Microvillus 110K-Calmodulin: Effects of Nucleotides on Isolated Cytoskeletons and the Interaction of the Purified Complex with F-Actin

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ABSTRACT Microvilli isolated from intestinal epithelial cells contain a cytoskeletal M_r 110,000 polypeptide complexed with calmodulin (110K-CM) that is believed to link the microfilament core bundle laterally to the plasma membrane. Previous work has shown that physiological levels of ATP can partially solubilize the 110K-CM complex from isolated microvillus cytoskeletons or isolated microvilli. However, once extracted, the 110K-CM complex has been found to be difficult to maintain stably soluble in aqueous buffers. This is due to the presence of an endogenous ATPase (~100 nmol Pi/min per mg at 37°C) in microvillus cytoskeletal preparations that depletes the ATP with subsequent precipitation of 110K-CM. Addition of ATP to such precipitates resolubilizes 110K-CM. Inclusion of an ATP regenerating system in the solubilization of 110K-CM from cytoskeletons, or membrane-bound brush borders, increases the amount of 110K-CM solubilized.

Solubilization of 110K-CM from microvillus cytoskeletons was found to require a divalent cation (Mg^{2+}, Mn^{2+}, or Co^{2+}, but not Zn^{2+}) and a nucleoside triphosphate (ATP, GTP, CTP, or ITP). ADP did not solubilize 110K-CM, but could partially inhibit ATP-dependent solubilization. Solubilized 110K was phosphorylated during extraction of microvillus cores with [γ-^{32}P]ATP, but this was unrelated to the solubilization of 110K-CM as the endogenous kinase was specific for ATP, whereas the solubilization was not.

The 110K-CM was purified using ATP extraction of brush border cytoskeletons in the presence of an ATP regenerating system, gel filtration of the solubilized extract, an ATP depletion step to specifically precipitate 110K-CM with F-actin, and resolubilization followed by phosphocellulose chromatography. The purified complex was stably soluble in aqueous buffers both in the presence and absence of ATP. It bound almost quantitatively to F-actin in the absence of ATP, and showed nucleotide solubilization characteristics from F-actin similar to that found for solubilization of 110K-CM from microvillus cores. At low ATP levels, the binding to F-actin was increased in the presence of ADP. These results suggest that the purified complex has been isolated in a native form. The data confirm and extend the studies of Howe and Mooseker (1983, *J. Cell Biol.*, 97:974–985) using a partially purified preparation of 110K-CM and further emphasize that 110K-CM is a stably water soluble complex and not an integral membrane protein.

Microfilaments are ubiquitous structures of eucaryotic cells that contribute to the determination of cell shape as well as being involved in many aspects of cell motility (reviewed in reference 1). Owing to the complexity of microfilaments in whole cells, the molecular details of their organization and membrane attachments are still poorly understood. We have chosen the intestinal microvillus as a model system in which to study both a defined microfilament arrangement and its attachment to the plasma membrane. The microvillus cytoskeleton, isolated in the absence of Ca^{2+}, is a highly ordered structure of 20–30 bundled actin filaments which, together with four major and some minor accessory proteins, can be

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isolated ultrastructurally intact in quantities sufficient for biochemical analysis (2–5). Two of these proteins, villin (M_r 95,000) and fimbrin (M_r 68,000), are internal proteins that cross-link the actin filaments (6–10). Projecting from the F-actin bundle are "lateral arms" or "cross-filaments" that spiral around the core bundle with a regular periodicity of 33 nm (3, 11, 12). These lateral arms link the core to the membrane and are currently the best-documented example of lateral attachment of microfilaments to the plasma membrane.

In 1979 Matsudaira and Burgess (3) made the important observation that treatment of microvillus cytoskeletons with Mg-ATP resulted in the dissociation of the lateral arms and the partial solubilization of a major accessory protein, the M_r 110,000 polypeptide (110K).[1] Subsequently, calmodulin was identified as the fourth major accessory protein (4, 13). At least part of the calmodulin was found to be associated with 110K based on co-solubilization studies and the finding that ^{125}I-calmodulin bound 110K in gel overlay experiments (14). Antibodies to 110K have been used in immunoferritin electron microscopy on brush border cytoskeletons and show that this polypeptide is restricted to the part of the microvillus cytoskeleton that used to be attached to the microvillus membrane (15; but see reference 16 for an opposing view). Thus, the evidence, although still circumstantial, supports the notion that 110K is a major component of the lateral arms.

Little is known about the attachment of the lateral arms to the microvillus membrane. The original work of Mukherjee and Staehelin (11) demonstrated that the lateral arms associate with electron-dense patches on the inner surface of the membrane. More recent experiments demonstrated that ATP treatment of membrane-intact microvilli resulted in the specific partial solubilization of 110K and calmodulin as well as in the loss of ultrastructurally observable lateral arms from the microvilli (17–19). In similar experiments with membrane-intact brush borders, 110K and calmodulin are among the proteins solubilized by ATP, and ultrastructural studies again indicate the loss of the lateral arms (20, 21). Indeed, Howe and Mooseker (20) have recently used the soluble extract obtained after ATP treatment of membrane-intact brush borders as starting material for the partial purification of a complex composed of 110K and calmodulin (110K-CM complex). All of these experiments suggest that ATP can dissociate the lateral arms from both the membrane and the core bundle, and that the 110K protein is not an integral membrane protein (17–20). These findings and conclusions have recently been challenged by Glenney and Glenney (22). These authors have reported that 110K is an integral membrane protein that requires the presence of an ionic detergent for solubilization. These conflicting views are evaluated in the Discussion.

Several laboratories have attempted to isolate the 110K protein and have been plagued by problems in maintaining its solubility (3, 17, 18, 20). While acknowledging these problems, Howe and Mooseker (20) were able to obtain a partially purified preparation of the 110K-CM complex and to characterize it. Another approach was taken by Glenney and Glenney (22) who explored the use of detergents and were able to obtain a partially purified preparation of the 110K polypeptide. To understand the basis for these problems before trying to purify 110K-CM ourselves, we decided to explore the characteristics of the ATP-induced solubilization of 110K from isolated cytoskeletons. The studies described in this paper provide the basic characteristics of the solubilization of 110K-CM from microvillus cytoskeletons and an explanation for the previous problems encountered in maintaining the solubility of the complex. This information, in turn, is used as a foundation for the purification and partial characterization of the 110K-CM complex.

[1] *Abbreviations used in this paper*: AMP·PNP, 5′-adenylyl-imidodiphosphate; DTT, dithiothreitol; 110K-CM complex, a 110,000-mol-wt cytoskeletal polypeptide complexed with calmodulin.

MATERIALS AND METHODS

Purification of Microvillus Cytoskeletons (Cores): Microvilli were prepared from chicken intestinal epithelial cells (2) as modified in reference 14. Cores were prepared by treatment with 1% Triton X-100 (2) and resuspended in solution I (75 mM KCl, 1 mM EGTA, 0.1 mM $MgCl_2$, 0.25 mM phenylmethylsulfonyl fluoride, 10 mM imidazole-HCl, pH 7.4) or in solution A (solution I containing 5.1 mM $MgCl_2$). In some experiments, brush border preparations were treated to reduce proteolysis with 5 mM diisopropyl fluorophosphate (Aldrich Chemical Co., Milwaukee, WI) for 10 min at 4°C prior to the first sucrose gradient.

Nucleotide Treatments: Brush borders, microvilli, or their cytoskeletons were treated with nucleotides at 4°C for 15 min, or as specified, and centrifuged at 25,000 *g* for 15 min at 4°C, and the pelleted and supernatant fractions were prepared for gel electrophoresis (23). Gels were stained with Coomassie Brilliant Blue and destained. Gel scans were performed on a Quick Scan R & D (Helena Laboratories, Beaumont, TX). Except for the experiment shown in Fig. 4, all nucleotide additions were made from stocks equimolar in $MgCl_2$ at pH 7.4. All nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO).

Phosphorylation Studies: Microvillus cytoskeletons were suspended in solution A and incubated with [γ-^{32}P]ATP at 0.05 Ci/mmol (Amersham Corp., Arlington Heights, IL) and treated as described above. Autoradiography was performed on dried gels at −70°C using Kodak X-Omat AR x-ray film.

Purification of 110K-CM: Sucrose gradient–purified brush borders were extracted in solution I for 20 min at 25°C with 2% Triton X-100; all subsequent steps were performed at 4°C. The resulting brush border cytoskeletons were washed several times in solution I and extracted in solution I with 10 mM ATP, 10 mM $MgCl_2$, and an ATP regeneration system consisting of 1 mg/ml phosphocreatine and 0.1 mg/ml creatine phosphokinase (120 U/mg protein; Sigma Chemical Co.) for 10 min. After centrifugation at 47,000 *g* for 15 min, the extract was adjusted to 0.5 M NaCl and 0.1 mM dithiothreitol (DTT) and then subjected to gel filtration chromatography on a 150-ml Sepharose 4B column at a flow rate of 7 ml/h. The column was equilibrated and eluted with buffer G (10 mM ATP, 10 mM $MgCl_2$, 0.5 M NaCl, 0.1 mM DTT, 1 mM NaN_3, 75 mM KCl, 1 mM EGTA, 10 mM imidazole, pH 7.4). Column fractions (2.5 ml each) were analyzed by SDS PAGE, and 110K peak fractions were pooled and dialyzed against 6 liters of solution I with 0.1 mM DTT overnight. The dialysate was then centrifuged at 47,000 *g* for 15 min. The pelleted material was resuspended in PC buffer (75 mM NaCl, 2 mM ATP, 2 mM $MgCl_2$, 0.1 mM DTT, 10 mM PIPES, pH 6.5) and then centrifuged at 47,000 *g* for 15 min. The supernatant fraction was loaded on a 1-ml PE 11 phosphocellulose column (Whatman Laboratory Products Inc., Clifton, NJ) equilibrated in PC buffer. The column was then washed with 20 ml of PC buffer adjusted to 100 mM NaCl. The 110K-CM was then eluted with 2 ml PC buffer adjusted to 1 M NaCl and immediately dialyzed against solution I for use. Purified 110K-CM was stored at 4°C in solution I.

Other Procedures: ATPase assays were performed in solution A according to Taussky and Shorr (24). Protein concentrations were determined by the method of Bradford (25) using ovalbumin as standard. Hexokinase from Baker's yeast (460 U/mg protein) was purchased from Sigma Chemical Co. Rabbit skeletal muscle actin was purified according to Spudich and Watt (26) as modified by Maclean-Fletcher and Pollard (27). Centrifugation of samples shown in Figs. 11 and 12 was performed with a Beckman Airfuge (Beckman Instruments, Inc., Palo Alto, CA) operating at 24 psi (130,000 *g*). Phalloidin was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN) and used at approximately a fivefold molar excess over actin.

RESULTS

ATP-induced Solubilization of the 110K-CM Complex from Isolated Microvillus Cytoskeletons: Effect of Endogenous ATPase Activity

Our initial experiments to examine the role of ATP in the solubilization of 110K-CM from microvillus cytoskeletons

gave variable results unless protein concentrations were carefully controlled. Fig. 1 *A* shows results of extracting microvillus cytoskeletons at either 0.25 or 1.0 mg/ml protein with various concentrations of ATP under our standard conditions. In these experiments cytoskeletons were treated with ATP for 15 min at 4°C, the samples were centrifuged for 15 min at 4°C, and the resulting supernatant and pellet fractions were prepared for gel electrophoresis. A dramatic difference in the efficiency of extraction was seen at low ATP concentrations; for example, at an initial concentration of 0.1 mM ATP, <10% of the 110K-CM was solubilized at 1.0 mg/ml cytoskeletal protein, whereas >50% was solubilized at 0.25 mg/ml protein (Fig. 1 *B*). In addition, as we have noted previously (18), the amount of 110K-CM solubilized by ATP was much reduced if the extraction temperature was raised. As the initial ATP concentrations in all these experiments were in a vast molar excess over 110K-CM, the results indicated that our preparations might contain an endogenous ATPase that hydrolyzed ATP during the course of the experiment. Assays revealed that our preparations have a reproducible Mg-ATPase activity of ~100 nmol Pi/min per mg at 37°C or 8 nmol Pi/min per mg at 4°C. Thus the activity present in 1 mg/ml cytoskeletons is capable, for example, of hydrolyzing 0.5 mM ATP in ~5 min at 37°C or 0.1 mM ATP in ~12 min at 4°C.

If endogenous ATPase activity is responsible for the differences seen in the curves in Fig. 1 *B*, 110K-CM must require ATP not only for solubilization in this system but also to maintain it in a soluble state thereafter. This property of

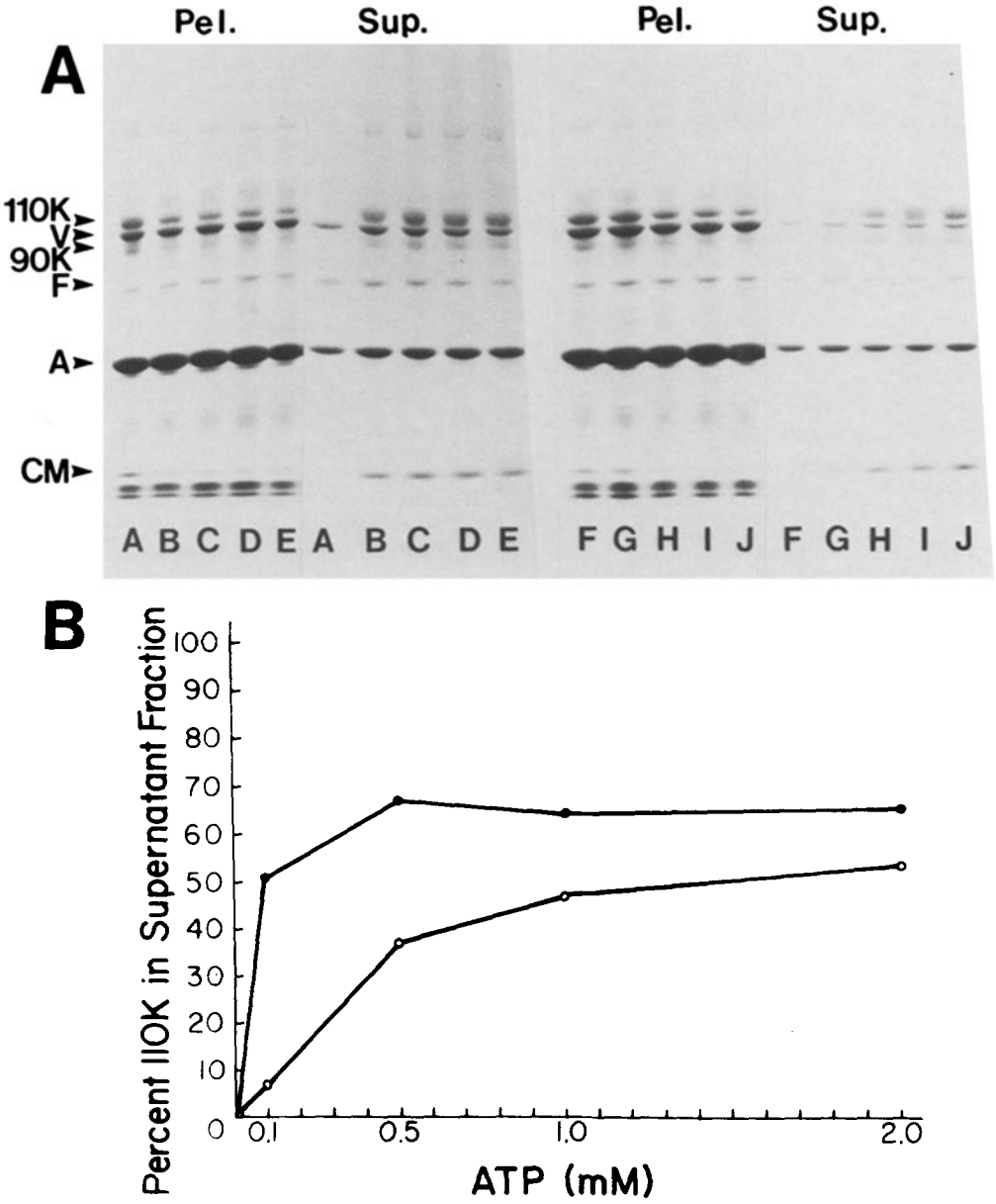

FIGURE 1 ATP concentration-dependent release of 110K from microvillus cytoskeletons. (*A*) SDS PAGE (5–20%) of pelleted (*Pel.*) and supernatant (*Sup.*) fraction of microvillus cores after treatment with various concentrations of ATP in solution I at either 0.25 mg/ml (lanes *A–E*) or 1.0 mg/ml (lanes *F–J*) core protein. ATP concentrations were 0 (lanes *A* and *F*), 0.1 mM (lanes *B* and *G*), 0.5 mM (lanes *C* and *H*), 1.0 mM (lanes *D* and *I*), and 2.0 mM (lanes *E* and *J*). *V*, villin; *90K*, 90,000-mol-wt polypeptide; *F*, fimbrin; *A*, actin; *CM*, calmodulin. (*B*) Dependence of 110K release from microvillus cores on ATP concentrations at either 0.25 mg/ml (●) or 1.0 mg/ml (○) core protein. Percentage release was obtained from gel scans of the pelleted and supernatant fractions shown in *A*.

110K-CM was directly demonstrated. Three identical aliquots of cytoskeletons at 1 mg/ml protein were treated with 1 mM ATP and incubated either for 15 min before centrifugation, for 90 min before centrifugation, or for 90 min followed by the addition of another 1 mM ATP and 15 min further incubation before centrifugation. The results, displayed in Fig. 2, clearly show that 110K was soluble 15 min after the addition of 1 mM ATP (lanes *A*), but was then rendered insoluble at 90 min (lanes *C*), presumably due to ATP hydrolysis, and could then be rendered soluble again by the addition of more ATP (lanes *B*). Therefore, in this system 110K requires ATP both for initial solubilization from the microvillus core bundle and for maintenance of solubility thereafter.

Effect of Enzymatic Depletion of ATP on the Solubility of 110K-CM in ATP Extracts

The experiment shown in Fig. 2 suggested that as the ATP concentration was depleted during the 90-min incubation, the 110K polypeptide either reassociated with the core structure or precipitated independent of the core. To distinguish between these possibilities, we examined the effect of ATP depletion on the solubility of 110K-CM, after removal of the residual core material (Fig. 3). Isolated cores at 8 mg/ml core protein (Fig. 3, lane *A*) were treated with 5 mM ATP, and the solubilized material (Fig. 3, lane *C*) was separated from the insoluble fraction (Fig. 3, lane *B*) by centrifugation. Analysis of the ATPase activity of the two fractions revealed that 89% of the original activity was recovered in the pellet fraction. Therefore, to reduce the ATP concentration in the solubilized fraction, it was necessary to add an ATP hydrolyzing system. Preliminary experiments showed that the addition of 22 mM glucose and 0.1 mg/ml hexokinase rapidly reduced the ATP levels. Incubation of the material solubilized from cores by ATP for 1 h at 4°C in the presence of glucose and hexokinase resulted in the precipitation of most of the 110K-CM, which was recovered by centrifugation (Fig. 3, lane *E*). This result indicates that the presence of intact core bundles is not required for precipitation of 110K-CM upon ATP depletion. The precipitation of 110K-CM together with some actin is rather specific; hexokinase and some other polypeptides re-

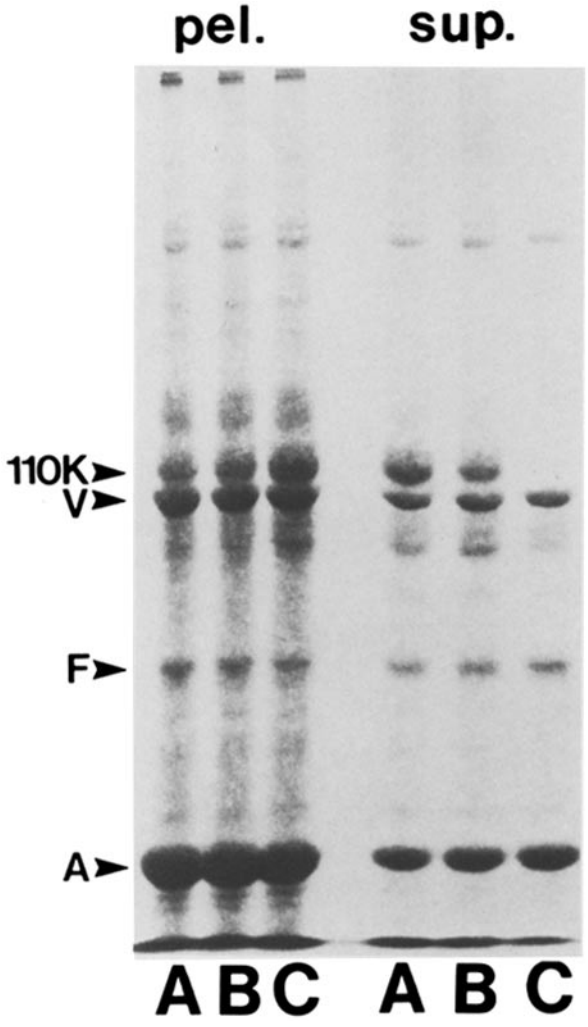

FIGURE 2 Effect of incubation time and second ATP challenge on ATP-dependent 110K solubilization from microvillus cytoskeletons. SDS PAGE (7%) of pelleted (*pel.*) and supernatant (*sup.*) fractions of microvillus cytoskeletons at 1 mg/ml treated with 1 mM ATP for (*A*) 15 min, (*C*) 90 min, or (*B*) 90 min followed by the addition of another 1 mM ATP and an additional 15 min incubation. Incubations were performed at 4°C. *V*, villin; *F*, fimbrin; *A*, actin.

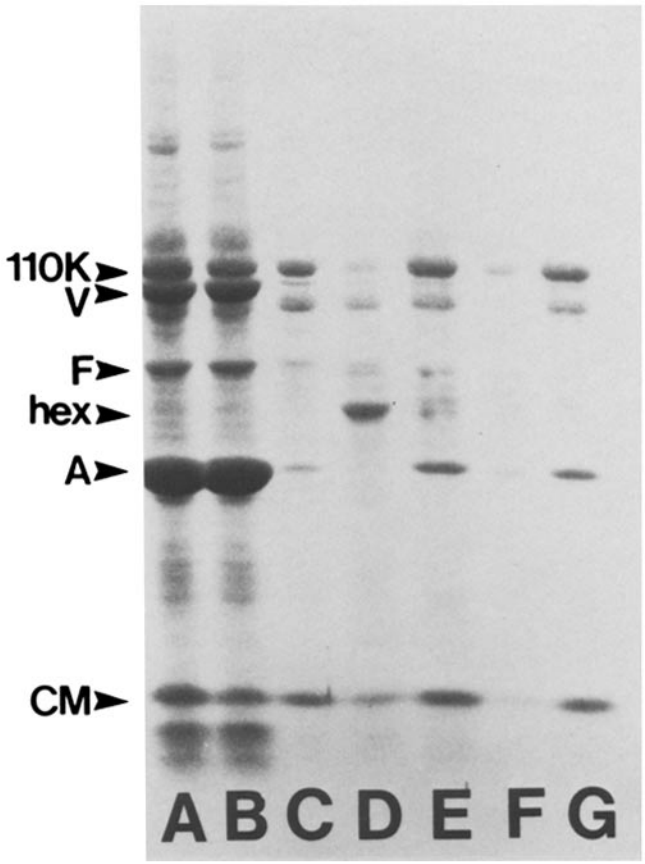

FIGURE 3 Effect of enzymatic depletion of ATP from an ATP extract of microvillus cytoskeletons. SDS PAGE (5–20%). Microvillus cytoskeletons (*A*) at 8 mg/ml were extracted with 5 mM ATP in solution A for 15 min at 4°C. The pellet (*B*) and supernatant (C) fractions were separated by centrifugation for 15 min at 25,000 *g* at 4°C. The extracted material was adjusted to 22 mM glucose and 0.1 mg/ml hexokinase and incubated at 4°C for 1 h. After centrifugation the supernatant fraction (*D*) was discarded and the pellet (*E*) was resuspended in solution A made 10 mM in ATP. The resuspended pellet was centrifuged for 15 min and the soluble fraction (*G*), enriched in 110K-CM, was separated from the pelleted fraction (*F*). *V*, villin; *F*, fimbrin; *hex*, hexokinase; *A*, actin; *CM*, calmodulin.

mained in the soluble fraction (Fig. 3, lane *D*). The material precipitated by ATP depletion was washed and resuspended in ATP-containing buffer. Following centrifugation, the 110K-CM complex was recovered almost quantitatively in the soluble fraction (Fig. 3, lane *G*). It should be noted that the actin initially solubilized (Fig. 3, lane *C*) follows 110K-CM after both ATP depletion (Fig. 3, lane *E*) and subsequent resolubilization (Fig. 3, lane *G*).

Characterization of 110K-CM Solubilization from Microvillus Cytoskeletons

DIVALENT CATION REQUIREMENT: To investigate the mechanism of action of ATP in the release of 110K-CM from microvillus cytoskeletons, we have begun to examine the specific requirements for this process. Microvillus cores prepared in solution I (which contains 0.1 mM $MgCl_2$ and 1 mM EGTA) were collected by centrifugation and resuspended in solution I lacking both $MgCl_2$ and EGTA. Various divalent cations, or EDTA, were added to aliquots of the cytoskeletons and then each sample was made 1 mM in ATP. After 15 min the samples were centrifuged, and the soluble and insoluble material was prepared for gel electrophoresis. The results (Fig. 4) show that there is an absolute requirement for a divalent cation, with Co^{2+} or Mn^{2+}, but not Zn^{2+}, being able to substitute for Mg^{2+} in this system. However, Mn^{2+}, and to a lesser extent Co^{2+}, also lead to an increased solubilization of other polypeptides. Identical experiments performed on phalloidin-stabilized cores gave essentially the same results presented in Fig. 4 except that less actin was recovered in the supernatant fraction for each treatment (not shown).

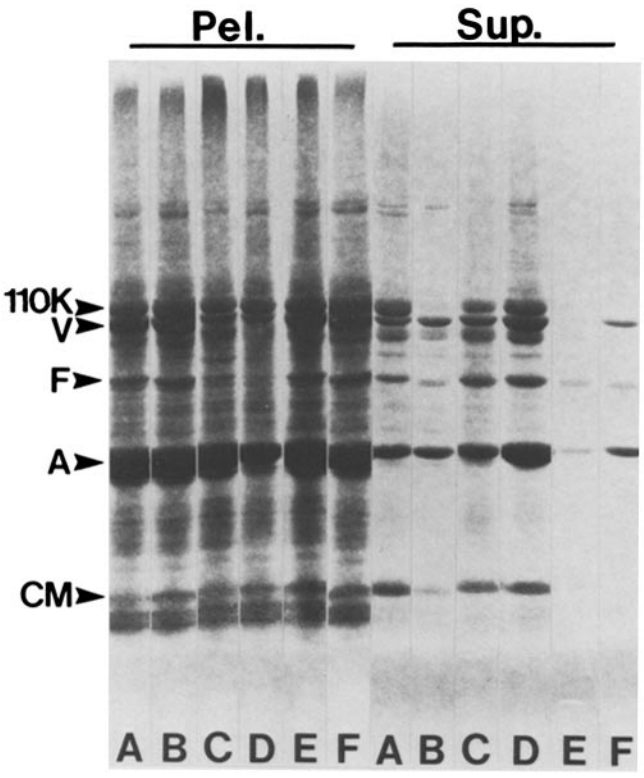

FIGURE 4 Effect of divalent cations on the ATP-dependent solubilization of 110K-CM from microvillus cytoskeletons. SDS PAGE (5–20%). Microvillus cytoskeletons (1 mg/ml) in 75 mM KCl, 1 mM EGTA, 10 mM imidazole (pH 7.4) were incubated for 15 min at 4°C in 1 mM ATP with either (*A*) 5 mM $MgCl_2$, (*B*) 5 mM EDTA, (*C*) 5 mM $CoCl_2$, (*D*) 5 mM $MnCl_2$, (*E*) 5 mM $ZnCl_2$, or (*F*) no added ATP. After treatment, the samples were centrifuged and the proteins in the pellet (*Pel.*) and supernatant (*Sup.*) analyzed. *V*, villin; *F*, fimbrin; *A*, actin; *CM*, calmodulin.

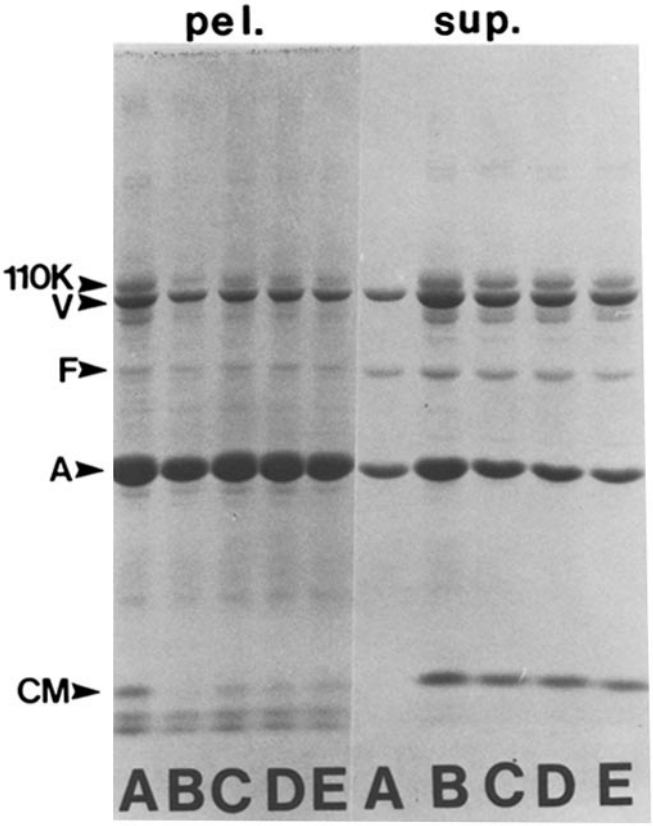

FIGURE 5 Effects of various nucleoside triphosphates on 110K-CM solubilization from microvillus cytoskeletons. SDS PAGE (5–20%) of pellet (*pel.*) and supernatant (*sup.*) fractions of microvillus cytoskeletons (0.5 mg/ml) in solution A after treatment for 15 min at 4°C with (*A*) no additions, (*B*) 1 mM ATP, (*C*) 1 mM GTP, (*D*) 1 mM CTP, or (*E*) 1 mM ITP. In this gel twice as much of the supernatant fraction was loaded compared with the pelleted fraction. *V*, villin; *F*, fimbrin; *A*, actin; *CM*, calmodulin.

NUCLEOTIDE SPECIFICITY: The results presented above demonstrate that Mg-ATP can lead to the solubilization of 110K-CM from microvillus cytoskeletons. We have therefore examined the nucleoside triphosphate specificity for 110K-CM solubilization from microvillus cytoskeletons. Isolated cytoskeletons at 0.5 mg/ml protein were treated with 1 mM ATP, 1 mM GTP, 1 mM CTP, 1 mM ITP, or no nucleotide. The samples were incubated for 15 min at 4°C and centrifuged, and the soluble and insoluble fractions were subjected to SDS PAGE (Fig. 5). The results indicate that ATP and GTP treatments induce substantial solubilization of 110K-CM, with the other nucleoside triphosphates also leading to significant solubilization.

EFFECTS OF ADP AND AMP·PNP ON ATP-INDUCED SOLUBILIZATION: The ATP requirement for the solubilization of 110K-CM could not be replaced by either 1–2 mM ADP or 1–2 mM of the nonhydrolyzable ATP analogue adenylyl-imidodiphosphate (AMP·PNP). However, both of these nucleotides had a dramatic effect on 110K-CM solubilization when added in combination with ATP.

Fig. 6 shows the effect of various concentrations of ADP on the solubilization of 110K by 0.1 mM ATP at 0.25 mg/

ml cytoskeletal protein. As can be seen, ADP strongly depressed the solubilization, with only ~20% of the control value being solubilized at an initial 20-fold molar excess of ADP.

The effect of increasing concentration of AMP·PNP on the solubilization of 110K-CM from cores by 0.1 mM ATP is shown in Fig. 7. Although alone unable to solubilize 110K-CM, AMP·PNP greatly enhanced 110K-CM solubilization in the presence of 0.1 mM ATP. One possible explanation for this effect is that AMP·PNP inhibits the endogenous ATPase activity. Direct measurement of the ATP remaining after 15-min incubations showed that AMP·PNP significantly reduced ATP hydrolysis (Fig. 7), with about twice as much ATP remaining in the presence of 2 mM AMP·PNP as in its absence.

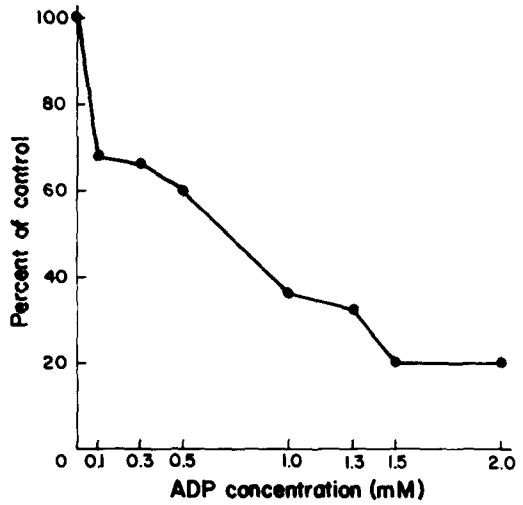

FIGURE 6 Effect of ADP in the presence of a low concentration of ATP on the ATP-induced solubilization of 110K from microvillus cytoskeletons. Microvillus cytoskeletons (0.25 mg/ml) were incubated for 15 min in solution A at 4°C in 0.1 mM ATP and increasing concentrations of ADP. The samples were centrifuged at 25,000 *g* for 15 min and the supernatant fractions were subjected to SDS PAGE. The amount of 110K solubilized at each ADP concentration was estimated from gel scans and is presented as the percentage of a control in which 0.1 mM ATP was present with no added ADP.

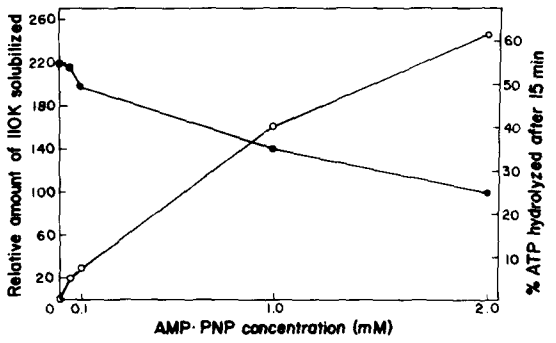

FIGURE 7 Effect of AMP·PNP in the presence of a low concentration of ATP on the ATP-induced solubilization of 110K and on ATP hydrolysis in microvillus cytoskeletons. Microvillus cytoskeletons (1 mg/ml) in solution A were incubated at 4°C in the presence of 0.1 mM ATP and increasing concentrations of AMP·PNP. After 15 min an aliquot of each sample was taken to determine the percentage of the initial ATP that was hydrolyzed. The samples were then centrifuged at 25,000 g for 15 min and the supernatant fractions were subjected to SDS PAGE. Gel scans were performed on the resulting gel, and the relative amount of 110K solubilized by each treatment was determined. ○, 110K solubilized; ●, ATP hydrolysis.

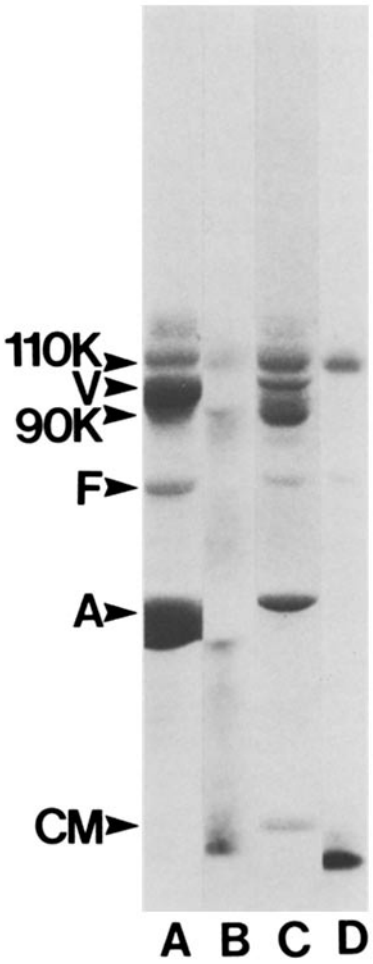

FIGURE 8 *In vitro* phosphorylation of microvillus core proteins. Microvillus cores (1.0 mg/ml) in solution A were incubated for 15 min at 4°C in 1.0 mM [γ-^{32}P]ATP (0.05 Ci/mmol). The pelleted and supernatant fractions were analyzed by SDS PAGE (5–20%) and autoradiography was performed on the dried gel. (*A*) Coomassie stain and (*B*) autoradiograph of pelleted fraction; (*C*) Coomassie stain and (*D*) autoradiograph of supernatant fraction.

ROLE OF 110K PHOSPHORYLATION: One possible role for ATP in the solubilization of 110K-CM is that it leads to the phosphorylation of one of these polypeptides and that this phosphorylation is required either for the release from the core bundle or to maintain the complex in a soluble state. Microvillus cytoskeletons were therefore incubated for 15 min at 4°C with 1 mM [γ-^{32}P]ATP, and the label incorporated into the soluble and insoluble fractions was examined (Fig. 8). A comparison of the protein profiles (Fig. 8, lanes *A* and *C*) with the fluorograms (Fig. 8, lanes *B* and *D*) shows that 110K and fimbrin were the major proteins labeled in the supernatant fraction. In the pellet fraction, a small amount of label is seen associated with the 110K polypeptide, although clearly with a much lower specific activity than the 110K present in the supernatant. Labeling of villin, actin, or calmodulin was never observed in either fraction, nor has the fimbrin that remained in the pellet fraction ever been observed to be labeled. Labeling was presumably due to protein phosphorylation since no labeling occurred when [α-^{32}P]ATP was substituted for [γ-^{32}P]ATP in otherwise identical experiments.

The finding that ATP-solubilized 110K is more phosphor-

ylated than unextracted 110K raised the possibility that 110K phosphorylation was required for its solubilization. To examine this question further, we incubated microvillus cytoskeletons with 0.1 mM [γ-^{32}P]ATP and 1 mM ATP, 1 mM GTP, 1 mM CTP, or 1 mM ITP and determined the amount and degree of phosphorylation of the solubilized 110K. These results are shown in Table I. As expected, the addition of cold ATP increased the solubilization of 110K somewhat and reduced the specific activity of the phosphorylated 110K by about 10-fold. Addition of any of the other three cold nucleotides led to increased solubilization of 110K and an increase in the amount of labeling of the solubilized 110K polypeptide. Thus, these nucleotides do not compete for the endogenous kinase to phosphorylate 110K, although they were able to solubilize 110K on their own (see above). This result strongly indicates that 110K phosphorylation is not required for its solubilization from microvillus cores. Moreover, when cytoskeletons were incubated with [γ-^{32}P]GTP under identical conditions, the 110K polypeptide was solubilized but not phosphorylated.

TABLE I

Effect of Unlabeled Nucleoside Triphosphates on 110K Solubilization and Phosphorylation by [γ-^{32}P]ATP

Treatment	110K in supernatant fraction	110K phosphorylation
	%	% of control
Control	48	100
ATP	68	10
GTP	65	168
CTP	60	111
ITP	63	163

Microvillus cytoskeletons (0.25 mg/ml) in solution A were incubated for 15 min at 4°C in 0.1 mM [γ-^{32}]ATP (0.05 Ci/mol) either alone or with the addition of 1 mM ATP, GTP, CTP, or ITP. The samples were centrifuged at 25,000 g for 15 min and the pelleted and supernatant fractions were subjected to SDS PAGE. The resulting gel was scanned to determine the percentage of 110K solubilized by each treatment. The gel was then dried and processed for autoradiography. The autoradiograph was scanned to determine the relative amount of 110K phosphorylation. Phosphorylation data are presented as a percentage of the control in which 0.1 mM [γ-^{32}P]ATP alone was present.

Finally, in experiments where 110K was extracted from cores with [γ-^{32}P]ATP and subsequently induced to precipitate by ATP depletion, autoradiographs revealed that 110K was phosphorylated both before and after precipitation (not shown), indicating that phosphorylation of 110K is not sufficient to maintain its solubility in the absence of ATP in this system.

In the experiment shown in Fig. 8, the ATP-solubilized material contained a large amount of a 90,000-mol-wt polypeptide. The preparation used for this experiment had not been treated with diisopropyl fluorophosphate to reduce proteolysis. This 90,000-mol-wt polypeptide, visible in variable amounts in all our preparations, is probably a degradation

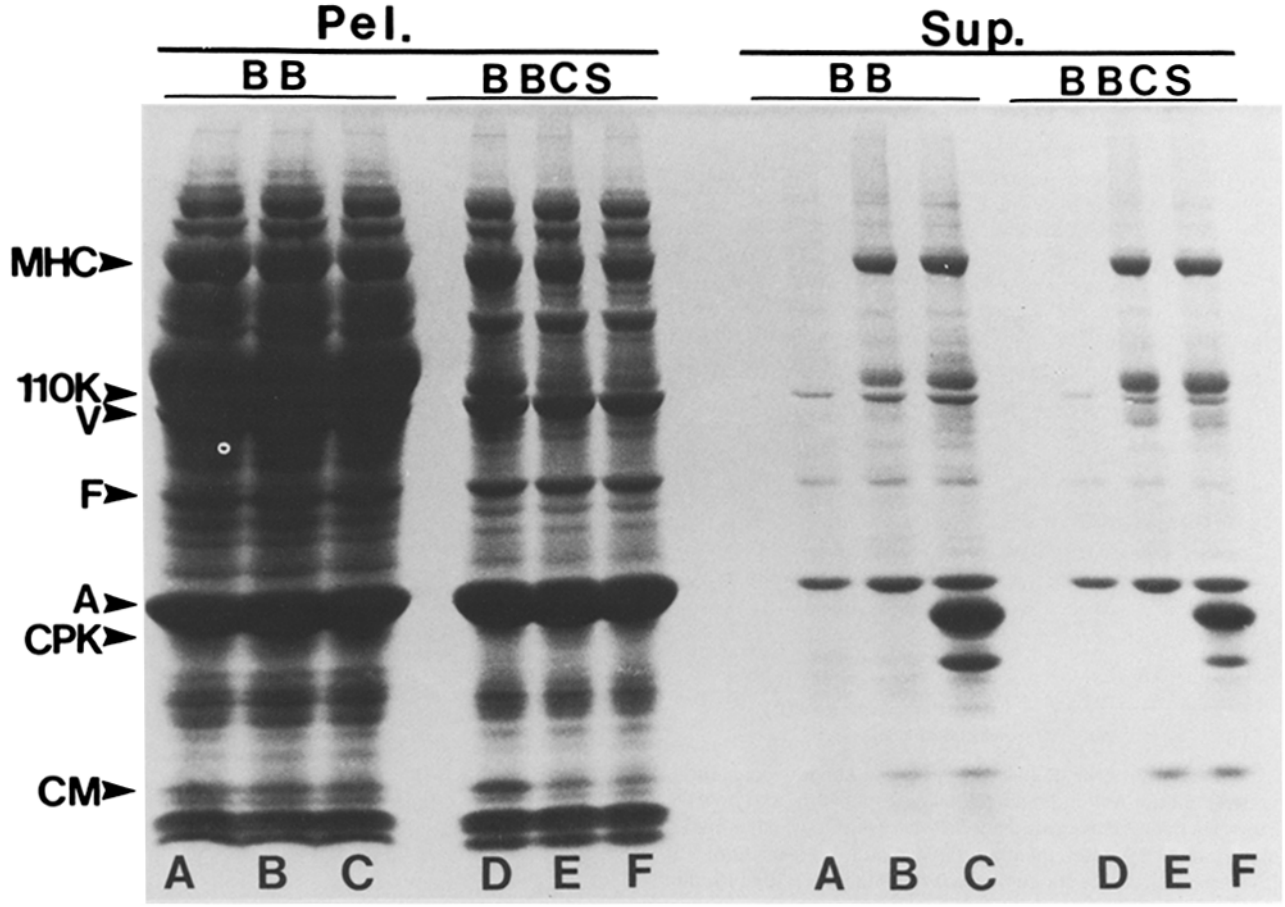

FIGURE 9 ATP extraction of brush borders and brush border cytoskeletons. SDS PAGE (5–20%) of pelleted (*pel.*) and supernatant (*sup.*) fractions of brush borders (*BB*) or brush border cytoskeletons (*BBCS*) at 2 mg/ml protein concentration in solution A. Treatments were no additions (lanes *A* and *D*), 1 mM ATP (lanes *B* and *E*), 1 mM ATP with 1 mg/ml phosphocreatine, and 0.1 mg/ml creatine phosphokinase (lanes C and *F*). *MHC*, myosin heavy chain; *V*, villin; *F*, fimbrin; *A*, actin; *CPK*, creatine phosphokinase; *CM*, calmodulin.

product of 110K as (*a*) it is more evident in preparations not treated with diisopropyl fluorophosphate; (*b*) it is released from the core in an ATP-dependent manner (Fig. 1); (*c*) it can be induced to precipitate upon ATP depletion (see Fig. 2); and (*d*) it cross-reacts with antibody to 110K and shares cyanogen bromide–generated fragments in common with 110K (not shown). However, this fragment is not phosphorylated when solubilized by ATP (Fig. 8), possibly because

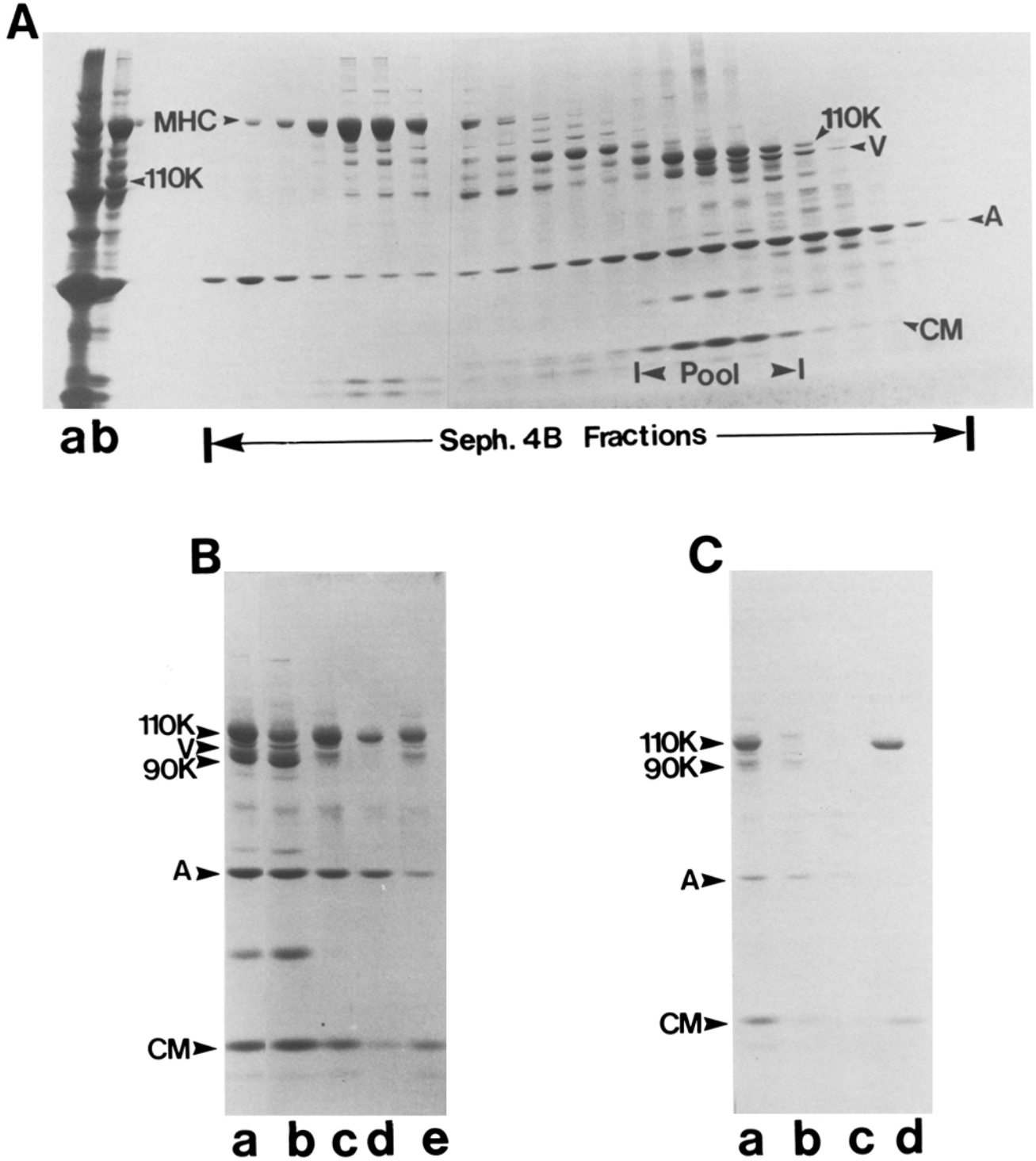

FIGURE 10 Purification of 110K-CM from brush border cytoskeletons. (*A*) SDS PAGE (5–20%). Following ATP extraction of brush border cytoskeletons in the presence of an ATP regeneration system and centrifugation at 47,000 *g*, the supernatant fraction (lane *b*) was separated from the pelleted fraction (lane *a*) and subjected to gel filtration on a Sepharose 4B column equilibrated in buffer G. *MHC*, myosin heavy chain; *V*, villin; *90K*, 90,000-mol-wt polypeptide; *A*, actin; *CM*, calmodulin. (*B*) SDS PAGE (5–20%). The 110K-CM fractions indicated in *A* were pooled (lane *a*) and dialyzed against solution I and then centrifuged at 47,000 *g*. The supernatant fraction (lane *b*) was discarded and the pelleted material (lane *c*) was resuspended in 75 mM NaCl-PC buffer. After centrifugation at 47,000 *g*, the supernatant fraction (lane *e*) was separated from the pelleted fraction (lane *d*). (*C*) The supernatant fraction in 75 mM NaCl-PC buffer (lane *a*) was loaded on a 1-ml phosphocellulose column equilibrated in 75 mM NaCl-PC buffer. The flow-through (lane *b*) was discarded. The column was washed with 20 column volumes of 100 mM NaCl-PC buffer (lane *c*). 110K-CM was eluted with 1 M NaCl-PC buffer (lane *d*).

the phosphorylation site present on 110K has been proteolytically removed.

Solubilization of 110K-CM from Brush Borders and Brush Border Cytoskeletons: Effect of an ATP-regenerating System

Having characterized the solubilization of 110K-CM from microvillus cores, we wished to purify the complex from brush borders or brush border cytoskeletons instead of microvillus cores since a significant percentage of the microvilli are always lost during their purification. It was therefore necessary to characterize and optimize extraction of 110K-CM from brush borders and their cytoskeletons. Hydrolysis assays revealed that our brush border and brush border cytoskeleton preparations contained Mg-ATPase activities of 188 and 84 nmol Pi/min per mg at 37°C, respectively. Since we had shown that the ATP level has to be maintained to solubilize 110K-CM from microvillus cores, we assessed the value of including an ATP-regenerating system on the outcome of the ATP extraction step (Fig. 9). Brush borders or their cytoskeletons at 2 mg/ml were extracted for 15 min at 4°C with either no added ATP (Fig. 9, lanes *A* and *D*), 1 mM ATP (Fig. 9, lanes *B* and *E*), or 1 mM ATP and a regenerating system consisting of 1 mg/ml phosphocreatine and 0.1 mg/ml creatine phosphokinase (Fig. 9, lanes *C* and *F*). To allow a good comparison of the extraction methods, we adjusted the amount of proteins loaded on the gel in Fig. 9 so that the brush border samples and brush border cytoskeleton samples contained the same amount of total cytoskeletal protein. Under the conditions used, we obtained nearly quantitative extraction of 110K-CM from cytoskeletons whether or not the regeneration system was used (Fig. 9, lanes *E* and *F*). However, the amount of 110K-CM extracted from membrane intact brush borders, as estimated from gel scans, was increased about twofold in the presence of the regenerating system (Fig. 9, lanes *B* and *C*). At higher protein concentrations, the effect was more dramatic with no apparent 110K-CM solubilization from either brush borders or cytoskeletons in the presence of ATP alone, but efficient extraction in the presence of the regenerating system.

Purification of 110K-CM from Brush Border Cytoskeletons

On the basis of the above findings, we developed a method for the purification of 110K-CM from brush border cytoskeletons (Fig. 10). Cytoskeletons were extracted in a minimal volume in the presence of ATP and the ATP regenerating system (Fig. 10*A*, lane *b*). The soluble extract was subjected to gel filtration on a Sepharose 4B column which separated the myosin and some other polypeptides from 110K-CM (Fig. 10*A*). Under these conditions, essentially all the calmodulin eluted with 110K. The fractions rich in 110K-CM were pooled and dialyzed to reduce the salt and ATP concentrations. The majority of 110K-CM precipitated and was recovered by centrifugation (Fig. 10*B*, lane *c*). This precipitation step left many contaminating polypeptides soluble (Fig. 10*B*, lane *b*), including most of the 90,000-mol-wt degradation product of 110K. When the precipitated 110K-CM complex was resuspended in ATP-containing buffer most of it was resolubilized, (Fig. 10*B*, lane *e*) although some (Fig. 10*B*, lane *d*) remained insoluble. Final purification was achieved on a phosphocellulose column to which 110K-CM bound tightly and was separated from the remaining contaminants (Fig. 10*C*, lanes *b* and *c*) before being eluted with a 1 M NaCl step (Fig. 10*C*, lane *d*). Purified 110K-CM was immediately dialyzed against solution I in which it remained stably soluble for periods of a month or more.

Interaction of 110K-CM with F-actin

EFFECT OF NUCLEOTIDES: Having purified the 110K-CM complex, we wished to test whether it was still native by comparing its interaction with F-actin with the results presented in the previous sections. Initial experiments showed that 110K-CM remained soluble in solution I in the presence and absence of ATP. The purified 110K-CM bound almost quantitatively (>95%) to F-actin in the absence of ATP, but did not bind in the presence of ATP. Attempts to saturate the binding of 110K-CM to F-actin have so far shown >90% binding up to an equal mass of 110K-CM to F-actin. We therefore arbitrarily chose a polypeptide molar ratio of 1:20 (110K/actin) to examine the interaction of the complex with F-actin in more detail. The effect of various nucleotides on the binding of purified 110K-CM to F-actin was examined (Fig. 11). In the absence of added nucleotide, all the 110K-CM was recovered in the pellet after high speed centrifugation. Of the nucleoside triphosphates tested, ATP clearly had the greatest effect on 110K-CM binding to F-actin, followed by GTP, CTP, and ITP. ADP led to a low level (~10%) of solubility of 110K-CM, regardless of the concentration used. AMP·PNP resulted in no detectable solubilization of the complex. 110K-CM alone in the absence or presence of any of the nucleotides tested remained soluble and could not be pelleted by high speed centrifugation.

EFFECTS OF ADP AND AMP·PNP ON ATP-INDUCED SOLUBILITY: As described above, ADP and AMP·PNP have dramatic effects on the ATP-dependent solubilization of 110K-CM from isolated microvillus cores. We therefore examined whether similar effects were present in the purified 110K-CM F-actin system. For these experiments we chose a concentration of 0.1 mM ATP that gave ~50% binding of

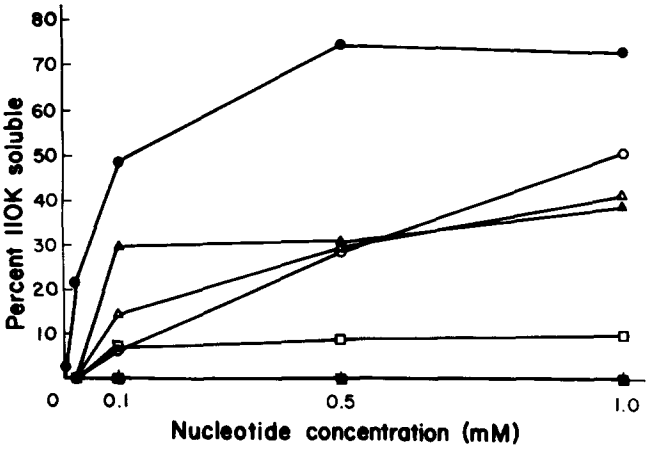

FIGURE 11 Effect of nucleotides on binding of 110K-CM to F-actin. Phosphocellulose-purified 110K-CM was mixed with purified rabbit skeletal muscle F-actin (0.05 mg/ml 110K-CM, 0.43 mg/ml actin) in solution A. Increasing concentrations of various nucleotides were added and the samples were incubated for 15 min at 25°C. After high speed centrifugation (130,000 *g*) for 20 min, the pelleted and supernatant fractions were separated and prepared for SDS PAGE. The percent of 110K in each fraction was determined by gel scans. ●, ATP; ○, GTP; ▲, CTP; △, ITP; ■, AMP·PNP; □, ADP.

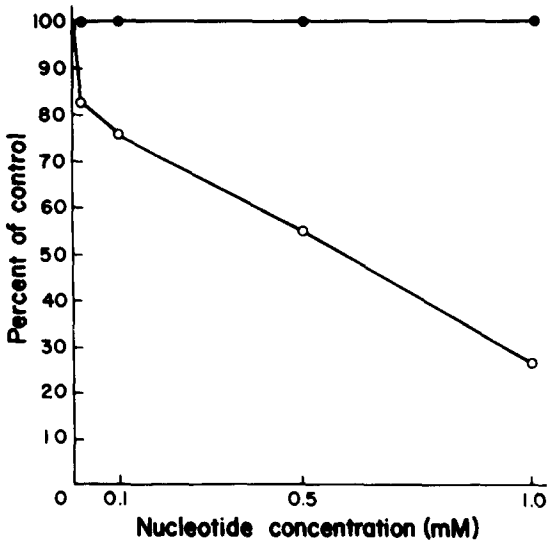

FIGURE 12 Effect of ADP and AMP·PNP on ATP-induced solubility of 110K-CM in the presence of F-actin. Phosphocellulose-purified 110K-CM was mixed with purified rabbit skeletal muscle F-actin (0.05 mg/ml 110K-CM, 0.43 mg/ml actin) in solution A. Each reaction was made 0.1 ATP. Increasing concentrations of either AMP·PNP (●) or ADP (○) were added and the reactions were incubated for 15 in at 25°C. After high speed centrifugation (130,000 g) for 20 min, the pelleted and supernatant fractions were subjected to SDS PAGE. The resulting gels were scanned to determine the amount of 110K in the supernatant fraction. The results are presented as the percentage of a control containing 0.1 mM ATP with no added AMP·PNP or ADP.

110K-CM to F-actin (Fig. 11). To each reaction an increasing amount of either ADP or AMP·PNP was added. The effects on 110K-CM binding to F-actin after high speed centrifugation are shown in Fig. 12. As with microvillus cores, increased concentrations of ADP led to reduced solubilization of 110K-CM, with about one quarter as much 110K-CM soluble at a 10-fold molar excess of ADP over ATP. By contrast, the inclusion of up to 1 mM AMP·PNP had no effect on the solubility of 110K-CM.

DISCUSSION

In this report we have examined some of the parameters involved in the solubilization of the 110K-CM complex from isolated microvillus cytoskeletons. Previous reports have consistently drawn attention to the variable nature of the ATP-induced solubilization of 110K-CM and problems in maintaining it in a soluble state (3, 17, 18, 20). Very recently, the apparent insolubility of the 110K polypeptide has resulted in the notion that it is an integral membrane protein that requires the presence of an ionic detergent for its solubilization (22). The data presented here indicate an alternative explanation for the apparent insolubility of the 110K-CM complex and demonstrate that the complex is, under appropriate conditions, freely water soluble.

The insolubility of the 110K-CM complex after ATP extraction of microvillus cytoskeletons can be traced to the presence of endogenous ATPase activity. This activity can deplete ATP in extraction buffers and, in the presence of even a trace of F-actin, result in the precipitation of initially solubilized 110K-CM. Moreover, 110K-CM initially solubilized by ATP and then precipitated by the endogenous ATPase activity, or by added enzymatic ATP depletion, can be resolubilized by the addition of more ATP. Finally, the addition of an ATP regenerating system to ATP extractions of membrane-intact brush borders or their cytoskeletons in the absence of detergent increases the degree of 110K-CM solubilization. Taken together with our earlier results (17, 18), and those of other laboratories (3, 20), the data show that 110K-CM is not an integral membrane protein and does not require the presence of a detergent to maintain solubility. At present we have no information on how the 110K-CM complex might interact with the membrane; however, Courdrier et al. (28) have implicated a membrane glycoprotein in this interaction. The claim by Glenney and Glenney (22) that 110K is an integral membrane protein stemmed from their inability to solubilize any 110K by ATP treatment of microvillus cytoskeletons and led them to explore the use of ionic detergents. To explain the results of others, they suggested that ATP-induced solubilization of 110K in the absence of detergent could be a result of ATP-dependent proteolysis of a small hydrophobic domain of 110K. Our evidence, particularly our ability to render 110K-CM soluble, then insoluble, and soluble again by simply manipulating ATP levels in the absence of added detergent, indicates that ATP can solubilize 110K-CM under appropriate conditions and that this ATP-mediated process does not involve proteolysis of 110K. Moreover, we have not been able to detect any ATP-stimulated proteolysis of 110K even after extensive exposure to ATP. We would suggest that in the experiments of Glenney and Glenney (22) the ionic detergent may have inhibited endogenous ATPase activity or perhaps disassembled or denatured the 110K-CM complex thereby giving it apparently hydrophobic properties.

We have begun to examine the role of ATP in the solubilization of 110K-CM from microvillus cytoskeletons. Earlier work (3) indicated that GTP could partially substitute for ATP and that EDTA inhibited the process. We have confirmed and extended these findings. ATP, GTP, CTP, and ITP can all lead to the solubilization of 110K-CM from microvillus cytoskeletons. However, since interconversion between these nucleotides may be possible in cytoskeletal preparations, we reexamined the nucleotide requirement in a purified 110K-CM F-actin system and obtained essentially the same results. These results, specifically the solubility of purified 110K-CM and its ATP-modulated interaction with F-actin, are in complete agreement with those reported by Howe and Mooseker (20) who partially purified and characterized the complex using a different method.

Neither ADP nor AMP·PNP is able to solubilize 110K-CM from microvillus cytoskeletons, although both have a significant effect on its solubilization by low levels of ATP. ADP inhibits the solubilization of the complex by ATP, suggesting that it might compete with ATP for its site of action in 110K-CM solubilization. By contrast, we were initially very surprised to find that AMP·PNP stimulated the solubilization of 110K-CM at low ATP levels, since alone it was ineffective in inducing solubilization. This indicates that AMP·PNP does not compete with ATP for its site of action in the solubilization process. The apparent stimulation of ATP-dependent solubilization by AMP·PNP is probably the result of partial inhibition of endogenous ATPase activity, thereby resulting in a slower rate of ATP hydrolysis. The effect of ADP in the purified 110K-CM F-actin system was essentially the same as that seen in microvillus cytoskeleton samples, with ~75% inhibition of 110K-CM solubilization when a 10-fold excess of ADP to ATP was present. On the other

hand, AMP·PNP neither stimulated nor inhibited 110K-CM solubility in the purified system, again indicating that AMP·PNP does not compete with ATP for sites in the solubilization process.

The demonstrated requirement for a nucleotide triphosphate in the solubilization of 110K-CM led us to investigate whether protein phosphorylation was an obligatory part of this process. We were able to show that in the presence of [γ-^{32}P]ATP the 110K polypeptide released from microvillus cytoskeletons was preferentially phosphorylated. However, when solubilization was performed with a low level of [γ-^{32}P]-ATP in combination with either 1 mM GTP, 1 mM CTP, or 1 mM ITP the amount of 110K solubilized increased as did the amount of label found in the solubilized 110K. These data indicate that although GTP, CTP, and ITP can substitute for ATP in the solubilization of 110K-CM, they are not substrates for the endogenous kinase in this system. Moreover, 110K solubilized and phosphorylated by [γ-^{32}P]ATP remained phosphorylated after precipitation following ATP depletion. Finally, incubation of microvillus cytoskeletons with [γ-^{32}P]GTP lead to the solubilization of 110K-CM but not to the phosphorylation of the 110K polypeptide. In combination, these results indicate that microvillus cytoskeletons contain a protein kinase that uses ATP and can phosphorylate the 110K polypeptide with some specificity, but that this protein phosphorylation is unrelated to the ATP-dependent solubilization of 110K-CM from the structure.

The protocol we have developed to purify 110K-CM was derived, in large part, from observations on ATP-mediated 110K solubilization from microvillus cytoskeletons. By repeating several key experiments using a purified 110K-CM F-actin system, we have shown that the effects observed in 110K-CM solubilization from microvillus cytoskeletons are likely directly related to a 110K-CM/F-actin interaction. The similar characteristics of the cytoskeletal and purified protein systems is an indication that the 110K-CM complex has been purified in a native state. Using our purification procedure, we are able to obtain a preparation of the 110K-CM complex that, in the absence of F-actin, is soluble in aqueous buffer for extended periods and should facilitate its further characterization.

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