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 (St) 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, NH₂-terminal tryptic fragment of chicken skeletal St, 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 St, 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.

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Each microvillus of the intestinal brush border (BB) 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 (S₁) 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
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 the 110K-CM complex has mechanchemical 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, NH2-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 gg of electroeluted I10K protein, precipitated by the ELISA method using subclass-specific antisera (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 coedentration 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.

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 gg/ml) to a mixture of 110K-CM (0.2 mg/ml) and F-actin (0.1 mg/ml) stabilized by addition of phalloidin (10 gg/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 coedentration 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-32P]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 uM radioactive nucleotide and 40 uM unlabeled ATP containing 2 mM Mg++. The reaction mixture was incubated on ice and irradiated by holding a gernicidal 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.).
Results

The 110-kD Subunit of 110K-CM Shares Antigenic Determinants with the 50-kD Actin-binding Domain of Skeletal Muscle S1

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 S1 (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 110-kD 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 S1

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, S1, light meromyosin, and rod) revealed that the epitope(s) recognized by these 110K mAbs resides in the S1 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 S1 subdomains. Limited digestion of S1 produced three major cleavage products: an NH2-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 NH2-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, NH2-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-32P]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-
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
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 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 125I-CM (2 x 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.

Discussion

The results presented in this report demonstrate that the 110-kD subunit of the 110K-CM complex share antigenic determinants with the S1 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 NH2-terminal, 25-kD tryptic fragment of S1 (Fig. 3), while the cross-reactive, myosin II mAbs bind to epitopes within the central, 50-kD fragment of S1. 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 CX1-5 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 S1, 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 S1 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 S1 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 S1. Assuming that the 36-kD, ATP-binding peptide of 110K-CM is derived from the NH2 terminus of the molecule, it may be significant that tryptic cleavage of Acanthamoeba myosin IA yields an NH2-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 S1 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 Haffner (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 (Conzemul 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 contains 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 S1 domain of myosin, it is possible to roughly align the various tryptic fragments characterized in this study into the presumed NH2- 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

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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 mechanochernical 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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