The V-ATPase Subunit C Binds to Polymeric F-actin as Well as to Monomeric G-actin and Induces Cross-linking of Actin Filaments*

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Previously, we have shown that the V-ATPase holoenzyme as well as the V₁ complex isolated from the midgut of the tobacco hornworm (Manduca sexta) exhibits the ability of binding to actin filaments via the V₁ subunits B and C (Vitavska, O., Wieczorek, H., and Merzendorfer, H. (2003) J. Biol. Chem. 278, 18499–18505). Since the recombinant subunit C not only enhances actin binding of the V₁ complex but also can bind separately to F-actin, we analyzed the interaction of recombinant subunit C with actin. We demonstrate that it binds not only to F-actin but also to monomeric G-actin. With dissociation constants of ~50 nM, the interaction exhibits a high affinity, and no difference could be observed between binding to ATP-G-actin or ADP-G-actin, respectively. Unlike other proteins such as members of the ADF/cofilin family, which also bind to G- as well as to F-actin, subunit C does not destabilize actin filaments. On the contrary, under conditions where the disassembly of F-actin into G-actin usually occurred, subunit C stabilized F-actin. In addition, it increased the initial rate of actin polymerization in a concentration-dependent manner and was shown to cross-link actin filaments to bundles of varying thickness. Apparently bundling is enabled by the existence of at least two actin-binding sites present in the N- and in the C-terminal halves of subunits C, respectively. Since subunit C has the possibility to dimerize or even to oligomerize, spacing between actin filaments could be variable in size.

V-ATPases are ubiquitous and highly conserved proton pumps that acidify specific organelles such as endosomes, lysosomes, or secretory vesicles in every eukaryotic cell (1). They also are found in plasma membranes of many specialized animal cells where they either are involved in pH homeostasis or in membrane energization (2). V-ATPases consist of two complexes, a peripheral V₁ complex whose catalytic part faces the cytosol and a membrane-bound proton-conducting V₀ complex. In the midgut of the tobacco hornworm (Manduca sexta), the V₁ complex of the plasma membrane V-ATPase contains eight different subunits, A–H, whereas the V₀ complex consists of the four different subunits a and c–e (3). Under special physiological conditions V-ATPase activity is down-regulated by reversibly dissociating the V₁ complex from the membrane as was shown in the tobacco hornworm as well as in yeast (4, 5).

Subunit C appears to be released into the cytoplasm during this process, because the purified V₁ complex lacks most of it (3, 6).

Dissociation of subunit C from the V₁ complex and its support of holoenzyme reassembly indicate that this subunit may play a crucial role in the regulation of V-ATPases. Another role, previously detected by us in the M. sexta midgut, is its ability to bind to the actin cytoskeleton (7). In feeding tobacco hornworms, actin filaments co-localize with the V-ATPase at the apical membrane of midgut goblet cells. Like in osteoclasts (8), actin binding occurs via the V₁ subunit B; however, binding to F-actin also comprises subunit C as we showed recently (7). Subunit C is of special interest, as its detaching from the V₁ complex during V₁V₀ dissociation at starvation or molt results in decreased binding capacity of the V₁ complex to F-actin. The intracellular distribution of F-actin reflects this fact since in starving tobacco hornworms it appeared to be different from that of the V₁ complex or of subunit C.

Actin-binding proteins usually have been classified according to their effect on actin organization and dynamics (9). This led to categories such as e.g. cross-linking proteins, capping proteins, or G-actin-binding proteins. However, this classification appears to be an oversimplification since, in recent years, several actin-binding proteins, among them cofilin or gelsolin, have been shown to exhibit multifunctional properties of actin binding. In this paper we analyze the interaction of the V-ATPase subunit C with actin and show that it binds to both G- and F-actin, that it stabilizes actin filaments, and increases the polymerization rate of actin. Finally, we provide evidence that subunit C is able to cross-link actin resulting in bundles of actin filaments.

EXPERIMENTAL PROCEDURES

Insects—M. sexta (Lepidoptera, Sphingidae) was reared under long day conditions (16 h of light) at 27 °C. Larvae (tobacco hornworms) were fed a synthetic diet modified according to Bell and Joachim (10).

Detection of Subunit C in the Cytosol—For crude extract preparations, the midguts of four feeding tobacco hornworms were suspended in buffer F (100 mM Tris-HCl (pH 7.5), 50 mM KCl, 2 mM MgCl₂, and 1 mM ATP) supplemented with 5 mM Pefabloc SC (Biomol) and homogenized gently in a glass homogenizer. After centrifugation at 10,000 g for 5 min at 4 °C to remove cell debris and the following centrifugation of the supernatant at 200,000 × g for 1 h at 4 °C to remove actin filaments and membranes containing the V₁V₀ holoenzyme, the cytosolic supernatant was applied onto a discontinuous sucrose gradient and centrifuged as published previously (37). Fractions of interest (pooled fractions containing the V₁ complex and the 10% sucrose fraction containing free monomeric subunit C as revealed by control runs with recombinant subunit C) were collected, and equal aliquots were separated by SDS-PAGE (17% T, 4% C). After transfer of the proteins onto a nitrocellulose membrane, blocking was performed for 1 h in buffer B (20 mM Tris-HCl (pH 7.5), 0.5 mM NaCl, 0.05% Tween 20, and 0.02% NaN₃) containing 3% gelatin. To detect subunit C, the membrane was incubated for 1 h with a monoclonal antibody to subunit C (clone 12C7), diluted 1:20 in buffer B containing 1% gelatin. After washing three
times for 5 min in buffer B, the membrane was incubated for 1 h with anti-mouse antibodies (Sigma, whole molecule) conjugated with alka-line phosphatase at a dilution of 1:10,000 in buffer B containing 1% gelatin. After washing three times, the membrane was incubated with the color reaction mixture by incubation of the membrane in a 0.34% nitro blue tetrazolium, 0.18% 5-bromo-4-chloro-3-indolyl phosphate in a buffer containing 50 mM Tris-HCl (pH 9.5), 0.1 M NaCl, and 50 mM MgCl2.

Preparation of Recombinant Proteins—We expressed recombinant M. sexta proteins in Escherichia coli cells using the pET expression system of Novagen. The complete subunit C was expressed and purified as described previously (7). N- and C-terminal halves of subunit C (N-terminal half, amino acids 1–194; C-terminal half, amino acids 190–385; for sequences see Ref. 3), respectively, were prepared as follows. A pBluescript SK(−) plasmid containing the cDNA encoding the M. sexta subunit C served as the template for PCR. The N-terminal part of the pBluescript SK(−) plasmid was amplified with the forward primer 5′-TACTCACTGAGGAGCCGCCTTCTCGATCATGT-3′, containing an Ndel site (underlined), and the reverse primer 5′-TACTCACTGAGGAGCCGCCTTCTCGATGTC-3′, containing an XhoI site (underlined). For PCR of the C-terminal part, the forward primer was 5′-TACTCACTGAGGAGCCGCCTTCTCGATGTC-3′ (XhoI site underlined). The PCR products were ligated into the Ndel and XhoI sites of the pET22a(+) vector. Each of the recombinant plasmids was transformed into E. coli BL21 cells, and expression was induced with 0.4 mM isopropyl 1-thio-D-galactopyranoside. Both recombinant proteins were purified from inclusion bodies according to the manufacturer’s protocol (Novagen).

Overlay Blots with G-actin and with F-actin—After transfer of the proteins onto a nitrocellulose membrane via the slot-blot technique, blocked with 1% gelatin in PBS buffer containing 3% glycerol, the membrane was then incubated with G- or F-actin, respectively, in buffer B containing 1% gelatin for 1 h at a monomer concentration of 2.2 μM (F-actin was produced by polymerization of 10 μM G-actin stabilized with 10 μM phaloidin; G-actin was taken from the supernatant after centrifugation of actin at 100,000 × g for 1 h at 4 °C). After incubation for 1 h at room temperature, the membrane was washed three times for 5 min in buffer B. To detect bound actin, the membrane was incubated for 1 h with a monoclonal anti-actin antibody (Sigma A4700), diluted 1:100 in buffer B containing 1% gelatin. Washing of the membrane, incubation with anti-mouse antibodies, and the color reaction were performed as described above.

G-actin-binding Assays—The change in the fluorescence of NBD-labeled G-actin was used to monitor the binding of the recombinant subunit C to actin monomers (11, 12). Actin was labeled by NBD-CI (Fluka) as described by Detmers et al. (13). NBD-actin was prepared as Mg-ATP-actin according to Pollard (14) by adding 50 mM MgCl2 and 0.2 mM EGTA to NBD-G-actin. NBD-actin was prepared as Mg-ADP-actin by incubating Mg-ATP-actin with hexokinase–agarose beads (Sigma) and 1 mM glucose for 4 h at 4 °C. The final actin concentration in the assays was 0.2 μM. Experiments with varying concentrations of subunit C were carried out at room temperature in a buffer containing 20 mM Tris-HCl (pH 8.0), 40 mM NaCl, 0.2 mM MgCl2, 0.5 mM Mg-g-glutamate, 0.1 mM DTT, and 0.1 mM ATP or ADP, respectively. NBD fluorescence was excited at 470 nm, and the emission was recorded at 530 nm using a Luminescence Spectrometer LS 50 B (PerkinElmer Life Sciences). The Kd values were calculated according to Valtinainen et al. (12) by fitting the normalized change in fluorescence as a function of subunit C concentration, obtaining binding curves for a complex with a 1:1 stoichiometry.

Depolymerization Assays—Actin was polymerized for 1 h in buffer F, and F-actin was collected by centrifugation at 200,000 × g for 1 h at 20 °C. It was resuspended in 10 mM Tris-MES at pH values of 6.0, 7.0, and 8.0, and centrifuged for 2 h at 25 °C with 2 μM recombinant subunit C. Controls for unspecific binding were performed in the presence of 2 μM bovine serum albumin (same buffer, pH 7.0) or only with 10 mM Tris-MES buffer (pH 7.0). After centrifugation at 200,000 × g for 1 h at 20 °C, the proteins in the supernatant were separated by SDS-PAGE (17% T, 0.4% C) and stained with 10 μM phalloidin. Each of the recombinant plasmids was transformed into E. coli BL21 cells, and expression was induced with 0.4 mM isopropyl 1-thio-D-galactopyranoside. Both recombinant proteins were purified from inclusion bodies according to the manufacturer’s protocol (Novagen).

Rate Zonal Centrifugation—To analyze the potential oligomerization of subunit C, we performed a linear 5–20% (w/v) sucrose gradient containing 16 mM Tris-HCl, 0.32 mM EDTA, and 0.2 M KCl (pH 8.1). Recombinant subunit C (0.1 mg) was applied on top of the gradient before centrifugation at 309,000 × g (av) for 3.5 h at 4 °C in a vertical rotor. Gradients were fractionated in 0.5-ml steps from the bottom of the tubes. A protein mixture (~0.6 mg low molecular weight calibration kit from Amersham Biosciences) was used for molecular mass standardization. The protein distribution in the fractions was analyzed after SDS-PAGE (17% T, 0.4% C) by silver staining. After dialyzing the pooled fractions 7 and 8 against the gradient buffer, a second centrifugation under the same conditions was performed. The fractions obtained from four gradients were pooled and concentrated by using a 100-kD cut-off filter. Following three cycles of washes and SDS-PAGE, the protein distribution was visualized by silver staining.

RESULTS

Subunit C Binds Not Only to F-actin but Also to G-actin—Recently we had shown that the recombinant subunit C binds to F-actin (7). In feeding tobacco hornworms, subunit C is bound to the VV0 holoenzyme, whereas in starving tobacco hornworms subunit C appears to occur mainly as the free cytosolic form because it evidently leaves the V0 complex during the V0V1 dissociation (3, 6). However, also in feeding tobacco hornworms free V0 complexes are found in the cytosol (24). Therefore, we checked whether free subunits C occur in the cytosol of feeding tobacco hornworms too. Indeed, subunit C was found in the cytosol, and most of it was not bound to the V1 complex (Fig. 1). This result gave rise to the speculation that in the cell free subunit C may also bind to actin. Since cellular actin occurs not only in its filamentous polymeric, but also in its globular monomeric form, we raised the new point whether subunit C could bind also to monomeric G-actin.

To solve this question, we performed overlay blots by incubating the recombinant subunit C with either F-actin or G-actin, using rabbit aldolase as a positive control for F-actin-
binding protein and bovine serum albumin as a negative control. The result, visualized with the aid of a monoclonal anti-actin antibody, was quite clear, indicating that subunit C binds to both forms of actin (Fig. 2). As expected, the control blots showed that aldolase binds only to F-actin, whereas bovine serum albumin binds neither to F-actin nor to G-actin.

Binding of subunit C to G-actin was independently monitored by experiments with NBD-labeled G-actin. Fluorescence of NBD-G-actin had been shown to be modulated on binding of ADF/cofilin or twinfilin, for example (11, 25). Since the first experiments indicated that subunit C increased the fluorescence of NBD-G-actin (not shown), we used this parameter to determine the equilibrium dissociation constant of the complex of subunit C and G-actin. To differentiate between binding to the ATP form of G-actin (which is present mostly at the plus end of filaments) and binding to the ADP form of G-actin (which is present mostly at the minus end of filaments), we measured the fluorescence of NBD-labeled ADP-G-actin as well as ATP-G-actin in the presence of variable concentrations of subunit C. In both cases we found an increase of fluorescence that was dependent on the concentration of subunit C (Fig. 3). The $K_v$ values of 62 nM for ADP-G-actin and 52 nM for ATP-G-actin, calculated on the basis of a 1:1 stoichiometry, were similar, indicating that there is no preference for one form of actin over the other.

**Subunit C Prevents Actin Filament Depolymerization and Increases the Initial Rate of Actin Polymerization**—Among the few actin-binding proteins with high affinity to both G- and F-actin, the ADF/cofilin family members are prominent examples (9). They stimulate in vitro the depolymerization of actin filaments in a pH-dependent manner (26). The evidence that subunit C also binds to both F- and G-actin prompted us to check whether it also exhibits actin depolymerizing activity. We therefore transferred actin filaments collected by centrifugation into an actin-depolymerizing buffer of either pH 6.0, 7.0, or 8.5 and incubated them in the presence or in the absence of subunit C. After an incubation period of 2 h followed by a centrifugation step to pull down actin filaments, G-actin could be detected in the supernatant when no subunit C had been added (Fig. 4). However, neither G-actin nor subunit C were found in the supernatant when subunit C had been incubated together with F-actin (Fig. 4). From these results we conclude that subunit C, irrespective of the pH, stabilizes actin filaments under conditions that normally lead to their depolymerization.

As subunit C evidently influences the stability of actin filaments, the question arose whether it also affects actin polymerization. Therefore, we investigated the rate of polymerization as a function of subunit C concentration by measuring the intensity of right angle light scattering, which is linearly proportional to the polymer mass concentration (15, 16). As shown in Fig. 5A, the polymerization rate was clearly dependent on the concentration of subunit C. The initial increase in light scattering was linear (Fig. 5B), with a sharp maximum at a subunit C concentration of $-0.4 \mu M$ (Fig. 5C).

**Subunit C Cross-links Actin Filaments**—From the positive influence of subunit C on actin polymerization as observed by light scattering, we could not deduce whether single filaments were formed or whether more complex structures such as bundles emerged. Therefore, we wanted to test the potential of subunit C to cross-link actin filaments. Sedimentation of single actin filaments requires high centrifugal forces (100,000 × g for 1 h). By contrast, bundled filaments are pelleted already at lower centrifugal forces (20,000 × g for 20 min). We capped actin filaments by use of gelsolin, stabilized them with phalloidin, and incubated them together with subunit C for 1 h. The following centrifugation at low centrifugal force resulted in the co-sedimentation of F-actin and subunit C (Fig. 6). On the contrary, after separate incubation of F-actin and subunit C, respectively, we could not find them in the pellet after low force centrifugation.

To visualize actin cross-linking mediated by subunit C, we looked for F-actin by using FITC-labeled phalloidin that exclusively binds to actin filaments but not to G-actin. As shown in Fig. 7A, single actin filaments with expected lengths could be observed by fluorescence microscopy in the absence of subunit C. However, when subunit C was present in the incubation
mixture, a completely different picture was obtained. After 30 min of incubation at room temperature, mesh-like structures consisting of thicker and thinner bundles of actin filaments were observed (Fig. 7B).

**Subunit C Exhibits at Least Two Actin-binding Sites**—The overlay blots in Fig. 2 had shown that subunit C binds to both F- and G-actin. In order to exhibit bundling activity, subunit C has to possess more than one actin-binding site. To address this question, we heterologously expressed two recombinant halves of subunit C, the first extending from the N terminus to amino acid 194 and the second extending from amino acid 190 to the C terminus. Overlay blots supported our hypothesis of at least two binding sites, since both the N- and the C-terminal halves, respectively, reacted with F-actin as well as with G-actin (Fig. 8).

Two binding sites are evidently sufficient to enable cross-linking actin filaments. However, a second mode of cross-linking could be achieved by oligomerization of subunit C as, for instance, has been shown to be the case for human cofilin (27). Indeed, not only monomers but also dimers and oligomers were observed after SDS-PAGE of subunit C under oxidizing conditions, whereas only a single monomeric band was obtained under reducing conditions (Fig. 9A). Dimerization under oxidizing conditions was also obtained after SDS-PAGE of the N- and C-terminal halves of subunit C (Fig. 9B). These results indicate that oligomerization may occur by the formation of disulfide bonds. The finding that the N- and C-terminal halves, respectively, only migrated as dimers and not as oligomers may easily be explained: the N-terminal half contains only one cysteine, and the two cysteines in the C-terminal half are in close proximity at amino acid positions 289 and 291. The results obtained with denatured subunit C could also be confirmed for the native protein. Rate zonal centrifugation of the native subunit C in a sucrose density gradient without reducing DTT revealed a protein distribution consistent with the presence of at least monomeric, dimeric, and trimeric forms (Fig. 10B). To test whether the populations obtained are stable or whether they re-equilibrate to form new heterogeneous populations, we pooled fractions 7 and 8 (containing, based on the distribution of standard proteins, mainly dimers) and re-centrifuged them under the same conditions. As shown in Fig. 10C, subunit C was found along different densities.
Previously, we have shown that actin filaments are co-localized with the plasma membrane V-ATPase in the midgut of the tobacco hornworm (7). As in osteoclasts (8), the V-ATPase binds to F-actin via its V1 subunit C. Moreover, we could demonstrate for the first time that the V1 subunit C also binds to F-actin. This fact was rather intriguing, since subunit C appears to play a significant role during the reversible V1V0 disassembly. Upon dissociation of the V1 complex from the membrane-bound V0 complex, subunit C gets lost from the V1 complex but is necessary for reassembly of the two complexes into a functional holoenzyme (6). The latter observation, which had been made in yeast, is in line with the finding that the V1 complex isolated from tobacco hornworm midgut contains subunit C in substoichiometric amounts at the most (28). Since the reassembly of the V1V0 holoenzyme does not require biosynthesis of new subunits (29), as in yeast, C subunits from the cytosolic pool have to merge with the V1 and V0 complexes during reassembly. As shown here, an appreciable amount of free subunit C is detectable in the cytosol and is not bound to the V1 complex. Thus free subunit C could bind within the cell to actin without being part of the V-ATPase. However, the question remains whether there is sufficient free subunit C available in the cytosol to be a significant player in cellular actin dynamics. Gräf et al. (24) reported previously that the concentration of the cytosolic V1 complex in Manduca midgut is rather high and makes up more than 1% of the total cytosolic protein. As discussed above, the molar cytosolic concentration of subunit C should at least be as high as that of the V1 complex. A rough estimation taking into account data from Manduca midgut (intracellular versus extracellular space (30), total protein concentration per midgut fresh weight, volume of goblet cells containing the plasma membrane V-ATPase versus volume of columnar cells, the second cell type constituting the midgut epithelium) indicates that the cytosolic concentration of the V1 complex should clearly be more than 1 μM. A comparable result is obtained when published cytosolic protein concentrations of other cells (31) are used for the calculation. Therefore, we conclude that there is enough free subunit C for the cellular interplay with actin. This suggestion is corroborated by our present in vitro results that clearly show that the interaction of subunit C and actin exhibits a high affinity in the submicromolar range.

We demonstrated previously that the isolated recombinant subunit C can bind to F-actin, but here we show that it can also bind to monomeric G-actin. With Kd values of ~50 nM, the interaction exhibits a high affinity and is not dependent on the phosphorylation state of the nucleotide bound to actin. In this respect subunit C differs from prominent actin monomer binding proteins such as profilin and β-thymosin, both of which interact with higher affinity with G-actin in its ATP- than its ADP-bound state (32), and such as members of the ADF/cofilin family including twinfilins, all of which exhibit higher affinity to G-actin in its ADP-than its ATP-bound state (33). Since all G-actin-binding proteins known so far play an important role by controlling the size and location of the cytoplasmic G-actin pool and by regulating the incorporation of actin monomers into filaments, we assume that the V1 subunit C has a similar function.

**DISCUSSION**

**Interaction of the V-ATPase Subunit C with G- and F-actin**

Previously, we have shown that actin filaments are co-localized with the plasma membrane V-ATPase in the midgut of the tobacco hornworm (7). As in osteoclasts (8), the V-ATPase binds to F-actin via its V1 subunit B. Moreover, we could demonstrate for the first time that the V1 subunit C also binds to F-actin. This fact was rather intriguing, since subunit C appears to play a significant role during the reversible V1V0 disassembly. Upon dissociation of the V1 complex from the membrane-bound V0 complex, subunit C gets lost from the V1 complex but is necessary for reassembly of the two complexes into a functional holoenzyme (6). The latter observation, which had been made in yeast, is in line with the finding that the V1 complex isolated from tobacco hornworm midgut contains subunit C in substoichiometric amounts at the most (28). Since the reassembly of the V1V0 holoenzyme does not require biosynthesis of new subunits (29), as in yeast, C subunits from the cytosolic pool have to merge with the V1 and V0 complexes during reassembly. As shown here, an appreciable amount of free subunit C is detectable in the cytosol and is not bound to the V1 complex. Thus free subunit C could bind within the cell to actin without being part of the V-ATPase. However, the question remains whether there is sufficient free subunit C available in the cytosol to be a significant player in cellular actin dynamics. Gräf et al. (24) reported previously that the concentration of the cytosolic V1 complex in Manduca midgut is rather high and makes up more than 1% of the total cytosolic protein. As discussed above, the molar cytosolic concentration of subunit C should at least be as high as that of the V1 complex. A rough estimation taking into account data from Manduca midgut (intracellular versus extracellular space (30), total protein concentration per midgut fresh weight, volume of goblet cells containing the plasma membrane V-ATPase versus volume of columnar cells, the second cell type constituting the midgut epithelium) indicates that the cytosolic concentration of the V1 complex should clearly be more than 1 μM. A comparable result is obtained when published cytosolic protein concentrations of other cells (31) are used for the calculation. Therefore, we conclude that there is enough free subunit C for the cellular interplay with actin. This suggestion is corroborated by our present in vitro results that clearly show that the interaction of subunit C and actin exhibits a high affinity in the submicromolar range.

We demonstrated previously that the isolated recombinant subunit C can bind to F-actin, but here we show that it can also bind to monomeric G-actin. With Kd values of ~50 nM, the interaction exhibits a high affinity and is not dependent on the phosphorylation state of the nucleotide bound to actin. In this respect subunit C differs from prominent actin monomer binding proteins such as profilin and β-thymosin, both of which interact with higher affinity with G-actin in its ATP- than its ADP-bound state (32), and such as members of the ADF/cofilin family including twinfilins, all of which exhibit higher affinity to G-actin in its ADP- than its ATP-bound state (33). Since all G-actin-binding proteins known so far play an important role by controlling the size and location of the cytoplasmic G-actin pool and by regulating the incorporation of actin monomers into filaments, we assume that the V1 subunit C has a similar function.

**Functional Similarities and Differences to Members of the ADF/Cofilin Family—**Unlike other G-actin-binding proteins like profilin or β-thymosin, subunit C also interacts with actin filaments. In this respect, it partially resembles the ADF/cofilins that bind to both G- and F-actin (32). However, ADF/
cofilins exert distinct effects on actin depending on the pH; below pH 7, they bind along actin filaments with a stoichiometry of one molecule per actin monomer, whereas at pH values near 8 they assume a destructive role by binding actin monomers and sequestering them, thus preventing actin polymerization (26). Like ADF/cofilins at slightly acidic pH, subunit C binds to F-actin monomers with a stoichiometry of 1:1 (7). However, unlike ADF/cofilins, subunit C does not destabilize F-actin, neither at slightly alkaline pH values like the ADF/cofilins nor at slightly acidic pH values. By contrast, at pH values ranging from 6.0 to 8.5, subunit C unequivocally stabilizes actin filaments. Moreover, it increases the initial rate of actin polymerization and cross-links actin filaments.

Cross-linking appears to be enabled by at least two binding sites, one of them is located in the N-terminal half and the other is located in the C-terminal half of subunit C. Cross-links, especially of variable size, may be facilitated by dimerization or oligomerization. The latter findings resemble properties of human cofilin which, after having been stably oligomerized with the zero-length cross-linker Ellman’s reagent, exhibited actin bundling activity, whereas its monomeric form lacked this property (27).

Our results obtained by repeated rate zonal centrifugation of subunit C unequivocally stabilize actin filaments. Moreover, it increases the initial rate of actin polymerization and cross-links actin filaments.

Recently, a profilin-like sequence of 11 amino acids has been reported to occur in the human V-ATPase B1 and B2 subunits (35). Peptides containing this sequence interacted with actin and competed with profilin for actin binding. In line with these findings, heterologously expressed fusion proteins containing subunits B with a spacer instead of the profilin-like sequence did not bind to actin. A sequence highly similar to the profilin-like sequence is also found in B subunits from other sources, including M. sexta (Fig. 11). Most interestingly, a similar sequence of 11 amino acids with 55% identity containing a phenylalanine essential for actin binding is also found in subunit C from M. sexta. This sequence occurs nearly identically in the human subunit C, less similar in subunit C from Caenorhabditis elegans, but not in subunit C from Arabidopsis thaliana and Saccharomyces cerevisiae, respectively (Fig. 11). Whereas the profilin-like sequence is at the beginning of the N-terminal half of subunit B, it is localized in the middle of the C-terminal half of subunit C. It remains to be shown whether the profilin-like sequence in subunit C is an actin binding domain as it is in subunit B.

The Question for a Biological Function of Subunit C in Actin Binding—The goblet cell apical membrane of the tobacco hornworm midgut is densely populated by the V-ATPase with ~5,000 copies per µm². In accordance with this, SDS-PAGE of highly purified goblet cell apical membranes reveals the well-defined pattern of V-ATPase subunits, and no other proteins are found in higher concentrations (23). Therefore, we speculated previously (7) that the V-ATPase takes over an actin-anchoring function from proteins such as spectrin, which usually link membrane proteins to the actin cytoskeleton and which are absent in the apical area of goblet cells (36). The finding of appreciable amounts of free subunit C in the cytosol of midgut cells allows us to extend our speculation. Provided that subunit C binds to ADP-actin as well as to ATP-actin not only as we found in G- but also in F-actin, it could bind equally everywhere to actin filaments without being restricted to their plus or to their minus ends. Nonpreferential binding makes subunit C an ideal candidate for cross-linking actin filaments in the apical microvilli and/or in the apical terminal web of goblet cells. As F-actin at least partly changes its localization in goblet cells during starvation and molt, subunit C could also play an important role in controlling the dynamics of the actin cytoskeleton.

Acknowledgments—The monoclonal antibody against the recombinant subunit C of M. sexta was produced by Sabine Buchmeier in the research group of Brigitte Jockusch (University of Braunschweig, Germany). We thank our colleagues for providing us with the hybridoma clone 12C7.

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