Primary proton transport by V-ATPases is regulated via the reversible dissociation of the V$_1$V$_0$ holoenzyme into its V$_1$ and V$_0$ subcomplexes. Laser scanning microscopy of different tissues from the tobacco hornworm revealed co-localization of the holoenzyme and F-actin close to the apical membranes of the epithelial cells. In midgut goblet cells, no co-localization was observed under conditions where the V$_1$ complex detaches from the apical membrane. Binding studies, however, demonstrated that both the V$_1$ complex and the holoenzyme interact with F-actin, the latter with an apparently higher affinity. To identify F-actin binding subunits, we performed overlay blots that revealed two V$_1$ subunits as binding partners, namely subunit B, resembling the situation in the osteoclast V-ATPase (Holliday, L. S., Lu, M., Lee, B. S., Nelson, R. D., Solivan, S., Zhang, L., and Gluck, S. L. (2000) J. Biol. Chem. 275, 32331–32337), but, in addition, subunit C, which gets released during reversible dissociation of the holoenzyme. Overlay blots and co-pelleting assays showed that the recombinant subunit C also binds to F-actin. When the V$_1$ complex was reconstituted with recombinant subunit C, enhanced binding to F-actin was observed. Thus, subunit C may function as an anchor protein regulating the linkage between V-ATPase and the actin-based cytoskeleton.

V-ATPases are ubiquitous proton pumps that are found in the endomembranes of all and the plasma membranes of many specialized eucaryotic cells (1, 2). They comprise at least 12 subunits that are part of two different subcomplexes, i.e. a peripheral, catalytic V$_1$ complex with subunits A$_{12}$B$_3$C$_6$D$_{ex}$E$_x$F$_2$G$_y$H and a membrane-bound, proton-conducting V$_0$ complex with subunits a$_{de}$. In the midgut of the tobacco hornworm (Manduca sexta) they are localized in the apical membrane of goblet cells where they exclusively energize all secondary active transport processes across the epithelium (3). During starvation or molt, pump activity abolishes due to the secondary active transport processes across the epithelium (3). During starvation or molt, pump activity abolishes due to the secondary active transport processes across the epithelium (3).

In vivo experiments indirectly suggested that microtubules might be involved in glucose-dependent dissociation of the yeast V-ATPase (18). However, V-ATPases are also associated with the actin-based cytoskeleton. During osteoclast activation ruffled membrane V-ATPase directly binds to F-actin, evidently linking V-ATPase transport to the reorganization of the actin cytoskeleton (19). Binding of actin and V-ATPase may occur in systems where the V-ATPase is sorted into the plasma membrane of a cell; latrunculin, which disrupts actin filaments, had no effect on glucose-dependent dissociation of the V-ATPase from the endomembranes (18).
To investigate whether the interaction between actin and plasma membrane V-ATPases reflects a general phenomenon, we performed co-localization studies, co-pelleting assays, and overlay blots using the plasma membrane V-ATPase of Manduca midgut goblet cells. We show that, in vitro, both the V₁ complex and the V₁V₀ holoenzyme bind directly to F-actin. In vivo, however, this interaction seems to be restricted to the holoenzyme. The interaction is mediated by subunits B and C; the latter subunit may function as a regulatory linker protein between the V-ATPase and the actin-based cytoskeleton.

EXPERIMENTAL PROCEDURES

Insects—Larvae of M. sexta (Lepidoptera, Sphingidae) were reared under long day conditions (16 h of light) at 27°C between the V-ATPase and the actin-based cytoskeleton.

MEMBRANE V-ATPase and F-Actin Co-localize at the Apical Membranes

For analyzing bound proteins, the beads were spun down in a microfuge, resuspended in Laemmli buffer (125 mM Tris-HCl, 5% sucrose, 2% SDS, 2% β-mercaptoethanol, and 0.05% bromphenol blue) and boiled for 1 min. Identification of actin was performed by Western blotting after SDS-PAGE using polyclonal antibodies directed to a highly conserved actin domain (Sigma, A-2068).

Linking V-ATPase to the Actin Cytoskeleton

Identification of actin was performed by Western blotting after SDS-PAGE using polyclonal antibodies directed to a highly conserved actin domain (Sigma, A-2068).

V-ATPase and F-Actin Co-localize at the Apical Membranes of Epithelial Cells from Midgut, Malpighian Tubules, and Salivary Glands—The midgut epithelium of the tobacco hornworm consists of columnar cells forming an extensive microvillar brush border, goblet cells with large cavities, and pluripotent stem cells from which both cell types derive (29). The microvilli of columnar cells are supported by actin filaments that are arranged along their longitudinal axes. Because the goblet cell...
plasma membrane V-ATPase may be associated with the actin cytoskeleton. To examine this possibility, we performed in vitro co-pelleting assays using the purified V₁V₀ ATPase and F-actin from rabbit muscle. For this purpose, we used phalloidin-stabilized F-actin at a concentration which was below the critical concentration for actin polymerization. The critical concentration in our assays was about 0.4 μM, a value that is in good agreement with previously published concentrations ranging from 0.1 to 1 μM (32). After centrifugation at 200,000 × g for 1 h, the proteins in the pellet were separated by SDS-PAGE and silver stained. In contrast to controls without actin, a significant portion of the holoenzyme was found in the pellet together with F-actin (Fig. 2A). The same result was obtained when we used the purified V₁ complex instead of the holoenzyme (Fig. 2B). In both cases, binding to F-actin was a saturable process. Our findings imply that both the holoenzyme and the V₁ complex bind directly to F-actin.

To evaluate the stoichiometries of the interaction with actin, we determined molar protein concentrations after co-pelleting, taking into account the known actin amounts of the pellet. Although actin appeared to exhibit a slightly higher (2–3-fold) affinity to the V₁V₀ holoenzyme as compared with the V₁ complex, binding reached its saturation at concentrations of about 50 nM for both the V₁V₀ holoenzyme and the V₁ complex if the concentration of F-actin monomers was 200 nM (Fig. 2C). Thus, the molar ratio was estimated to be about 1 V-ATPase to 4–5 F-actin monomers. Because the V₁V₀ holoenzyme as well as the V₁ complex have more than 10-fold molecular masses as compared with the actin monomer, these results suggest that maximum binding capacity is limited, which is likely due to steric hindrance of F-actin binding to the V-ATPase. The determined stoichiometry was in the same range as those reported previously for the bovine V-ATPase, which was estimated to be 1:8 (19).

Subunits B and C Mediate Binding to F-actin—Recently, Holliday et al. (20) demonstrated that the bovine V-ATPase subunit B contains an F-actin binding site. To determine whether subunit B is responsible also for the binding of F-actin to the Manduca V-ATPase, we performed overlay blots. For that purpose, we separated the subunits of the V₁V₀ holoenzyme and the V₁ complex by SDS-PAGE and stained them, after transfer onto nitrocellulose, with either Ponceau S or F-actin (Fig. 3, A and B, respectively). Immunodetection of bound F-actin revealed a protein band of 56 kDa for both enzyme preparations, confirming that subunit B interacted with actin filaments. To our surprise, we found an additional polypeptide of ~40 kDa in the V₁V₀ holoenzyme preparation that was intensively labeled by anti-actin antibodies. Control reactions performed in the absence of F-actin showed no labeled protein bands (Fig. 3C). Based on the observed molecular mass, we assumed that the labeled polypeptide was the V₁ subunit C, which we had identified previously to be part of the Manduca V-ATPase (6). Because this subunit is present in preparations of the Manduca V₁ complex in substoichiometric amounts at the most (7), we were not astonished to observe labeling only in the V₁V₀ holoenzyme. To confirm our assumption and to discriminate the labeled band from the V₀ subunit d, which has a similar molecular mass (33), we also tested the recombinant subunit C (Crec) for F-actin binding. As expected, Crec reacted with actin filaments in the overlay blots (Fig. 3, lane 4). Moreover, it co-pelleted together with F-actin in a saturable manner (Fig. 4A). Estimation of the binding stoichiometry revealed a molar Crec/F-actin-monomer ratio of ~1:1 (Fig. 4B).

To date, we have demonstrated that subunit C of the purified V₁V₀ ATPase as well as the recombinant subunit C...
bind to rabbit muscle actin. However, binding between V-ATPase and actin may depend on the source of the microfilaments, although rabbit skeletal muscle actin shares more than 93% identical amino acids with Manduca non-muscle actin, whose sequence (submitted to the EMBL/GenBank database under accession no. AJ519536) we have recently deduced from a J9261-Zap clone isolated from a Manduca midgut cDNA library (34).

To confirm that subunit C also binds to Manduca non-muscle actin, we covalently coupled the recombinant subunit C to beads of Affi-Gel 10 and incubated them with a cytoplasmic extract from the larval midgut. SDS-PAGE of proteins attached to subunit C revealed one major band that was identified immunologically in a Western blot as actin (Fig. 5). Thus, we could show that C rec not only binds to rabbit muscle actin but also to non-muscle actin from the midgut of the tobacco hornworm. This finding also suggests that the described interaction between C rec and actin may occur in vivo in the Manduca midgut.

Recombinant Subunit C Increases Binding of F-actin to the V1 Complex—The isolated V1 complex does not contain subunit C in significant amounts (see also Fig. 2B) and thus lacks one...
of the F-actin binding subunits. Therefore we hypothesized that the evidently lower affinity of the V1 complex to F-actin as compared with that of the V1V0 holoenzyme (see Fig. 2C) might be due to the lack of subunit C. Because the V1 complex can be supplemented by incubation with Crec resulting in a V1C complex containing significant amounts of subunit C (Ref. 24; see also Fig. 6A), we tested the capability of V1C to bind F-actin. For this purpose, we performed F-actin co-pelleting assays with either the V1C complex or the V1 complex lacking subunit C (see “Experimental Procedures”). Reconstitution of the V1 complex with Crec led to a significant increase in the amount of V1-ATPase subunits found in the pellet as compared with the V1 complex lacking subunit C (Fig. 6, B and C). This result indicated an increase in binding capacity due to the presence of an additional actin binding subunit.

**V1 Complexes That Have Detached from the Membrane Do Not Co-localize with F-actin**—The above-mentioned results raised the question of whether binding of F-actin mediated solely by subunit B is sufficient to allow binding to V1 complexes in vivo. To answer this question, we labeled cryosections of the posterior midgut from starving tobacco hornworms with either a monoclonal antibody to the V1 subunit A to detect V1 complexes, a monospecific antiserum to subunit C, or FITC-phalloidin to visualize F-actin, respectively. In accordance with previous biochemical studies, the V1 complexes became detectable in the cytoplasm of goblet cells due to dissociation of the V1V0 holoenzyme during starvation (Fig. 7A). As expected, subunit C was found in the cytoplasm, too (Fig. 7B). The cytoplasmic fluorescence signals resulting from immunoreactions with subunit A and C were not distinguishable so that both, free V1 complexes and C subunits, appeared to be evenly distributed throughout the cytoplasm of the goblet cells. By contrast, actin filaments were localized almost exclusively near the brush border membrane or close to the basal membrane (Fig. 7C). Thus, neither the detached V1 complexes nor the C subunits appeared to co-localize with F-actin, suggesting that binding only to subunit B may not be sufficient for the attachment of actin filaments to the V-ATPase in vivo.

**DISCUSSION**

In the larval midgut of *M. sexta*, high amounts of non-muscle actin filaments are found in the microvilli of the columnar cell-derived brush border (Ref. 31; see also Fig. 1). Here we show that actin filaments also reside at the goblet cell apical membranes, which are organized in a microvillus-like fashion (29). Because goblet cell apical membranes contain V-ATPase at high densities, we asked whether this enzyme is linked to the actin-based cytoskeleton. Co-localization studies and different binding assays performed in this work clearly indicated...
that the *Manduca* V-ATPase is able to interact with F-actin. Our results are in good agreement with those from previous studies demonstrating interaction between F-actin and V-ATPase during osteoclast activation (19). Like the *Manduca* midgut V-ATPase, the osteoclast V-ATPase resides in the apical plasma membrane. Thus, the interaction of a plasma membrane V-ATPase with cellular actin may reflect a general phenomenon that could be of functional relevance.

Overlay blots performed with rabbit muscle actin and either the V1V0 holoenzyme or the V1 complex confirmed the results of Holliday et al. (20) demonstrating that subunit B is involved in actin binding. To our surprise, however, we found that not only subunit B but also subunit C, which is a constituent part of the V1V0 holoenzyme but not of the V1 complex (5, 6, 7), binds to F-actin. In contrast to our results, previous studies with V-ATPase immunoprecipitated from metabolically labeled mouse marrow cultures or immunopurified from bovine kidney supplied no indications for subunit C to be involved in actin binding (19). Consequently, either binding properties of C subunits differ between insects and mammalian V-ATPases, or subunit C did not bind to actin in the mammalian system because it was not present in the V-ATPase preparations used for these binding studies. A reasonable explanation for the absence of subunit C may be based on the purification procedure using anti-V1 antibodies for immunopurification. Because cytoplasmic V1 complexes and membrane-bound holoenzymes are in dynamic equilibrium, immunopurification via anti-V1 antibodies might have favored enrichment of V1 complexes rather than enrichment of the holoenzyme. Consistently, none of the reported V-ATPase preparations from mouse marrow and bovine kidney showed the presence of either subunit C or any V0 subunit (19). Thus, it seems possible that mainly the cytoplasmic V1 complex instead of the V1V0 holoenzyme had been investigated for actin binding and, therefore, only subunit B could be detected as actin binding protein.

In contrast to some other V-ATPase subunits, the function of the V1 subunit C is still elusive. Subunit C is believed to be a peripheral stalk component that may not be essential for enzyme activity in a reconstituted system (35) but is important for assembly of the V1 complex (36, 37). Genetic disruption of the gene VMA5 encoding subunit C in yeast leads to a typical VMA− phenotype with cells that are unable to grow at neutral pH and in the presence of higher calcium concentrations. Vacuolar vesicles isolated from VMA5Δ yeast strains showed no V-ATPase activity and only poor assembly of the peripheral V1 portion onto the vacuolar membrane (37). Because the deletion of either subunit C or H disrupts the ability for assembly of the remaining V1 subunits, it was suggested that subunits C and H might play a role in bridging the V1 and V0 complexes. Upon reversible disassembly of the V1V0 complex, subunit C is the only protein that dissociates from the resulting subcomplexes (5). Both, the peripheral localization as well as its ability to dissociate from cytoplasmic V1 complexes and reattach to the holoenzyme make subunit C an ideal candidate for regulation of the enzyme by the interaction with other cellular proteins. In contrast to other V-ATPase subunits, to date no cellular protein has been reported that would directly interact with subunit C. Thus, actin is the first cellular protein known hitherto that binds to subunit C.

Currently, we can only speculate on the meaning of the interaction between actin and V-ATPase. Our results indicate that the V1V0 holoenzyme is bound to the filamentous actin cytoskeleton lining goblet cell apical membranes. Reduced F-actin binding capacity of V1 complexes in comparison to V1C complexes and missing co-localization of cytoplasmic V1 complexes and actin filaments suggest that, upon enzyme dissociation, V1 complexes do not only detach from the membrane but also from actin filaments due to the loss of subunit C. Consequently, subunit C may support subunit B to retain the holoenzyme at the actin cytoskeleton and assist in releasing V1 complexes into the cytoplasm. In conclusion we hypothesize that subunit C plays a crucial role not only in supporting V-ATPase assembly but also in controlling its linkage to the actin-based cytoskeleton in those cases where V-ATPase resides in the plasma membrane. By contrast, V-ATPase residing in the endomembrane system appears to be linked exclusively to microtubules because it was found that nocodazole, which disrupts microtubules, partially blocks dissociation of the V-ATPase in response to glucose depletion in yeast, whereas latrunculin, which disrupts actin filaments, had no effect (18). Independent of the type of filament, however, the interaction between V1 subunits of the active holoenzyme and cytoskeletal proteins could assist the stator subunits of the V-ATPase, such as V0 subunit a (2), in compensating the torque resulting from the rotation of central subunits.

The goblet cell apical membrane contains V-ATPase molecules at a very high density, which can be estimated by electron microscopy of isolated vesicles to be about 5,000 complexes/μm². The high density may lead to a lack of space for other transmembrane proteins. Indeed, SDS-PAGE of highly purified goblet cell apical membranes only shows the well defined protein pattern for V-ATPases without any indications for the presence of additional proteins at higher concentrations (28). Therefore, transmembrane proteins that are able to link the plasma membrane to the spectrin-based actin cytoskeleton via ankyrin, such as different antiporters, P-type ATPases, or ion channels (38), might be absent as well. For instance, the *Manduca* midgut completely lacks a Na+/K+ ATPase (39), which had been presumed to bind to ankyrin in the *Drosophila* midgut (40). Other putative anchor proteins in the goblet cell apical membrane may be present at densities that are too low for anchoring the apical plasma membrane at the actin cytoskeleton. Based on these considerations, it appears plausible that the V-ATPase takes over this anchor function. For *Drosophila*, variability between different epithelia with respect to the organization of the membrane skeleton and its transmembrane anchors has been suggested (40). However, if there is variability regarding the choice of the anchor protein, then it would not be surprising if the linker protein connecting the anchor to the actin filaments is variable also. Subunit C may act as such a linker between the goblet cell apical membrane and the cytoskeleton, bypassing the necessity of further proteins because it directly binds to F-actin. Consistently, immunolocalization of spectrin in the *Manduca* midgut showed no signal at the goblet cell apical region, whereas spectrin was clearly localized in the region of the terminal web of the columnar cell brush border (31).

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