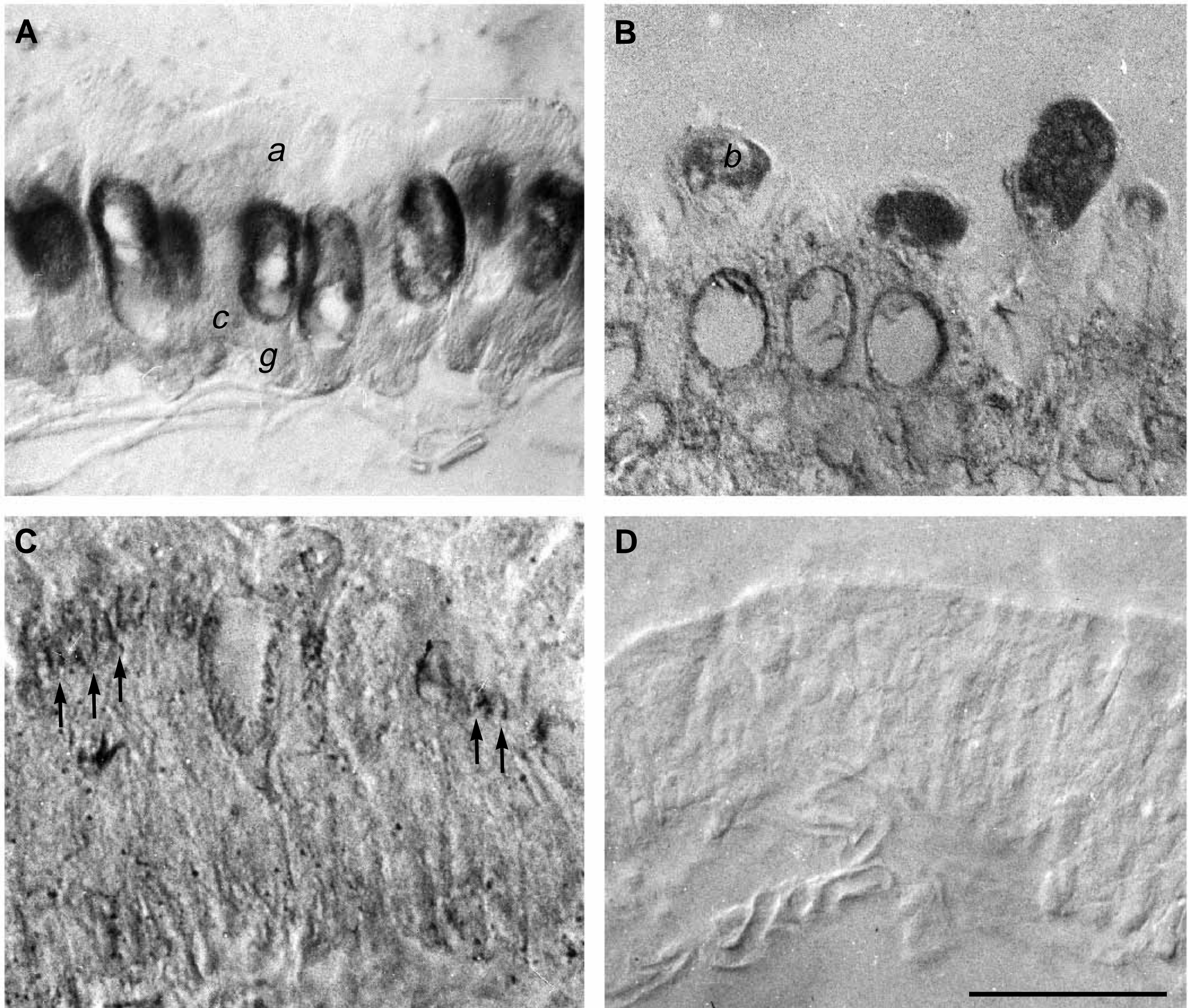


Fig. 4. Localization of V-ATPase protein in the midgut during the moult. Immunocytochemistry with monoclonal antibodies to the *M. sexta* V-ATPase, viewed under DIC. (A) Fifth-instar intermoult larva, showing the immunoreactivity of the goblet cell apical membrane surrounding the large mature goblets (antibody clone 221-9). (B) Moulting stage C, with immunoreactivity in the goblet cell apical membrane and in the cytosol of the apical blebs of the columnar cells (antibody clone 224-3). (C) In stage F before ecdysis, immunoreactivity appeared in the young goblets and was reconstituted in the mature goblets; the new goblets (arrows) are considerably smaller than the mature ones (antibody clone 221-9). (D) Control incubation with secondary antibodies only. *a*, apical; *b*, apical bleb; *c*, columnar cell; *g*, goblet cell. Scale bar, 50 μm.

distribution paralleled that of rough endoplasmic reticulum, whereas subunit A mRNA distribution paralleled that of free ribosomes: rough endoplasmic reticulum was abundant around the central nucleus but also extended into the entire cytosol, whereas free ribosomes were highly concentrated at the apical portion of the cell (Fig. 5A–C). In Malpighian tubules, however, subunit A mRNA (Fig. 3A) as well as the proteolipid mRNA (not shown) were evenly distributed.

The level and distribution of subunit A mRNA changes during moulting

At the beginning of the moult (stages B and C), the distribution of subunit A mRNA in mature cells was the same as the pattern found in intermoult larvae (Fig. 1A–C). In stages D and E, the apical mRNA concentration in the columnar cells disappeared; the pattern in goblet cells remained unchanged (Fig. 1D). During late moult (stage F) and shortly after ecdysis (stage G), this apical concentration reappeared in all cells (Fig. 1E). In contrast to the distribution of subunit A mRNA, the level and the spatial distribution of proteolipid mRNA did not change during the moulting process (Fig. 2B).

V-ATPase signals occur very early in all developing cells

In differentiating cells, mRNA encoding subunit A or the

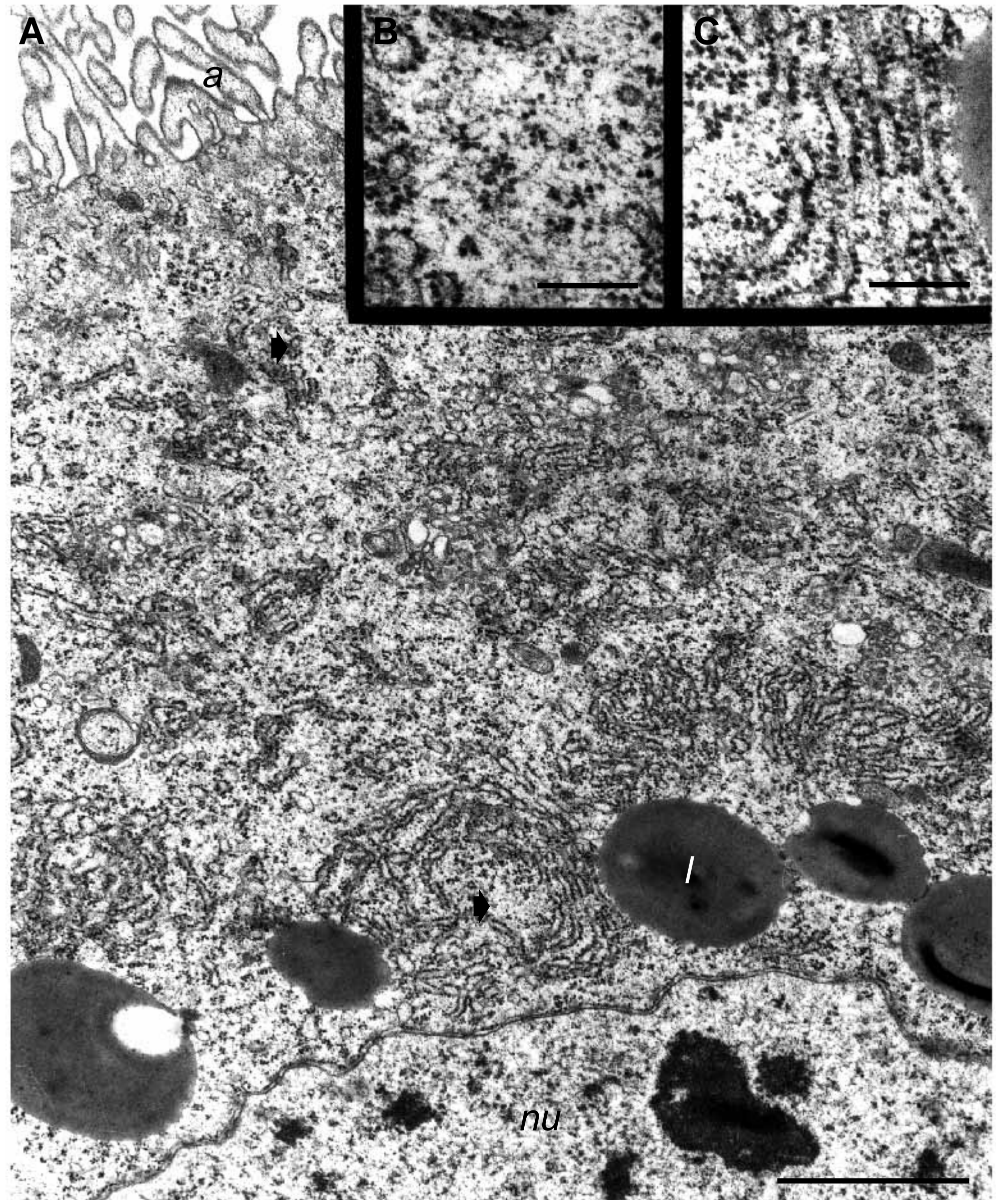


Fig. 5. Fine structural details of the moulting midgut epithelium of *M. sexta.* Electron micrographs. (A) The distribution of ribosomes in the cytoplasm of a columnar cell. Free ribosomes (B) are located preferentially in the apical region of the cell. Rough endoplasmic reticulum (C) occurs throughout the cytoplasm but is particularly abundant around the nucleus. The positions of insets B (apical) and C (close to the nucleus) are marked by arrows. *a*, apical; *l*, lipid droplet *nu*, nucleus. Scale bars, A, 1 μm; B,C, 0.2 μm.

proteolipid was present at the beginning of proliferation (Figs 1B, 2B). No specialized pattern of distribution was seen in these cells until late moult (stage E, Fig. 1D). V-ATPase immunoreactivity was already present in the membrane of the GCPC by stage F and, at the same stage, re-appeared in the apical membrane of the mature goblet cells (Fig. 4C).

Discussion

Is the V-ATPase mRNA in columnar cells a biosynthetic product of housekeeping gene activity?

In the midgut of *Manduca sexta*, a well-known model epithelium for ouabain-insensitive ion transport (Harvey *et al.* 1983), a proton-transporting V-ATPase was identified as the primary ion pump, thus demonstrating for the first time that a proton-motive force energizes secondary transport in animal plasma membranes (Wieczorek *et al.* 1989, 1991; Wieczorek, 1992). Since biochemical and immunocytochemical evidence demonstrated the presence of V-ATPase at high levels only in the goblet cell apical membrane (Wieczorek *et al.* 1986; Schweikl *et al.* 1989; Klein *et al.* 1991), the presence of large amounts of V-ATPase mRNA in both mature midgut cell types of intermoult and moulting larvae as revealed by the present study was unexpected. However, V-ATPases are part of the housekeeping enzyme repertoire of all cells and, therefore, their mRNA may be expected to occur in all cells. Our comparative hybridization screening of larval tissues exhibited pronounced mRNA signals not only in several epithelia known to possess or suspected of possessing a plasma membrane V-ATPase (Klein, 1992), but also in fat body and nerve cell somata, which are thought to be enriched in secretory acidic organelles. Furthermore, the V-ATPase-energized transporting epithelium of the Malpighian tubules (for a review, see Nicolson, 1993) showed regional variations in signal intensity

and thus in mRNA content. Therefore, it appears that our hybridization method reports only high concentrations of mRNA.

By refining our tissue preparation techniques we are able to demonstrate immunolabelling in apical cell protrusions of the columnar cells. Immunolabelling seems to be located in the cytosol and is only excluded from the numerous lipid droplets and from areas that seem to be rich in proteasome-like particles when inspected by conventional electron microscopy (D. Jaeger, H. Wieczorek and U. Klein, unpublished observations). Ultrathin sections failed to demonstrate a large number of lysosomes or other endosomes in these bleb regions but confirmed the presence of large numbers of free ribosomes. Cytosolic immunoreactivity may be due to soluble V_1 subunits, which have been found in yeast (Doherty and Kane, 1993) and rat renal papilla cells (Sabolic *et al.* 1992), or may even represent fully assembled V_1 sectors, which have been demonstrated to be present as a cytoplasmic pool in a bovine kidney cell line (Myers and Forgac, 1993). The function of such cytosolic material is, so far, unknown. In summary, the high level of plasma membrane V-ATPase in mature goblet cells readily explains their mRNA content, whereas the presence of the V-ATPase mRNA signal in mature columnar cells and the origin of the cytosolic V_1 immunoreactivity remain enigmatic. However, quantitative conclusions have to be drawn with caution, since nothing is known about post-transcriptional mechanisms regulating the amount of V-ATPase mRNA and of V-ATPase protein.

Membrane domain definition and V-ATPase sorting in developing cells

From the results presented here, it is not surprising that all undifferentiated cells exhibit intense mRNA signals for V-ATPase subunits of both holoenzyme sectors even as proliferation begins. Developing goblet cells already show V-ATPase immunolabelling of the GCPC membrane, but the biosynthesis of this V-ATPase-rich membrane does not cause a significant mRNA peak during cell differentiation, which would allow us to distinguish between precursors of columnar or goblet cells at early developmental stages. Even though it is impossible to demonstrate by light microscopy, and difficult to demonstrate by electron microscopy (Baldwin and Hakim, 1991), it may be assumed that the GCPCs are not fused with the apical membrane before ecdysis. Since the young goblet cavities grow and increase in V-ATPase content after ecdysis, as demonstrated by their increasing size and increased immunoreactivity (Fig. 4), the question of how V-ATPases are sorted to different target membranes arises. In particular, the nature of the GCPC membrane is ambiguous. If the GCPC membrane is already considered to be an apical plasma membrane, targetting into this membrane would be similar before and after fusion with the apical membrane domain, and may differ from the endosome-directed sorting processes. Alternatively, sorting into the GCPC membrane before ecdysis may follow the endosomal signals, but then switch to different sorting signals after ecdysis if the nature of the GCPC membranes changes to a plasma membrane after fusion.

mRNA of peripheral and membrane-integral subunits is differentially localized

Another striking result of our *in situ* hybridization study was the apically enhanced hybridization signal of the mRNA encoding subunit A of the peripheral V_1 sector, which contrasted with the even distribution of mRNA encoding the proteolipid of the integral V_0 sector. A similar uneven mRNA distribution had been demonstrated previously by *in situ* hybridization using DNA probes for mRNA encoding other subunits of the V_1 sector (F. J. S. Novak and D. Jaeger, unpublished observations). The spatial pattern of the V_1 mRNA in the columnar cells paralleled the apically enhanced distribution of free ribosomes, whereas the distribution of proteolipid mRNA paralleled the distribution of the rough endoplasmic reticulum. The polarized mRNA localization evidently depends on the ultrastructural compartmentation of the epithelial cell and may not be exclusive for V-ATPase subunits. Although an uneven distribution of the translational machinery may be appropriate in polarized cells such as the midgut cells, it is not invariably the case. For instance, V-ATPase mRNA distribution in the Malpighian tubules (Fig. 3A) or in the hindgut epithelium (U. Klein, M. Timme, F. J. S. Novak, A. Lepier, W. R. Harvey and H. Wieczorek, in preparation) is similar for both holoenzyme sectors; it is found all over the cytosol. The transient disappearance of the polarized pattern of subunit A mRNA and the resulting decrease in mRNA signal intensity may easily be explained by the loss of apical cell material by apocrine secretion.

The mRNA localization found in the midgut cells has several implications for the assembly process of the V-ATPase multisubunit complex and poses important problems regarding the regulation and maintenance of plasma membrane composition which need to be addressed. (i) The findings support the view that V_1 subunits are synthesized at free ribosomes, whereas the V_0 subunits are synthesized at ribosomes bound to the endoplasmic reticulum. (ii) Localized mRNA may indicate mRNA sorting during the biogenesis of localized proteins, a process well-documented in other systems (Wilhelm and Vale, 1993). (iii) Since translation sites for the V_1 and V_0 subunits are spatially separated, where do the subunits of the V_1 and V_0 sectors meet? Little is known about the biogenesis and assembly of V-ATPases in general. (iv) The striped hybridization signal seen in larval moult stage D could be due to a slight increase in mRNA in the goblet cells, associated with increased synthesis of V_1 subunits (Fig. 1D). Biochemical, physiological and immunocytochemical studies had demonstrated that V-ATPase activity is regulated during early moult by stripping of the V_1 sector of the V-ATPase from its V_0 sector, which remains in the goblet cell apical membrane (Sumner *et al.* 1995). Thus, the recovery of V-ATPase activity in mature goblet cells after inactivation during moulting may be achieved by the association of newly synthesized V_1 sectors after ecdysis. However, we cannot exclude the possibility that

there is an association of 'old' existing V_1 sectors or a complete turnover of both V-ATPase sectors.

This work was supported by the DFG (Wi 698 and Graduierten-Kolleg 'Zelluläre und molekulare Aspekte der Entwicklung'), the EC (SC1*-CT90-0480) and the NIH (AI 22444).

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