# Mapping of the Microvillar 110K–Calmodulin Complex (Brush Border Myosin I). Identification of Fragments Containing the Catalytic and F-Actin-Binding Sites and Demonstration of a Calcium Ion Dependent Conformational Change†

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ABSTRACT: In intestinal microvilli, the 110K–calmodulin complex is the major component of the cross-bridges which connect the core bundle of actin filaments to the membrane. Our previous work showed that the 110-kDa polypeptide can be divided into three functional domains: a 78-kDa fragment that contains the ATPase activity and the ATP-reversible F-actin-binding site, a 12-kDa fragment required for binding calmodulin molecules, and a terminal 20-kDa domain of unknown function [Coluccio, L. M., & Bretscher, A. (1988) *J. Cell Biol. 106*, 367–374]. By analysis of limited $\alpha$-chymotryptic cleavage products, we now show that the molecular organization is very similar to that described for the $S_1$ fragment of myosin. The catalytic site was identified by photoaffinity labeling with [5,6-$^3$H]UTP, and fragments binding F-actin were identified by cosedimentation assays. Cleavage of the 78-kDa fragment yielded major fragments of 32 and 45 kDa, followed by cleavage of the 45-kDa fragment to a 40-kDa fragment. Of these, only the 32-kDa fragment was labeled by [5,6-$^3$H]UTP. Physical characterization revealed that the 45- and 32-kDa fragments exist as a complex that can bind F-actin, whereas the 40-kDa/32-kDa complex cannot bind actin. We conclude that the catalytic site is located in the 32-kDa fragment and the F-actin-binding site is present in the 45-kDa fragment; the ability to bind actin is lost upon further cleavage of the 45-kDa fragment to 40 kDa. Peptide sequence analysis revealed that the 45-kDa fragment lies within the molecule and suggests that the 32-kDa fragment is the amino terminus. Cleavage sites in the 78-kDa fragment which generate the 45-, 40-, and 32-kDa peptides are protected when the fragment is bound to F-actin; moreover, in the absence of $Ca^{2+}$, the 110-kDa polypeptide is not readily cleaved by $\alpha$-chymotrypsin to the 78-kDa fragment or beyond. These results suggest that the 12-kDa calmodulin-binding region confers a $Ca^{2+}$-dependent accessibility to the F-actin-protectable cleavage site in the $S_1$-like domain. This is the first evidence for a $Ca^{2+}$-regulated structural change in the $S_1$-like domain of the 110K–calmodulin complex.

Microvilli contain a core bundle of actin filaments which exhibits helically arranged lateral links extending to the membrane (Mukherjee & Staehelin, 1971; Tilney & Mooseker, 1971). These links are comprised of a 110-kDa polypeptide complexed to calmodulin (Matsudaira & Burgess, 1979; Coluccio & Bretscher, 1989). Isolated 110K–calmodulin complex resembles myosin in its ability to bind F-actin in a calcium ion and adenosine 5′-triphosphate (ATP)[1]-sensitive manner (Howe & Mooseker, 1983; Verner & Bretscher, 1985; Coluccio & Bretscher, 1987; Conzelman & Mooseker, 1987) and to bind to actin filaments with a defined polarity (Coluccio & Bretscher, 1987). A partial cDNA clone for the 110-kDa polypeptide from chicken has been obtained (Garcia et al., 1989). Its deduced amino acid sequence is similar to the sequences of a bovine protein (Hoshimaru & Nakanishi, 1987), known to be a major protein of the intestine, and to the single-headed myosins found in *Acanthamoeba* (Pollard & Korn, 1973; Jung et al., 1987) and *Dictyostelium* (Jung et al., 1989). In addition, the 110K–calmodulin complex is a mechanochemical molecule as determined by in vitro motility assays (Mooseker & Coleman, 1989; Collins et al., 1990).

In an earlier study, we described the functional domains of the isolated 110K–calmodulin complex by analysis of fragments resulting from controlled proteolytic cleavage (Coluccio & Bretscher, 1988). Limited proteolysis of the 110K–calmodulin complex with $\alpha$-chymotrypsin in the presence of EGTA results in the generation of a 90-kDa–calmodulin complex which binds F-actin, decorates actin filaments, and hydrolyzes ATP in a similar manner to the intact 110K–calmodulin complex. Cleavage of 110K–calmodulin with $\alpha$-chymotrypsin in buffer containing $Ca^{2+}$ generates a 78-kDa fragment with no associated calmodulin molecules (Coluccio & Bretscher, 1988). The 78-kDa fragment is then subsequently cleaved to major fragments of 45, 40, and 32 kDa. The 78-, 45-, and 32-kDa fragments will sediment with F-actin; the 78 kDa and its fragments can also hydrolyze ATP (Coluccio & Bretscher, 1988). These data indicate that the calmodulin-binding domain is distinct from both the nucleotide-binding and F-actin-binding domains and, thus, binding to F-actin does not require calmodulin.

Here we identify the catalytic site on the 110-kDa polypeptide using the method of direct photoaffinity labeling with [5,6-$^3$H]UTP as previously described (Maruta & Korn, 1981). Actin-binding assays using $\alpha$-chymotryptic fragments of the 110-kDa polypeptide which were photoaffinity-labeled allow

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; CTP, cytidine 5′-triphosphate; DTT, dithiothreitol; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GTP, guanosine 5′-triphosphate; ITP, inosine 5′-triphosphate; PMSF, phenylmethanesulfonyl fluoride; $S_1$, myosin subfragment 1; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; UTP, uridine 5′-triphosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; UV, ultraviolet.

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us to align the actin-binding region, the catalytic site, and the calmodulin-binding domain.

We show that the 78-kDa fragment has a functional organization very similar to that of the $S_1$ fragment of myosin. In addition, analysis of the cleavage products generated in the presence and absence of $Ca^{2+}$ and/or F-actin has revealed a $Ca^{2+}$-regulated change in protease accessibility induced in the 78-kDa fragment by the adjacent 12-kDa calmodulin-binding domain.

## Experimental Procedures

*Protein Purification.* Fresh chicken intestines were obtained from the local poultry houses in either Odessa, NY, or Gainesville, GA. 110K–calmodulin was isolated from chicken intestinal brush borders as previously described (Coluccio & Bretscher, 1987). Rabbit skeletal muscle actin was prepared as described by Spudich and Watt (1971) and further purified by gel filtration according to MacLean-Fletcher and Pollard (1980). Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

*Photoaffinity Labeling.* Purified 110K–calmodulin complex at 0.5 mg/mL was incubated with 10 $\mu$M [5,6-$^3$H]UTP (Amersham Corp.) in buffer containing 10 mM Tris, pH 8.0, 100 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, and either 1 mM EGTA or 0.2 mM $CaCl_2$. Unlabeled UTP at 40 $\mu$M was included in the experiment described in Figure 5. The samples were UV-irradiated while on ice for 30 min using a General Electric G8T5 germicidal lamp positioned 8 cm away. Samples were then separated by SDS–PAGE and analyzed by fluorography.

*Limited Proteolytic Cleavage.* The 110K–calmodulin complex was dialyzed into buffer containing 10 mM Tris, pH 8.0, 100 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, and either 0.2 mM $CaCl_2$ or 1 mM EGTA. Controlled cleavage of the 110K–calmodulin complex with $\alpha$-chymotrypsin was performed essentially as described earlier (Coluccio & Bretscher, 1988). Briefly, the 110K–calmodulin at 0.3 mg/mL was incubated at room temperature with $\alpha$-chymotrypsin at a 1:50 w/w ratio. For time courses, aliquots at certain time intervals were removed and boiled in 5× concentrated sample buffer containing final concentrations of 80 mM Tris-HCl, pH 6.8, 1% $\beta$-mercaptoethanol, 5 mM EGTA, 10% glycerol, 0.3 mM PMSF, and 0.5 mM benzamidine.

*Actin-Binding Assays.* Column-purified rabbit skeletal muscle actin in buffer containing 10 mM Tris, pH 8.0, 1 mM DTT, 0.2 mM $CaCl_2$, and 0.5 mM ATP was polymerized by the addition of KCl and $MgCl_2$ to 100 mM and 1 mM, respectively. The ATP was then removed by dialysis against the same buffer without ATP. The F-actin at 0.3 mg/mL was incubated at room temperature with 110K–calmodulin at 0.3 mg/mL for 30 min. Samples were then centrifuged in a Beckman TL-100 centrifuge using a TLA-100 rotor at 75000 rpm for 20 min. Pellets were resuspended in 25 $\mu$L of 1 M Tris, pH 8.7. Supernatants were precipitated with 10% trichloroacetic acid and then resuspended in 25 $\mu$L of 1 M Tris. Both pellets and supernatants were then prepared for gel electrophoresis using 2× concentrated sample buffer; equivalent amounts were always loaded onto the gels. To show reversibility of binding of the 110K–calmodulin complex to F-actin in the presence of nucleotide (Figure 3), 15 min prior to centrifugation, ATP or UTP was added to 5 mM.

*Gel Filtration of $\alpha$-Chymotryptic Fragments.* Three hundred fifty microliters of sample resulting from cleavage of the 110K–calmodulin complex with $\alpha$-chymotrypsin in buffer containing $Ca^{2+}$ was chromatographed on a 7-mL column containing Sephadex G150 Superfine equilibrated in buffer containing 10 mM Tris, pH 8.0, 1.0 mM $MgCl_2$, 100 mM KCl, and 1 mM DTT. Fractions of 150 $\mu$L were collected and precipitated with 10% trichloroacetic acid. The pellets were solubilized in 1 M Tris, pH 8.7, boiled in 2× sample buffer, and then separated by SDS–PAGE.

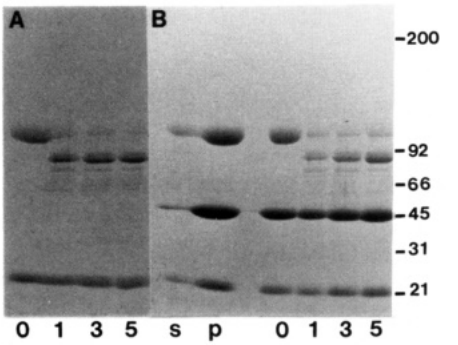


FIGURE 1: Cleavage of the 110K–calmodulin complex in buffer containing 1 mM EGTA. Purified 110K–calmodulin at 0.3 mg/mL in either the absence (A) or the presence (B) of 0.3 mg/mL F-actin was incubated with $\alpha$-chymotrypsin at a 1:50 w/w ratio in buffer containing 1 mM EGTA. Aliquots were removed at the times indicated (minutes), boiled in sample buffer containing PMSF and benzamidine, and separated by SDS–PAGE on split gels containing 7.5%/15% acrylamide. No difference in cleavage patterns was observed over the next 60 min. Cosedimentation assays (s, supernatant; p, pellet) indicate that the 110K–calmodulin complex associates with the F-actin under the buffer conditions used. Molecular weight markers are indicated at the right ($\times 10^{-3}$).

*Other.* SDS-PAGE was performed according to Laemmli (1970) on split mini-gels containing 7.5% and 15% acrylamide (Coluccio & Bretscher, 1987), or when indicated, on gels containing 12% acrylamide. Molecular weight markers used in electrophoresis were as follows: myosin, 200K; phosphorylase, 92K; bovine serum albumin, 66K; carbonic anhydrase, 31K; soybean trypsin inhibitor, 21K. For fluorography, after being stained in Coomassie brilliant blue, gels were destained in 7.5% methanol and 10% acetic acid containing 1 M sodium salicylate, dried under vacuum, and then placed at −70 °C with Kodak X-OMAT AR film; exposure was usually for several days. Peptide sequencing was done on fragments electroblotted onto polyvinylidene membranes (Immobilon; Millipore Corp., Bedford, MA) as described by Matsudaira (1987). Polyclonal antibodies were raised in rabbits against the 110-kDa polypeptide eluted from an SDS–polyacrylamide gel. Western immunoblotting was done according to Towbin et al. (1979). Detection was by horseradish peroxidase conjugated goat anti-rabbit IgG prepared by Cappel (OrganonTeknika-Cappel, Durham, NC).

## Results

We have previously shown that incubation of the 110K–calmodulin complex with $\alpha$-chymotrypsin in buffer containing EGTA results in the formation of a 90-kDa–calmodulin complex (Coluccio & Bretscher, 1988). We now show that the same cleavage pattern is observed when the 110K–calmodulin complex is bound to F-actin (Figure 1A,B).

Cleavage of the 110K–calmodulin complex in buffer containing calcium ions resulted in the generation of a major fragment of 78 kDa followed by cleavage to fragments of 45, 40, and 32 kDa and a 38-kDa fragment found in lesser amounts (Coluccio & Bretscher, 1988). In the presence of F-actin, the 78-kDa polypeptide was protected from further cleavage by $\alpha$-chymotrypsin even after prolonged incubation times (Figure 2A,B). Control actin-binding assays were performed to demonstrate that the 110K–calmodulin complex interacts with actin filaments under the buffer conditions used

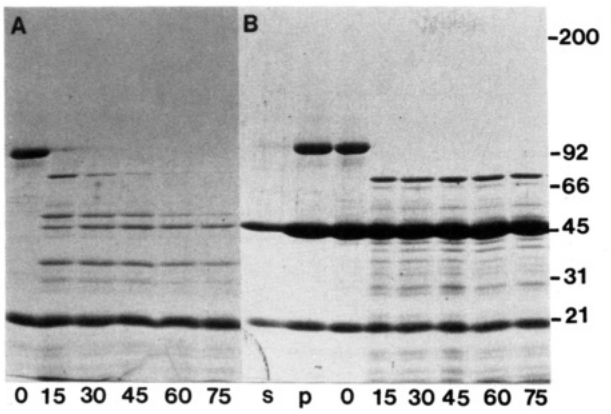


FIGURE 2: Cleavage of the 110K–calmodulin complex in buffer containing 0.2 mM $CaCl_2$. Purified 110K–calmodulin complex at 0.3 mg/mL in either the absence (A) or the presence (B) of 0.3 mg/mL F-actin was incubated with $\alpha$-chymotrypsin at a 1:50 w/w ratio in buffer containing 0.2 mM $CaCl_2$. Aliquots were removed at the times indicated (minutes), boiled in sample buffer containing PMSF and benzamidine, and separated by SDS–PAGE on 7.5/15% acrylamide gels. Cosedimentation assays (s, supernatant; p, pellet) indicate that the 110K–calmodulin complex associates with F-actin under the conditions used. Molecular weight markers are indicated at the right ($\times 10^{-3}$).

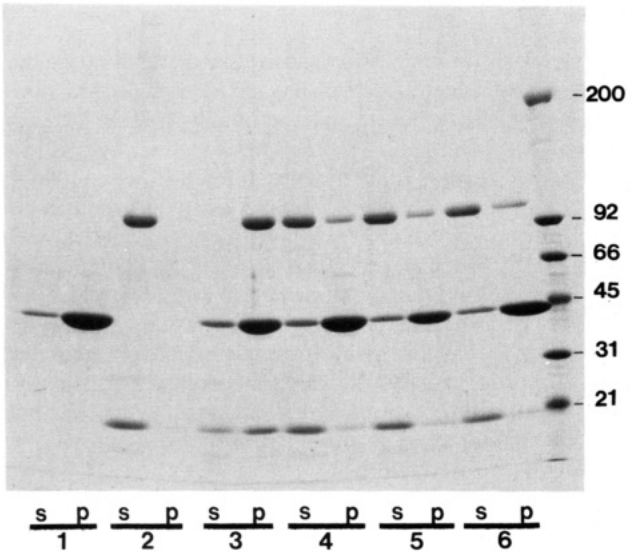


FIGURE 3: Binding of the 110K–calmodulin complex to F-actin is reversible in the presence of UTP. UTP at 5 mM (sample 5, s and p) or 10 mM (sample 6, s and p) was added to F-actin at 0.3 mg/mL after incubation with 0.3 mg/mL 110K–calmodulin. After centrifugation, supernatants and pellets were analyzed by gel electrophoresis on a 7.5%/15% polyacrylamide gel. Control samples contained F-actin only (sample 1, s and p), 110K–calmodulin complex only (sample 2, s and p), F-actin + 110K–calmodulin complex (sample 3, s and p), and F-actin + 110K–calmodulin complex followed by the addition of ATP to 5 mM (sample 4, s and p). Molecular weight markers ($\times 10^{-3}$) are indicated in the last lane.

(Figures 1B and 2B; s, supernatant; p, pellet).

The observed difference in cleavage patterns in buffer containing $CaCl_2$ vs EGTA was not due to a calcium ion sensitivity of $\alpha$-chymotrypsin. To demonstrate this, 110K–calmodulin complex in $Ca^{2+}$-containing buffer was cleaved with $\alpha$-chymotrypsin to the 78-kDa product. The sample was then divided into two aliquots, and EGTA was added to one of the aliquots; cleavage of both samples was allowed to proceed. No difference in the extent of cleavage was observed over a 90-min time course (data not shown).

In experiments done to test whether UTP could substitute for ATP, we found that the behavior of the 110K–calmodulin complex in the presence of UTP mimicked that observed in the presence of ATP. Binding of the 110K–calmodulin complex to F-actin was reversible by the addition of either ATP (Figure 3, sample 4) or UTP (Figure 3, samples 5 and 6). In addition, the 110K–calmodulin complex could hydrolyze UTP, albeit to a smaller extent than that observed with ATP [162 vs 352 nmol of $P_i$ (mg of protein$^{-1}$ min$^{-1}$)]. These results indicate that UTP is binding to the same nucleotide-binding site as can be occupied by ATP and that UTP can serve as a substrate for the 110K–calmodulin complex.

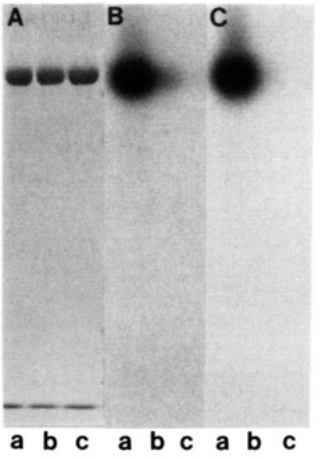


FIGURE 4: Photoaffinity labeling of the 110K–calmodulin complex with [5,6-$^3$H]UTP in the presence of unlabeled UTP or ATP. 110K–calmodulin complex at 0.5 mg/mL was incubated with 10 $\mu$M [5,6-$^3$H]UTP and increasing amounts of (B) unlabeled UTP or (C) unlabeled ATP; fluorographs are shown in (B) and (C). Concentrations of competing ligand are (a) 0 $\mu$M, (b) 50 $\mu$M, and (c) 250 $\mu$M. The Coomassie blue stained 10% polyacrylamide gel is shown in (A). Note that on this percentage acrylamide gel, calmodulin runs with the dye front.

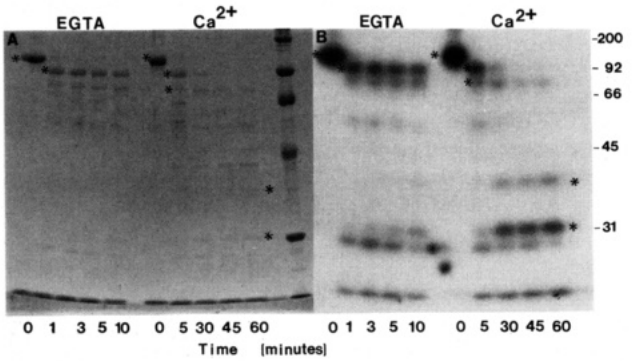


FIGURE 5: [5,6-$^3$H]UTP photoaffinity labeling of the 110K–calmodulin complex followed by cleavage with $\alpha$-chymotrypsin. Following labeling, the 110K–calmodulin complex was cleaved in buffer containing 1 mM EGTA or 0.2 mM $CaCl_2$ and analyzed by SDS–PAGE on 10% polyacrylamide gels (panel A). The corresponding fluorograph is shown in (B). The number of minutes after introduction of $\alpha$-chymotrypsin is indicated at the bottom. The 110-kDa polypeptide and major fragments at 90, 78, 38, and 32 kDa were labeled; the positioning of these bands on each of the panels is indicated by asterisks.

To investigate the location of the catalytic site, the 110K–calmodulin complex was photoaffinity-labeled with [5,6-$^3$H]UTP (Figure 4A). Only the 110-kDa polypeptide was labeled. The amount of labeling of the 110-kDa polypeptide chain decreased when either unlabeled UTP (Figure 4B) or unlabeled ATP (Figure 4C) was included in the mixture. In another experiment, the 110K–calmodulin complex was photoaffinity-labeled with [$\alpha$-$^{32}$P]ATP; the amount of labeling of the 110-kDa polypeptide was decreased when unlabeled UTP was present during the incubation (data not shown).

Photoaffinity labeling of the 110K–calmodulin complex by [5,6-$^3$H]UTP was followed by cleavage of the 110K–calmo-

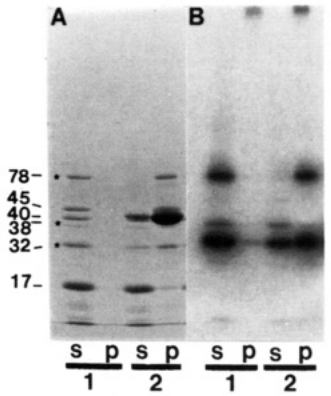


FIGURE 6: Cosedimentation of [5,6-$^3$H]UTP-labeled fragments with F-actin. 110K–calmodulin complex was photoaffinity-labeled with [5,6-$^3$H]UTP and then incubated with $\alpha$-chymotrypsin in buffer containing 0.2 mM $CaCl_2$. The resulting fragments were then allowed to incubate with F-actin for 30 min before centrifugation. Supernatants (s) and pellets (p) were separated by SDS–PAGE on 7.5/15% polyacrylamide gels and then analyzed by fluorography. Sample 1, fragments only; sample 2, fragments incubated with F-actin. The Coomassie brilliant blue stained gel is indicated in (A); fluorograph, (B). Fragments are identified by their molecular weight ($\times 10^{-3}$) at left. Positioning of the radiolabeled fragments identified by fluorography (i.e., the 78-, 38-, and 32-kDa fragments) is indicated by asterisks on the polyacrylamide gel.

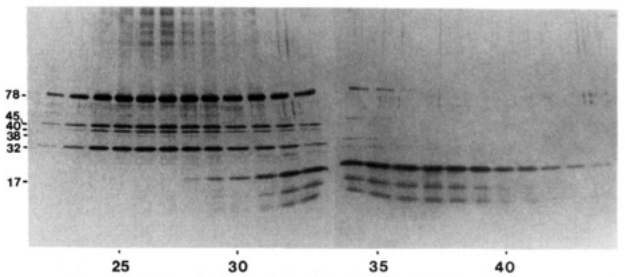


FIGURE 7: Gel filtration of the $\alpha$-chymotryptic fragments. 110K–calmodulin complex was digested in the presence of 0.2 mM $CaCl_2$ to yield fragments of 78, 45, 40, 38, and 32 kDa along with calmodulin (17 kDa) and some fragments of molecular weight less than that of calmodulin. The sample was chromatographed over Sephadex G150 Superfine, and 150-$\mu$L fractions were collected, precipitated with 10% trichloroacetic acid, separated by SDS–PAGE, and visualized by silver stain. Fraction numbers are indicated at the bottom. The major fragments are identified at the left by their molecular weight ($\times 10^{-3}$).

dulin complex with $\alpha$-chymotrypsin in buffer containing either $CaCl_2$ or EGTA to determine which proteolytic fragments contain the catalytic site (Figure 5). The 90-kDa product generated by proteolysis in the presence of EGTA retains the UTP label (Figure 5B). Controlled proteolysis in the presence of $CaCl_2$ resulted in labeling of the 90-, 78-, 38-, and 32-kDa fragments (Figure 5B). Binding assays were performed with the photoaffinity-labeled fragments to investigate the relationship between the nucleotide and actin-binding sites (Figure 6). The 78-kDa fragment, 45-kDa fragment, and ~60% of the 32-kDa fragment pelleted with F-actin; the 40- and 38-kDa fragments did not associate with the actin filaments.

We have used gel filtration (Figure 7) and sucrose gradients (with identical results; data not shown) in order to investigate the physical properties of the fragments. The objective was to determine whether different cleavage fragments can self-associate in a noncovalent manner. If so, then a fragment lacking the actin-binding site could cosediment with actin. Analysis of the fragments by gel filtration and sucrose gradients shows that the calmodulin chains can be resolved from the major fragments of the 110-kDa polypeptide, confirming our original finding that calmodulin is not associated with the 78-, 45-, 40-, 38-, or 32-kDa fragments. The 78-, 45-, 40-, 38-, and 32-kDa fragments migrated together, indicating that the fragments smaller than 78 kDa are cleavage products of the 78-kDa polypeptide and remain tightly associated through noncovalent bonds.

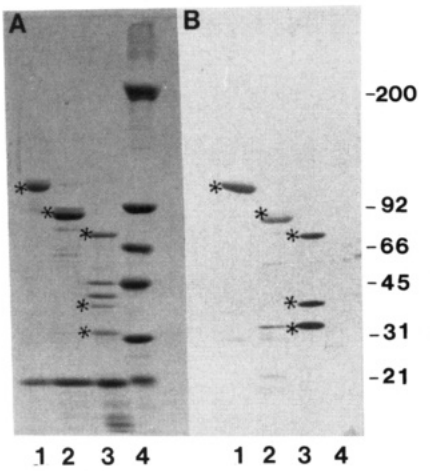


FIGURE 8: Immunoblot of the $\alpha$-chymotryptic fragments using rabbit anti-110-kDa antiserum. Lane 1, 110K–calmodulin complex; lane 2, sample resulting from cleavage of the 110K–calmodulin complex in the presence of 1 mM EGTA, predominantly the 90-kDa–calmodulin complex; lane 3, sample resulting from cleavage of the 110K–calmodulin complex in the presence of $Ca^{2+}$; lane 4, molecular weight markers ($\times 10^{-3}$). (Panel A) Coomassie blue stained gel; (panel B) immunoblot. The polyclonal antibodies recognize the 110-kDa polypeptide and the 90-, 78-, 38-, and 32-kDa fragments. The asterisks indicate corresponding positions on each of the panels.

The 110K–calmodulin complex, the 90-kDa–calmodulin complex, and the cleavage sample containing primarily the 78-kDa fragment (together with smaller polypeptides) were analyzed by electrophoresis, transferred electrophoretically to nitrocellulose, and then immunoblotted with a polyclonal antibody raised against the 110-kDa polypeptide chain. The 110-, 90-, 78-, 38-, and 32-kDa fragments were recognized by the antiserum; however, the 45- and 40-kDa fragments were not (Figure 8). This indicates that the 38- and 32-kDa fragments are likely related and are contained in the larger fragments of 78 and 90 kDa.

## Discussion

Earlier studies have shown that ATP or GTP (Matsudaira & Burgess, 1979), CTP or ITP (Verner & Bretscher, 1985), can dissociate the 110K–calmodulin complex from microvillar cytoskeletons. In addition, studies on the isolated 110K–calmodulin complex have shown that the 110K–calmodulin complex will hydrolyze ATP (Collins & Borysenko, 1984; Conzelman & Mooseker, 1987; Krizek et al., 1987) as well as GTP, UTP, CTP, and ITP (Conzelman & Mooseker, 1987). The evidence presented here indicates that isolated 110K–calmodulin complex can hydrolyze UTP and validates the use of [5,6-$^3$H]UTP as a photoaffinity label for the nucleotide-binding site found on the 110-kDa polypeptide. Our selection of [5,6-$^3$H]UTP rather than [$\alpha$-$^{32}$P]ATP as a marker for the nucleotide-binding site was influenced by a previous report that indicated that UTP bound more efficiently than ATP to *Acanthamoeba* myosin 1A (Maruta & Korn, 1981). By direct photoaffinity labeling of the nucleotide-binding site and through actin-binding assays, we can assign the relative positions of the ATP- and actin-binding domains within the 110-kDa polypeptide.

We showed earlier that incubation of the 110K–calmodulin complex with $\alpha$-chymotrypsin in buffer containing EGTA results in the generation of a 90-kDa–calmodulin complex.

The 90-kDa–calmodulin complex can also be formed from the 110K–calmodulin complex when bound to F-actin, indicating that the cleavage site involved in generation of the 90-kDa polypeptide is still accessible to the α-chymotrypsin. The 90-kDa–calmodulin complex exhibits characteristics indistinguishable from the parent complex in terms of its ability to bind F-actin reversibly in the presence of ATP, and to hydrolyze ATP (Coluccio & Bretscher, 1988). As would be predicted by these functional criteria and predicted amino acid sequence (Garcia et al., 1989), photoaffinity labeling now indicates that the 90-kDa polypeptide contains the nucleotide-binding site. Interestingly, no further cleavage of the 90-kDa–calmodulin complex occurs in the absence of $CaCl_2$, whether bound to F-actin or not.

Conversely, in the presence of $CaCl_2$, the 110-kDa polypeptide is cleaved into a 78-kDa fragment that has ATPase activity and contains the ATP-reversible F-actin-binding site (Coluccio & Bretscher, 1988). Further α-chymotryptic cleavage of the calmodulin-free 78-kDa fragment results in the appearance of major fragments of 45, 40, and 32 kDa. A careful analysis of the time course of the appearance of these fragments suggests that the 40-kDa fragment is derived from the 45-kDa fragment (Figure 1). These cleavage sites are protected when the 78-kDa fragment is bound to F-actin. The smaller fragments comigrate with the 78-kDa fragment on gel filtration columns and on sucrose gradients, indicating that they remain associated and have physical properties similar to the 78-kDa fragment. We have tried to dissociate the fragments that bind actin filaments indirectly by increasing the salt concentration. In an earlier study, we showed that the 110K–calmodulin complex will associate with actin in 0.6 M KCl (Coluccio & Bretscher, 1988). Actin binding of the 45- and 32-kDa fragments was not reversed in the presence of 0.6 M KCl (data not shown). This indicates a tight association between the cleavage products and perhaps explains why our earlier attempts to separate them by ion-exchange chromatography were unsuccessful.

The 38- and 32-kDa fragments of the 110-kDa polypeptide contain the UTP-binding site and are recognized by polyclonal antibodies to the 110-kDa polypeptide (Figure 8), whereas the 45- and 40-kDa fragments are not labeled by UTP and are not recognized by the antibodies. This suggests that the four fragments represent two related sets of peptides: the 38- and 32-kDa peptides and the 45- and 40-kDa peptides.

From the kinetics of proteolysis and the functional studies described here, a tentative model of the positioning of the major α-chymotryptic fragments within the 110-kDa polypeptide can be made (Figure 9). α-Chymotrypsin cleaves the 78-kDa fragment 32 kDa from one end of the molecule to form a complex consisting of the 32- and 45-kDa fragments. The 45 kDa can be cleaved to 40 kDa with a subsequent loss in the ability to bind F-actin. The F-actin-binding site is envisaged as being destroyed by α-chymotrypsin to generate the 40-kDa fragment, a site that is protected when the 78-kDa intact peptide is bound to F-actin. The digest mixture is therefore predicted to contain two major complexes: the 32-kDa/45-kDa complex that contains the ATP-binding site in the 32-kDa domain and the F-actin-binding domain in the 45-kDa fragment, and the 32-kDa/40-kDa complex that has the ATP-binding site in the 32-kDa fragment and the 40 kDa that does not bind F actin. This is consistent with the observation that only part of the labeled 32-kDa fragment will associate with F-actin in sedimentation assays (Figure 6).

Following our initial study of the proteolytic fragments resulting from incubation with α-chymotrypsin, Carboni and

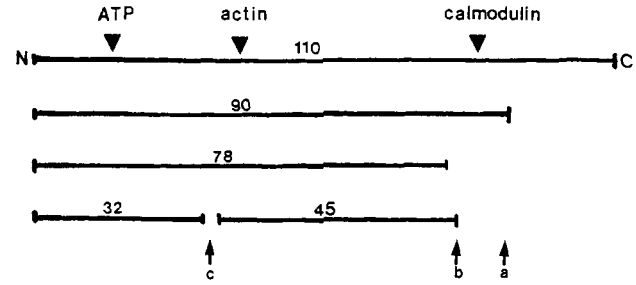


FIGURE 9: Proposed model for the organization of the α-chymotryptic fragments within the 110-kDa polypeptide. The 110-kDa polypeptide is cleaved to 90 kDa without affecting the ability of the chain to bind calmodulin. Further cleavage to the 78-kDa fragment results in loss of the ability to bind calmodulin, indicating that some site(s) within the 12-kDa fragment is (are) required for binding the calmodulin molecules. Further cleavage of the 78-kDa fragment results in fragments of 32 and 45 kDa which remain associated even in the presence of reducing agent. The nucleotide-binding site is present on the 110-kDa polypeptide and the 90-, 78-, and 32-kDa fragments. The sedimentation with F-actin of some of the 32 kDa is a consequence of its association with the 45-kDa fragment which contains the site involved in actin binding. Cleavage sites are indicated as follows: a, accessible in EGTA ± F-actin; b, accessible in $Ca^{2+}$ ± F-actin; c, protected by F-actin and not accessible in the 90-kDa–calmodulin complex in the absence of $CaCl_2$.

colleagues (Carboni et al., 1988) presented a study using monoclonal antibodies to examine tryptic cleavage fragments of the 110-kDa polypeptide. They identified a 36-kDa fragment which contains the nucleotide-binding site, but has no actin-binding activity, and cross-reacts with antibodies prepared against the nucleotide-binding site of myosin $S_1$. It is likely that the 32- and 38-kDa α-chymotryptic cleavage fragments described here are functionally similar to the 36-kDa tryptic fragment described by Carboni et al. (1988).

Peptide sequence analysis of the 32-kDa α-chymotryptic fragment suggests that it contains a blocked amino terminus, implying that the 32-kDa (and overlapping 38-kDa) fragments are derived from the amino-terminal end of the molecule (Figure 9). Our results indicate that the 32-kDa fragment contains the nucleotide-binding site. That the nucleotide-binding site is near the 110-kDa polypeptide's amino terminus is predicted by the amino acid sequence derived from the partial cDNA clone of the 110-kDa polypeptide recently published by Garcia and colleagues (Garcia et al., 1990). Within this predicted amino acid sequence for the 110-kDa polypeptide is the consensus sequence for nucleotide binding [see Warrick and Spudich (1987)] beginning at amino acid position 59. A similar consensus sequence for nucleotide binding has also been found in *Acanthamoeba* myosin 1's (Hammer et al., 1986; Jung et al., 1987, 1989b), *Dictyostelium* myosin 1's (Jung et al., 1989a), and a bovine cDNA clone corresponding to the 110-kDa polypeptide (Hoshimaru & Nakanishi, 1987).

To position the 45-kDa fragment produced by proteolysis with α-chymotrypsin within the intact molecule, we have obtained partial sequence of the fragment. The first seven amino acids at the amino terminus of the 45-kDa fragment produced by α-chymotrypsin are Gln-Ara-Ser-Gly-Met-Glu-Ala. This corresponds to amino acid 241 of the sequence predicted for the chicken protein (Garcia et al., 1989) and position 283 in the bovine sequence (Hoshimaru & Nakanishi, 1987). In both cases, this sequence is preceded by phenylalanine, an α-chymotryptic cleavage site. The amino acid sequence deduced from the chicken clone predicts that this region of the molecule contains the actin-binding site. Since the 45-kDa fragment binds actin, our results confirm the arrangement of the functional domains predicted by the derived amino acid se-

quence. On the basis of all our studies of the proteolytic fragments, we suggest that the 78-kDa amino-terminal domain of the 110-kDa polypeptide is functionally similar to the amino-terminal, or $S_1$, domain of myosin.

This similarity is extended to include our finding that the 32- and 45-kDa fragments remain associated after cleavage. Proteolytic attack of the $S_1$ fragment of myosin also cleaves the polypeptide chain with the resulting fragments remaining tightly associated (Muhlrad & Morales 1984; Burke & Kamalakannan, 1985).

Although the similarities between the 78-kDa fragment of the 110K–calmodulin complex and the $S_1$ fragment of myosin, both in sequence (Garcia et al., 1989) and in structure, are striking, there are some notable differences. The 78-kDa fragment has no associated light chains, and the biological activity is unknown. In addition, our results demonstrate a structural change in the molecule induced by the presence of $Ca^{2+}$. In the absence of $Ca^{2+}$, the 110-kDa polypeptide is cleaved by $\alpha$-chymotrypsin to 90 kDa; however, in the presence of $Ca^{2+}$, it is cleaved to an amino-terminal 78-kDa fragment and then at a site 32 kDa from the amino terminus. This second cleavage site is not available in the 90-kDa–calmodulin complex and is protected in the 78-kDa fragment when bound to F-actin. Therefore, $Ca^{2+}$ must induce a structural change that exposes this cleavage site in the $S_1$-like portion of the molecule. It may be that the 12-kDa fragment of the polypeptide (generated from proteolysis of the 90-kDa fragment to the 78-kDa fragment) with its associated calmodulins (Coluccio & Bretscher, 1988) confers this $Ca^{2+}$ sensitivity.

A number of properties of the 110K–calmodulin complex have been found to be regulated by $Ca^{2+}$ concentration. In addition to the interaction of the complex with F-actin and the interaction of the 110-kDa polypeptide with calmodulin (Coluccio & Bretscher, 1987), the mechanochemical activity of the isolated complex shows a $Ca^{2+}$ sensitivity. This was most recently described by Collins and colleagues (Collins et al., 1990), who demonstrated that in vitro translocation of F-actin by isolated 110K–calmodulin complex immobilized on coverslips is inhibited in buffers containing 0.1 mM $CaCl_2$ or higher. They attribute this to the loss of calmodulin molecules observed in the presence of calcium (Coluccio & Bretscher, 1987).

The work described here demonstrates that the ATP-binding site and the F-actin-binding site reside at the amino terminus whereas from previous work we know that the calmodulin molecules reside in the terminal third of the 110-kDa polypeptide. Positioning of the ATP-binding site and F-actin-binding site is also predicted from the deduced amino acid sequence determined by Garcia et al. (1989). Electron microscopy has indicated that the 110K–calmodulin complex has a globular head and a 12-nm tail region (Conzelman & Mooseker, 1987). The prediction would be that the globular head contains the actin-binding and ATP-binding sites whereas the calmodulin molecules are associated with the tail region. Nevertheless, it appears that the presence or absence of calmodulin can regulate the activity at the other end of the molecule in terms of both susceptibility to proteolytic cleavage and motility. This structural analysis is the first evidence that the non-$S_1$-like region of the 110-kDa polypeptide can be regulated by the rest of the molecule.

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