Structural and Immunological Characterization of the Myosin-like 110-kD Subunit of the Intestinal Microvillar 110K-Calmodulin Complex: Evidence for Discrete Myosin Head and Calmodulin-binding Domains

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Abstract. The actin bundle within each microvillus of the intestinal brush border is tethered laterally to the membrane by spirally arranged bridges. These bridges are thought to be composed of a protein complex consisting of a 110-kD subunit and multiple molecules of bound calmodulin (CM). Recent studies indicate that this complex, termed 110K-CM, is myosin-like with respect to its actin binding and ATPase properties. In this study, possible structural similarity between the 110-kD subunit and myosin was examined using two sets of mAbs; one was generated against *Acanthamoeba* myosin II and the other against the 110-kD subunit of avian 110K-CM. The myosin II mAbs had been shown previously to be cross-reactive with skeletal muscle myosin, with the epitope(s) localized to the 50-kD tryptic fragment of the subfragment-1 (S_1) domain. The 110K mAbs (CX 1–5) reacted with the 110-kD subunit as well as with the heavy chain of skeletal but not with that of smooth or brush border myosin. All five of these 110K mAbs reacted with the 25-kD, NH_2-terminal tryptic fragment of chicken skeletal S_1, which contains the ATP-binding site of myosin. Similar tryptic digestion of 110K-CM revealed that these five mAbs all reacted with a 36-kD fragment of 110K (as well as larger 90- and 54-kD fragments) which by photoaffinity labeling was shown to contain the ATP-binding site(s) of the 110K subunit. CM binding to these same tryptic digests of 110K-CM revealed that only the 90-kD fragment retained both ATP- and CM-binding domains. CM binding was observed to several tryptic fragments of 60, 40, 29, and 18 kD, none of which contain the myosin head epitopes. These results suggest structural similarity between the 110K and myosin S_1, including those domains involved in ATP- and actin binding, and provide additional evidence that 110K-CM is a myosin. These studies also support the results of Coluccio and Bretscher (1988. *J. Cell Biol.* 106:367–373) that the calmodulin-binding site(s) and the myosin head region of the 110-kD subunit lie in discrete functional domains of the molecule.

EACH microvillus of the intestinal brush border (BB)[1] contains a bundle of actin filaments that is tethered laterally to the microvillar membrane by periodic, spirally arranged bridges (Mooseker and Tilney, 1975; Matsudaira and Burgess, 1982). There is considerable evidence (reviewed in Mooseker, 1985) that indicates that these bridges are composed, at least in part, of a protein complex termed 110K-CM consisting of a 110-kD subunit and three to four molecules of calmodulin (CM). The long-held view that these bridges are purely structural has been challenged by recent studies that strongly suggest that the 110K-CM is a myosin-like mechanoenzyme (for reviews of myosin properties see Warrick and Spudich, 1987, and Korn and Hammer, 1988). Like myosin, 110K-CM interacts with actin in an ATP-dependent fashion (Howe and Mooseker, 1983; Collins and Borysenko, 1984; Conzelman and Mooseker, 1987; Coluccio and Bretscher, 1987). This includes the formation of "arrowhead"-like complexes on actin filaments (Coluccio and Bretscher, 1987) analogous to those observed with the head fragments of myosin, subfragment-1 (S_1) or heavy meromyosin, suggesting that the complex is structurally similar to myosin-I. Consistent with this suggestion, electron microscopy of rapid-freeze, rotary-shadowed preparations of the complex (performed by John Heuser, Washington University, St. Louis, MO) reveals that the 110K-CM protein complex is a tadpole-shaped molecule with a single, myosin-sized head and a very short (10–12 nm) "tail" (Conzelman and Mooseker, 1987). As first noted by Collins and Borysenko (1984), the 110K-CM complex is also a myosin-like

1. *Abbreviations used in this paper*: BB, brush border; CM, calmodulin; S_1, subfragment-1.

ATPase, exhibiting highest activity in either high (mM range) Ca^{++} or K^{+} EDTA, while having low activity under physiological conditions in the presence of Mg^{++} (Collins and Borysenko, 1984; Howe and Mooseker, 1983; Conzelman and Mooseker, 1987; Krizek et al., 1987). Most importantly, the MgATPase is activated by actin, albeit to a modest degree (Mooseker et al., 1986; Conzelman and Mooseker, 1987; Swanljung-Collins et al., 1987; Krizek et al., 1987). Finally, preliminary observations have been presented that suggest that the 110K-CM complex has mechanochemical activity (see Discussion).

Taken together, the data summarized above strongly support the notion that the 110K-CM complex is a member of the myosin family, perhaps representing a vertebrate member of the monomeric, tail-less myosin I family first characterized in *Acanthamoeba* (reviewed in Korn et al., 1988). Firm evidence for the presence of vertebrate myosins of the myosin I class has recently been provided by Hoshimaru and Nakanishi (1987). As described in detail below (see Discussion) these workers have isolated a bovine cDNA molecule encoding a 119-kD protein that is structurally homologous to *Acanthamoeba* myosin I, which may encode the bovine form of 110K.

In the present study, we have further examined the structural similarity between the 110-kD subunit of avian 110K-CM and myosin. Using two different sets of mAbs, one raised against the 110-kD subunit of the complex and the other against *Acanthamoeba* myosin II, we demonstrate that the 110-kD subunit shares antigenic determinants with skeletal muscle myosin. The common epitopes reside within two of the three subdomains of the myosin head: the 25-kD, NH_2-terminal domain that contains the ATP-binding site and the 50-kD, central domain that contains an actin-binding site. In addition, we provide further evidence to support the notion, first suggested by Coluccio and Bretscher (1988), that the CM-binding domain(s) of 110K may lie outside the "myosin-like" domain of the 110K molecule.

Materials and Methods

Production and Characterization of mAbs

Hybridoma cell lines producing mAbs were obtained from the fusion of spleen cells from a BALB/c mouse immunized with electroeluted 110K protein of the 110K-CM complex. 20–80 μg of electroeluted 110K protein, prepared by the method of Glenney and Glenney (1984), was emulsified with complete Freund's adjuvant (Difco Laboratories, Inc., Detroit, MI) and injected intraperitoneally into BALB/c mice. Mice were boosted intraperitoneally 2 wk later (20–80 μg of protein in incomplete Freund's adjuvant) and again 2 wk after the second injection. A final injection of 110K was given intraperitoneally in saline 3 d before fusion.

Screening of hybridoma culture supernatants was performed by the ELISA method using an enzyme immunoassay Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, CA) with purified 110K-CM as the antigen (Conzelman and Mooseker, 1987). The monospecificity of each antibody was determined by immunoblot analysis as described below. Isotype-specific subclasses of the five myosin cross-reactive mAbs (CX 1–5) were analyzed by the ELISA method using subclass-specific antisera (Boehringer Mannheim Biochemicals, Indianapolis, IN). All five antibodies were of the IgG_1 subclass (data not shown).

Gel Electrophoresis and Immunoblotting

SDS-PAGE was performed as described by Laemmli (1970) using minigels (either 5–15 or 12.5%; Matsuidaira and Burgess, 1978) or 7.5% standard size gels. Proteins were electrophoretically transferred to nitrocellulose paper (Towbin et al., 1979) and were processed further according to modifications of the procedures outlined in Shibayama et al. (1987). After incubation of the nitrocellulose strips with primary antibodies and subsequent washing, they were incubated with either alkaline phosphatase–conjugated anti-mouse IgG or anti-rabbit IgG (Promega Biotec, Madison, WI), and the reactive bands were visualized according to the procedures outlined by the manufacturer. Immunoblots incubated with myosin II mAbs (see Fig. 1) were incubated with ^{125}I-goat anti-mouse antibodies or ^{125}I-protein A. The membranes were washed, air-dried, and exposed to x-ray film for autoradiography at −80°C using an intensifying screen (DuPont Co., Wilmington, DE).

Protein Purifications

110K-CM complex: chicken intestinal BBs used for isolation of 110K-CM were prepared by procedures described elsewhere (Mooseker and Howe, 1982; Keller and Mooseker, 1982). The 110K-CM complex used for immunoblot studies was made according to the method of Conzelman and Mooseker (1987). The 110K-CM used for proteolytic digestion studies was made by the method of Coluccio and Bretscher (1987).

Myosin and subfragments: chicken gizzard myosin was isolated according to the methods of Sobieszek and Bremel (1975) and Sellers et al. (1981). Chicken skeletal muscle myosin and its subfragments heavy meromyosin and light meromyosin were isolated according to the method of Margossian and Lowey (1982). S_1 was obtained by proteolytic cleavage of myosin using alpha-chymotrypsin (Worthington Biochemical Corp., Freehold, NJ) at 50 μg/ml at 25°C (Weeds and Taylor, 1975; Weeds and Pope, 1977). As a by-product of S_1 digestion, a 120-kD fragment (presumably the rod portion of myosin) was obtained in the low salt pellet after centrifugation along with some undigested myosin (see Fig. 2).

Proteolytic Digestions of S_1 and 110K-CM

Tryptic digestion (Worthington Biochemical Corp.) of S_1 was performed for varying periods of time at 25°C in either 40 mM NaCl, 10 mM imidazole-Cl (pH 7.2), 1 mM dithiothreitol (DTT) using a trypsin to S_1 ratio (wt/wt) of 1:25 (see Fig. 3 *c*; Winkelmann and Lowey, 1986), or in 40 mM KCl, 10 mM imidazole-Cl (pH 7.2), 0.05 mM EGTA, 0.05 mM $MgCl_2$, 0.02% sodium azide using a trypsin to S_1 ratio (wt/wt) of 1:100 (Fig. 3 *a*). The reaction was stopped by the addition of a twofold excess of soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Missouri).

Purified 110K-CM was digested at a protein concentration of 0.5 mg/ml trypsin at an enzyme/protein ratio of 1:50 at 25°C for 15 and 30 min in Soln I (75 mM KCl, 10 mM imidazole-Cl [pH 7.2], 1 mM EGTA, 2 mM $MgCl_2$, 0.02% sodium azide).

Proteolytic Digestion of 110K-CM in the Presence of F-Actin

Digestion of 110K-CM in the presence of F-actin was performed by addition of trypsin (4 μg/ml) to a mixture of 110K-CM (0.2 mg/ml) and F-actin (0.1 mg/ml) stabilized by addition of phalloidin (10 μg/ml; Boehringer Mannheim Biochemicals). Digestion was performed at 25°C for 1–2 h and the reaction was stopped as described above. The ATP-dependent binding of tryptic fragments was assessed by cosedimentation of 110K tryptic digests with actin in the presence and absence of 5 mM ATP in Solution I. Mixtures of F-actin and 110K tryptic digests were spun for 20 min in an airfuge (Beckman Instruments, Inc., Palo Alto, CA) at 26 psi. The supernatant and pellet fractions were examined by SDS-PAGE and immunoblot analysis using mAb CX-1.

Photoaffinity Labeling of 110K-CM with ATP

110K-CM complex was labeled with [alpha-^{32}P]ATP (New England Nuclear, Boston, MA) according to the method of Maruta and Korn (1981). Photoaffinity labeling was carried out with 110K-CM at a concentration of 0.5 mg/ml to which was added 10 μM radioactive nucleotide and 40 μM unlabeled ATP containing 2 mM Mg^{++}. The reaction mixture was incubated on ice and irradiated by holding a germicical UV (254 nm) lamp at a distance of 12 cm for 20 min. An aliquot of the labeled mixture was removed before the addition of trypsin. Labeled tryptic digests were run on a 12.5% PAGE and processed for autoradiography using Kodak X-OMAT AR x-ray film with intensification (Dupont Co.).

CM Overlay of 110K-CM

SDS-PAGE of tryptic digests of 110K-CM was performed using 5–15%

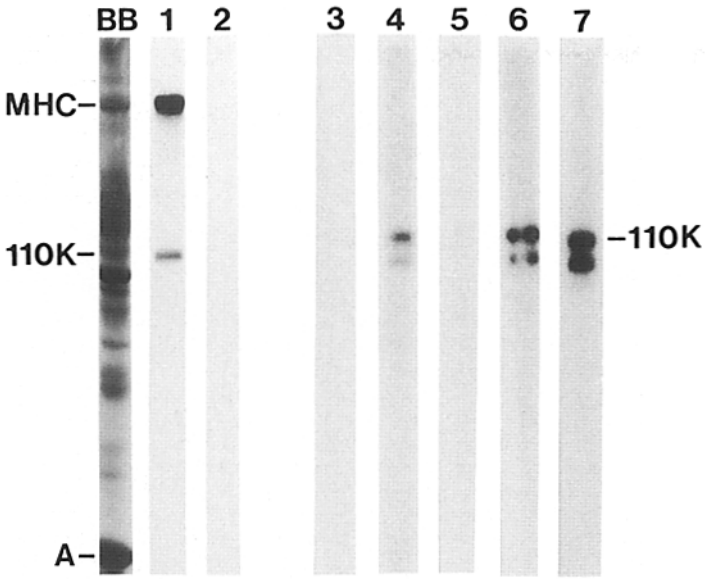

Figure 1. Cross-reactivity of mAbs generated against *Acanthamoeba* myosin II to avian 110K protein. *BB* is a Coomassie-stained gel of chicken BB proteins. Chicken BB proteins (lanes *1* and *2*) and purified 110K-CM (lanes *3–7*) were separated by SDS-PAGE and transferred to nitrocellulose paper. Lane *1* is an immunoblot of BB proteins incubated with a mixture of mAbs to *Acanthamoeba* myosin II (including M2.18 and M2.37). Lane *2* is an immunoblot of BB incubated with a mAb to *Acanthamoeba* alpha-actinin. Immunoblots of purified 110K-CM protein were incubated with anti-myosin II mAbs: lane *3*, M2.17; lane *4*, M2.18; lane *5*, M2.25; and lane *6*, M2.37. Lane *7* shows the reactivity of an affinity-purified polyclonal antibody to 110K. The positions of BB myosin heavy chain (*MHC*), the 110K subunit (*110K*), and actin (*A*) are shown on the *left*.

polyacrylamide gels. The gels were incubated with ^{125}I-CM (2×10^6 cpm/μg) according to the method of Carlin et al. (1980). One-half of the gel was incubated in buffer containing either 0.1 mM $CaCl_2$ or 1 mM EGTA. The CM-binding peptides were identified by autoradiography as described above. ^{125}I-CM was the gift of Dr. A. Harris (Department of Pathology, Yale University).

Results

The 110-kD Subunit of 110K-CM Shares Antigenic Determinants with the 50-kD Actin-binding Domain of Skeletal Muscle S_1

To probe for structural similarities between the myosin and the 110-kD subunit, we used a battery of mAbs raised by Kiehart et al. (1984) against *Acanthamoeba* myosin I and II. None of the myosin I mAbs tested were immunoreactive with 110K protein. The set of myosin II mAbs had previously been shown to react with vertebrate skeletal muscle myosin with epitopes mapped to the central, 50-kD tryptic fragment of myosin S_1 (Bhandari, Rimm, Kaiser, and Pollard, unpublished observations). Immunoblot analysis of isolated BBs using a cocktail of these cross-reactive mAbs (M2.17, M2.18, M2.25, M2.37, M2.42, and M2.43) revealed two immunoreactive species of 200 (BB myosin heavy chain) and 110 kD (Fig. 1). To determine if the 110K band was the 110-kD subunit of the 110K-CM complex and not a fragment of BB myosin, four of the *Acanthamoeba* mAbs were tested for immunoreactivity against purified 110K-CM (Fig. 1). Two of these mAbs (M2.18 and M2.37) reacted with the 110-kD subunit and its 90-kD proteolytic fragment; the other two mAbs (M2.17 and M2.25) were nonreactive.

A 36-kD ATP-binding Tryptic Peptide of 110K Shares Epitopes with the 25-kD, ATP-binding Peptide of S_1

Immunoblot analysis of myosin and its subfragments with 110K mAbs. We have generated a large battery (~50) of mAbs against the 110-kD subunit for use in probing the structure and function of this myosin-like complex. Among these we have identified a group of five 110K-specific mAbs (CX 1–5), all of which strongly cross react with the heavy chain of skeletal but not with that of smooth or brush border myosin (Fig. 2 *b*; the reactivity of CX-1 is shown and is comparable to the other mAbs listed). Immunoblot analysis of various proteolytic fragments of skeletal myosin (heavy meromyosin, S_1, light meromyosin, and rod) revealed that the epitope(s) recognized by these 110K mAbs resides in the S_1 domain of myosin (Fig. 2). As a control, a second mAb, CX-7, reacted specifically to 110K but not with myosin or its subfragments (Fig. 2 *c*).

Immunoblot analysis of S_1 subdomains. Limited digestion of S_1 produced three major cleavage products: an NH_2-terminal, 25-kD peptide that contains the ATP-binding site (Walker et al., 1982), a central 50-kD fragment, and a COOH-terminal 20-kD fragment, all of which are involved in actin binding (Mornet et al., 1981). Under mild digestion conditions CX-2 reacts with fragments of 75 and 25 kD, the latter most likely the NH_2-terminal domain that contains the ATP-binding site of myosin (Fig. 3, *a* and *b*). The other two stable tryptic fragments of 50 and 20 kD were not reactive. In a more complete digestion (Fig. 3, *c* and *d*) containing primarily the 25-, 50-, and 20-kD fragments, only the 25-kD, NH_2-terminal fragment was immunoreactive with CX-1. Identical results were observed with the other four myosin cross-reactive 110K mAbs (not shown).

ATP labeling and immunoblot analysis of 110K-CM tryptic digests. Tryptic digestion of 110K-CM was performed to determine if the 110K might be composed of trypsin-resistant domains that could be mapped with respect to ATP, actin, and CM binding, in addition to placement of the CX-1–5 epitope(s). These digestions were performed on 110K-CM preparations photoaffinity labeled with [alpha-^{32}P]ATP (Mooseker et al., 1984; Verner and Bretscher, 1985) to identify those tryptic fragments containing the ATP-binding site of the 110-kD subunit (Fig. 4). Using digestion conditions detailed in Materials and Methods, multiple fragments between 20 and 90 kD were observed (Fig. 4 *a*; the 90-kD proteolytic fragment is present before addition of trypsin). Of these, four prominent fragments of 90, 80, 54, and 36 kD contained the covalently linked ATP (Fig. 4, *b* and *d*). Moreover, all of these ATP-containing fragments were also im-

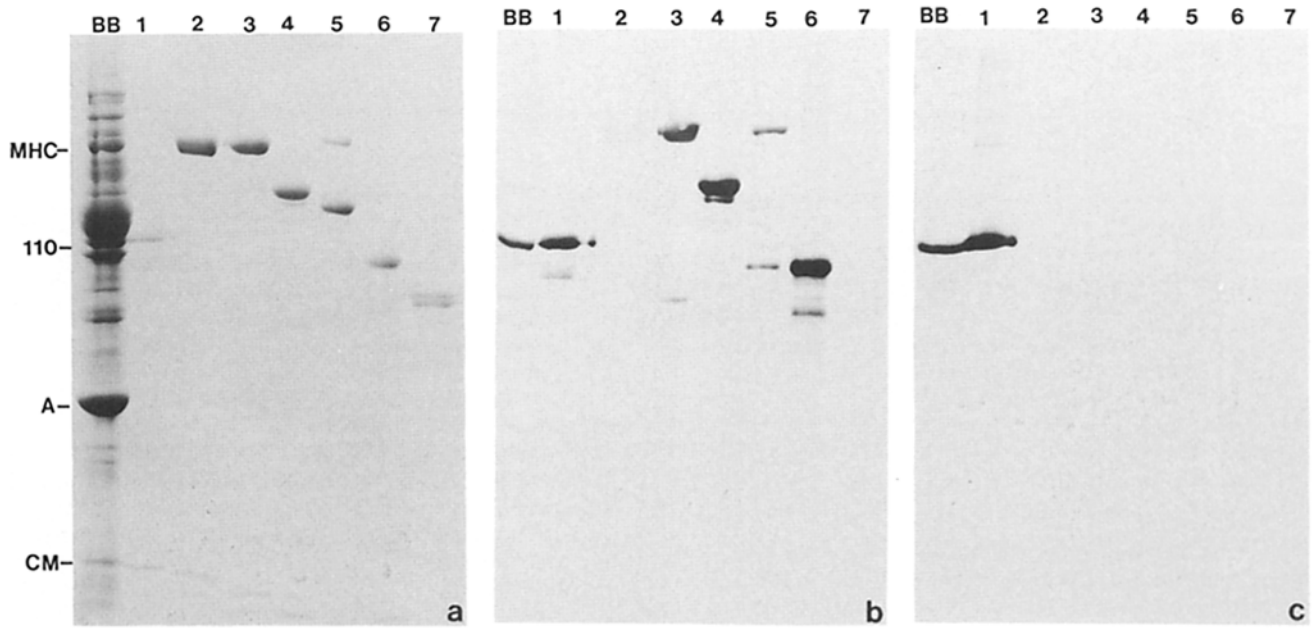

Figure 2. Reaction of 110K mAbs to chicken BB protein, purified 110K-CM, and various myosins. Proteins were separated by SDS-PAGE and transferred to nitrocellulose paper. Purified 110K-CM complex (lanes *1*), gizzard myosin (lanes *2*), chicken skeletal muscle myosin (lanes *3*), and subfragments of skeletal muscle myosin which are heavy meromyosin (lanes *4*), rod (lanes *5*), S_1 (lane *6*), and light meromyosin (lanes *7*). (*a*) A Coomassie-stained gel of the samples used for immunoblot analysis in *b* and *c*. The immunoblot was incubated with (*b*) CX-1 or (*c*) CX-7, a nonmyosin cross-reactive 110K mAb. The positions of BB myosin heavy chain (*MHC*), the 110K subunit (*110*), actin (*A*), and calmodulin (*CM*) are shown on the left.

munoreactive with CX-1 (Fig. 4, *c* and *d*), suggesting that the two smaller fragments of 54 and 36 kD are proteolytically derived from the larger 80- and 90-kD fragments. Thus, the shared epitope recognized by this mAb resides in the region of both myosin and 110K involved in ATP-binding, although it is not necessarily within the binding site itself.

Actin-binding Properties of 110K-CM Tryptic Peptides: The 80- and 54-kD, but not the 36-kD, Fragments Exhibit ATP-dependent Binding to F-actin

Another way to assess the functional properties of the CX-1 immunoreactive tryptic peptides is to examine their interaction with actin. The ATP-dependent actin-binding activity of

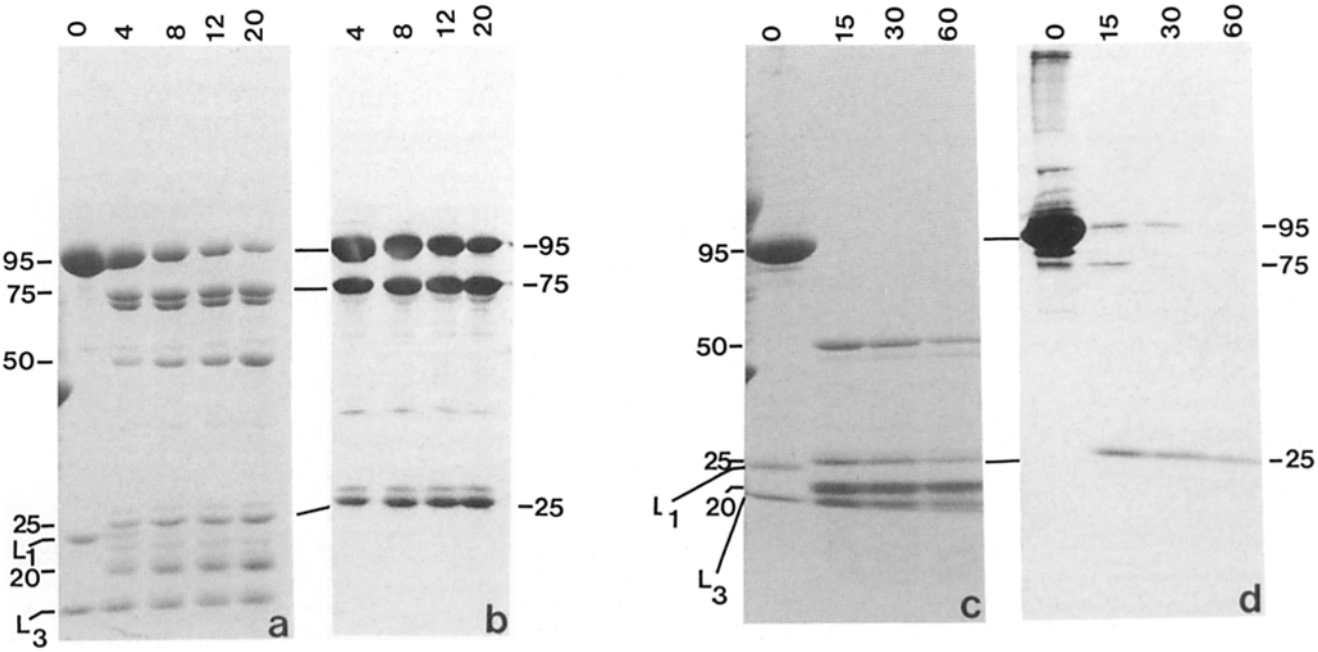

Figure 3. Binding of 110K mAbs to tryptic fragments of S_1. S_1 was digested with trypsin for various times (0–60 min) at two different enzyme/protein ratios. *a* and *c* are Coomassie-stained gels. *b* and *d* are the corresponding immunoblots. The digests were separated on a 5–15% SDS-PAGE, transferred to nitrocellulose, and then incubated with the (*b*) CX-2 or (*d*) CX-1. (*a*) trypsin/S_1 = 1:100; (*c*) trypsin/S1 = 1:25.

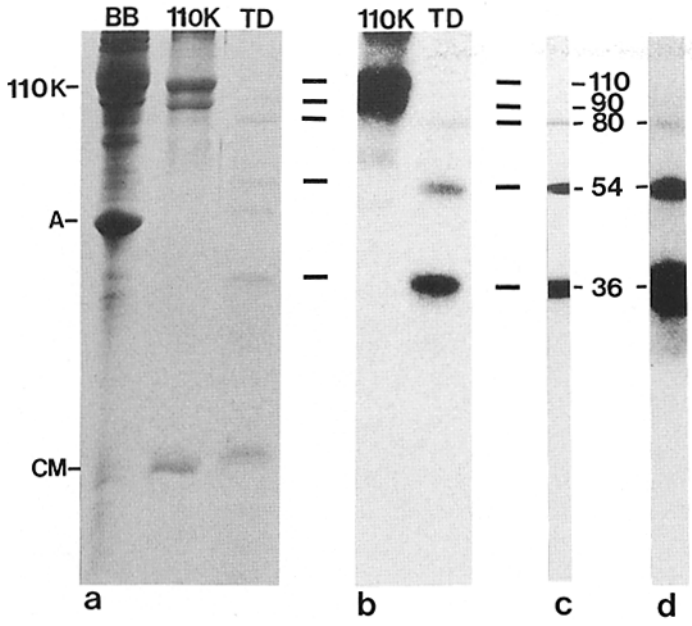

Figure 4. ATP-binding domains of tryptic digests of photoaffinity-labeled 110K-CM protein. (*a*) A Coomassie-stained gel of chicken *BB*, 110K-CM protein (*110K*), and tryptic digests of 110K (*TD*). (*b*) Autoradiogram of ATP-binding domains on intact 110K-CM and on 110K tryptic fragments. (*c*) Immunoblot analysis of 110K tryptic digests incubated with CX-1. (*d*) Autoradiogram of the immunoblot shown in *c*.

these tryptic fragments was assessed by cosedimentation assay and CX-1 immunoblot analysis of the resulting supernatant and pellet fractions (Fig. 5, *a* and *b*). The 36-kD ATP-binding peptide of 110K showed no detectable binding to F-actin in the absence or presence of ATP. Variable amounts (~50% of the experiment shown in Fig. 5 *a*) of the 54-kD fragment pelleted with actin in the absence but not the presence of ATP. This assay was done under conditions where 100% pelleting of equivalent concentrations of intact complex would occur, indicating that the 54-kD fragment either has lower affinity for F-actin, or that a proportion of the 54-kD peptides have lost binding activity.

At the suggestion of a reviewer, we also examined the tryptic digestion profile of 110K-CM bound to F-actin. We observed a marked increase in the resistance of 110K-CM to tryptic cleavage in the presence of actin, but only in the ab-

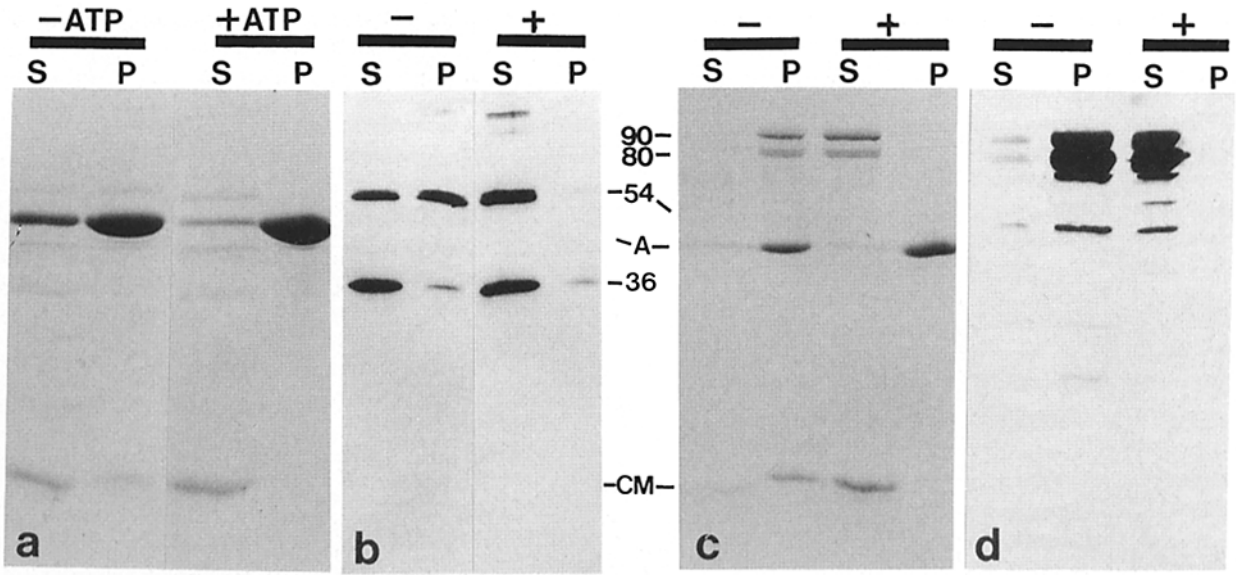

Figure 5. (*a* and *b*) ATP-dependent binding of 110K-CM tryptic fragments to actin. (*a*) SDS-PAGE analysis of supernatant (*S*) and pellet (*P*) fractions derived by cosedimentation of F-actin (*A*) with a mixture of 110K-CM tryptic fragments (see Fig. 4) in the presence (+*ATP*) and absence (–*ATP*) of 5 mM ATP in Solution I. (*b*) Immunoblot analysis of an SDS gel identical to that in *a*. The ATP-binding fragments of 54 and 36 kD are identified by reactivity with the mAb, CX-1. (*c* and *d*) Tryptic digestion of 110K-CM in the presence of F-actin. (*c*) SDS-PAGE of supernatant and pellet fractions derived from the sedimentation of a tryptic digest of 110K-CM (0.2 mg/ml) carried out in the presence of F-actin (0.1 mg/ml) and the absence of ATP. After digestion for 1 h, the digests were either sedimented directly (–*ATP*) or after addition of 5 mM ATP to Solution I (+*ATP*). (*d*) Immunoblot analysis of an identical SDS gel to that in *c* using mAb CX-1. The cleavage sites responsible for generation of the 36-, and, to a lesser extent, 54-kD fragments are protected from digestion when 110K-CM is bound to actin. Instead, larger trypsin fragments of 80–90 kD are produced, all of which exhibit ATP-dependent binding to F-actin.

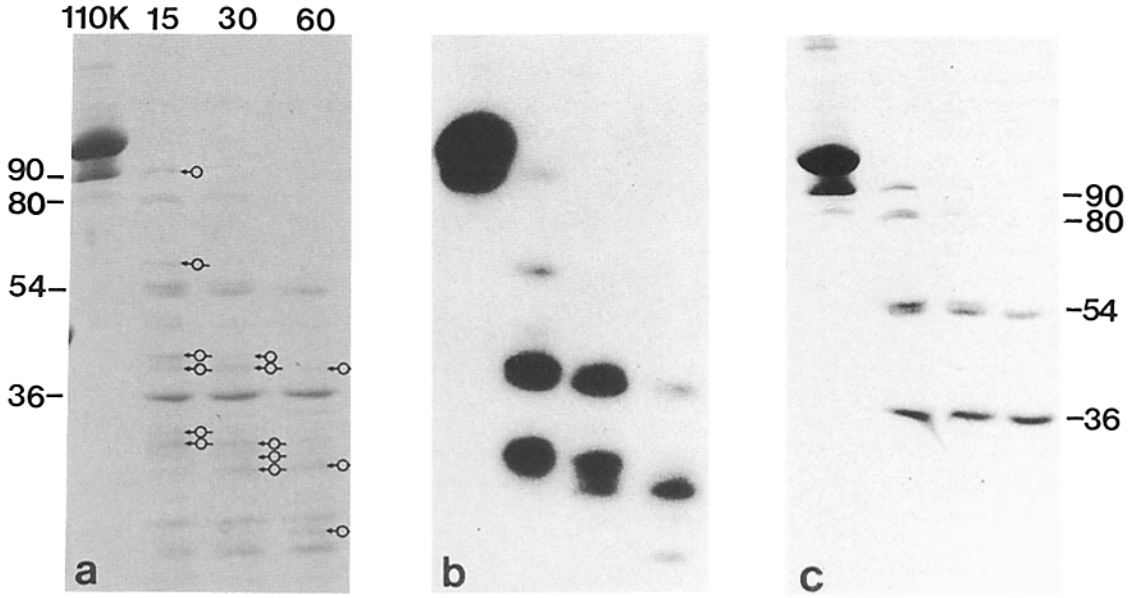

Figure 6. CM-binding domains of tryptic digests of 110K-CM protein. (*a*) Coomassie-stained gel of 110K before (*110K*) and after (*15*, *30*, and *60* min) digestion with trypsin. (*b*) Autoradiogram of the gel shown in *a* overlaid with ^{125}I-CM (2×10^6 cpm/μg). The 110K fragments that bind CM are indicated by the circles shown in *a*. (*c*) Immunoblot of the same samples used in *a* and incubated with CX-1. The molecular masses of the 110K peptides that bind to the myosin cross-reactive 110K mAbs are indicated on the sides.

sence of ATP; in the presence of ATP, the digest profile of 110K-CM was identical to that observed in the absence of actin. Prolonged digestion in the absence of ATP (up to 2 h) failed to produce the 36-kD fragment, and only small amounts of the 54-kD fragment were observed (Fig. 5, *c* and *d*). Instead, several fragments of 80–90 kD were produced, all of which retained ATP-dependent binding to F-actin. Unlike intact 110K-CM, only part of the CM in these digests cosedimented with actin, probably due to the fact that only the 90-kD fragment retained CM-binding activity (as assessed by CM-overlay techniques; results not shown but see Fig. 6). These results demonstrate that the trypsin-sensitive domains which give rise to both the 36- and, to a lesser extent, 54-kD peptides are protected in the presence of actin, providing further evidence that these peptides are derived from the region of the molecule involved in ATP-dependent binding to F-actin.

Analysis of CM-binding Activity Associated with Tryptic Fragments of 110-CM: The 80-, 54- and 36-kD ATP-binding Peptides Lack CM-binding Sites

CM binds to 110K fragments of 90, 60, 40, 29, and 18 kD (Fig. 6 *b*). Of these, only the 90-kD fragment was reactive with CX-1 (Fig. 6 *c*). Thus, those peptides of 80, 54, and 36 kD that are presumably derived from the portion of the 110K molecule which is structurally similar to myosin, either lack or have not retained CM-binding activity. As with intact 110K-CM, (e.g., see Howe et al., 1982; Glenney and Glenney, 1985) CM binding to these fragments was also observed in the absence of Ca^{++}, but the level of binding was much reduced (results not shown).

Discussion

The results presented in this report demonstrate that the 110-kD subunit of the 110K-CM complex share antigenic determinants with the S_1 domain of skeletal muscle myosin (Figs. 2 and 3). Most importantly, these shared epitopes are not restricted to a single domain of the myosin head. The 110K mAbs react with the NH_2-terminal, 25-kD tryptic fragment of S_1 (Fig. 3), while the cross-reactive, myosin II mAbs bind to epitopes within the central, 50-kD fragment of S_1. These results suggest that there is considerable structural homology between 110K and the head domain of myosin. This conclusion is strengthened by the observation that the epitopes recognized by the 110K mAbs are localized to the subdomain of both myosin and the 110K that contains the ATP-binding site of these molecules (Figs. 3 and 4, respectively); that is, this set of shared epitopes are localized within functionally analogous domains of both molecules. These results, together with previous functional studies summarized in the introduction to this report, provide strong support for the conclusion that the 110K-CM complex is a member of the myosin family of mechanoenzymes.

One puzzling aspect of these results, raised by a reviewer of this paper, concerns the reactivity of the 110K mAbs CX 1–5 with skeletal but not other types of myosins, particularly *Acanthamoeba* myosin I. Such a result could indicate that the 110K is structurally more similar to skeletal muscle myosin than to other types of myosin, at least within the region of the myosin head involved in ATP binding (the cross-reactivity of the *Acanthamoeba* myosin II mAbs is not relevant to this discussion since these antibodies react with a variety of myosin classes, including conventional nonmuscle myosin [Fig. 1]). If each of the five cross-reacting 110K mAbs were reactive with unique, structurally distinct epitopes within the 25-kD tryptic domain of S_1, then such a conclusion might be warranted. On the other hand, it is quite plausible that these mAbs are reactive with epitope(s) that lie within a single, relatively small region of the respective molecules; if so,

these immunochemical results need not be reflective of a greater degree of overall structural homology between 110-kD subunit and skeletal muscle myosin S_1 as compared to the head domain of other myosins. Another way to address this question is to compare the tryptic digestion of 110K-CM to that of the S_1 domain of skeletal muscle myosin. The placement of the trypsin-sensitive domains that give rise to the 20-, 25-, and 50-kD peptides is a conserved feature among skeletal muscle myosins; this is not true for nonmuscle and smooth muscle myosins, which exhibit diverse patterns of tryptic cleavage (for review see Warrick and Spudich, 1987). The tryptic digestion profile of 110K-CM is clearly quite distinct from skeletal muscle myosin S_1. Assuming that the 36-kD, ATP-binding peptide of 110K-CM is derived from the NH_2 terminus of the molecule, it may be significant that tryptic cleavage of *Acanthamoeba* myosin IA yields an NH_2-terminal, ATP-binding peptide of similar molecular mass (38 kD; for review, see Korn et al., 1988). However, such a comparison of digestion profiles is of limited value since there is great variability in the digestion patterns observed among various classes of myosin, all of which presumably show marked sequence similarity throughout their respective head domains (see Warrick and Spudich, 1987). Final resolution of this issue will await sequencing of the 110-kD subunit.

The conclusive identification of the 110K-CM complex as a myosin will, of course, require demonstration of mechanochemical activity and a determination of the primary structure of the 110-kD subunit. In fact, such sequence information may already be available for the analogue of 110K present in bovine intestine. Hoshimaru and Nakanishi (1987) have fortuitously isolated and sequenced a full-length, cDNA molecule whose sequence encodes a 119-kD protein with high sequence homology to the S_1 domain of myosin. Surprisingly, this cDNA shares its 3′ sequence (noncoding) with the 5′ sequence of preprotachykinin B mRNA resulting from an overlapping transcription of the same strand of the genomic DNA sequence. The structural organization, as well as primary sequence of this molecule is more similar to that of *Acanthamoeba* myosin I than conventional myosin. Hoshimaru and Nakanishi (1987) have proposed that the bovine molecule is a vertebrate member of the myosin I family, and have termed the molecule "myosin I heavy chain–like protein", or MIHC. In addition, the authors have suggested that MIHC is, in fact, the "110-kD" subunit of bovine 110K-CM, based on its high level of expression in intestinal tissues. Although this seems likely, results of Southern blot analysis revealed evidence for multiple genes that hybridize with MIHC probes. Thus, it is still critical to verify that the MIHC protein is the 110-kD subunit of bovine 110K-CM and not another, as yet uncharacterized, myosin I–like protein found in intestinal tissue. This is an important open question because recent studies by Rochette-Egly and Haffen (1987), using a polyclonal antiserum generated in our laboratory against avian 110K (for characterization see Shibayama et al., 1987) identified two immunoreactive polypeptides in developing rat intestine: a 110-kD form and a slightly higher molecular mass form of 130 kD that disappears at birth. Similarly, we (Peterson, Carboni, West, and Mooseker, unpublished observations) have observed both 110- and 120–130-kD immunoreactive forms in preparations of isolated BBs from human ileum and colon. These two immunoreactive species were detected with the same myosin–cross-reactive 110K mAbs used in the present study. These data demonstrate that multiple, immunoreactive forms of 110K are present in mammalian intestine. Additional study is required to determine if the 110-kD forms arise by proteolysis of the larger protein, or if two distinct but structurally related proteins are present.

Assuming that the 110K-CM complex is a myosin-like mechanoenzyme, one must also address questions concerning the function of CM in this complex. Enzymatic studies have shown that [Ca^{++}] in the micromolar range activates the MgATPase of 110K-CM in both the presence and absence of actin (Conzelman and Mooseker, 1987; Swanljung-Collins et al., 1987). Based on these observations, we have suggested that at least a fraction of the three to four molecules of CM in the complex might be functionally analogous to the Ca^{++}-sensitive light chains present on certain myosins (e.g., scallop myosin; Szent-Gyorgyi and Chantler, 1986). One would predict that at least some of the CM-binding sites would be present within the "myosin-like" domain of the 110K. On the other hand, such a conclusion is not supported by recent studies of Coluccio and Bretscher (1988), in which the actin-binding, ATPase activity, and CM binding of various sized chymotryptic fragments of 110K were examined. These workers noted that chymotryptic cleavage of 110K-CM in the absence of Ca^{++} results in production of a 90-kD fragment that retains both CM- and ATP-dependent actin-binding activities. Cleavage in the presence of Ca^{++} results in a major fragment of 78 kD that lacks CM-binding activity but, like the 90-kD fragment, retains ATP-dependent binding to actin. Based on these and other results, Coluccio and Bretscher (1988) have suggested that the CM-binding domain(s) lie outside the "myosin-like" domain of the 110K molecule and that CM may not contribute to the presumed mechanochemical activity of the complex (as assessed by actin-binding and ATPase activity). The results presented here (Fig. 6) are consistent with this conclusion. Of the 90-, 80-, 54-, and 36-kD fragments of 110K derived from the "myosin-like" domain of the 110K, only the 90-kD fragment retains CM-binding activity. It is quite likely that the 90- and 80-kD fragments obtained either by prolonged tryptic digestion of 110K-CM in the presence of F-actin (Fig. 5, *c* and *d*) or by brief digestion in the absence of actin (Fig. 4) are the structural equivalents of the 90- and 78-kD chymotryptic fragments observed by Coluccio and Bretscher (1988), since both pairs of peptides bind actin in an ATP-dependent fashion, but the 80-kD tryptic fragment, like the 78-kD chymotryptic fragment, lacks CM-binding activity.

Taken together, these results suggest that a large portion of the molecule (up to 80 kD) that contains the myosin-like epitope does not contain (or retain) CM-binding sites. Conversely, the four tryptic fragments of 60, 40, 29, and 18 kD that do show CM-binding activity are most likely derived from the other half of the 110K molecule (presumably the COOH-terminal half by analogy with myosin; see below), since none of these fragments react with the myosin-reactive 110K mAbs. If one assumes structural homology with the S_1 domain of myosin, it is possible to roughly align the various tryptic fragments characterized in this study into the presumed NH_2- and COOH-terminally derived domains as shown in the tentative model in Fig. 7. If correct, a logical conclusion from these results and the results of Coluccio and

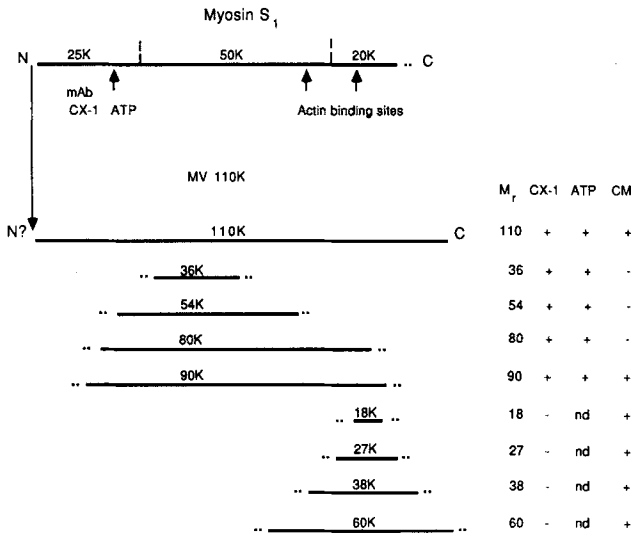

Figure 7. Functional properties of 110K tryptic fragments: evidence for discrete "myosin head" and CM-binding domains. Functional grouping of 110K tryptic peptides derived from the presumed NH_2- and COOH-terminal halves of the molecule, based on the presence of CX-1 epitope and ATP binding site. This tentative grouping is extrapolated from the proposed structural homology with the NH_2-terminal, S_1 domain of myosin heavy chain. The domain structure of myosin S_1 is shown for comparison; ATP and actin binding sites are indicated by arrows. Except for the 90-kD fragment (present in the 110K-CM preparation before digestion), none of the peptides from the presumed myosin head domain contain (retain) CM-binding activity. Conversely, there are numerous peptides (18–60 kD), probably derived from the opposite end of the molecule, that do bind CM, suggesting that the 110-kD subunit consists of discrete "myosin head" and CM-binding domains. Note that only the smallest molecular mass species for the two sets of the CM-binding fragments in the 38–40-kD and 27–29-kD range are listed (see Fig. 6).

Bretscher (1988) is that the 110K molecule is composed of two discrete functional domains consisting of a myosin head linked to a CM-binding domain(s) at its COOH-terminal end (it also seems reasonable to assume that the membrane-binding domain is at this end). It is important to note, however, that both tryptic and chymotryptic digestion could destroy CM-binding sites present in the "myosin-head" domain of the 110K. It is also possible that CM bound at the presumed COOH-terminal "tail" portion of the molecule might allosterically regulate the interaction of 110K with actin. In this regard, it will be important to determine if either the CM-free, 78-kD chymotryptic fragment described by Coluccio and Bretscher (1988) or the 80-kD tryptic fragment described here exhibit Ca^{++}-sensitive MgATPase activity characteristic of the intact complex (Conzelman and Mooseker, 1987).

The final chapter in the characterization of the 110K-CM as a myosin must include a demonstration that it is a mechanoenzyme. To this end, we are conducting studies using the in vitro motility assay of Sheetz and Spudich (1983) to assess the motile properties of 110K-CM. Our initial studies indicate that both purified 110K-CM (Mooseker, M., and T. Coleman, manuscript submitted for publication) and membrane-associated 110K-CM (Mooseker, M., M. Sheetz, K. Conzelman, T. Coleman, and J. Heuser, unpublished observations) promote the in vitro movement of beads along *Nitella* actin cables. These initial observations establish the mechanochemical potential of 110K-CM and should provide the experimental basis to examine the role of Ca^{++}, CM, and membrane association in the regulation of 110K-CM motility.

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