The Major Myosin-binding Domain of Skeletal Muscle MyBP-C (C Protein) Resides in the COOH-terminal, Immunoglobulin C2 Motif

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Abstract. A common feature shared by myosin-binding proteins from a wide variety of species is the presence of a variable number of related internal motifs homologous to either the Ig C2 or the fibronectin (Fn) type III repeats. Despite interest in the potential function of these motifs, no group has clearly demonstrated a function for these sequences in muscle, either intra- or extracellularly. We have completed the nucleotide sequence of the fast type isoform of MyBP-C (C protein) from chicken skeletal muscle. The deduced amino acid sequence reveals seven Ig C2 sets and three Fn type III motifs in MyBP-C. α-chymotryptic digestion of purified MyBP-C gives rise to four peptides. NH_2-terminal sequencing of these peptides allowed us to map the position of each along the primary structure of the protein. The 28-kD peptide contains the NH_2-terminal sequence of MyBP-C, including the first C2 repeat. It is followed by two internal peptides, one of 5 kD containing exclusively spacer sequences between the first and second C2 motifs, and a 95-kD fragment containing five C2 domains and three fibronectin type III motifs. The C-terminal sequence of MyBP-C is present in a 14-kD peptide which contains only the last C2 repeat. We examined the binding properties of these fragments to reconstituted (synthetic) myosin filaments. Only the COOH-terminal 14-kD peptide is capable of binding myosin with high affinity. The NH_2-terminal 28-kD fragment has no myosin-binding, while the long internal 100-kD peptide shows very weak binding to myosin. We have expressed and purified the 14-kD peptide in *Escherichia coli*. The recombinant protein exhibits saturable binding to myosin with an affinity comparable to that of the 14-kD fragment obtained by proteolytic digestion (1/2 max binding at $\approx$0.5 μM). These results indicate that the binding to myosin filaments is mainly restricted to the last 102 amino acids of MyBP-C. The remainder of the molecule (1,032 amino acids) could interact with titin, MyBP-H (H protein) or thin filament components.

A comparison of the highly conserved Ig C2 domains present at the COOH-terminus of five MyBPs thus far sequenced (human slow and fast MyBP-C, human and chicken MyBP-H, and chicken MyBP-C) was used to identify residues unique to these myosin-binding Ig C2 repeats.

THICK filaments from striated muscle contain myosin and a unique set of associated proteins (Offer et al., 1973; Craig and Offer, 1976; Reinach et al., 1982, 1983; Starr and Offer, 1983, Bahler et al., 1985*a*,*b*; Hartzell and Glass, 1984; Cooper and Trinick, 1984; Yamamoto and Moos, 1983; Price, 1987). Myosin-binding protein-C (MyBP-C),[1] C protein, was originally identified as an abundant 140-kD contaminant present in myosin preparations (Offer et al., 1973). MyBP-C is found in the inner two-thirds of the cross-bridge bearing region of the A band, with a 43-nm axial spacing (Craig and Offer, 1976; Bennett, 1986; Dennis et al., 1984; Bahler et al., 1985*b*). It was first estimated that two molecules of MyBP-C are present at each 43-nm repeat (Offer et al., 1973) although later studies by Morimoto and Harrington (1973, 1974) indicate that the value is closer to three per stripe. The interactions of MyBP-C with myosin filaments are complex: (*a*) there are different classes of myosin binding sites of different affinities (Davis, 1988); (*b*) MyBP-C lowers the critical concentration for myosin polymerization (Davis, 1988); and (*c*) MyBP-C binds to titin, another myofibrillar protein known to bind myosin (Fürst et al., 1992). Variant isoforms of MyBP-C are present in different muscles (Reinach et al., 1982) and are expressed in a regulated program during development (Obinata et al., 1984). In some muscles more than one isoform is present in a single sarcomere (Reinach et al., 1983) or even in a single 43-nm repeat along the thick filament (Dennis et al., 1984). MyBP-C has a rod-shaped appearance with a "hinge" region

1. *Abbreviations used in this paper:* FN, fibronectin; MyBPC, myosin-binding protein-C; PM, pectoralis muscle.

when visualized by rotary shadowing in the electron microscope (Swan and Fischman, 1986; Hartzell and Sale, 1986).

A partial cDNA for the fast-type isoform of MyBP-C from chicken pectoralis muscle has been isolated (Einheber and Fischman, 1990). Its deduced amino acid sequence revealed the presence of 9 internal domains, 6 homologous to Ig C2 motifs and three to the fibronectin (Fn) type III repeats. MyBP-C is therefore a member of the Ig superfamily (Cunningham et al., 1987; Williams, 1987; Williams and Barclay, 1988). The Ig C2 and Fn III repeats are conserved in human MyBP-C (Fürst et al., 1992) and often found in other myosin-associated proteins (Ayme-Southgate, 1991; Benian et al., 1989; Olson et al., 1990; Labeit et al., 1990; Price et al., 1991; Noguchi et al., 1992; Kobayashi et al., 1992; Vaughan et al., 1993*a*,*b*). Since the location of the myosin-binding site(s) in MyBP-C has not been established, the role of C2 or fibronectin motifs in myosin binding remains uncertain.

Here we describe the α-chymotrypsin proteolytic fragments of MyBP-C, map their position along the MyBP-C sequence, which we have now completed, demonstrate the presence of a seventh IgC_2 domain and determine which fragments are capable of binding to synthetic myosin filaments. In addition, we now report the bacterial expression of the myosin-binding domain and demonstrate its functional properties in a myosin filament binding assay.

Materials and Methods

Construction and Screening of a Primer Extension Library

Using the oligonucleotide 5′-GGATGAACGCTTCGCTCTTT-3′, derived from the partial sequence of MyBP-C (Einheber and Fischman, 1990) and mRNA from 1-wk posthatch chicken pectoralis muscle (PM), a primer extension cDNA library was prepared. First and second strand synthesis, methylation, polishing of free ends, and ligation of EcoRI linkers were performed as described (Reinach and Fischman, 1985). The cDNA was digested with EcoRI and size-fractionated by column chromatography using Sepharose 4B. cDNAs exceeding 500bp were ligated into λZAP and packaged. The library was screened by plaque hybridization (Sambrook et al., 1990) using a 300-bp fragment derived from the partial cDNA for MyBP-C (bases 500-800) (Einheber et al., 1990).

DNA Sequencing

The cDNAs were subcloned into M13 mp18 and mp19 for dideoxy sequencing (Sanger et al., 1977). Both strands were sequenced.

Crude Myosin Preparation

Crude myosin was prepared from chicken skeletal muscle as described (Reinach et al., 1982).

Purification of MyBP-C

A crude myosin preparation was used to prepare MyBP-C by a combination of DEAE-cellulose and hydroxyapatite as described (Reinach et al., 1982) or by a method which avoids the degradation of MyBP-C sometimes observed in the hydroxyapatite columns. In this new procedure, a crude myosin preparation was mixed with Q-Sepharose fast flow (Pharmacia, Uppsala, Sweden), equilibrated with 0.135 M KH_2PO_4, 0.015 M K_2HPO_4 (pH 7.0), 1 mM DTT, 2 mM EDTA, and 0.5 mM PMSF (Q buffer), and placed on a Buchner funnel containing 3 mm paper (Whatman Laboratory Products, Inc., Clifton, NJ). About 50 ml of preswollen resin was required to process 100 mg of crude MyBP-C. MyBP-C did not bind to Q-Sepharose under these conditions and was collected in the flow through volume. MyBP-C was concentrated with ammonium sulfate (27–42% saturation) and resuspended in Q buffer. After dialysis against the same buffer, the sample was cleared at 100,000 *g* for 2 h and loaded on a Q-Sepharose fast flow column equilibrated with Q-buffer (16 × 300-mm run at 2 ml/min). MyBP-C and MyBP-H were collected in the flow through (Bahler et al., 1986*a*; Vaughan et al., 1993*a*). Bound myosin was eluted with Q buffer containing 0.3 M KCl, concentrated by addition of ammonium sulfate (20–45% saturation) and dialyzed against 0.6 M KCl, 20 mM Tris-Cl (pH 7.5), 2 mM EDTA, and 2 mM DTT. Column-purified myosin was mixed with an equal volume of glycerol and stored at −20°C.

The crude fraction containing MyBP-C and MyBP-H was loaded on an Affigel blue column (16 × 200-mm run at 0.5 ml/min) (Bio-Rad Laboratories, Richmond, CA), equilibrated with 0.3 M KCl, 20 mM Imidazole-HCl (pH 7.0), 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 0.5 μg/ml leupeptin. MyBP-C was eluted with a linear gradient (0.3–1.0 M KCl in the same buffer). Up to 40 μg of the fraction from the Q-Sepharose column could be separated on each run of the Affigel blue column. Fractions containing MyBP-C were transferred to a dialysis bag and immersed in solid polyethylene glycol (M_r-20,000). Concentrated MyBP-C was then dialyzed against 0.3 M KCl, 20 mM Imidazole-HCl (pH 7.0), 1 mM DTT, 0.5 mM PMSF, 0.5 μg/ml leupeptin, and 50% glycerol and stored at −20°C.

Cleavage of MyBP-C with α-Chymotrypsin

Before α-chymotryptic cleavage of MyBP-C, PMSF was removed by dialysis or passage over a Sephadex G25 column. MyBP-C (0.5–1 μg/ml) was mixed with 1/500 wt/wt of α-chymotrypsin in 0.1 M KCl, 20 mM Imidazole-HCl (pH 7.0), and incubated at 23°C. The cleavage reaction was terminated at selected time points by addition of 1 mM PMSF.

Purification of α-Chymotryptic Peptides

To purify the fragments under nondenaturing conditions, products of the cleavage reaction were fractionated on a Superose 12 FPLC column (Pharmacia; 10 × 300-mm run at 0.3 ml/min) equilibrated with 0.1 M KCl, 20 mM Imidazole-HCl (pH 7.0), 1 mM DTT, 1 mM EGTA, and 0.5 mM PMSF. Proteolytic fragments were also purified under denaturing conditions for the determination of NH_2-terminal sequences. SDS (0.1%) and DTT (0.1 mM), were added to the digest which was then dialyzed against 0.12 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 0.5 mM DTT, and fractionated by gel filtration on a Sephacryl S-200 column (20 × 850 mm) equilibrated with the same buffer. Pooled fractions containing the proteolytic fragments and intact MyBP-C were dialyzed against water, lyophilized, and sequenced using automated Edman degradation.

Expression and Purification of the 14-kD Fragment in E. coli

Two mutagenic oligonucleotides and the full-length cDNA were used in a polymerase chain reaction to generate a DNA fragment encoding the last C2 domain of MyBP-C. The first oligonucleotide (5′-TTGAATTCGCTGACCCTAACCATGGTGCCCTAT-3′) creates an NcoI site before methionine 1027 in the protein sequence (see Fig. 1) and an EcoRI site further upstream. This methionine is located three residues before the α-chymotrypsin cleavage site that generates the 14-kD fragment. The second oligonucleotide: (5′-TTAAGCTTCTCTGCGATGGGATCCTCATTG-3′) creates a BamHI site just after the stop codon and a HindIII site further downstream. A PCR reaction was carried out for 30 cycles of 63°C (15 s) 72°C (20 s) 94°C (90 s) using standard conditions (Perkin Elmer Cetus CT, Eden Prairie, MN). The 300-bp fragment was purified from an agarose gel, digested with EcoRI and HindIII and cloned in pUC19. After sequencing the insert was excised with NcoI/BamHI and cloned in NcoI/BamHI cut pET3d (Studier et al., 1990) to produce the final construct (pET-C2).

Escherichia coli strain BL-21(DE3), transfected with pET-C2, were grown to OD_{600} = 0.6 in LB media, induced with 0.4 mM IPTG, and grown for another 3–4 h. Cells were collected by centrifugation (10,000 *g*), resuspended in 50 mM Tris-Cl pH 8.0, 2 mM EDTA. Lysozyme and Triton X-100 were added to a final concentration of 100 μg/ml and 0.1%, respectively. After a 10-min incubation at room temperature; the lysate was chilled on ice and sonicated for 30 s. After centrifugation (15,000 *g*, 15 min) the pellet was washed twice with lysis buffer without lysozyme, resuspended 8 M Urea, 50 mM Tris-Cl (pH 8.0), 1 mM EDTA, and loaded onto a DEAE-cellulose column equilibrated in the same buffer. Material eluting in the void volume was collected, diluted to a concentration below 0.5 mg/ml and renatured by slow dialysis. Two changes of 50 mM Tris-Cl (pH 7.5), 0.3 M NaCl, 2 mM DTT were followed by two changes of 50 mM Tris-Cl (pH 7.5), 0.15 M NaCl, 2 mM DTT, and 0.5 mM EDTA. At this step the protein could be concentrated and stored frozen. Circular dichroism measurements of the purified and renatured recombinant peptide (0.25 mg/ml in the

preceding buffer) were performed with a Jobin-Yvon MARK 5 CD Spectrometer in the 200-250-nm UV range.

Binding of MyBP-C and MyBP-C Fragments to Myosin

Myosin stored in glycerol was dialyzed against 0.1 M KCl, 20 mM Imidazole-HCl (pH 7.0), and 1 mM DTT to obtain synthetic filaments. MyBP-C or its α-chymotryptic peptides in the same buffer were mixed with preformed myosin filaments for 10 min in a final volume of 100 μl. Filaments were sedimented in an Airfuge (Beckman Instruments, Palo Alto, CA) at 22 P.S.I. for 20 min and the pellets analyzed with SDS-PAGE. Binding of the recombinant 14-kD peptide was performed as described above but in 0.1 or 0.15 M NaCl; 10 mM Tris-Cl (pH 7.5); 1 mM DTT, 0.5 mM EDTA. Under these conditions <1% of the 14-kD fragment was sedimented in the absence of myosin filaments and >95% of the added myosin filaments were sedimented. Gels were stained with Coomassie blue and proteins quantified by densitometry. Calibration was achieved with serial dilutions of known quantities of the same proteins loaded on the same gels. Scanning of the Coomassie blue-stained gels was performed in a scanner (GS300; Hoffer, San Francisco, CA) with a GS350 data processing system.

Results

Full-length Cloning and Sequencing of MyBP-C

Sixty independent cDNA clones encoding MyBP-C were isolated to obtain a cDNA containing the complete coding sequence. Of the 60 clones, 59 started at the primer used to construct the library. One began 500-bp down-stream, possibly the result of random priming. Of the 59 clones initiated by the specific primer, 58 ended within a region of 17 nucleotides (5′-GGAGGCGCCGCCCCCAC-3′), rich in G-C. This region may have formed stable secondary structures, resulting in premature termination of the reverse transcription reaction. Since these cDNAs lacked an open reading frame which would generate the amino acid sequence found in the 28-kD α-chymotryptic fragment (see below), it was apparent that none of these clones spanned the complete coding sequence of the cDNA. A single clone extended 100 bp beyond the G-C rich region, including 25 bp of the 5′-untranslated region and a partial sequence found in both the 28-kD chymotryptic fragment and in the 128-kD fragment of MyBP-C (Table I). The overlapping COOH-terminal and NH_2-terminal cDNA clones generated a continuous nucleic acid sequence of 3,488 bases, with an open reading frame of 1,132 amino acids shown in Fig. 1. The complete cDNA sequence has been deposited in Genbank (accession number U00922). Analysis of the deduced protein sequence indicates that chicken fast-type MyBP-C cDNA is composed of seven Ig C2 motifs and three Fn type III repeats (Fig. 1); no other recognizable motifs were identified. These domains have been numbered I-X, from the NH_2- to COOH-terminus. Six C2 motifs were identified in our initial report (Einheber and Fischman, 1990) but another C2 motif has been identified in the NH_2-terminal sequence completed in the present study.

Table I. Amino Acid Sequences of C-Protein Fragments

Fragment	Sequence
128 kD	PEPSKAAXKK
28 kD	PEPSKAAPKKEAKKKEEKKEEKKE
5 kD	LKKREVQVEEKKKKKTE
14 kD	KEVDLXAAPQ

Note: The underlined residues do not agree with the cDNA sequence possibly due to inaccuracies in the protein sequence determination or allelic variation.

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   1 MPEPSKAAPK KEAKKKEEKK EEKKEAPPPQ EHKDEAPDDV HPPETPDPEG LFLSKPQNVM

  61 VESGRDVTVS ARVAGAALPC APAVKWFKGK WAELGDKSAR CRLRHSVDDD KVHTFELTIT
       <---------------------------- C2 ----------------------------

 121 KVAMGDRGDY RCEVTAKEQK DSCSFSIDVE APRSSEGNVL QAFKRTGEGK DDTAGELDFS
     ------------------------------------>

 181 GLLKKREVQV EEKKKKKDED DQFPPEIWEL LKGVTKKSEY ERIAFQYGIT DLRGMLKRLK

 241 KVHVEPKKSE AFIRKLDPAY QVDKGNKIKL VVELSDPDLP LKWYKNGQLL KPSTKYVFEN
                          <-----------------------------------------

 301 VGLKRILTIH KCSLADDAAY ECRVNDEKCF TEVFVKEPPV TVVRGLEDQQ VVVGDRVVLE
     ---------- C2 ---------------------------------->  <-----------

 361 AEVSEEGAQV MWLKDGVDVT RDDAFKYRFK KDGKKHFLII NEAELSDSAH YKIMTNGGES
     ------------------------------------ C2 ----------------------

 421 EAELSVEEKQ LEVLQDMADL TVKASEQAVF KCEVSDEKVT GRWFRNGVEV KPSKRIHISH
     ------------------><-----------------------------------------

 481 NGRFHKLVID DVRPEDEGDY TFIPDGYALS LSAKLNFLEI KVEYVPKQEP PKIHLDCSGK
     ---------- C2 ---------------------------->

 541 AAENTIVVVA GNKVRLDVPI SGEPAPTVTW KRGDQLFTAT EGRVHIDSQA DLSSFVIESA
       <---------------------------- C2 ----------------------------

 601 ERSDEGRYCI TVTNPVGEDS ATLHVRVVDV PDPPQSVRVT SVGEDWAVLS WEAPPFDGGM
     ------------------------------>  <==============================

 661 PITGYLMERK KKGSMRWMKL NFEVFPDTTY ESTKMIEGVF YEMRVFAVNA IGVSQPSLNT
     ========== FN ================================================

 721 QPFMPIAPTS EPTHVVLEDV TDTTATIKWR PPERIGAGGV DGYLVEWCRE GSNEWVAANT
     ========>   <======================== FN ========================

 781 ELVERCGLTA RGLPTGERLL FRVISVNMAG KSPPATMAQP VTIREIVERP KIRLPRHLRQ
     ================================================>

 841 TYIRRVGEQV NLVIPFQGKP RPQVTWSREG GALPAEVQTR TSDVDSVFFI RSAARPLSGN
       <---------------------------- C2 ----------------------------

 901 YEMRVRIDNM EDCATLRLRV VERPGPPQAV RVMEVWGSNA LLQWEPPKDD GNAEISGYTV
     ----------------------> <==================================

 961 QKADTRTMEW FTVLEHSRPT RCTVSELVMG NEYRFRVYSE NVCGTSQEPA TSHNTARIAK
     ================================== FN ========================

1021 EGLTLKMVPY KERDLRAAPQ FLTPLVDRSV VAGYTVTLNC AVRGHPKPKV TWLKNSVEIG
     >                          <**********************************

1081 ADPKFLSRHG LGVLSLLIRR PGPFDGGTYG CRAVNEMGEA TTECRLDVRV PQ
     ********************************** C2 *****************>
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Figure 1. Complete amino-acid sequence of chicken MyBP-C and the location of the Ig C2 and Fn type III repeats. The bold amino acid sequences indicate those partial sequences obtained from the α-chymotryptic fragments of MyBP-C (Table I). Boundaries of the immunoglobulin C2 repeats (<---->), the fibronectin type III repeats (<===>), and the last C2 repeat (<***>) are marked. The complete cDNA sequence data has been deposited in the EMBL/GenBank databank under accession number U0092.

Characterization of MyBP-C Fragments

Chicken fast-type MyBP-C, from pectoralis muscle was digested with α-chymotrypsin, generating four proteolytic fragments with relative mobilities of 96, 28, 14, and 5 kD, respectively (Fig. 2 *a*). These four fragments were separated by gel filtration (Fig. 2 *b*) and purity assessed by SDS-PAGE (Fig. 2 *a*). Purified fragments of 28, 14, and 5 kD were partially sequenced (Table I) and then positioned along the predicted sequence established from the cDNA (Fig. 1). We then performed a time course study of chymotryptic digestion to obtain products of partial digestion that could be used for mapping the myosin-binding domain within the protein (Fig. 3, *A* and *B*). Within a minute of initiating the reaction, four peptides of 128, 100, 28, and 14 kD were detected. The 14-kD fragment increased in quantity during the first 5 min and then plateaued. The gradual disappearance of the large 128-kD fragment paralleled the appearance of 100- and 28-kD fragments. After 15 min, three major peptides (100, 28, and 14 kD) of approximately equal molar ratios could be detected. The 100-kD fragment was subsequently cleaved into two smaller products of 5 and 95 kD (Fig. 3, *a* and *b*). These results indicate that the site between the 95- and the 14-kD fragments is the first to be cleaved, followed by the 28/5-kD site and finally by the 5/95-kD site (Fig. 3, *c*). The partial sequence of the 128-kD fragment (Table I) is identical to the partial sequence obtained from the 28-kD fragment, confirming our assignment of the 28-kD fragment to the NH_2-terminus of the molecule, and the 14-kD peptide to the

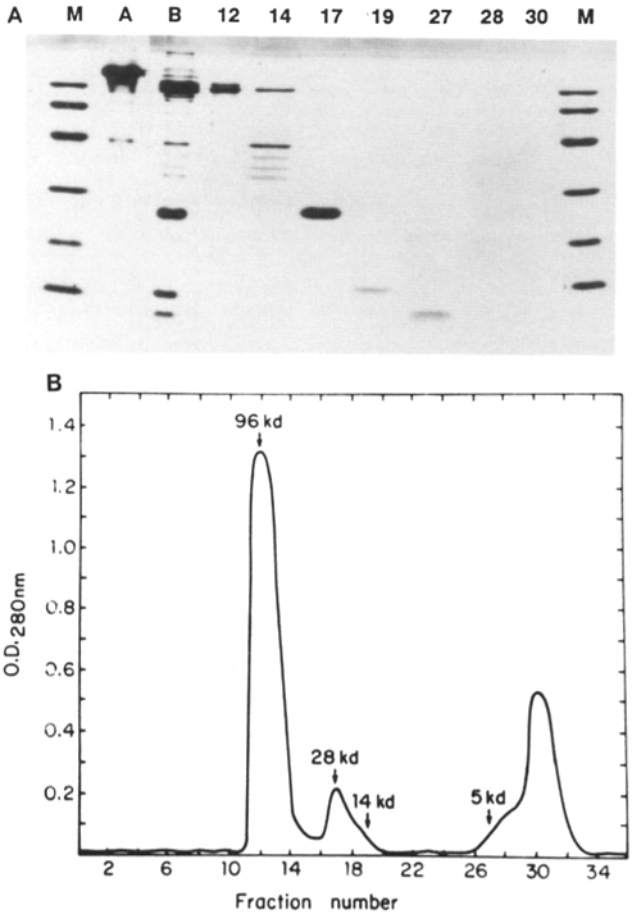

Figure 2. Purification of the α-chymotryptic fragments of MyBP-C. (*A*) SDS-PAGE of MyBP-C fractions recovered during isolation of the proteolytic fragments. Lane *M*, molecular weight markers (94, 68, 42, 30, 20, and 14 kD); lane *A*, purified chicken pectoralis muscle MyBP-C; lane *B*, purified MyBP-C after digestion with α-chymotrypsin; lanes *12–30*, samples of the column fractions with the corresponding fraction numbers. (*B*) Elution profile of α-chymotryptic fragments fractionated on a Sephacryl S-200 column. The elution position of the different fragments are indicated. Fraction numbers are the same as in *A*.

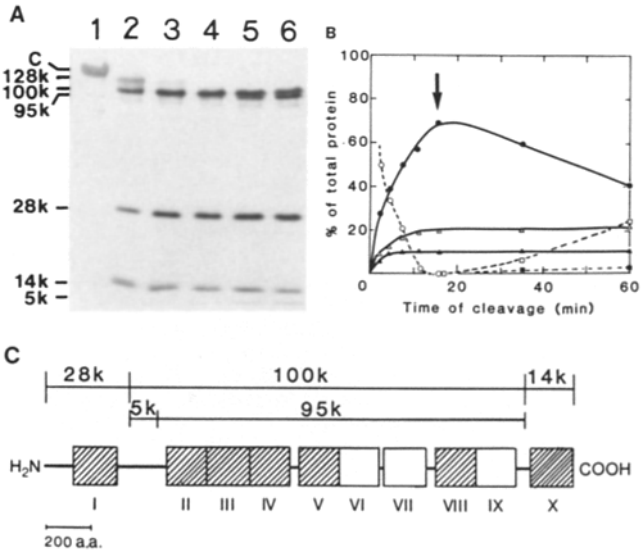

Figure 3. (*A*) SDS-PAGE analysis of the time course of MyBP-C cleavage with α-chymotrypsin. Lanes *1* to *6* show MyBP-C cleaved for 0, 4, 8, 16, 30, and 60 min. (*B*) The relative amount of each fragment generated during digestion was determined by densitometry of Coomassie blue stained gels the data plotted as a function of digestion time. 128-kD fragment (○, ---); 100-kD fragment (●); 95-kD fragment (□, ---); 28-kD fragment (△); 14-kD fragment (▲); 5-kD fragment (■, ---). Observe that after 15 min of cleavage, only three peptides (100, 28, and 14 kD) are present (*arrow*). (*C*) Schematic illustration of MyBP-C showing the internal Ig C2 (▨) and Fn type III (□) repeats and their relation to the proteolytic fragments.

COOH-terminus. The relative molecular masses of the α-chymotryptic fragments were established by SDS-PAGE. The sum of the relative masses of the four peptide came to 142 kD and matched the relative molecular weight of MyBP-C purified from chicken PM. The few mismatches between the deduced amino acid sequences and the peptide sequences (Table I) may reflect inaccuracies in the Edman peptide sequencing or allelic variations. Taken together, these data indicate that the order of the α-chymotryptic fragments in MyBP-C is: N-28 kD-5 kD-95 kD-14 kD-C.

The distribution of each fragment along the sequence is indicated in Fig. 1 and their positions within the domain structure of the molecule are shown in Fig. 3 *c*. Interestingly, all cleavage sites are located between domains, the 5-kD fragment containing only "linker" sequences between domains I and II (Fig. 3 *c*).

To further characterize the COOH-terminal Ig C2 domain of MyBP-C the last 106 residues of the chicken protein were expressed in *E. coli*. The fragment is expressed in large amounts and accumulates in inclusion bodies (Fig. 4). After purification the protein was renatured from urea in the presence of high salt and reducing agents as described for the troponin C and troponin I (Reinach and Karlsson, 1988; Quaggio et al. 1993). Up to 50 mg of pure recombinant 14-kD fragment could be obtained per liter of bacterial culture.

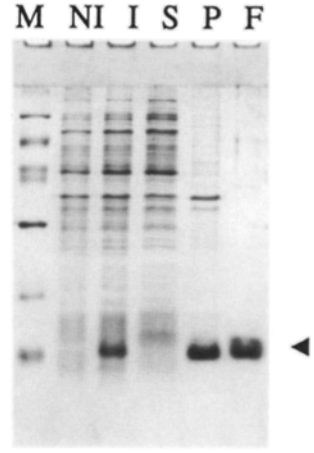

Figure 4. SDS-PAGE analysis of the protein fractions obtained during the purification of the recombinant 14-kD fragment. Total protein extract of *E. coli* BL21(DE3) containing the plasmid pET-C2 before induction (*NI*) and after induction (*I*) show the accumulation of the recombinant protein (*arrow*). After lysis the recombinant protein is found only in the pellet (*P*), none of the protein being present in the supernatant (*S*). The final product after DEAE-cellulose chromatography is shown in *F*. Molecular weight markers of 96, 68, 43, 24, 20, and 14 kD are shown in *M*.

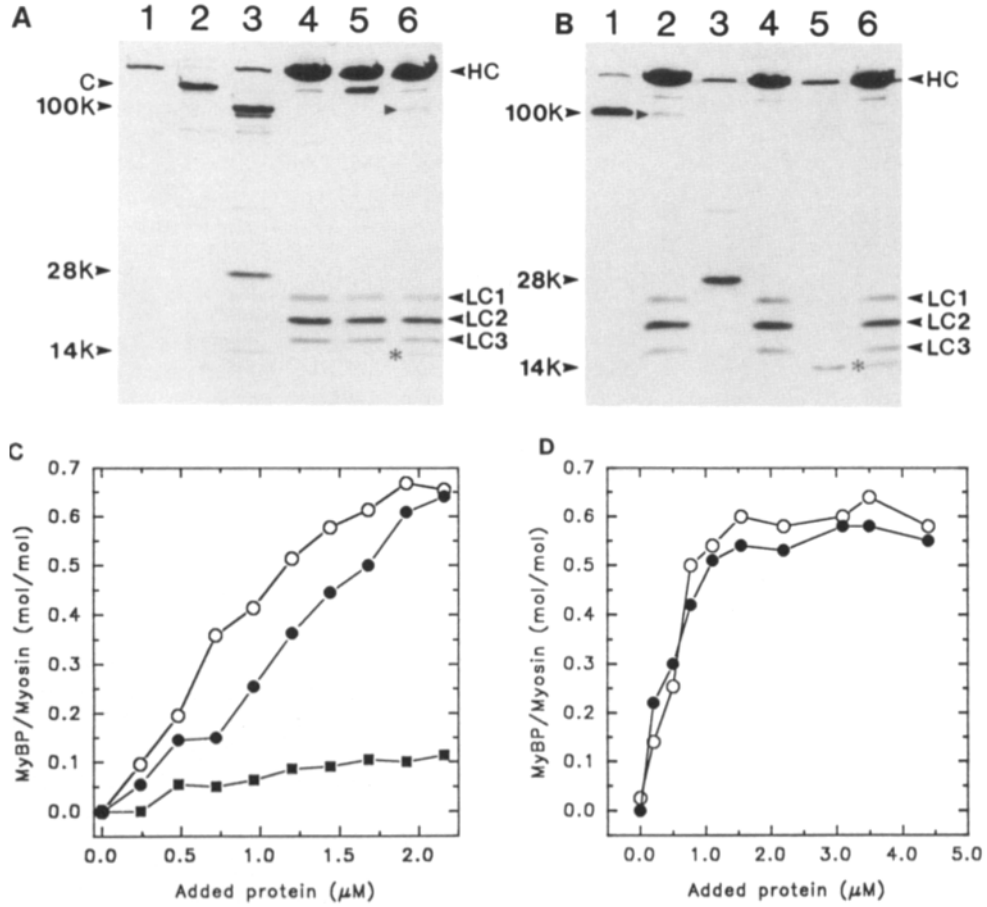

Figure 5. Binding of MyBP-C and MyBP-C fragments to synthetic myosin filaments. Fractions containing different combinations of myosin filaments, MyBP-C and the fragments of MyBP-C were sedimented in an air fuge. The supernatant and pellet of each fraction was analyzed using SDS-PAGE. The position of the myosin heavy chain (*HC*), myosin light chains (LC1, LC2, and LC3), MyBP-C (*C*), and the 100-, 28-, and 14-kD fragments (*100k*, *28k*, and *14k*) are indicated. (*A*) Supernatants (lanes *1*, *2*, and *3*) and pellets (lanes *4*, *5*, and *6*) of mixtures containing only 1.3 μM myosin filaments (lanes *1* and *4*) or 1.3 μM myosin and 1.8 μM intact MyBP-C (lanes *2* and *5*) or 1.3 μM myosin and 1.8 μM of a total digest of MyBP-C (lanes *3* and *6*) are shown. In lane *5* observe the precipitation of intact MyBP-C. In lane *6* observe that the 14-kD fragment is sedimented with the myosin filaments (*) but only a small amount of the 100-kD fragment cosediments with the myosin filaments (*arrowhead*). (*B*) Supernatants (lanes *1*, *3*, and *5*) and pellets (lanes *2*, *4*, and *6*) of mixtures containing 2.2 μM myosin filaments and 2.2 μM purified 100-kD (lanes *1* and *2*) or 2.2 μM myosin filaments and 2.2 μM purified 28-kD fragment (lanes *3* and *4*) or 2.2 μM myosin and 2.2 μM purified 14-kD fragment (lanes *5* and *6*) are shown. In lane *2* observe that only a small fraction of the 100-kD fragment binds to the filaments (*arrowhead*). In lane *6* note that almost half of the 14-kD fragment co-precipitates with the myosin filaments. (*C* and *D*) Binding curves of intact MyBP-C and MyBP-C fragments to myosin filaments were obtained as described under Materials and Methods. In *C*, binding of intact MyBP-C (○), purified 14-kD fragments obtained by proteolytic digestion of MyBP-C (●), and 100-kD fragment obtained by proteolytic digestion of MyBP-C (■) were measured in independent experiments. In these experiments myosin concentration was kept at 2.2 μM. In *D*, binding of recombinant 14-kD fragment was measured in either 0.1 M NaCl (○) or 0.15 M NaCl (●). Myosin concentration was kept at 1.5 μM and the concentration of 14-kD fragment was increased from zero to 4.4 μM (a threefold molar excess over myosin) to demonstrate saturation.

Binding of MyBP-C and MyBP-C Fragments to Myosin Filaments

We next examined the binding of MyBP-C and its α-chymotryptic fragments to synthetic myosin filaments using a centrifugation assay. Fig. 5 *A* shows the protein composition of supernatant and pellet fractions after intact MyBP-C or the chymotryptic digests are mixed with myosin filaments for 10 min at a 1:1 molar ratios and then sedimented. A large fraction of the MyBP-C cosediments with the myosin filaments. Densitometric analysis of the pellet reveal a MyBP-C to myosin ratio of 0.65:1 (Fig. 5 *A*). The fraction of bound MyBP-C is not increased by longer incubations with the myosin filaments. Using a total α-chymotryptic digest of MyBP-C, only the 14-kD COOH-terminal fragment shows significant cosedimentation with myosin (Fig. 5 *A*). The NH_2-terminal 28-kD peptide, which also contains a single Ig C2 repeat, had no detectable myosin binding. The large internal 100-kD peptide exhibits very low binding to myosin filaments (Fig. 5 *A*). To confirm the binding of the different fragments to myosin and to exclude the possibility that the binding of the 14-kD fragment was interfering with the binding of other fragments to the filaments, we mixed purified fragments, isolated under nondenaturing conditions, with the myosin filaments. The results were identical to those obtained with the total digests (Fig. 5 *B*), excluding the possibility that binding of the 14-kD peptide competes for a common binding site with the other fragments.

Binding curves of intact MyBP-C and the fragments to myosin filaments were obtained at a myosin concentration of 2.2 μM. At 1:1 myosin/MyBP-C (2.2 μM of intact MyBP-C) (Fig. 5 *C*), a stoichiometry of 0.7 moles of MyBP-C per mole of myosin was observed in the pellets. A similar stoichiometry (0.65 moles fragment/mole of myosin) was obtained with the 14-kD fragment, at near saturating conditions (Fig. 5 *C*). The binding of the 100-kD fragment to myosin filaments was very weak (Fig 5 *C*).

Because of difficulties in obtaining sufficient 14-kD fragment by proteolytic digestion of native MyBP-C, we used a recombinant 14-kD fragment to repeat the binding curves at high 14-kD peptide concentrations (Fig. 5 *D*). The results were similar to the ones obtained with the proteolytic fragment/saturation occurring at a 0.6:1 (moles of fragment/mol of myosin) with an apparent dissociation constant of ~0.5 μM. These data, showing that saturation occurs at molar ratios of 0.65 to 0.7 moles of 14-kD COOH-terminal proteolytic peptide or recombinant 14-kD fragment per mole of myosin is in agreement with previous measurements performed with purified rabbit MyBP-C (Moos et al., 1975). The concentration of MyBP-C and 14-kD peptide necessary to obtain half-saturation also agrees with previous studies using purified rabbit MyBP-C (Moos et al., 1975; Yamamoto and Moos, 1983). To assess the secondary structure of the renatured, recombinant peptide, we performed circular dichroism measurements. As expected from the high sequence homology of this domain with the Ig C2 domain of telokin, the CD analysis demonstrated ~30% β sheet, 30% turns, and 40% random structure; no α-helix was detected (data not shown). These results suggest that the peptide has refolded appropriately.

The fact that the 14-kD fragment retains most of the myosin-binding properties found in the intact MyBP-C molecule suggests that the major high affinity myosin-binding site of MyBP-C is restricted to a single C2 immunoglobulin repeat located in the last 102 amino acids of the MyBP-C molecule.

Two series of preliminary experiments were conducted to test whether the recombinant 14-kD peptide was capable of competing with MyBP-C for myosin binding. In both cases the results were negative (data not shown). In the first set, "crude" myosin, i.e., myosin before ionic exchange chromatography, at 2.2 μM, was mixed in 0.3 M KCl with 14-kD peptide at varying concentrations (0.1–2.0 μM). (The MyBP-C concentration in the crude myosin preparation was ~1/8th the molar concentration of myosin [~0.3 μM].) After reduction of the KCl to 0.1 M, the samples were incubated on ice for 15 min and spun at 22 P.S.I. in the airfuge as described for the binding assay above. The supernatant and pellets were then examined by SDS-PAGE and Coomassie blue staining. In none of three independent experiments was there any release of MyBP-C into the supernatant fractions from the myosin filament pellets. The second set of experiments was designed to test whether other thick filament–associated proteins, e.g., MyBP-H and titin, which were also present in the crude myosin preparations, could have affected the binding of MyBP-C to the myosin filaments. A separate set of experiments was conducted with column-purified myosin, MyBP-C, and 14-kD peptide. For these latter experiments, myosin filaments (1.5 μM) in the 0.1 M KCl-imidazole-DTT binding buffer were incubated with MyBP-C (1.5 μM) and varying concentrations of 14-kD peptide (0–20 μM) for 15 min on ice, and then pelletted in the airfuge. Pellets and supernatants were displayed by SDS-PAGE and stained with Coomassie blue. Again, no release of MyBP-C into the supernatant fractions was observed at any of these concentrations of the 14-kD peptide.

Discussion

We have completed the sequence of the fast isoform of chicken skeletal muscle MyBP-C and used this sequence to map the position of four α-chymotryptic fragments within the primary sequence. Then, using a myosin filament-binding assay, we established that only the 14-kD fragment, located in the COOH-terminal end of the molecule and comprising a single Ig C2 repeat, is capable of binding myosin with high affinity, ~0.5 μM. This result was confirmed using a recombinant 14-kD peptide produced in *E. coli*. This is the first direct demonstration of a functional role for these motifs in a myosin-associated protein. These data do not exclude the possibilities that secondary binding sites may exist in the intact molecule, or that these may contribute to the complex binding interactions with myosin filaments (Davis, 1988). The failure to release MyBP-C from reconstituted crude or pure myosin filaments with recombinant 14-kD peptide, even at 15-fold excess of the recombinant peptide, suggests that the interactions of MyBP-C with myosin filaments are complex. This is not unexpected: immunolocalizations of MyBP-C show that only a fraction (from 45–50%, depending on the muscle fiber type) of the 43-nm axial repeats in the C zone of the A band contain MyBP-C (Dennis et al., 1984). It has also been shown that a single sarcomere and single 43-nm repeat can contain more than one MyBP-C isoform (Reinach et al., 1983). Furthermore, the localization of a single isoform may differ in different fiber types (Reinach et al., 1983). These results indicate that that the interaction of MyBP-C must differ along the filament. This notion is supported by Davis's experiments (1987) demonstrating four separate classes of C protein: myosin filament binding sites which differ in affinity. Those experiments were interpreted as implying a role of MyBP-C in myosin polymerization and filament length regulation. Finally, MyBP-C is known to bind to titin (Fürst et al., 1992) and interactions may exist with other thick filament–associated proteins, e.g., MyBP-H. Thus, it is unlikely to expect that the 14 kD: myosin interaction can explain all of the associative properties between MyBP-C and thick filaments.

The production of a recombinant form of this Ig C2 repeat in *E. coli* should allow the mapping of the myosin binding surface in this domain and the subsequent identification of the Ig C2 binding site in the myosin rod.

The presence of both Ig C2 and Fn type III repeats in protein sequences was first observed in cell surface molecules involved in cell–cell and cell–substrate adhesive interactions (Cunningham et al., 1987; Williams and Barclay, 1988) but was soon demonstrated in growth factor receptors (Yarden and Ullrich, 1988), and proteins involved in viral binding and parasitic uptake (Ockenhouse et al., 1992). In each of these molecules, the repeats have been limited to extracellular compartments. Over the past two years, cDNA cloning and primary sequence comparisons have shown that an increasing number of intracellular, myosin-associated proteins in both vertebrates and invertebrates exhibit a similar repeat structure. These include: twitchin in *C. elegans* (Benian et al., 1989), chicken fast type MyBP-C (Einheber and Fischman, 1990), chicken MyBP-H (Vaughan et al., 1993*a*), chicken M protein (Noguchi et al., 1992), mouse skelemin (Price et al., 1991), rabbit titin (Labeit et al., 1990), *Drosophila* projectin (Ayme-Southgate et al., 1991; Fyrberg et al., 1992), human fast and slow type MyBP-C (Fürst et al., 1992), human MyBP-H (Vaughan et al., 1993*b*), and smooth muscle myosin light chain kinase (MLCK) of chicken and bovine (Olson et al., 1990; Kobayashi et al., 1992) sources. Although most members of this protein family bind myosin,

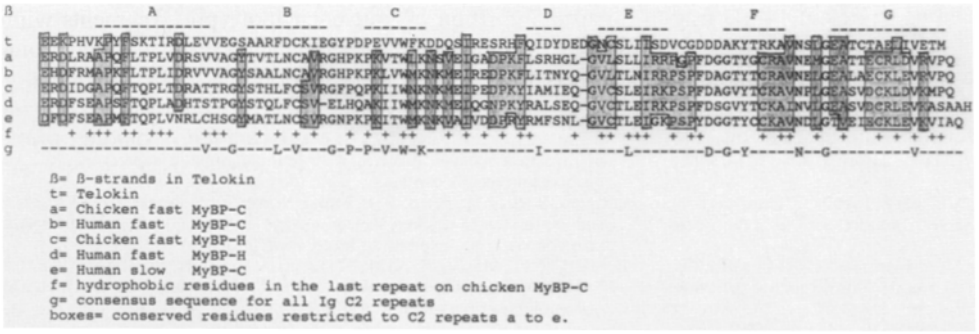

Figure 6. Alignment of the amino acid sequences of the COOH-terminal 14-kD section of the MyBP family and their comparison with the telokin (*t*) sequence. β regions of β strand structure present in the telokin structure (Holden et al., 1992); (*a*) residues 1031-1132 of chicken fast muscle MyBP-C; (*b*) residues 959-1060 of human fast muscle MyBP-C (Weber et al., 1993); (*c*) residues 437-539 of chicken fast muscle MyBP-H (Vaughan et al., 1993*a*); (*d*) residues 374-476 of human fast muscle MyBP-H (Vaughan et al., 1993*b*); (*e*) residues 1036-1138 of human slow muscle MyBP-C (Fürst et al., 1992); (*f*) crosses (+) indicate the hydrophobic residues present in the chicken MyBP-C; (*g*) indicate the consensus sequence for all Ig C2 repeats present in MyBP-C, MyBP-H, twitchin, smooth muscle MLCK, twitchin, and projectin. Amino acid residues conserved on these five potential myosin-binding C2 motifs but not present in most C2 motifs are boxed. These highly conserved residues might be involved in myosin binding.

no consensus myosin-binding motif has been identified, nor have the Fn or Ig motifs been shown previously to participate in the myosin-binding reactions.

To identify such a motif we decided to compare the COOH-terminal Ig repeats of the MyBP-C and MyBP-H family, since this is a conserved region of these proteins and is now known to contain the myosin-binding site in at least one member of the family, chicken fast type MyBP-C. To date, most sequence comparisons of the myosin-associated proteins have been extended to include all C2 motifs in the molecule (Benian et al., 1989; Olson et al., 1990; Einheber et al., 1990; Vaughan et al., 1993*a*,*b*; Price et al., 1991; Labeit et al., 1991; Fürst et al., 1992; Ayme-Southgate et al., 1991; Noguchi et al., 1992; Kobayashi et al., 1992). As such, the comparisons have identified those amino acids conserved within all of these C2 motifs but have not identified residues which are potentially involved in myosin binding. To do so, we have now restricted this comparison to the COOH-terminal motifs of chicken fast type MyBP-C (Einheber and Fischman, 1990), human fast and slow type MyBP-C (Fürst et al., 1992; Weber, F. E., unpublished results) and chicken and human fast type MyBP-H (Vaughan et al., 1993*a*,*b*). In Fig. 6, we have aligned the amino acid sequences of the COOH-terminal regions of these proteins. Secondary structure predictions with the Chou-Fasman algorithm (Chou and Fasman, 1978), and direct analogy with the C2 motif present in telokin, in which the crystal structure has been established (Holden et al., 1992), have been used to determine the tentative positions of seven potential β strands which constitute the β sheets characteristic of immunoglobulins (Williams, 1987). Surprisingly, more than 60 out of 100 amino acids are conserved within these COOH-terminal repeats of the MyBPs, irrespective of their species or fiber type muscle origin. A similar comparison using all intracellular C2 motifs shows a much lower degree of sequence conservation, 39% (Weber et al., 1993). Of these 60 residues we excluded those amino acids which are shared by all Ig C2 motifs and are considered determinants of the C2 "signature" (see Fig. 6). The remaining 42 amino acids are conserved only in the C2 motifs believed to bind myosin (Fig. 6). Those residues are organized in clusters along β strands E, F, and G, and in the loop regions between β strands C and D (Fig. 6). In β strands A and C, we also found a small consensus region. When the position of the conserved side chains are mapped on the telokin structure, we find that some of these residues form two elongated patches on opposite sides of the telokin β barrel. A search of other Ig C2 motifs present in MLCK, twitchin and projectin with the amino acid clusters characteristic of the COOH-terminal domain did not reveal any homologies. This analysis suggests that MyBP-C and MyBP-H share a similar C2 immunoglobulin motif in the COOH-terminus of the molecule which evolved into a myosin binding site. In support of this notion, unpublished data from our laboratory indicate that MyBP-H and cardiac MyBP-C contain comparably sized proteolytic fragments which bind to myosin filaments (Alyonycheva, T., T. Mikawa, and D. A. Fischman. 1993). Furthermore, this C2 motif is likely to have a similar three-dimensional structure to that of telokin. The production of recombinant 14-kD fragment in *E. coli* should allow us to determine the structure of this fragment and map the interface between the myosin rod and MyBP-C.

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