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Myofibrillar interaction of cytosolic creatine kinase (CK) isoenzymes: allocation of N-terminal binding epitope in MM-CK and BB-CK

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SUMMARY

The molecular origin of the isoenzyme-specific interaction of cytosolic creatine kinase isoenzymes, muscle-type creatine kinase and brain-type creatine kinase, with myofibrillar structures has been studied by confocal microscopy in an functional in situ binding assay with chemically skinned, unfixed skeletal muscle fibers using wild-type and chimeric creatine kinase isoproteins. The specific interaction of both wild-type isoforms with the sarcomeric structure resulted in a stable, isoform-characteristic labeling pattern with muscle-type creatine kinase bound exclusively and tightly to the sarcomeric M-band while brain-type creatine kinase was confined to the I-band region. Chimeric proteins of both muscle-type and brain-type creatine kinases were constructed to localize the corresponding binding domain(s). Exchanged domains included the N-terminal part (residues 1-234), the region containing an isoenzyme 'diagnostic box' (residues 235-285) and the C-terminal part (residues 286-380). The purified recombinant proteins were all fully intact and enzymatically active. All chimeric proteins containing the N-terminal region (amino acid 1-234) of muscle-type or brain-type creatine kinase were always specifically targeted to the sarcomeric M-band or I-band, respectively. We therefore propose that the relevant epitope(s), determining the isoenzyme-specific targeting in skeletal muscle, are entirely located within the N-terminal regions of both cytosolic creatine kinase isoforms.

Key words: Creatine kinase isoenzyme, Protein targeting, Skeletal muscle association, M-band, I-band, Interaction epitope, Confocal microscopy

INTRODUCTION

Cells and tissues with intermittently high and fluctuating energy requirements, such as skeletal and cardiac muscle, brain, retina and spermatozoa, depend on the immediate availability of vast amounts of energy. In these cells and tissues, the enzyme creatine kinase (CK; ATP: creatine N-phosphoryl transferase, EC 2.7.3.2) plays a key role in the cellular energy metabolism (Wallimann et al., 1992) by replenishing ATP through the reversible transfer of the phosphoryl group from phosphorylcreatine (PCr) to ADP in the reaction: $PCr^{2-} + MgADP^{-} + H^{+} \leftrightarrow Cr + MgATP^{2-}$ (for review see Kenyon and Reed, 1983; Watts, 1973). Creatine kinases constitute a family of different oligomeric isoforms with tissue-specific expression and isoenzyme-specific subcellular localization. Two cytosolic subunit isoforms, 'ubiquitous' brain-type B-CK and 'sarcomeric' muscle-type M-CK, as well as two mitochondrial subunit isoforms, ubiquitous Mi_a-CK and sarcomeric Mi_b-CK, are synthesized in a tissue-specific manner. The latter are associated with the inner mitochondrial membrane (Schlegel et al., 1988, 1990). In vivo, the cytosolic M-CK and B-CK subunits (≈43 kDa) combine to form enzymatically functional homodimers and heterodimers, MM-CK, MB-CK and BB-CK isoenzymes, whereas the mitochondrial protomers combine to form two different interconvertible oligomeric forms: homodimers and, preferentially, homo-octamers (Schlegel et al., 1988). In fully differentiated skeletal muscle, MM-CK is the predominant isoform occurring in appreciable amounts together with mitochondrial Mi_b-CK, whereas BB-CK is the more widely distributed ubiquitous isoform present in brain, smooth muscle, heart and a variety of other tissues (Eppenberger et al., 1967; Trask and Billadello, 1990). Together, all three cytosolic isoforms co-exist only during myogenesis (Caravatti et al., 1979; Perriard et al., 1978; Trask et al., 1988) and to some extent also in mammalian heart, while MB-CK and BB-CK are undetectable in mature skeletal muscle (Turner et al., 1973; Wallimann et al., 1977, 1983a). Biochemical fractionation and in situ immunolocalization techniques on skeletal and cardiac muscle have shown that cytosolic MM-CK is not evenly distributed within muscle cells. A small but significant amount of cytosolic MM-CK (5-10%, depending on muscle-fiber type and preparation) is specifically bound to the myofibrillar M-band, whereas the soluble main fraction can be extracted by buffers of physiological ionic strength (Turner et al., 1973; Wallimann et al., 1977, 1983a). This property is unique to MM-CK and is not shared by BB-CK or the heterodimeric MB-CK (Wallimann et al., 1983a; Schäfer and Perriard, 1988). The M-band-bound fraction of MM-CK is functionally coupled to

the myofibrillar actin-activated Mg^{2+}-ATPase as an efficient intramyofibrillar ATP regenerator (Ventura-Clapier et al., 1994; Wallimann et al., 1984). Besides the enzymatic function, immunoelectron microscopic data have proposed a structural role of the M-band-bound MM-CK, in forming the interlinking M4 (M4′) bridges (Strehler et al., 1983; Wallimann et al., 1983b), important for the structural integrity of the myosin filament lattice within the myofibrils (Luther et al., 1981; for a review, see Wallimann and Eppenberger, 1985). Despite the functional relevance of this MM-CK-specific myofibrillar interaction, little is known about the structural basis, reflected by differences in the MM-CK and BB-CK sequences, that cause this isoform-specific association with the sarcomere. The experiments of an earlier report, involving microinjection of in vitro generated mRNA of M-CK/B-CK hybrid constructs into living differentiating embryonic chicken heart cells, which are naturally devoid of MM-CK, suggested that the M-band-binding property may reside within the C-terminal half of the MM-CK molecule (Schäfer and Perriard, 1988). In the work presented here, we were aiming at identifying and characterizing the responsible molecular interaction sites of both cytosolic CK isoforms in more detail.

Reconstitution of M-band-bound MM-CK by incubation of muscle fibers (Kraft et al., 1995) or extracted myofibrils (Wallimann et al., 1977) with externally added MM-CK has been used before for the localization of MM-CK and to study the function of M-band-bound MM-CK (Wallimann and Eppenberger, 1985). Here, we use an in situ biochemical approach with single skinned muscle fibers, from which the sarcolemma membrane has been removed by detergent treatment, to study the binding of fluorescently labeled wild-type isoforms, MM-CK and BB-CK, as well as chimeric constructs thereof, to myofibrillar subregions directly and on-line by laser confocal microscopy. In contrast to isolated myofibrils, these fibers are nearly intact, showing normal contraction and calcium regulation properties (Kraft et al., 1995), and thus are better suited to study weak interactions of proteins with as much intact sarcomeric superstructure as possible. The lack of M-band binding of BB-CK, observed when added as extrinsic protein into skeletal muscle (Kraft et al., 1995) or when present during myogenesis (Kraft et al., 1995; Wallimann et al., 1977), provided us with a suitable reference protein to evaluate the functional role of different CK domains for the isoenzyme-specific binding to subsarcomeric regions. The present work provides convincing evidence for the localization of the relevant epitope(s) for the isoenzyme-specific interaction of MM-CK and BB-CK with the sarcomeric M-band and I-band, respectively, both within the N-terminal region of the respective cytosolic CK isoforms.

MATERIALS AND METHODS

E. coli strains, plasmids and DNA manipulation

E. coli strain BL21(DE3)pLysS and expression vector *pET-3b* have been described elsewhere (Studier et al., 1990). *pRF5* is identical to *pET-3b* except a deleted *Eco*RV/*Eco*RI fragment. *E. coli* XL-1 blue (Bullock et al., 1987), media and standard DNA manipulations have already been described (Ausubel, 1994). Plasmid *pRF182*, containing the chicken B_b*-CK* cDNA (EMBL access No. X03509; Hossle et al., 1986), was a gift from Dr Rolf Furter (this Institute; unpublished result). The construction of plasmid *pMCK7-14*, containing the chicken *M-CK* cDNA (EMBL access No. X00954, M35380; Kwiatkowski et al., 1984; Ordahl et al., 1984), also a gift from Dr J.-C. Perriard, will be described elsewhere.

Protein sources and fluorescently labeled antibodies

A rabbit anti-chicken M-CK antibody (Wallimann et al., 1977, 1983b; Caravatti et al., 1979) and a monoclonal mouse antibody against the 185 kDa M-band protein myomesin from chicken (Grove et al., 1984) were generated by standard protocols at the Institute of Cell Biology, ETH Zürich, Switzerland and kindly provided by Dr H. M. Eppenberger and Dr J.-C.Perriard, respectively. Cyanin-5-conjugated goat anti-mouse IgG (Jackson, Immuno Research: Dianova, Hamburg, Germany) and FITC-conjugated swine anti-rabbit antibody (Nordic, Lausanne, Switzerland) were used as secondary antibodies. Commercially available cytosolic MM-CK from rabbit muscle (Boehringer Mannheim, Germany) and cytosolic BB-CK from rabbit brain (Sigma, Buchs, Switzerland) were used without further purification. Chicken MM-CK, chicken BB-CK and chimeric derivates thereof were overexpressed in *E. coli* and purified to homogeneity as will be described in a separate work.

Site-directed mutagenesis and construction of CK isoprotein chimerae

Polymerase chain reaction (PCR) was used for site-directed mutagenesis (Kadowaki et al., 1989) of the chicken *M-CK* and chicken *B-CK* cDNA. Synthetic oligonucleotides were designed, such that common restriction sites, *Mlu* I and *Sfu* I, were introduced at homologous positions to either side of the isoenzyme-specific 'diagnostic box' (corresponds to amino acid 258-270) in both cDNAs, but without changing the encoded amino acid sequence. The necessary site-specific mutations were generated using the 'megaprimer' PCR method described elsewhere (Sarkar and Sommer, 1990). Plasmid *pMCK7-14*, containing the chicken *M-CK* cDNA, and *pRF182*, containing the chicken B_b*-CK* gene, were used as initial templates. The reaction was initiated by adding Taq DNA polymerase to the denatured DNA template at 95˚C. Routinely, 30 PCR cycles were performed. General amplification conditions were: 100 μM dNTPs, 1.5 mM $MgCl_2$, 15 pmol primer (each), 1-10 ng template DNA, 1.5 U Taq DNA Polymerase (Promega or Appligene). $MgCl_2$ concentration was varied between 1.5 and 4.5 mM, and DMSO between 0 and 5%, the latter to improve yield and specificity of the PCR reaction (Saiki et al., 1988). Annealing temperatures and extension times depended on the primers used and the length of the amplified fragment, respectively. The resulting PCR products were gel purified, using the GENECLEAN KIT (Bio101 Inc., La Jolla, CA; Vogelstein and Gillespie, 1979), digested with flanking restriction enzymes, *Nde*I and *Bam*HI, subcloned into expression vector *pRF5*, transformed into *E. coli* XL1-blue, plasmid DNA prepared as described elsewhere (Birnboim, 1983) and analyzed using standard procedures. The complete coding region of these constructs was subsequently sequenced, employing the dideoxy chain termination method (Sanger et al., 1977), to ascertain the absence of random mutations. The obtained plasmids, *pT17*, containing the modified gene for chicken *M-CK*, and *pT23*, containing the modified gene for chicken B_b*-CK*, were used for the construction of different chimeric CK constructs. Both vectors were cut at one of the newly generated common restriction sites, *Mlu*I and *Sfu*I, and at one of the common cloning sites in the vector, *Nde*I or *Bam*HI. Using standard cloning techniques, the different gel-purified fragments were used to exchange homologous cDNA regions between the two CK isoforms, while maintaining the correct reading frame. A summary of the different chimeric constructs is given in Fig. 3.

Fluorescent labeling of MM-CK, BB-CK and chimeric derivates thereof

Specific labeling of proteins at their accessible sulfhydryl groups of cysteine residues was done with iodoacetamide-based fluorescent dyes, rhodamine iodoacetamide (Rhod-IA; Molecular Probes Inc., Eugene, OR, USA) or 5′ iodoacetamide fluorescein (5′ IAF; Molecular Probes). 4 mg protein in CK storage buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.2 mM EDTA, 2 mM β-mercaptoethanol) was incubated in the presence of 10 mM DTT at 37˚C for 1 hour to activate the cysteine sulfhydryl groups. Subsequently, DTT was removed and the buffer exchanged against 100 mM H_3BO_3 pH 8.5, 0.5 mM EDTA by size exclusion chromatography on a Fast Desalting FPLC column (Pharmacia, Uppsala, Sweden). The protein was then concentrated with a CENTRICON-30 ultrafiltration unit (Amicon Corp., Lexington, MA, USA) to a final volume of 0.5 ml and incubated with a 2.2-fold molar excess of Rhod-IA or 5′ IAF at room temperature for 2 hours in the dark under gentle agitation. The labeling reaction was then quenched by adding 1 mM DTT to the reaction. Finally, unconjugated fluorescent dye was removed and the buffer exchanged for 50 mM NH_4HCO_3 pH 7.9, a volatile buffer system, either by extensive dialysis at 4˚C or by size exclusion chromatography on homemade SEPHADEX G-25 columns (Pharmacia, Uppsala, Sweden). Small aliquots of the labeled proteins were then lyophilized and stored at –20˚C, protected from light. The ratio dye/protein was evaluated spectrophotometrically at pH 7.9 (Simon and Taylor, 1986), using a molar extinction coefficient of 20,000 $M^{-1}cm^{-1}$ at 575 nm for Rhod-IA and 72,200 $M^{-1}cm^{-1}$ at 490 nm for 5′IAF, respectively, and $E_{280}^{0.1\%}$ = 0.88 for MM-CK and $E_{280}^{0.1\%}$ = 0.83 for BB-CK, respectively.

Functional in situ binding assay

All binding experiments were performed on single, chemically skinned fibers of rat *musculus psoas major* (Stolz, 1997), prepared according to Brenner (1983) and Yu and Brenner (1989) and stored in chilled skinning solution (5 mM KH_2PO_4, 3 mM magnesium acetate, 5 mM EGTA, 3 mM Na_2ATP, 50 mM PCr, 2 mM DTT, 8 mM NaN_3 and pH 6.8 at 4˚C) supplemented with protease inhibitors (10 μM Leupeptin, 10 μM Pepstatin A, 10 μM E-64, 10 μM Antipain, 1 μg/ml Aprotinin, all from Sigma, Buchs, Switzerland; 1 mM AEBSF, Calbiochem-Novabiochem, La Jolla, CA) at the day of the experiment (Kraft et al., 1995). The fibers were mounted in a flat, homemade, flow-through chamber on a microscopic slide as described in detail elsewhere (Stolz, 1997). All subsequent incubation and washing steps of the mounted fibers, as well as all protein dilutions were performed with relaxation solution (10 mM imidazole, 2 mM $MgCl_2$, 3 mM EGTA, 2 mM DTT, 2 mM MgATPγS (Calbiochem-Novabiochem), 0.25 mM p1,p5-di(adenosine-5′)-pentaphosphate (Boehringer, Mannheim, Germany), 100 mM glucose and 0.1 units/ml hexokinase (Boehringer); Kraft et al., 1995). The ionic strength was adjusted to 120 mM with potassium propionate and the pH to 6.8 at the experimental temperature of 22˚C. Fluorescently labeled molecules were diluted routinely to 25-50 μg/ml and unlabeled proteins, used in chase experiments, to 5 mg/ml. The laser confocal system consisted of a Zeiss AXIOPHOT fluorescence microscope and a Bio-Rad MRC-600 confocal scanner unit. It allowed us to follow the time course of equilibration of fluorescently labeled molecules in a longitudinal optical section through the different parts of the sarcomeres, as well as the exchange of the fluorescently labeled molecules in chase experiments with unlabeled molecules, in principal as described earlier (Kraft et al., 1995). Unfixed fibers, as used in the experiments of the present study, were still able to contract, but did not tolerate the use of reagents to reduce photobleaching. Therefore, the observation time was kept as short as possible and the excitation light intensity was kept as low as possible. If scanning occurred more frequently, e.g., in exchange/chase experiments, images were taken at neighboring spots, wherever possible, to ensure that a decrease in the intensity was not due to photobleaching.

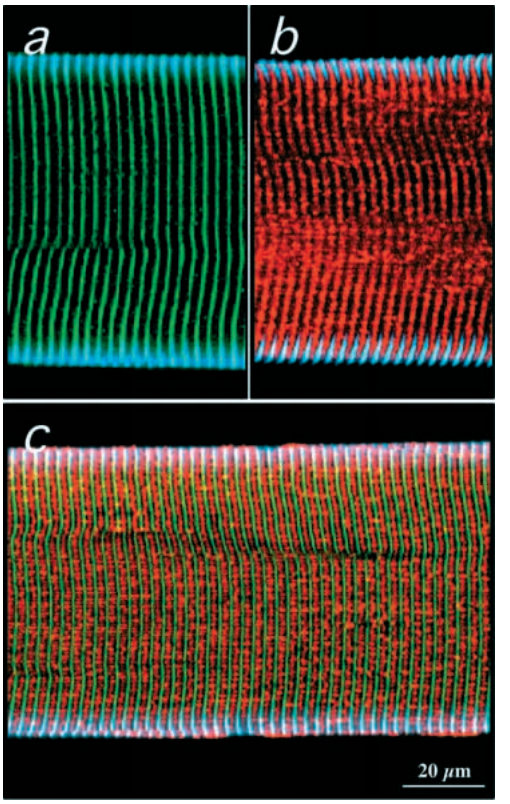

Fig. 1. In situ labeling pattern of fluorescently labeled MM-CK and BB-CK in a skinned muscle fiber. Confocal images of longitudinal optical sections through the core region of unfixed skinned muscle fibers after equilibration with fluorescently labeled CK isoforms (25 μg/ml). The fibers were preincubated with an antibody against the M-band-specific protein myomesin for at least 2 hours, followed by a second, cyanin-5-labeled antibody for another 2 hours. Fluorescence channels: in green, 5′ IAF-conjugated chicken MM-CK; in red, rhodamine-conjugated chicken BB-CK; in blue, cyanin-5-labeled anti-myomesin antibody. (a) Fiber incubated with labeled MM-CK alone; (b) fiber incubated with labeled BB-CK alone; (c) fiber co-labeled with MM-CK and BB-CK. Note that, in an alternating labeling pattern, MM-CK binds specifically to the M-band, whereas BB-CK is confined to the I-band region.

RESULTS

Association of MM-CK with the M-band and confinement of BB-CK to the I-band

The established in situ binding assay, essentially described by Kraft et al. (1995), allowed us to follow the diffusion of exogenously added fluorescently labeled CK isoforms into a single skinned muscle fiber on-line by confocal light microscopy. Details of the instrumental set-up has been described elsewhere (Stolz, 1997).

The average ratio of the cysteine-specific dye to protein was 0.9 to 3.3 for MM-CK (8 cysteines) and 0.9 to 4.1 for BB-CK (10 cysteines), respectively. The high spatial resolution allowed the identification of subsarcomeric structures like the I-bands, formed by the actin filaments emanating from the Z-line, as well as the central M-bands interlinking the myosin filaments. Specific binding of the labeled proteins to myofibrillar

structures cause an increased fluorescence intensity in the respective region within each sarcomere, resulting in a characteristic regular cross-striated binding pattern (Fig. 1). Furthermore, the time course of the myofibrillar interaction of wild-type CK isoenzymes could be analyzed within each sarcomere. By co-localization with myomesin, a M-band-specific protein (Grove et al., 1984), we could show that fluorescein-conjugated muscle-type creatine kinase (5′IAF MM-CK) was bound specifically to sites corresponding to the M-band (Fig. 1a). This binding was strong and not affected by extensive washing with relaxation buffer, performed routinely after equilibration. Contrary to in situ chemically fixed, cryosectioned muscles, showing, besides the M-band staining, a broad and intense staining in the I-band region as well (Wegmann et al., 1992), there was no binding of MM-CK in the I-band of skinned fibers. In contrast to MM-CK, rhodamine-conjugated brain-type creatine kinase (Rhod BB-CK) was bound exclusively in the I-band region (Fig. 1b). In both cases, the observed binding was fairly strong, highly specific and resisted extensive washing. A similar labeling of the I-band region by BB-CK was reported before (Kraft et al., 1995) and (Wallimann et al., 1977) but, contrary to the observations reported here, it has been described as weak and easy to wash out. We also noticed a marked variability in the binding strength of fluorescently conjugated BB-CK to the I-band, depending on the batch of BB-CK used for labeling.

A specific antibody against myomesin (Grove et al., 1984), used as intrinsic marker for the sarcomeric M-band, unambiguously identified the different regions in each sarcomere. However, a complete equilibration of the fiber with this antibody could not be achieved within the time of incubation (between 2 hours and overnight), due to its size and slow diffusivity. The labeling of only the outermost fiber layers is in accordance to an earlier study comparing the diffusion of different fluorescently labeled molecules into skinned muscle fibers (Kraft et al., 1995).

Time course of equilibration and reversibility of myofibrillar interaction

Time series of single optical sections through the core region of chemically skinned, unfixed rat psoas muscle fibers were recorded to determine the rate of equilibration, for the wild-type CK isoforms. The time was determined from the first appearance of the fluorescently labeled CK protein in the outermost parts of the fiber until no further change in the ratio of fluorescence intensity in the core versus the outer fiber layers could be detected. An equilibration time of about 8 minutes could be determined for MM-CK, whereas BB-CK was equilibrated after about 10 minutes. These values for MM-CK and BB-CK in rat fibers are consistent with those published for rabbit psoas muscle fibers (Kraft et al., 1995).

The reversibility of this isoenzyme-specific myofibrillar interaction of the CK isoenzymes could be demonstrated by an in situ chase protocol. The time course of disappearance of the

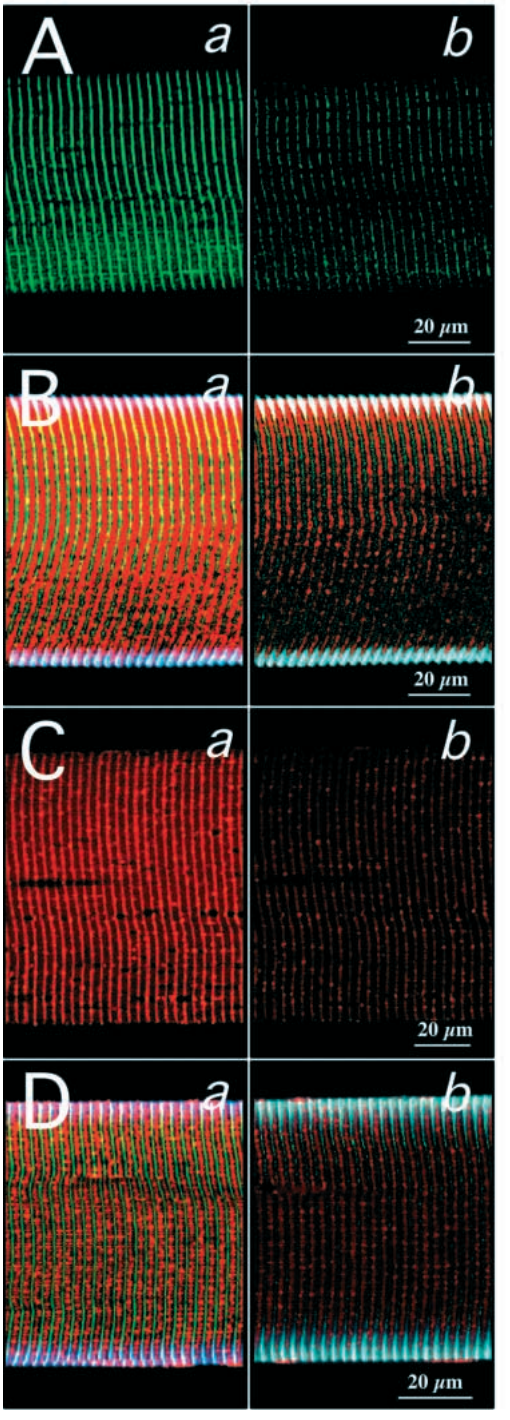

Fig. 2. Reversibility of in situ labeling patterns in the presence of an excess of unlabeled exogenously added CK. Confocal images of longitudinal optical sections through the core region of unfixed skinned muscle fibers before (a) and after competition (b) with unlabeled CK at 5 mg/ml for about 40 minutes. Fluorescence channels: in green, 5′IAF-conjugated chicken MM-CK; in red, rhodamine-conjugated chicken BB-CK; in blue, M-band-specific immunolabeling with antibody against myomesin.
(A) Exchange/chase with rabbit MM-CK of fiber equilibrated only with 5′IAF MM-CK. (B) Triple fluorescence pattern; exchange/chase with rabbit MM-CK of fiber equilibrated with 5′IAF MM-CK and Rhod BB-CK. (C) Exchange/chase with rabbit BB-CK of fiber equilibrated only with Rhod BB-CK. (D) Triple fluorescence pattern; exchange/chase with rabbit BB-CK of fiber equilibrated with 5′IAF MM-CK and Rhod BB-CK. Note the significant exchange of M-band MM-CK (A,B) and I-band BB-CK (C,D) which is seen with excess of unlabeled homologous CK isoenzymes, as indicated by a decrease in the fluorescence intensity in the (b) versus (a) panels.

specific fluorescence obtained with 5′ IAF MM-CK or Rhod BB-CK, or both together, was followed, while the fiber was incubated in relaxation solution containing unlabeled CK [5 mg/ml], either rabbit MM-CK or rabbit BB-CK (Fig. 2).

Both, unlabeled CKs showed a displacing effect on both fluorescent patterns of their respective homologues. Whereas unlabeled MM-CK had a strong displacing effect upon 5′ IAF MM-CK (Fig. 2A), the effect was less pronounced on Rhod BB-CK (Fig. 2B). After about 40 minutes most of the 5′IAF MM-CK labeling was displaced from the M-band whereas the Rhod BB-CK labeling in the I-band had lost only part of its initial labeling intensity.

Unlabeled BB-CK had a similar effect. The labeling of Rhod BB-CK in the I-band was strongly decreased by excess of homologous BB-CK after about 40 minutes (Fig. 2C). Within that time, a significant decrease in the 5′IAF MM-CK labeling could also be seen by excess BB-CK (Fig. 2D). It is noteworthy to mention that, in double labeling experiments with 5′IAF MM-CK and Rhod BB-CK, differences in the labeling strength were observed depending on the order of incubation, e.g. if BB-CK was diffused in first, the MM-CK did not bind as well to the M-band, and vice versa. The second isoform was always hampered somewhat in its binding to the specific target sites, suggesting some kind of interaction between both CK isoforms or an unspecific competition for defined target sites.

Chimeric CK constructs generated

In an effort to identify the regions in the CK isoforms responsible for the isoenzyme-specific binding property to sarcomeric substructures, a new approach with CK isoprotein chimerae was used. Their design was based on amino acid sequence comparisons between the cytosolic CK isoforms, MM-CK and BB-CK, especially of the C-terminal half of the enzymes where the M-band-binding epitope for MM-CK was postulated to reside (Schäfer and Perriard, 1988). There, an isoform-characteristic motif of 13 amino acids (residues 258 to 270) was identified, which is the most distinct region between both highly homologous isoforms. Unique restriction sites were introduced to both sites of this motif at homologous positions of the corresponding cDNAs by site-directed mutagenesis, giving the ‘diagnostic box’ region (residues 235 to 285). This allowed the exchange of three different parts of the CK isoform cDNAs while maintaining the correct reading frame (Fig. 3). The different chimeric proteins were expressed in *E. coli*, purified, fluorescently labeled and then used to investigate the possible role of the exchanged regions constituting the N-terminal fragment, the ‘diagnostic-box’ and the C-terminal fragments of both M-CK and B-CK, in determining the isoprotein-specific localization in skeletal muscle.

Characterization of the CK isoprotein chimerae

All chimeric constructs containing the C-terminal end of chicken M-CK were highly enriched in the form of inclusion bodies, whereas all chimeric constructs containing the C-terminal end of chicken B-CK accumulated in the soluble fraction of the cell lysate. All purified recombinant proteins showed a single band of 42 to 45 kDa (Fig. 4) and one-dimensional IEF confirmed the calculated charge shift of the constructs (Stolz, 1997; not shown here). The specific activities of the different recombinant chimeric CK proteins is shown in

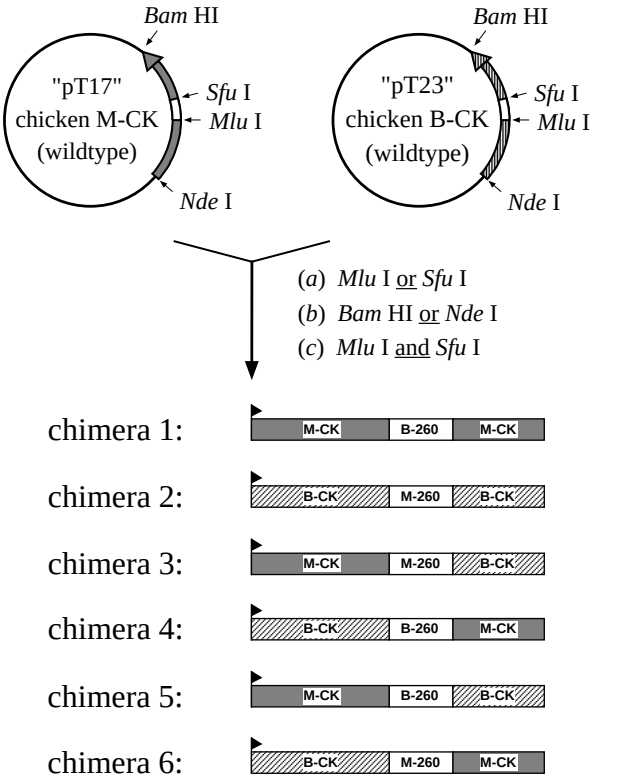

Fig. 3. CK isoprotein chimerae generated by recombinant technologies. The details of construction are given under Materials and Methods. cDNA fragments of chicken *M-CK* (grey tinted areas) and of chicken B_b-*CK* (crosshatched areas) were excised using a suitable combination of restriction enzymes. The exchange of these fragments generated six chimeric constructs, maintaining correct reading frames. The *Mlu*I/*Sfu*I fragment, which corresponds to amino acid 235 to 285, contained the isoform characteristic ‘diagnostic box’ region (amino acid 258 to 270). In the schematic drawing of the chimeric constucts, this fragment is symbolised by the boxed region: ‘M-260’, *Mlu*I/*Sfu*I fragment from chicken *M-CK*; ‘B-260’, *Mlu*I/*Sfu*I fragment from chicken B_b-*CK*. The start codon is symbolised by a small flag.

Table 1. All chimeric proteins were enzymatically highly active in both directions of the CK reaction with the same or even a higher activity as compared to the parental wild-type CKs, except for chimera 6, which is somewhat less active.

Spatial distribution of CK isoprotein chimerae in skinned muscle fibers

The recombinant chimeric CK proteins were used in the functional in situ binding assay, described above. The resulting labeling pattern of the chimerae (see Table 2) was directly compared with that of two reference labels, wild-type chicken MM-CK and an antibody against the M-band protein myomesin (Grove et al., 1984). The labeling pattern of the chimeric proteins was stable throughout the different steps used in our in situ binding assay protocol. Neither the final labeling pattern nor the reference labeling with wild-type MM-CK was affected by extensive washing with relaxation buffer.

Chimerae 1 and 2 were designed to study the functional role of the isoform characteristic ‘diagnostic box’ region for the

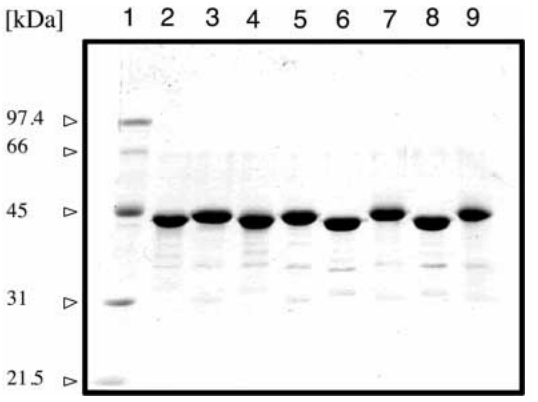

Fig. 4. SDS-PAGE of expressed CK isoprotein chimerae and their parental wild-type CKs. Individual fractions, each 10 µg of purified recombinant protein (lane 2 to 9), were separated on a 11% SDS-polyacrylamide gel and stained with Coomassie Blue. All proteins were expressed in an inducible bacterial system. The recombinant proteins were purified either from inclusion bodies (wild-type chicken MM-CK and chimera 1, 4 and 6) or purified as soluble protein from the cell lysate (wild-type chicken BB-CK and chimera 2, 3 and 5). Lane 1, positions of molecular size markers (Bio-Rad, low M_r standards); lane 2, wild-type chicken MM-CK; lane 3, wild-type chicken BB-CK; lane 4, chimera 1; lane 5, chimera 2; lane 6, chimera 3; lane 7, chimera 4; lane 8, chimera 5; lane 9, chimera 6.

Table 1. Summary of the specific enzyme ativities of CK constructs

CK construct	Specific activity reverse reaction (IU/mg protein)	Specific activity forward reaction (IU/mg protein)
chicken MM-CK (wild type)	200	26.9
chicken BB-CK (wild type)	204	32.7
chimera 1	186	27.4
chimera 2	310	46.4
chimera 3	321	52.5
chimera 4	223	28.3
chimera 5	282	48.0
chimera 6	80	10.7

The table shows representative specific enzyme activities of the purified chimeric CK proteins and their parental wild-type proteins. The recombinant proteins were all expressed in *E. coli* BL21(DE3)pLysS. Protein was purified either from inclusion bodies (chimera 1, 4 and 6 and wild-type chicken MM-CK) or purified in soluble form from the cell lysate (chimera 2, 3 and 5 and wild-type chicken BB-CK). The protein concentration was determined by Bio-Rad protein assay, using BSA as a standard. The enzyme activies were determined with the pH-stat assay (Milner-White and Watts, 1971; Wallimann et al., 1984) in the reverse (ATP production) and the forward (PCr production) direction. An international unit (IU) of enzyme activity is equal to 1 µmol of phosphorylcreatine transphosphorylated per minute at pH 7.0 and 25˚C in the reverse reaction.

isoprotein-specific localization in skeletal muscle. Chimera 1 contained a B-CK-specific 'diagnostic box' in a M-CK protein, whereas chimera 2 contained a M-CK-specific 'diagnostic box' in an otherwise complete B-CK protein. The in situ binding assay showed that both chimeric proteins have the same binding properties as their parental wild-type CKs (see Table 2), i.e., chimera 1 showed the characteristic M-band-binding

Table 2. Summary of the CK chimerae binding properties in chemical skinned muscle fibers

	Binding after diffusion		Binding after wash		Binding after reference labeling with Rhod MM-CK	
CK construct	M-band	I-band	M-band	I-band	M-band	I-band
chick. MM-CK	+++	–	+++	–	+++	–
chick. BB-CK	–	+++	–	+++	±	+++
chimera 1	+++	–	++	–	++	–
chimera 2	–	+++	–	++	–	++
chimera 3	+++	–	++	–	++	–
chimera 4	–	+++	–	++	±	++
chimera 5	+++	–	++	–	++	–
chimera 6	–	+++	–	+	–	+

The resulting labeling pattern of the chimerae was stable, after the fiber was equilibrated with the fluorescently labeled chimeric protein. Only minor changes, mainly in the fluorescence intensity, could be observed. Intensity of fluorescence: +++, very strong; ++, strong; +, weak; ±, trace; –, none. Note that the N-terminal sequence of M-CK leads to a conversion of BB-CK to behave like a genuine wild-type MM-CK with respect to M-band binding (chimerae 3 and 5), whereas replacement of the N-terminal domain of M-CK by B-CK leads to a loss of M-band interaction of MM-CK, but conveys I-band interaction (chimerae 4 and 6).

ability of wild-type MM-CK. The labeling was highly specific and did not show any affinity for the I-band region. The homologous chimera 2 showed selective I-band-binding ability, known from wild-type BB-CK, without any affinity towards the M-band.

Chimera 3 and 4, designed to study the functional role of the exchanged C-terminal CK regions for the isoprotein-specific localization in skeletal muscle, showed also the same binding properties as their parental wild-type CK protein (see Table 2). Chimera 3, M-CK with C-terminal part from B-CK, still showed the characteristic M-band-binding pattern as did wild-type MM-CK. This highly specific labeling did not show any affinity for the I-band region in muscle. The homologous chimera 4, B-CK with C-terminal part from M-CK, showed the same I-band-binding properties known from wild-type BB-CK. A minor affinity for the M-band could be observed but only after reference labeling with rhodamine-labeled MM-CK.

A final set of chimerae was used to study the functional role of the N-terminal region for the isoprotein-specific localization in skeletal muscle. The resulting labeling patterns of chimerae 5 and 6 is shown in Fig. 5. In contrast to all other chimerae, these two displayed the binding property of the parental CK isoform accounting for the N-terminal region in the chimeric protein. Chimera 5, B-CK with the N-terminal part from M-CK but the entire rest of B-CK, showed the strong, highly specific M-band-binding property of wild-type MM-CK without any affinity at all towards the I-band region in muscle. The homologous chimera 6, M-CK with N-terminal part from B-CK but the entire rest of M-CK, showed similar I-band-binding properties as was demonstrated for wild-type BB-CK. The somewhat weaker binding strength of chimera 6, an observation also made with wild-type BB-CK, was again variable and depending on the protein batch used for labeling.

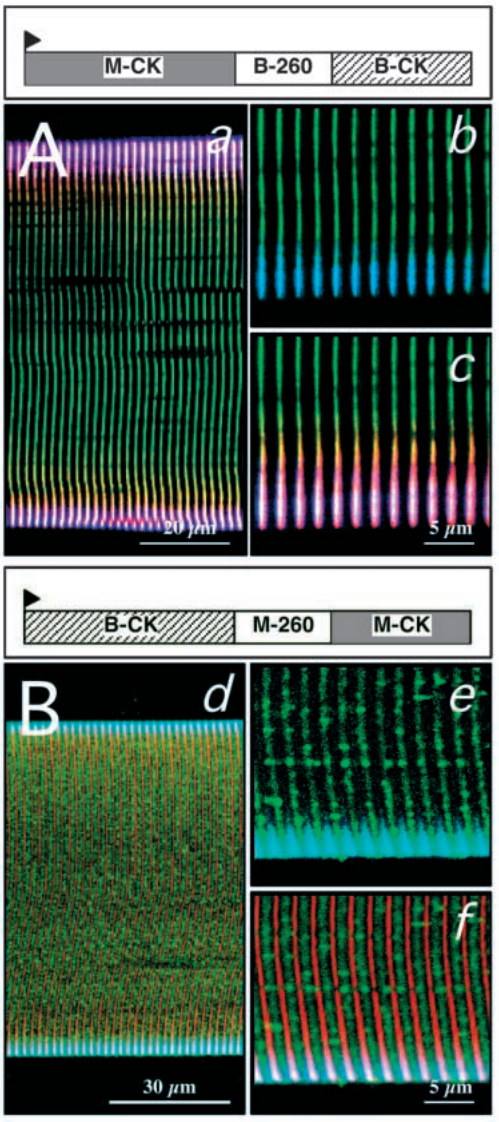

Fig. 5. In situ labeling pattern of N-terminal M-CK and B-CK chimerae in a skinned muscle fiber. Confocal images of longitudinal optical sections through the core region of unfixed skinned muscle fibers after equilibration with 5′IAF fluorescently labeled chimeric protein (25 μg/ml): (A) chimera 5, a B-CK with a M-CK N-terminal stretch; (B) chimera 6, a M-CK with a B-CK N-terminal stretch. The schematic drawing on top of each panel depicts the chimeric construct: grey tinted areas, parts derived from M-CK; crosshatched areas, parts derived from B-CK; 'M-260', 'diagnostic box' region from M-CK; 'B-260', 'diagnostic box' region from B-CK; small flag; N-terminus. Images were taken after washing and reference labeling with rhodamine-labeled chicken MM-CK. The fiber was preincubated with an antibody against the M-band-specific protein myomesin for at least 2 hours, followed by a second, cyanin-5-labeled antibody for another 2 hours. Fluorescence channels: in green, 5′ IAF-labeled chimeric protein; in red, rhodamine-labeled MM-CK; in blue, anti myomesin antibody. (a) Triple fluorescence pattern of chimera 5; (b) double-fluorescence pattern with chimera 5 at higher magnification; (c) triple fluorescence pattern of chimera 5 at higher magnification. (d) triple fluorescence pattern of chimera 6; (e) double-fluorescence pattern with chimera 6 at higher magnification; (f) triple fluorescence pattern of chimera 6 at higher magnification. Chimera 5 shows the same strong and specific M-band-binding property as wild-type MM-CK (b,c). In contrast, chimera 6 binds specifically in the I-band region, like wild-type BB-CK (e,f).

DISCUSSION

Myofibrillar binding properties of MM-CK and BB-CK

MM-CK and BB-CK isoenzymes have similar biochemical properties and catalyze the same enzymatic reaction with similar kinetic constants (Stolz, 1997). This is also reflected in an extensive sequence homology (Babbitt et al., 1986; Mühlebach et al., 1994). However, both isoenzymes are expressed in a tissue-specific fashion and show a highly specific and very distinct intracellular targeting to specific sites within the myofibrillar organization (Wallimann et al., 1983a), which was corroborated in this study with a newly established functional in situ binding assay using skinned muscle fibers. The pattern obtained with 5′ IAF MM-CK in this assay was identical to the pattern obtained by in situ immunohistochemical localization of MM-CK in muscles that were permeabilized and washed before fixation in order to remove soluble CK (Wegmann et al., 1992) and with that obtained from isolated myofibrils (Wallimann and Eppenberger, 1985; Wallimann et al., 1977). In contrast, in fixed cryosectioned muscles, and hence intact muscle, besides the strong antibody staining of the M-band, most of the soluble MM-CK protein was found in the I-band region (Wegmann et al., 1992). This I-band fraction of MM-CK was completely lost during the course of the skinning process. In the in situ assay, MM-CK by itself did not show any intrinsic affinity towards the I-band region on its own. Only in presence of phosphofructokinase or aldolase, or both, could it be shown that some of the labeling of MM-CK in the I-band region can be restored (Kraft et al., 1996). This indicates that the association of MM-CK at this location is mediated via glycolytic enzymes which were, however, lost from the muscle upon permeabilization and which are located at this subsarcomeric region (Arnold and Pette, 1968; Wegmann et al., 1992). In contrast, Rhod BB-CK alone showed a different but specific labeling restricted to the I-band region when introduced into skeletal muscle, which was somewhat more variable in its binding strength. Previous reports (Kraft et al., 1995) already reported a low affinity binding of BB-CK to sites in the I-band region which, however, was lost from the muscle fiber upon washing. This is probably also the case in the transgenic 'switch-mouse' expressing only BB-CK in muscle (Roman et al., 1997). We observed a rather strong binding of BB-CK to the I-band region, although the binding strength was largely dependent on the batch of the fluorescently labeled BB-CK used. Strong binding batches of BB-CK had a high ratio of dye to protein compared to weak binding batches, indicating a higher number of modified sites in the protein. Hence, this variability may be explained by an interference of the attached fluorescent dye with the specific interaction site of the BB-CK in the I-band region, which could, of course, occur by different mechanisms. Regardless of the variability in the binding strength of labeled BB-CK, its interaction with the I-band region is supported by the consistency in the labeling patterns observed with the isoprotein chimerae and corroborated by earlier findings, based on in situ immunofluorescent labeling

of chicken skeletal and cardiac myofibrils with CK isoenzyme-specific antibodies (Wallimann et al., 1977).

Dynamic nature of myofibrillar interaction of CK isoenzymes

The exchange/chase studies supported the rather high affinity of both CK isoenzymes to bind distinct sites within the subsarcomeric structures, with no or little removal of bound CK by simple washing with relaxation buffer, a buffer of physiological ionic strength. For fluorescently labeled MM-CK, some 8-10 hours of washing with this buffer was required to observe removal of a significant fraction from the M-band (Kraft et al., 1995). This basically was also true in our hands for I-band-bound BB-CK, since we could not see a significant difference in this behaviour between MM-CK and BB-CK. However, as mentioned above, in earlier attempts with Rhod BB-CK, the latter disappeared within a few minutes of washing, because of a weak binding strength to sites in the I-band region. In contrast, for MM-CK, a brief extraction with a low ionic strength buffer was necessary to remove nearly all of the M-band-bound MM-CK fraction (Wallimann et al., 1977). This supports the idea that the myofibrillar interaction of CK isoforms is not only of ionic nature. Most importantly, in the exchange/chase experiments both isoforms could be displaced fairly well by an excess of unlabeled homologous CK protein, indicating a dynamic binding and dissociation behaviour of endogenous myofibrillar CK in situ. It also showed that the displacement of CK in skinned muscle fibers is not just due to simple diffusion but a process that is strongly affected by interactions of the CK isoproteins with the respective structural muscle proteins, as well as of the CK molecules themselves. This is in good agreement with recent photobleaching experiments showing that, in cultured muscle cells, the cytosolic diffusion rate of MM-CK is lower than that of dextran beads of equivalent size and that a significant fraction of MM-CK shows a very low mobility, attributed to the exchange between free and bound MM-CK (Arrio-Dupont et al., 1997).

Three-dimensional model of the structural relationship in CK isoprotein chimerae

The location of the three domains, between chicken MM-CK and BB-CK, that had been exchanged by genetic engineering, are shown for the dimeric form of the highly homologous, mitochondrial Mi_b-CK (Fig. 6) from chicken (66-67% amino acid identity), whose three-dimensional X-ray structure has been solved recently (Fritz-Wolf et al., 1996). The structural similarity with chicken Mi_b-CK has already allowed others to solve by molecular replacement the structure of rabbit MM-CK as well as that of arginine kinase (Kabsch and Fritz-Wolf, 1997). Therefore, this structure is also a suitable model to describe the approximate structure of the chimerae produced. The N-terminal region (amino acid 1 to 234) forms the central core of the dimeric molecule and contains the complete monomer/monomer interface and the main part of the catalytic subunit involved in the substrate binding. The 'diagnostic box' region (amino acid 235 to 285), which contains an isoform characteristic sequence motif, forms mainly a long α-helix located laterally at the surface of the molecule, well exposed to the surrounding media. Another part of this domain is also involved in forming the active site

Fig. 6. The putative molecular structure of dimeric CK isoprotein chimerae. The image was created with the program 'WebLab Viewer' V2.0 (Molecular Simulations, Inc., San Diego, CA, USA) and shows a dimeric CK molecule in a sideview. The model visualizes the homologous regions that were exchanged between the parental wild-type CKs, chicken MM-CK and BB-CK, to generate the different chimeric constructs: pale blue, N-terminal region (amino acid 1 to 234); green, part containing the 'diagnostic box' region (amino acid 235 to 285); orange, C-terminal region (amino acid 286 to 380). The putative dimer structure is based on the three-dimensional dimer structure of the homologous chicken Mi_b-CK octamer (Fritz-Wolf et al., 1996). For better orientation, bound ATP is shown in red, identifying the catalytic center in each monomer. The numbering used corresponds to the primary amino acid sequence of the cytosolic isoforms, including the start methionine.

of the enzyme, e.g. cysteine 283 (Furter et al., 1993). The C-terminal region (amino acid 286 to 380) is located as a 'cap' at both ends of the longitudinal axis of the dimer molecule and contains a highly flexible loop (321 to 331), which might be important for the catalytic mechanism, and a long α-helix associated with the flexible C-terminal end. Major parts of this region are exposed at the molecule surface. However, as our data clearly show, this large C-terminal region from residue 235 to 380, containing also the isoenzyme-specific 'diagnostic box', has no influence on the binding of CK isoenzymes to subsarcomeric regions.

Allocation of the myofibrillar binding epitope in the CK isoforms

We have used a new in situ approach with CK isoprotein chimerae to identify the regions in the protein, responsible for the isoprotein-specific localization in skeletal muscle. The

high enzymatic activity and biochemical integrity of the chimeric proteins, including those purified from enzymatically inactive inclusion bodies, were a good indication that the recombinant chimerae used were all fully intact and correctly folded. Three different CK-domains were exchanged and the resulting chimerae tested in the in situ binding assay with skinned muscle fibers. Although the exchanged domain in chimera 1 and 2 contained major amino acid sequence differences between the cytosolic isoforms, a functional role of the 'diagnostic box' region (residues 235 to 285) for the isoform-specific targeting could be excluded, unambiguously. Similar, with the second set of chimerae, chimera 3 and 4, a functional role of the C-terminal region (residues 286 to 380) for the isoform-specific targeting could be excluded. Finally, with the third set of chimerae, chimera 5 and 6, it was possible to show definitely that the N-terminal part of the CK molecule (residues 1 to 234) plays the essential role in determining the isoprotein-specific binding property in skeletal muscle for both cytosolic isoforms. Most important, B-CK could be converted into a competent M-band-binding protein, which was not seen before, by exchanging its N-terminal segment for the homologous M-CK segment. This is in contrast to an earlier study (Schäfer and Perriard, 1988) using microinjection of in vitro generated mRNA for CK and CK hybrid constructs into differentiating chicken heart cells in culture and detection of the translation products with isoprotein-specific antibodies. While the MM-CK specificity for M-band association was confirmed by the above authors, their experiments with CK hybrids suggested that the C-terminal half of MM-CK is responsible for the interaction with the M-band. However, for various technical reasons, this earlier study cannot be compared directly with ours.

To summarize, our results fully corroborate the differential isoenzyme-specific binding of the cytosolic CK isoforms to myofibrillar substructures, reported earlier in vitro and in situ (Kraft et al., 1995; Wallimann et al., 1977, 1983a; Wegmann et al., 1992), as well as in vivo (Schäfer and Perriard, 1988) and are compatible with data obtained with a transgenic 'switch-mouse' (Roman et al., 1997). This system, together with chimeric CK proteins, was successfully used to unambiguously allocate the relevant myofibrillar-binding epitope(s) entirely in the N-terminal part of both cytosolic isoforms. However, in this part of the CK molecule, isoenzyme-specific sequences are not as prevalent as for example in the 'diagnostic box' and thus the M-band interaction epitope may be rather complex, involving residues from different locations within this region, which is currently under investigation.

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