A Novel Insect V-ATPase Subunit M9.7 Is Glycosylated Extensively*

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Plasma membrane V-ATPase isolated from midgut and Malpighian tubules of the tobacco hornworm, *Manduca sexta*, contains a novel prominent 20-kDa polypeptide. Based on N-terminal protein sequencing, we cloned a corresponding cDNA. The deduced hydrophobic protein consisted of 88 amino acids with a molecular mass of only 9.7 kDa. Immunoblots of the recombinant 9.7-kDa polypeptide, using a monoclonal antibody to the 20-kDa polypeptide, confirmed that the correct cDNA had been cloned. The 20-kDa polypeptide is glycosylated, as deduced from staining. Treatment with N-glycosidase A resulted in the appearance of two additional protein bands of 16 and 10 kDa which both were immunoreactive to the 20-kDa polypeptide-specific monoclonal antibody. Thus, extensive N-glycosylation of the novel *V*<sub>o</sub> subunit M9.7 accounts for half of its molecular mass observed in SDS-polyacrylamide gel electrophoresis. M9.7 exhibits some similarities to the yeast protein *Vma21p* which resides in the endoplasmic reticulum and is required for the assembly of the *V*<sub>o</sub> complex. However, as deduced from immunoblots as well as from activities of the V-ATPase and endoplasmic reticulum marker enzymes in different membrane preparations, M9.7 is, in contrast to the yeast polypeptide, a constitutive subunit of the mature plasma membrane V-ATPase of *M. sexta*.

H<sup>+</sup> V-ATPases are a class of ion transport proteins that couple ATP hydrolysis to the movement of protons across membranes. In endomembranes they function, in concert with chloride channels, as acidifiers of intracellular compartments, whereas in plasma membranes their roles are dependent on the cell type. V-ATPases consist of a peripheral *V*<sub>i</sub> complex, which is responsible for the hydrolysis of ATP, and a membrane-bound *V*<sub>o</sub> complex which is responsible for the translocation of protons. Although the subunit composition may depend upon the source of the enzyme, at least seven subunits of the *V*<sub>i</sub> complex, subunits A to G, appear to be universal V-ATPase components (1). By contrast, the subunit composition of the *V*<sub>o</sub> complex is less clear. There is no doubt that a 16–17-kDa proteolipid, the proton “channel,” is a major constituent of the *V*<sub>o</sub> complex. A membrane-associated subunit in the 40-kDa range and an ~100-kDa transmembrane subunit may be two additional essential *V*<sub>o</sub> components (1). Recently, a novel 9.2-kDa membrane sector-associated polypeptide was reported from bovine chromaffin granules (2). Its sequence and structure show some similarity to *Vma21p*, a yeast protein involved in the assembly of the V-ATPase; whether or not it is a constitutive V-ATPase subunit remains an open question.

In the larval midgut epithelium of the model insect, *Manduca sexta* (Lepidoptera, Sphingidae), a plasma membrane V-ATPase is present in the apical membranes of goblet cells where it energizes the alkalination of the gut lumen to a pH of more than 11 (3). For the *V*<sub>i</sub> complex, amino acid sequences of five insect subunits A, B, E, F, and G have been deduced from cloned cDNAs (4), and evidence for the existence of subunit D has been derived from partial amino acid sequence analysis. For the *V*<sub>o</sub> complex, only sequences of the 17-kDa proteolipid and of the subunit M40 have been derived from cDNAs to date (5, 6), although evidence for a 100-kDa subunit is appearing on the horizon.

Based on a partial amino acid sequence obtained from a 20-kDa polypeptide band that is present in gels after SDS-PAGE of the insect holoenzyme, we have cloned and sequenced the cDNA encoding a 9.7-kDa protein that is remarkably similar to the bovine 9.2-kDa *V*<sub>o</sub>-associated protein. The insect protein is glycosylated extensively, with sugar residues contributing to half of its apparent molecular mass. We provide evidence here that the 9.7-kDa protein is a constitutive subunit of the *V*<sub>o</sub> complex of the mature V-ATPase holoenzyme.

**EXPERIMENTAL PROCEDURES**

**Insects**—Larvae of *M. sexta* (Lepidoptera, Sphingidae) were reared under long day conditions (16 h of light) at 27 °C using a synthetic diet modified according to Bell et al. (7).

**N-terminal Protein Sequencing—V-ATPase** was isolated from the goblet cell apical membranes of larval *M. sexta* midgut as described previously (8, 9). Three hundred μg of purified V-ATPase were subjected to preparative SDS-PAGE and stained with Coomassie Blue. The 20-kDa band was excised from the gel and concentrated as described by Rider et al. (10) but using 5% polyacrylamide as spacer and a 15% polyacrylamide gel underneath. The resulting protein spot was blotted onto a polyvinylidene difluoride membrane (Immobilon P) using a buffer system consisting of 10 mM NaHCO<sub>3</sub> and 3 mM Na<sub>2</sub>CO<sub>3</sub>. After staining with Amido Black, the spot was excised and installed into the N-terminal Protein Sequencing cartridge of a model 473A protein sequencer (Applied Biosystems), staining with Amido Black, the spot was excised and installed into the N-terminal Protein Sequencing cartridge of a model 473A protein sequencer (Applied Biosystems), and its amino acid sequence was determined as described previously (11).

**Screening of an *M. sexta* cDNA Library and Sequencing of Clones**—The N-terminal amino acid sequence of the *M. sexta* 20-kDa protein was used to design the degenerate primer pM20-deg (5'-ATGGC(T/C)TTCT-TCTG(T/C)CCAAT/ACT/ACC/CTGTTTCT-3') which was optimized according to the codon usage of *M. sexta* proteins (12). Direct PCR (13) was

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank<sup>®</sup> (13) or EBI Data Bank with accession number(s) AJ006029.

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1 M. Huss, R. Schmid, W. R. Harvey, and H. Wieczorek, unpublished data.

2 The abbreviations used are: ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; MBP, maltose-binding protein; bp, base pair; DCCD, dicyclohexylcarbodiimide.
performed in the presence of the primers pM20-deg (100 pmol) and pT7 (20 pmol, 5’-AAATCAGCTACTATAGGCG-3’), the latter corresponding to the T7 promoter of the α-Zap II DNA and 2 × 10^6 plaque-forming units from an M. sexta larval midgut α-Zap II cDNA library (14). The reaction was carried out with AmpliTaq DNA polymerase (Perkin Elmer-Applied Biosystems). The first 19 bases of the coding sequence (5’-TATCCAGCAT-GGGTGTCCCTTTGTTGCG-3’; 0.15 μM), the first 15 bases of the downstream primer (5’-CTATCCAGCTACTATAGGCG-3’) corresponding to the T7 site of pBluescript SK–). The PCR product was digested with NcoI at the 5’ end and with KpnI at the 3’ end, purified by gel electrophoresis in 0.75% agarose, and extracted from the gel using the QIAquick gel extraction kit from Qiagen. The fragments were cloned into the NcoI/KpnI site of the pcM20–3REV (5’-TATCAGCTACTATAGGGC-3’) vector, prepared for in vitro transcription/translation with SP6 polymerase and [35S]methionine (20 μCi; Amersham Pharmacia Biotech) and sequenced using the TNT-rabbit reticulocyte system from Promega. After SDS-PAGE and Coomassie Blue staining, the gel was incubated in enhancer solution (ENDANCE, DuPont) for 1 h, washed in cold water for 1 h, and dried on Whatman filter paper. [35S]Methionine-labeled protein bands were visualized by phosphorimaging.

**Results**

**Fractionation—**Midguts from fifth instar larvae were homogenized with a glass Teflon homogenizer in a buffer containing 0.32 M sucrose, 0.02 M Tris-HCl (pH 7.6), 1 mM EGTA, 3 mM MgCl2, and 5 mM Pefabloc® Sc. After filtration through cotton gauze the homogenate was centrifuged at 700 x g for 10 min at 4 °C. The resulting supernatant was again centrifuged at 7000 x g for 10 min at 4 °C. The microsomal pellet was obtained by spinning the 7000 x g supernatant at 100,000 x g for 100 min at 4 °C. It was resuspended in a buffer containing 0.02 μM Tris-HCl (pH 7.6), 1 mM EGTA, 3 mM MgCl2, and 5 mM Pefabloc® Sc, layered onto a discontinuous 20–60% (w/w) sucrose density gradient (10 steps), and centrifuged overnight in a swing-out rotor (SW41TI, Beckman) at 25,000 rpm and at 4 °C. Fractions obtained were spun down and frozen in liquid nitrogen.

**Purification of Golgi Membranes—**Golgi membranes from either midgut goblet cell apical membranes or Malpighian tubule membranes were isolated as described previously (6, 9, 23, 24). V-ATPase was assayed as enzyme activity sensitive to 1 μM bafilomycin A1 (Fluka) according to Wieczorek et al. (9). The activities of NADPH-cytochrome c reductase and glucose-6-phosphatase were determined following modified protocols (25, 26). To inhibit unspecific hydrolysis of glucose-6-phosphate by alkaline phosphatases, 1 mM levamisol was added to the assay mixtures. Cytoplasmic expression of the 9.7-kDa polypeptide as an MBP fusion protein in E. coli was performed according to Graef et al. (27) using the pMal-c2 expression system from New England Biolabs. The vector that was obtained by inserting the coding sequence and the 3′ untranslated region of pcM20BSK-A into the multiple cloning site of pMal-c2 was named pM9.7Mal-c2.

**RESULTS**

The M. sexta V-ATPase Contains a 20-kDa Polypeptide as Part of Its V Complex—In immunoblots of V-ATPase isolated from highly purified goblet cell apical membranes, a polyclonal antiserum to the M. sexta V-ATPase revealed a prominent protein band with an apparent molecular mass of ~20 kDa (lane 1 in Fig. 1, see also Ref. 28). The polypeptide could also be visualized by silver staining (lane 2 in Fig. 1; see also Ref. 22), whereas staining with Amido Black (lane 3 in Fig. 1) as well as with Coomassie Blue (not shown) usually did not reveal detectable amounts of polypeptide. In immunoblots of V-ATPase purified from either midgut goblet cell apical membranes or Malpighian tubule membranes, the 20-kDa V-ATPase band recognized by the monoclonal antibody 224-3 (lanes 4 and 5 in Fig. 1, respectively), which is specific for this polypeptide but also shows slight cross-reactivity to subunit B (22). Since the 20-kDa polypeptide was isolated, together with known V-ATPase subunits, from two different types of highly purified plasma membranes originating from different tissue sources, midgut and Malpighian tubules, it appears to...
be a constitutive subunit of the insect V-ATPase.

Two previous experiments already had indicated that the 20-kDa polypeptide may be a member of the $V_o$ complex. First, it remained in the membrane fraction after peripheral subunits were stripped off by chaotropic iodide (24). Second, its relative amount in goblet cell apical membranes was enriched during moult when the $V_1$ complex was released from the membrane (22, 29).

If the 20-kDa polypeptide is a $V_o$ subunit, it should be present in the free $V_o$ complex together with the established $M. sexta$ $V_o$ subunits c (5) and M40 (6). Partially purified goblet cell apical membranes from starving larvae turned out to be a good source for the isolation of the $V_o$ complex because, as in membranes from moulting larvae, they contain enriched free $V_o$ complexes from which the $V_o$ complexes have been detached.

After solubilization of goblet cell apical membranes and zonal centrifugation in a discontinuous sucrose density gradient (8, 9), the two established $V_o$ subunits c and M40 as well as the putative novel 20-kDa subunit were found not only in the upper 30% fraction as part of the remaining $V_o V_1$ holoenzyme but also in the upper 20% fraction as part of the integral $V_o$ complex (Fig. 2). The strictly similar distribution of the 20-kDa polypeptide and the $V_o$ subunits c and M40 indicates that the novel polypeptide is a member of the $V_o$ complex.

Protein Sequencing of the 20-kDa Polypeptide Leads to the Isolation of a cDNA Encoding a 9.7-kDa Protein.—The 20-kDa polypeptide was isolated by SDS-PAGE, and its N-terminal protein sequence was determined to be (MG)AXFVPTTVFLTIILXXXYGOI. The first 10 amino acids (underlined) were chosen to design a codon-optimized, degenerate primer pM20-deg, assuming that the initial amino acid was methionine and that the third one was possibly phenylalanine (see “Experimental Procedures”).

PCR using the $M. sexta$ $\lambda$-Zap II cDNA library as a template resulted in the specific amplification of a 750-bp fragment that was obtained only in the presence of both primers, pM20-deg and pT7 (Fig. 3). Sequencing of the cloned PCR fragment revealed that the correct cDNA encoding the 20-kDa protein had been amplified; the sequence and position of the deduced N-terminal amino acids perfectly matched with those that were determined from protein sequencing at positions 11–19, a section that could not be attributed to the upstream primer.

To obtain a full-length cDNA clone, the $M. sexta$ $\lambda$-Zap II cDNA library was screened by hybridization with a digoxigenin-11-dUTP-labeled probe corresponding to the PCR fragment that was related to the 20-kDa polypeptide. After three screening steps, two independent cDNA clones were isolated and turned out to be identical in their nucleotide sequences. The cDNA sequence comprised 765 base pairs and was terminated by a poly(A) tail of 31 bp (Fig. 4). Unexpectedly, the open reading frame which corresponded to the data obtained from protein sequencing was only 264 bp in length, encoding a hydrophobic protein of 88 amino acids with a calculated molecular mass of 9.67 kDa and an isoelectric point at pH 9.04.

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determined according to Skoog and Wichman (30). Prediction of hydropathic properties and secondary structure based on different algorithms (31–35) showed a high probability for one membrane-spanning α-helix in the range of amino acid positions 40–60. A second predicted hydrophobic α-helix within the first 20 amino acids may possibly be too short to span the membrane. The C terminus was more hydrophilic than the N terminus and is likely to be located at the extracellular surface since, according to the PROSITE data base, it contains two potential glycosylation sites at positions 68–70 and 84–86 (Fig. 4).

Although the protein deduced from cDNA cloning was only approximately half the size of the 20-kDa polypeptide identified by SDS-PAGE, four findings suggest that the 20-kDa protein was encoded by the open reading frame. First, the deduced N-terminal 19 amino acids were in entire agreement with the data obtained from sequencing of the 20-kDa protein (Fig. 4), except for the missing N-terminal methionine in the mature protein. Moreover, the initiator ATG (nucleotide positions 64–66) of the open reading frame was embedded in a sequence environment that is similar to that of other cloned cDNAs (5, 14, 24, 27, 36) and matched closely the Kozak consensus sequence for translational initiation by eucaryotic ribosomes (37). Second, cDNA sequencing was performed three times with cDNAs cloned independently, each in both directions, rendering unlikely sequencing mistakes or cloning artifacts leading to a frameshift. Even if nucleotides were deleted or inserted, the resulting reading frames would not increase substantially the molecular masses of the corresponding polypeptides. Third, the initial PCR performed on the λ-Zap II DNA resulted in only one PCR fragment of the indicated length even at relatively low stringency. Thus, a possible splice variant encoding an expanded open reading frame seems not to exist in the cDNA library. This conclusion was supported by Northern blots of poly(A) RNA isolated from midgut and Malpighian tubules; in both cases only one mRNA species of approximately 800 bp in length was observed (Fig. 5). Moreover, this experiment placed the identified mRNA precisely in those tissues in which the putative 20-kDa V-ATPase subunit was expressed (Fig. 1). Fourth and finally, both the deduced 9.7-kDa protein and the 20-kDa polypeptide appear to be hydrophilic membrane proteins.

To supply direct and definitive evidence that the cloned cDNA encoded the 20-kDa polypeptide, we expressed the recombinant 9.7-kDa protein as a fusion protein in E. coli. After SDS-PAGE the fusion protein was blotted and immunostained with the monoclonal antibody 224-3. In contrast to the unfused maltose-binding protein (MBP) with a molecular mass of 42.7 kDa, the 9.7-kDa/MBP-fusion protein had an electrophoretic mobility corresponding to 53 kDa and was immunoreactive to the monoclonal antibody 224-3 (see Fig. 7, lanes 5 and 6).

A BLASTP search revealed that the deduced M. sexta amino acid sequence was 46% identical and 69% similar to the 9.2-kDa membrane sector-associated protein of V-ATPase subunit recently cloned from human, bovine, and murine sources (Fig. 4), which differ from each other in only one amino acid at position 22 (2). Similarities were also detected to the unidentifed open reading frames of Caenorhabditis elegans chromosome IV (44% identity, 70% similarity) and of Drosophila melanogaster chromosome III (29% identity, 55% similarity), both reported recently (2), and to the yeast Vma21p protein (38) (24% identity, 51% similarity) and the E. coli Ucn gene product (39) (21% identity, 56% similarity). In general, all similarities observed were accompanied by a similarity of the predicted hydropathic properties (not shown).

The 20-kDa Polypeptide Is Glycosylated—The deduced amino acid sequence of the M. sexta M9.7 polypeptide. The M. sexta protein (GenBank accession number AJ006029) is compared with the recently identified human V-ATPase subunit M9.2 (GenBank accession number Y15286) and with an unidentified open reading frame of D. melanogaster (GenBank accession number L07835). Identical amino acids are indicated by vertical bars and similar ones by dots. Identities between all three sequences are highlighted by gray shading. Putative polyadenylation signals in the 3′-untranslated region of the cDNA sequence are underlined. Possible N-glycosylation sites of the M. sexta protein are double underlined. In addition, the N-terminal amino acid sequence obtained from protein sequencing is shown in the gray box.
acid sequence of the M9.7 protein contains two potential N-glycosylation sites near the C terminus. Thus it appeared plausible that the discrepancy between the molecular mass of 9.7 kDa calculated from the open reading frame and that of 20-kDa determined from SDS-PAGE of the V-ATPase was due to the posttranslational processing of the 9.7-kDa protein by N-glycosylation.

Glycosylation of the 20-kDa protein had already been suggested previously (28), because the band detected by immunostaining after SDS-PAGE consistently appeared rather broad and diffuse (Fig. 1). To check for glycosylation of the 20-kDa polypeptide, we deglycosylated purified M. sexta V-ATPase by treatment with a mixture of endoglycosidase F and N-glycosidase F (40). After SDS-PAGE and Western blotting, putative glycoproteins were visualized by staining with concanavalin A. Whereas lectin staining of the untreated control resulted in the appearance of two major bands at 40 and 20 kDa, respectively, deglycosylation of the V-ATPase resulted in a complete loss of reactivity to concanavalin A (Fig. 6). Thus both lectin staining and the susceptibility to endoglycosidase F/N-glycosidase F treatment suggested that the 20-kDa polypeptide of the M. sexta V-ATPase is an N-linked glycoprotein.

Deglycosylation of the 20-kDa Polypeptide Leads to 16- and 10-kDa Products—The 20-kDa polypeptide band from SDS-PAGE was excised from the gel and treated with glycosidase. To cleave N-linked glycans carrying a fucose α1,3-linked to asparagine N-acetylgalactosamine, a motif present in lepidopteran glycoproteins (41), we used N-glycosidase A which is capable of hydrolyzing most types of asparagine-bound N-glycans including α1,3-bound core fucose residues (42). After a further SDS-PAGE separation and Western blotting, deglycosylation products were detected by the monoclonal antibody 224-3. Treatment of the isolated 20-kDa polypeptide with N-glycosidase A resulted in the appearance of two additional immune reactive bands at approximately 16 and 10 kDa, whereas the untreated, fully glycosylated polypeptide still migrated at 20 kDa (Fig. 7). Since the control polypeptide showed no signs of degradation, and since N-glycosidase A did not exhibit protease activity (not shown), these results provided clear evidence that the 20-kDa polypeptide was glycosylated to a high degree. In addition, the lower deglycosylated protein band migrated in SDS-PAGE at exactly the same molecular mass as the recombinant 9.7-kDa protein obtained from coupled in vitro transcription/translation of the cloned open reading frame (Fig. 7). Finally, the recombinant 9.7-kDa protein obtained as a fusion protein with maltose-binding protein by expression in E. coli exhibited the same immunoreactivity as the 20-kDa polypeptide (see above). Thus the remarkable difference between the calculated and the apparent molecular masses of 10 and 20 kDa appears to be due to extensive posttranslational processing of the 9.7-kDa protein by N-glycosylation, contributing to half of the molecular mass of the 20-kDa polypeptide observed in SDS-PAGE.

The 20-kDa Polypeptide Is a Subunit of the Mature V-ATPase—Copurification of the 20-kDa polypeptide with established V-ATPase subunits in preparations of the V1V0 complex and the partially purified V0 complex already had indicated that it is a member of the plasma membrane V-ATPase of M. sexta (see above). By contrast, the putative yeast homologue Vma21p evidently is not a constituent part of the V-ATPase. Instead, it resides in the membranes of the endoplasmic reticulum, where it is required for the assembly of the integral membrane sector of the V-ATPase (38). To exclude the possibility that the presence of the Manduca polypeptide in goblet cell apical membranes results from contaminating ER membranes, we partially purified midgut microsomal membranes and assayed V-ATPase activity and the activities of the ER marker enzymes, NADPH-cytochrome c reductase and glucose-6-phosphatase in the various fractions from sucrose density gradient centrifugation as well as in purified goblet cell apical membranes. The highest activities of both NADPH-cytochrome c reductase and glucose-6-phosphatase were found in the 20/30% sucrose fraction, whereas V-ATPase activity in this fraction was less than 10% that in purified goblet cell apical membranes (Fig. 8A). By contrast, the 30/40% sucrose fraction contained less ER marker enzyme activity and considerably higher V-ATPase activity than the 20/30% sucrose fraction. The highest V-ATPase activity was found in purified goblet cell apical membranes, which exhibited only ~20% of the activity of the ER marker enzymes. Immunoblots after SDS-PAGE using equal protein amounts of the different membrane preparations indicated that the 20-kDa polypeptide, which was detected by the monoclonal antibody 224-3, was heavily enriched in the goblet cell apical membranes, whereas the 20/30% sucrose fraction contained only minor amounts (Fig. 8B). Since the same result was observed for the constitutive V-ATPase subunit E, which was detected by monoclonal antibody 90-7 (43), we conclude that, in contrast to its yeast homologue Vma21p, the 20-kDa polypeptide is not retained in the endoplasmic reticulum. We designate the 20-kDa polypeptide as subunit M9.7, in accordance with the common nomenclature for V0 subunits, because these results demonstrate that it is a constituent part...
of the mature holoenzyme that resides predominantly in the plasma membrane.

**DISCUSSION**

**M9.7 Is a Constitutive V, Subunit of the Mature V-ATPase**—
The cDNA encoding a prominent 20-kDa polypeptide in V-ATPase preparations from two different tissues of *M. sexta* larvae was cloned after N-terminal sequencing. Although the deduced hydrophobic protein exhibits a molecular mass of only 9.7 kDa, expression of the recombinant M9.7 protein in *E. coli* revealed immunoreactivity to a monoclonal antibody directed to the 20-kDa polypeptide and thus confirmed that the cloned cDNA encoded the 20-kDa polypeptide. The remarkable difference between the theoretical molecular mass of 9.7 kDa and the electrophoretic mobility at 20 kDa was shown to be caused by extensive N-glycosylation.

The poor reactivity of the 20-kDa band to Coomassie Blue or Amido Black may be a consequence of the less than 5% of positively charged amino acids in the 9.7-kDa protein. In this respect the 9.7-kDa protein resembles the proteolipid, subunit c, which is also poorly detectable by both dyes, due to a low (4.5%) content of lysine and arginine. In any case, the faint staining behavior of the M9.7 (20-kDa) protein does not argue against our conclusion that it is a constitutive V-ATPase subunit. By contrast, it appears to be a genuine V, subunit of the mature V-ATPase, since it copurifies in strictly reproducible amounts with highly purified V-ATPase from midgut and Malpighian tubules and since it is a significant component not only of the holoenzyme but also of the partially purified V, complex.

**M9.7 and Its Mammalian Counterpart May Differ in Glycosylation**—Deglycosylation of the 20-kDa polypeptide with N-glycosidase A resulted in the weak immunostaining with the monoclonal antibody 224-3 of a 10-kDa polypeptide and a strong staining of a 16-kDa polypeptide, whereas the staining intensity of the 20-kDa band decreased. This finding suggests that deglycosylation by N-glycosidase A may occur at two different reaction rates as follows: a fast step resulting in the decrease of the molecular mass from 20 to 16 kDa, and a slow step reducing the molecular mass by an additional 6 kDa. This conclusion is consistent with the existence of two potential N-glycosylation sites on the deduced amino acid sequence of the 9.7-kDa protein, one at positions 68–70 and the other at positions 84–86, close to the C terminus. By contrast, the amino acid sequences of the mammalian 9.2-kDa protein contain only one of the two potential N-glycosylation sites present in the *M. sexta* protein (see Fig. 3). The bovine 9.2-kDa protein was reported not to be affected by glycosidase F treatment (2). However, several observations suggest that the V-ATPase isolated from chromaffin granules may also contain a glycosylated 9.2-kDa protein. Thus SDS-PAGE of bovine V-ATPase obtained by electrophoresis in blue native gels revealed a membrane-bound 16-kDa polypeptide that was not susceptible to internal protein sequencing (2). It was tentatively assigned to subunit M20, a putative V, polypeptide first reported for the coated vesicle V-ATPase (44, 45). The results obtained from deglycosylation of the *M. sexta* 20-kDa polypeptide suggest that the 16-kDa protein represents an intermediate product resulting from preferential deglycosylation at only one glycosylation site. Thus, the bovine 16-kDa polypeptide may represent the N-glycosylated version of the 9.2-kDa polypeptide, being processed at the glycosylation site that is also conserved in the 9.7-kDa protein of *M. sexta* at amino acid positions 68–70. Consequently, the conserved glycosylation sites could contribute to approximately 6 kDa of the total molecular mass of both the bovine and the *M. sexta* polypeptides, whereas the unique C-terminal site of the *M. sexta* protein may contribute to an additional 4 kDa, resulting in a total molecular mass of 20 kDa in SDS-PAGE.

*M. sexta* Subunits M9.7 and B May Share an Epitope—The monoclonal antibody 224-3 not only cross-reacts with the V, subunit M9.7 but also with the V, subunit B of the *M. sexta* V-ATPase (22). However, no immunocytochemical labeling was detected in golget cell apical membranes of the mounting midgut, which lacks V, subunits, including subunit B, but contains all subunits of the V, complex, including M9.7. The cross-reactivity of antibody 224-3, observed in immunoblots after SDS-PAGE, with subunits B and M9.7 may be the result of the similar epitope LA/MLTAA, with five of six identical amino acids that are present in subunit B (positions 261–266) as well as in subunit M9.7 (positions 39–44). The epitope of subunit M9.7 is localized in a region that is predicted to be in the transmembrane a-helix. Consequently, the lack of immunoreactivity of subunit M9.7 in cryosections of *M. sexta* midgut may result from insufficient accessibility of the epitope.

The Function of Subunit M9.7 Remains Enigmatic—Sequence and structural similarities of bovine M9.2 with yeast Vma21p already had suggested that these proteins may be potential homologues (2). The yeast protein is not a V-ATPase subunit but has been assigned to the endoplasmic reticulum membrane where it may be involved in the assembly of V, subunits (38). By contrast, the extensive glycosylation of the *M. sexta* polypeptide argues against such a localization. Moreover, we have clearly demonstrated its presence in high amounts in two preparations of purified target membranes of the insect V-ATPase, the golget cell apical membrane from midgut, and the brush border membrane from Malpighian tubules, and its presence in low amounts in preparations enriched with endoplasmic reticulum membranes.

Although the localization of the bovine polypeptide remains questionable (2), like the *M. sexta* 9.7 polypeptide, it lacks a di-lysine motif (46), the signal for retention in the endoplasmic reticulum; on the other hand, this motif occurs in the yeast protein (38). In addition, the topology of the yeast protein seems to be inverse to that of the mammalian and insect proteins: glycosylation at the C terminus of the *M. sexta* protein indicates that the C terminus is exposed to the extracellular surface, whereas the C terminus of Vma21p appears to be localized on the cytosolic side of the membrane (38).
Significant similarities were also observed to the uncI gene product of *E. coli*. *uncI* precedes the eight ATP synthase genes and encodes a hydrophobic protein of 14.5 kDa which may be part of the F0 complex (39, 47). However, no function for *uncI* has been described to date with the exception that it may specifically affect the expression of the F-ATPase subunit encoded by *uncB* (48).

The *M. sexta* 9.7-kDa protein is more similar to the mammalian 9.2-kDa V-ATPase subunit than to the putatively homologous protein from *D. melanogaster* that was deduced from an unidentified open reading frame. Since *Manduca* and *Drosophila* V-ATPase subunits usually exhibit identities and similarities of 77–98 and 86–99%, respectively, the putative *Drosophila* protein may not be the exact correlate of the *M. sexta* 9.7-kDa protein.

Prediction of the secondary structure of the mammalian V-ATPase subunit M9.2 and its putative homologous counterparts in *C. elegans*, *D. melanogaster*, and yeast as well as that of the *E. coli* of the UncI protein of *E. coli* reveals a high probability for only one hydrophobic α-helix at a central position similar to that known for the homologous proteins. A second transmembrane helix at the N terminus, such as that predicted for the mammalian proteins, and therefore *uncI* protein be-

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