doi:10.1016/S0022-2836(03)00921-5 J. Mol. Biol. (2003) 332, 877–887

JMB

Available online at www.sciencedirect.com

SCIENCE DIRECT®

ELSEVIER

Muscle-type Creatine Kinase Interacts with Central Domains of the M-band Proteins Myomesin and M-protein

Thorsten Hornemann[1], Stefan Kempa[2], Mirko Himmel[2], Katrin Hayeß[2] Dieter O. Fürst[2] and Theo Wallimann[1]*

[1]*Institute of Cell Biology Swiss Federal Institute of Technology, HPM-F39 ETHZ-Hönggerberg, CH-8093 Zürich, Switzerland*

[2]*Department of Cell Biology University of Potsdam Lennéstr. 7a, D-14471 Potsdam Germany*

**Corresponding author*

Muscle-type creatine kinase (MM-CK) is a member of the CK isoenzyme family with key functions in cellular energetics. MM-CK interacts in an isoform-specific manner with the M-band of sarcomeric muscle, where it serves as an efficient intramyofibrillar ATP-regenerating system for the actin-activated myosin ATPase located nearby on both sides of the M-band. Four MM-CK-specific and highly conserved lysine residues are thought to be responsible for the interaction of MM-CK with the M-band.

A yeast two-hybrid screen led to the identification of MM-CK as a binding partner of a central portion of myomesin (My7-8). An interaction was observed with domains six to eight of the closely related M-protein but not with several other Ig-like domains, including an M-band domain, of titin.

The observed interactions were corroborated and characterised in detail by surface plasmon resonance spectroscopy (BiaCore). In both cases, they were CK isoform-specific and the MM-CK-specific lysine residues (K8. K24, K104 and K115) are involved in this interaction. At pH 6.8, the dissociation constants for the myomesin/MM-CK and the M-protein/MM-CK binding were in the range of 50–100 nM and around 1 μM, respectively. The binding showed pronounced pH-dependence and indicates a dynamic association/dissociation behaviour, which most likely depends on the energy state of the muscle. Our data propose a simple model for the regulation of this dynamic interaction.

Keywords: creatine kinase; myomesin; M-protein; sarcomeric M-band; titin

Introduction

Cross-striated muscle cells possess a unique, highly ordered lattice of thick and thin myofilaments. While thin filaments are structurally anchored in the Z-disc, thick filaments are interconnected in the middle of a sarcomere, at the so-called M-band. This sarcomeric region is a complex structure that transverses the center of the A-band[1] and is supposed to provide physical stability between thick filaments during contraction. Specific adaptations of this basic structural concept to distinct muscle fiber types have led to the assumption that the M-band might, in addition, serve a physiological role.[2] Until now, relatively few structural components of the M-band have been identified unambiguously. These include, besides the bare zone of the myosin rods, the 185 kDa protein myomesin,[3] the 165 kDa M-protein[4] and the 250 kDa C-terminal region of titin.[5] The giant protein titin stretches all the way from the Z-disk into the M-band where it interacts specifically with myomesin in a phosphorylation-dependent manner.[6] While titin and myomesin are constitutive M-band components, the myomesin-related M-protein is limited in its distribution to the M-band of fast skeletal muscle fibers and to cardiomyocytes. The approximate molecular layout of titin, myomesin and M-protein within the M-band was determined by electron microscopy.[5]

Present addresses: T. Hornemann, University Hospital Zürich IKC, Rämisstrasse 100, 8091 Zürich, Switzerland; S. Kempa, Max-Planck-Institute for Molecular Plant Physiology, Am Mühlenberg 1, 14476 Golm, Germany.

Abbreviations used: CK, creatine kinase; MM-CK, muscle-type CK; BB-CK, brain type CK; MB-CK, muscle/brain heterodimer CK.

E-mail address of the corresponding author: theo.wallimann@cell.biol.ethz.ch

In addition to these structural proteins, the M-band contains significant amounts of specifically bound muscle-type creatine kinase (MM-CK). Creatine kinase (CK; ATP: creatine *N*-phosphoryl transferase, EC 2.7.3.2) plays an important role in cellular energy metabolism by replenishing ATP from phosphocreatine.[7] Three cytosolic isoforms, brain type BB-CK, muscle-type MM-CK and the MB-CK heterodimer,[8] as well as two mitochondrial isoforms are synthesized in a tissue-specific and developmentally regulated manner.[9,10] In fully differentiated skeletal muscle, MM-CK is the predominant isoform, whereas BB-CK is the more widely distributed ubiquitous isoform present in brain, smooth muscle, heart and a variety of other tissues.[8,11] The ability to interact with the myofibrillar M-band is a unique property of MM-CK and is not shared by the highly homologous BB-CK.[12,13] Recently, we demonstrated that four highly conserved MM-CK-specific lysine residues (K8. K24, K104 and K115) are responsible for this isoenzyme-specific interaction.[14] However, up to now, no data are available about the appropriate interaction partners of MM-CK within the M-band. In a systematic search for proteins interacting with myomesin by yeast two-hybrid analysis, MM-CK was found to interact specifically with a central myomesin fragment (My7-8) and with the closely related M-protein (M-Pr6-8). No interaction of MM-CK was found, however, with fragments of titin. The molecular details of the MM-CK interaction with myomesin and M-protein were subsequently analysed by surface plasmon resonance spectroscopy and yielded a model for the regulation of MM-CK interaction with its binding partners in a physiological context.

Results

Human myomesin interacts with human MM-CK in yeast two-hybrid analysis

In order to identify proteins binding to human myomesin, we performed yeast two-hybrid analysis using My6-8 as a bait and human skeletal and cardiac muscle cDNA libraries. For unknown reasons, the number of transformants and of positive clones was always extremely small in comparison to all the other baits that we have been using successfully (approximately 270,000 doubly transformed clones). Thus, the screens yielded only between five and ten HIS3 positive clones, out of which three to five were also positive in β-gal assays. Determination of their DNA sequences revealed that the only cDNA that appeared in all three screens (once, twice and three times, respectively) was one encoding full-length MM-CK. Since both myomesin and MM-CK are found at the myofibrillar M-band, this result prompted us to investigate this interaction in greater detail. First, forced yeast two-hybrid analysis was used to narrow the site of interaction within the myomesin polypeptide. The three domain construct My6-8 was therefore subcloned into fragments comprizing either two domains or a single domain of this region. Resulting baits were co-transfected into yeast cells with constructs encompassing MM-CK. As negative controls, baits encoding other portions of myomesin and several Ig-like domain-containing titin fragments were included in this experiment; the results are summarized in Figure 1. Thus, we could clearly map the interaction of MM-CK to the two myomesin domains My7-8, while neither single domain was sufficient for binding. Similarly, the portions My4-6 and My9-13 were negative by this criterion. Solely the amino-terminal fragment My1-3 could not be analysed in this way, due to autoactivation of the construct. None of the titin constructs revealed any binding to the MM-CK prey, thus ruling out an unspecific Ig-module interaction of MM-CK.

In the subsequent experiment, an interaction of the human myomesin construct with the non-muscle creatine kinase isoform BB-CK (both human and chicken), as well as with chicken MM-CK was tested. This clearly demonstrated binding solely between human My7-8 and human MM-CK, but not with human or chicken BB-CK. Conversely, exclusively chicken myomesin My6-8 gave an interaction with chicken MM-CK. Finally, we tested whether M-protein, a polypeptide closely related to myomesin that also is located in the myofibrillar M-band, would also be capable of interacting specifically with MM-CK. Indeed, the forced two-hybrid experiments indicated an interaction similar if not identical with that indicated for myomesin (the results are summarized in Figure 1).

In summary, the yeast two-hybrid data suggested an interaction between a central fragment of the two related M-band proteins myomesin and M-protein and the muscle isoform of creatine kinase, MM-CK. The binding under these conditions is CK isoenzyme-specific and does not occur with the brain-specific enzyme BB-CK. No interaction of MM-CK was found with any of a variety of titin fragments, including hTm1-m3 located at the M-band.

Interaction of myomesin, M-protein and MM-CK *in vitro*

For a thorough biochemical investigation of the interactions indicated by the yeast two-hybrid analysis, we performed binding assays using surface plasmon resonance spectroscopy. The cDNA portions encoding hMy7-8, cMy6-8 and cM-Pr6-8 were subcloned in a pET vector containing the fusion sequence for an N-terminal T7 tag and a C-terminal His_6 tag. The proteins were expressed in BL21(DE3)pLysS and purified by metal chelate chromatography. Full-length myomesin was purified from chicken pectoralis muscle tissue as described in Materials and Methods.

a)

	hMM-CK	cMM-CK	hBB-CK	cBB-CK
hMy4-6	-	n.d.	n.d.	n.d.
hMy6-8	+++	-	-	-
hMy6-7	+/-	-	-	-
hMy7-8	+++	-	-	-
hMy6	-	n.d.	-	n.d.
hMy7	-	n.d.	-	n.d.
hMy8	-	n.d.	-	n.d.
hMy9-11	-	n.d.	n.d.	n.d.
hMy11-13	-	n.d.	n.d.	n.d.
cMy6-8	-	+++	-	-
hMp2-3	-	n.d.	-	n.d.
hMp6-8	+++	-	-	-
hMp9-13	-	n.d.	-	n.d.
cMp6-8	+/-	+++	-	-
hTz1-z3	-	n.d.	n.d.	n.d.
hTz7-z8	-	n.d.	n.d.	n.d.
hTa1-a4	-	n.d.	n.d.	n.d.
hTm1-m3	-	n.d.	n.d.	n.d.

b)

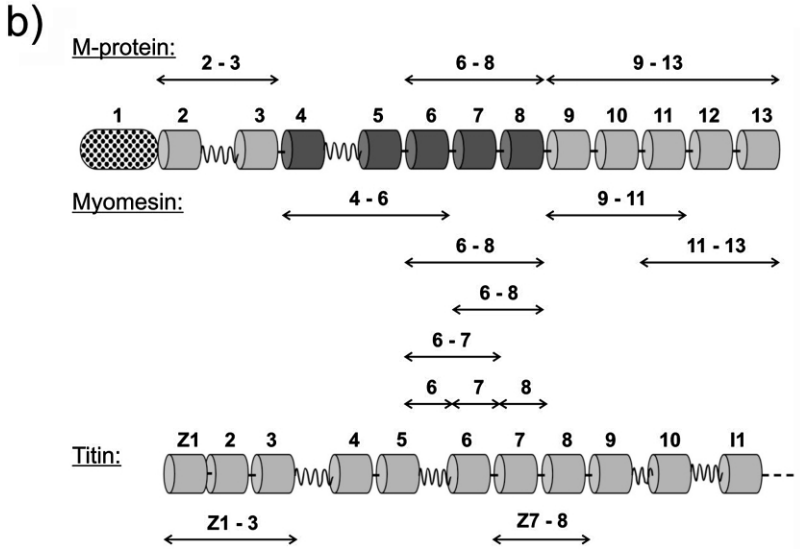

Figure 1. Analysis of the CK interactions by the yeast two-hybrid assay and the domain structures of myomesin, M-protein and the Z-disc portion of titin. (a) The results of the forced yeast two-hybrid analysis: +++ indicates strongly growing co-transformants; +/− few and weakly growing colonies; − no growth; n.d., not determined. To facilitate the nomenclature of the constructs, we have adopted the following scheme. The first letter indicates the species the respective construct is derived from (c, chicken; h, human). The subsequent letters refer to the protein: My, myomesin; Mp, M-protein; T, titin (z, a and m specify that constructs are derived from the Z-disc, A-band and M-band, respectively); MM-CK and BB-CK, muscle and brain-specific creatine kinase. (b) Illustration of the modular structures of myomesin and M-protein that are joined to a unique aminoterminal domain (1), followed by two Ig-like repeats (2 and 3), five Fn-like modules (4–8) and five Ig-like domains (9–13). The M-protein (Mp) constructs used are indicated by arrows. Below this sketch, the arrows indicate positions of myomesin constructs used in this study to narrow the myomesin (My) domains interacting with MM-CK. The strongest interaction is seen with My6-8 and My7-8 (see (a)). Finally, we show a sketch illustrating the modular structure of the Z-disc portion of titin, which is composed largely of ten Ig-like domains (Z1 to Z10). The positions of constructs used are again indicated by arrows.

Interestingly, on SDS-PAGE, the purified cMy6-8 fragments showed an apparent mass that was approximately 10 kDa smaller than theoretically expected (Figure 2). Western blotting revealed that the N-terminal T7 tag was missing, while the C-terminal His_6 tag was still present. Sequence analysis of this region did not indicate a potential alternative internal translation start sequence. N-terminal microsequencing of this fragment showed that the first 100 residues of the expected fragment were missing and instead the fragment started at myomesin residue 793 (QAAI…), i.e. just prior to domain 7. An explanation for this apparent post-translational cleavage could be the close proximity to a prokaryotic signal peptide cleavage site (QDSEPIEVQ). Fortunately, only domain 6 of myomesin, a domain obviously not involved in the interaction with MM-CK, was affected by this cleavage. Therefore, this shortened fragment was still suitable for our planned interaction studies. The fragments of hMy7-8 and M-Pr6-8 migrated during SDS-PAGE according to their calculated size, and both contained the N-terminal T7 tag as well as the C-terminal His_6 tag.

All fragments mentioned above were immobilized separately on a Bia CM5 chip *via* amine coupling and assayed for binding to CK. The interaction kinetics of the different expressed fragments with MM-CK and BB-CK is depicted in Figure 3. Thus, a clearcut concentration-dependent binding of MM-CK to all immobilized fragments as well as full-length myomesin was evident. No binding of MM-CK or BB-CK to a control chip without

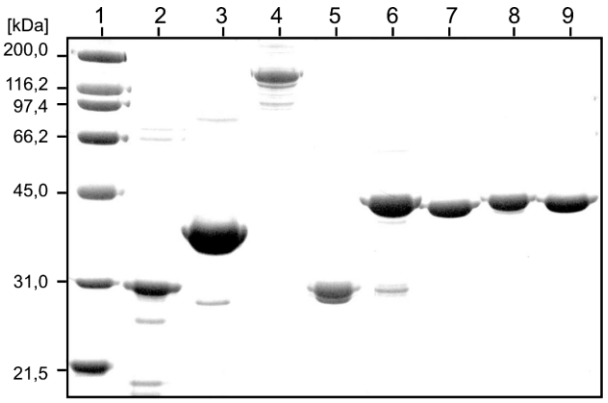

Figure 2. Coomassie blue-stained SDS-PAGE of the heterologous expressed and purified proteins and fragments used for the plasmon resonance spectroscopy analysis. Lane 1, molecular size marker; 2, cMy6-8; 3, cMp6-8; 4, purified chicken myomesin; 5, hMy7-8; 6, cBB-CK; 7, cMM-CK; 8, hBB-CK; 9, hMM-CK.

immobilized protein was observed. Most importantly, the interaction was isoform-specific for MM-CK, since no interaction of any of the myomesin and M-protein fragments was observed with the highly homologous BB-CK isoform. Only with immobilized full-length myomesin (and to a much smaller extent with cMy6-8) could one see a slight tendency for an interaction with BB-CK (Figure 3(b)). However, this interaction is negligible in comparison to the MM-CK association at the same concentration. The kinetic data obtained fit very well to a model of a single phase/single binding site interaction. The dissociation rate constant (k_d) was determined by an iterative fit according to the equation:

$$R_d = R_0 \exp(-k_d(t - t_0))$$

The association rate constant (k_a) was calculated by a fit according to:

$$R_a = R_{eq}(1 - \exp(-(k_a Cn + k_d)(t - t_0)))$$

The dissociation constant K_d was resolved by the quotient $K_d = k_d/k_a$. A representative residual plot of such a fit is shown in Figure 3(e). The calculated apparent K_d values are summarized in Table 1. The K_d for myomesin was found to be in the range of 50–120 nM, whereas the interaction with the M-protein fragment appeared to be about an order of a magnitude weaker (~1 μM).

Interestingly, an association of CK with the immobilized myomesin or M-protein fragments *in vitro* was observed only after a first initial unfolding/refolding step by a standard regeneration cycle (binding buffer + 0.1% (w/v) SDS). Without this initialization, no reproducible binding of MM-CK to the fragments or the full-length protein was observed. The strength of this protein–protein interaction did not vary significantly between an ionic strength from 40 mM to 200 mM sodium propionate (data not shown). However, at low ionic strength (10 mM Hepes, pH 8.0) the same interaction appeared significantly weaker, which is in good agreement with earlier findings that buffers of low ionic strength and high pH are the method of choice to specifically extract MM-CK from the myofibrillar M-band.[15] The interactions of CK with the two M-band proteins were CK isoenzyme-specific, but not strictly species-specific, e.g. similar binding constants were obtained with human MM-CK bound to chicken cMy6-8 and *vice versa* (Table 1). This supports earlier findings, that cMM-CK could be bound *in situ*, across species barriers, to skinned rabbit fibers,[14,16,39] indicating that the M-band binding of MM-CK is an evolutionarily highly conserved feature (Table 1 and see Figure 4).

The interaction of MM-CK with myomesin is strongly pH-dependent

In a systematic search for parameters that might influence the interaction between myomesin and MM-CK, we found a surprisingly strong effect of pH on binding strength. This effect was identical for purified, full-length myomesin and for the fragment hMy7-8. If the pH was lowered from 7.0 to 6.0, the association constant ($K_a = 1/K_d$) increased dramatically, by three orders of magnitude (Figure 5). No binding at all was observed above pH 7.5. The most pronounced change of K_a, respectively K_d, was evident between pH 6.7 and 7.0, which is exactly the range of intracellular pH changes that occur upon moderate muscle activation under physiological conditions.[17]

MM-CK specific lysine residues K8, K24, K104 and K115 are important for the isoform-specific interaction

In an earlier work, we showed that the region that mediates the isoform-specific interaction of MM-CK with the sarcomeric M-band is located within the N-terminal region of MM-CK.[18] Furthermore, we identified four M-CK-specific and highly conserved lysine residues (K8, K24, K104 and K115) that are responsible for this interaction (see Figure 4).[14] Those lysine residues form, pairwise, a weak (K8/24) and a strong (K104/115) M-band interaction site. An exchange of these lysine residues by site-directed mutagenesis between MM-CK and BB-CK led to a loss of interaction for the M-type mutants and a gain of interaction for the B-type mutants bearing the introduced lysine residues (Figure 5).

These mutants were tested for their interaction on isolated cMy6-8. The observed affinity changes of these mutants towards isolated cMy6-8 (Figure 6(a) and (b)) were fully consistent with the results obtained earlier on structurally intact skinned muscle fibers.[14] The introduction of the K8/24 pair alone into BB-CK (bck1) did not lead to a significant interaction with My6-8, whereas the BB-CK mutant containing the K104/115 site did show a clear gain of interaction function. The

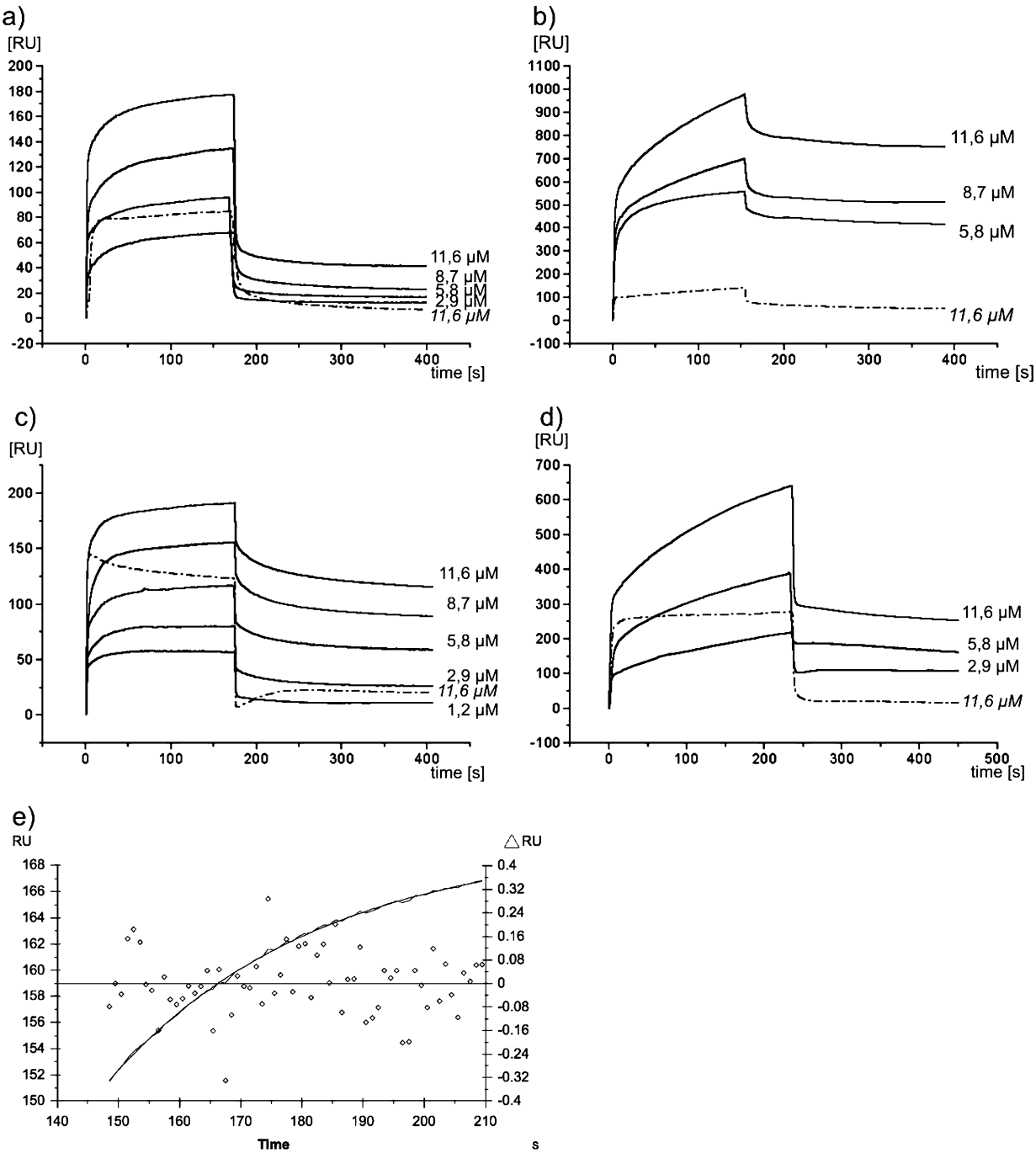

Figure 3. Concentration-dependent interaction kinetics of MM-CK with immobilized M-band proteins and fragments thereof. The MM-CK concentration used (in µM) was calculated for the CK dimer. Interactions were measured at pH 6.8. The dot-dash line shows the curve obtained with the highly homologous BB-CK isoform. (a) Ligand: cMy6-8, analyte: cMM-CK; (b) ligand: chicken myomesin (full length), analyte: cMM-CK; (c) ligand: hMy7-8, analyte: hMM-CK; (d) ligand: cM-Pr, analyte: cMM-CK; (e) representative residual plot (e.g. cMy6-8 and MM-CK—8.7 µM) of the difference between the mathematical fit and the data points. No systematic divergence between the measured and fitted curve was observed. This shows that the single phase/single binding site equation model is suitable to describe the measured interaction data in mathematical terms.

most pronounced effect was observed after the introduction of all four lysine residues. This mutant showed strong binding to the immobilized cMy6-8 fragment with an affinity comparable to that of the MM-CK wild-type protein. *Vice versa*, the successive replacement of the lysine pairs in the MM-CK mutants (mck1-mck3) led to a stepwise reduction of the binding affinity. However, a minute residual affinity could be observed even after the replacement of all four lysine residues of MM-CK. This indicates a minor involvement of further, hitherto unknown, amino acid residues of

Table 1. Dissociation constants (K_d) of different combinations between immobilized M-band proteins (ligands) and MM-CK (analyte)

Ligand	Analyte	k_d (M)	SE
cMy_full	cMM-CK	0.7×10^{-7}	4.3×10^{-8}
cMy6-8	cMM-CK	0.5×10^{-7}	4.3×10^{-8}
cMy6-8	hMM-CK	1.2×10^{-7}	7.1×10^{-8}
hMy7-8	hMM-CK	1.2×10^{-7}	1.7×10^{-8}
cM-Pr6-8	cMM-CK	1.2×10^{-6}	4.2×10^{-7}

The constants were calculated from an iterative fit of the measured kinetic dataset (see also Figure 1(a)–(e)) assuming a single phase/single site interaction model (see the text).

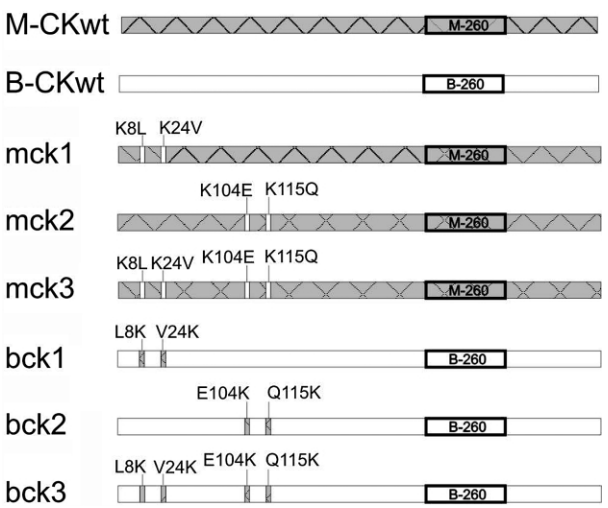

Figure 4. A summary sketch illustrating muscle-type M-CK and brain-type B-CK wild-type isoforms (M-CKwt and B-CKwt), as well as the CK point mutants (described in detail by Hornemann *et al.*[14]), used here for the protein/protein interaction experiments by BiaCore. Besides the isoenzyme-specific diagnostic box of 12 amino acid residues (258–270) in M-CK (M-260) and B-CK (B-260), which differ markedly between the two isoforms, the otherwise highly homologous M- and B-CK isoforms show additional evolutionary highly conserved isoenzyme-specific residues in their N-terminal regions; that is, K8 and K24, as well as K104 and K115. These are specific for M-CK and form two positive "charge clamps" shown to be responsible for targeting M-CK to the M-band.[14] If one of the two (see mutants mck1 and mck2) or both of these lysine pairs (see mutant mck3) are replaced by uncharged amino acids, binding of these M-CK mutants to the M-band is lost[1] and interaction with myomesin or M-protein is hampered or abolished completely (see Figure 3). If at the corresponding amino acid residue of B-CK, the wild-type of which does not interact with the M-band,[14] these M-CK-specific lysine residues are built-in by site-directed mutagenesis, e.g. if L8K plus V24K (bck1 mutant) or E104K plus Q115K (bck2 mutant) B-CK mutants are generated, or all four of these residues are exchanged into lysines (bck3 mutant), a gain of function in terms of binding of these B-CK mutants to the M-band is seen.[14] We show here that upon introduction of these changes, an interaction with myomesin and M-protein is observed (see Figure 3).

MM-CK in this interaction, which was also fully in line with earlier results using the intact skinned fiber assays.[14,39] Similar results were observed between MM-CK and M-Pr6-8, although the effect of each of the lysine pairs K8/24 or K104/115 alone was less pronounced compared to the effects seen with myomesin (Figure 6(c)). Here, an interaction with the BB-CK type mutants was detectable only if all four lysine residues were introduced. However, the affinity of the BB-CK mutant containing all four lysine residues (bck3) was again in the same range as the MM-CK wild-type protein, clearly demonstrating the gain of binding function of mutated BB-CK, with the two lysine pairs introduced (Figure 4).

Discussion

The isoenzyme-specific interaction of MM-CK with the M-band in sarcomeric muscle is a well documented phenomenon, first studied 30 years ago.[19] Many biochemical, immunohistological, as well as ultrastructural studies have been performed on this topic. CK was shown to be located at the M4/M4′ substructure within the M-band.[12,20] The fraction of CK that is bound to the M-band is functionally coupled to the myofibrillar actin-activated Mg^{2+}-ATPase[21,22] and only the muscle-specific M-type MM-CK, but not the highly homologous B-type BB-CK, is able to interact with this sarcomeric structure.[18] However, all attempts to identify the appropriate interaction partner(s) of CK within the M-band have failed so far. Until now, four structural components of the M-band have been identified unambiguously. These are, besides the bare zone of the myosin rod, the 250 kDa C-terminal region of titin, the 185 kDa protein myomesin and the closely related 165 kDa M-protein as well as MM-CK. Additional M-band components were reported to be enolase,[23] AMPK[24] and calpain/p94.[25] Further components, however, might remain unidentified.

In this work, we could for the first time show unequivocally a strong interaction of MM-CK with myomesin and with the highly homologous M-protein. Titin as a third major component of the M-band shares with myomesin and M-protein the typical Ig-domain and Fn-domain structure.[26] Although all these titin domains display a high degree of similarity with the myomesin and M-protein domains, we did not observe an interaction of MM-CK with purified titin in the BiaCore analysis (data not shown). However, due to the technical difficulties when working with a protein of this molecular size (>1500 kDa), we could not exclude the possibility of titin as an additional interaction partner. This was the reason for performing the forced yeast two-hybrid analysis using several cDNAs derived from the Z-disc, A-band as well as the M-band portions of titin, respectively. Since none of these constructs exhibited binding to MM-CK, we excluded the

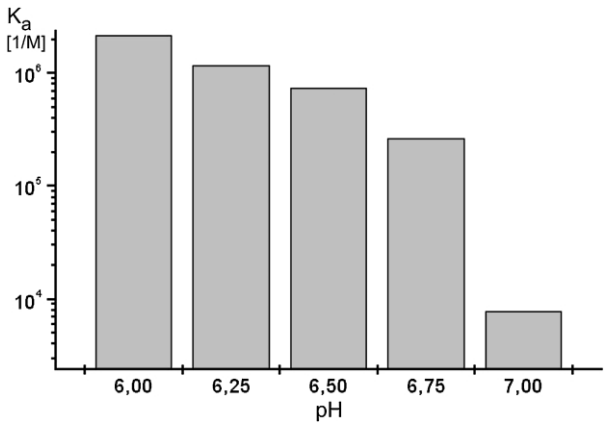

Figure 5. pH-dependence of the interaction of hMM-CK with myomesin fragment hMy7-8. The association constant (K_a) was determined for different pH values between 6.0 and 9.0. No binding could be observed above pH 7.5 (data not shown). The K_a decreased by an order of three magnitudes after increasing the binding buffer pH from 6.0 to 7.0.

possibility of unspecific binding of MM-CK to Ig-like domains and favour the conclusion that MM-CK does not interact directly with titin. The specific binding of MM-CK to two interaction partners within the sarcomeric M-band, although with different affinities, may indicate either that the enzyme is able to form part of the 3D structure of the M-band with precise geometry or, in contrast, that MM-CK might bind to the M-band *via* highly specific interactions with several partner molecules, which would represent the structural elements. The first hypothesis would emphasize a plain structural role of MM-CK, e.g. to represent the M4 and M4′ M-bridges as proposed earlier.[27] The second proposition would imply a rather flexible and dynamic interaction of MM-CK with the M-band scaffold involving one or more partner molecules, most likely depending on the precise physiological state of the muscle. This is also in line with the finding that MM-CK interacts with myomesin and M-protein with very distinct binding strengths. It may thus be important to have a

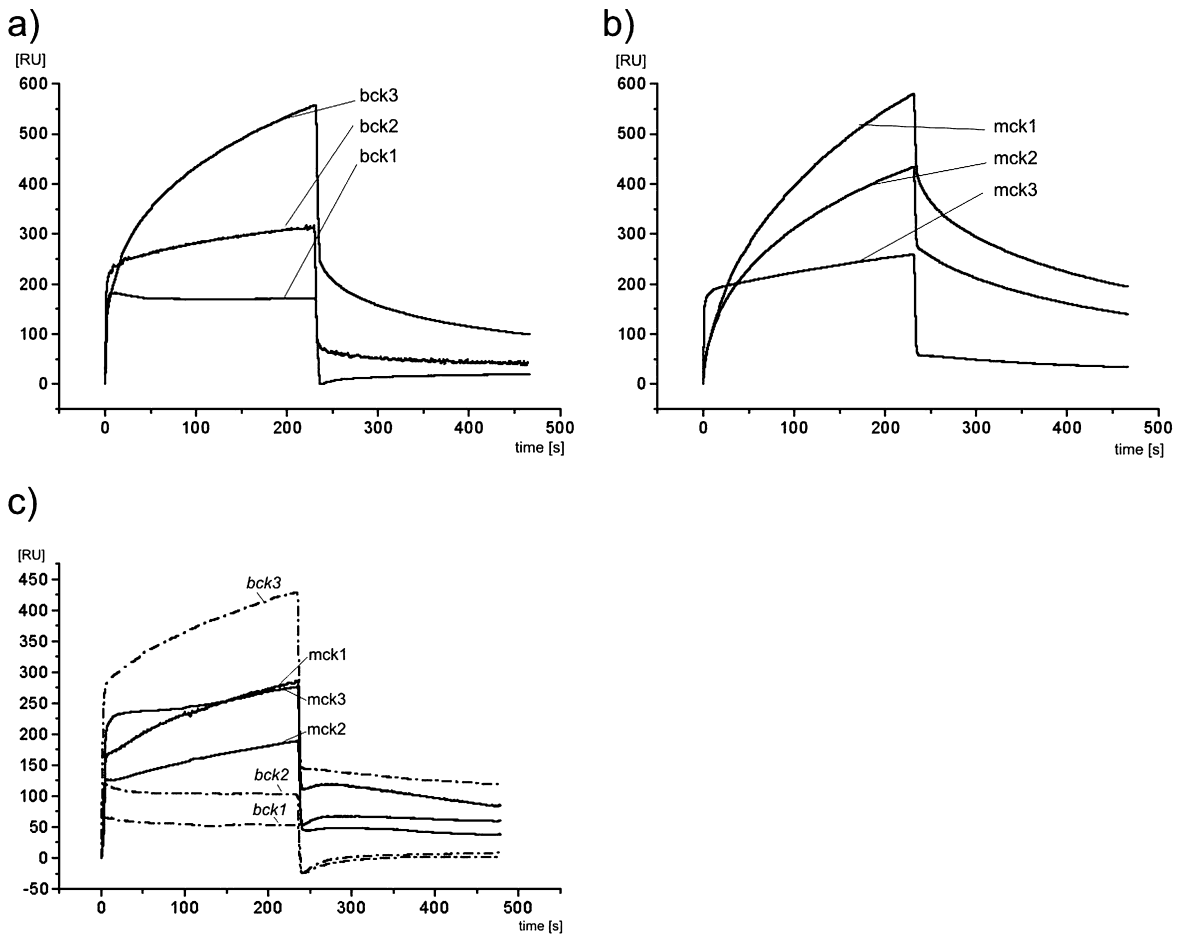

Figure 6. Interaction kinetics of CK point mutants (see Figure 3) with myomesin fragment cMy6-8 (Figure 4(a) and (b)) and M-protein fragment cMp6-8 (Figure 4(c)). All mutants were measured at a protein concentration of 5.8 μM and at pH 6.8. The successive introduction of the lysine pairs K8/24 (bck1), K104/115 (bck2) or both pairs (bck3) into the B-CK isoform leads to a stepwise increase in the affinity towards the myomesin fragment cMy6-8 (a), whereas the replacement of those lysine residues in the M-CK isoform leads to a stepwise decrease of binding strength (b). The same behaviour, but to a lesser extent, can be observed with immobilised M-protein fragment cMp6-8 (c).

second binding partner for MM-CK (albeit with lower affinity) in fast and cardiac muscle fibers, i.e. the only fiber types that contain M-protein,[3,28] particularly during times of higher demand for energy in the form of ATP.

Some of our findings offer a simple explanation, why earlier interaction studies between CK and myomesin (and other proteins), like pull down assays or immunoprecipitations did not lead to any significant and reproducible result. The main reason for this failure most likely has its reason in the strong pH dependency of this interaction. Strong binding of MM-CK to myomesin was detected only below or up to pH 7.0. No such association was seen at or above pH 7.5. Since many of those earlier studies were done at pH 7.5 or higher, it is explainable why no interaction could be seen then. Secondly, a first partial unfolding/refolding step was important to observe a reproducible interaction between CK and the immobilised ligand. The reason for this might be found in the structure of myomesin, which is built mostly of repeating Ig-like and Fn-like domains. NMR studies have revealed that the single Ig-like domains within the titin molecule exhibit significantly distinct degrees of stability and, although no structural information is available about myomesin domains, distinct stability is quite likely also true for the Ig-like domains in myomesin. It is therefore feasible that at least some of these domains might contract and convolute upon displacement from their appropriate structural environment. This, in turn, could lead to a masking of the CK binding sites, which are then accessible only after a previous unfolding step. The fact that the observed interaction of MM-CK and its lysine mutants, as well as BB-CK and its mutants, behaved in an isoenzyme-specific manner with binding characteristics exactly as observed in the skinned muscle fiber assays, indicates strongly that the interaction is specific and not simply an artefact of protein denaturation.

It is remarkable that the pH range in which the interaction occurs is exactly within the physiological pH range, in which the intramuscular pH oscillates between rest and activation. Therefore, it is likely that the observed interactions are of dynamic nature *in vivo*, depending largely on the intracellular pH and therefore indirectly on the energy state of the muscle cell. This would suite very well the role of CK as an accessory energy supply system during high and fluctuating energy requirements. The tendency to form protein–protein complexes at moderately acidic pH seems to be an intrinsic feature of MM-CK and has been observed earlier (our unpublished observations).[28] This would favour the following, simple model for the regulation and recruitment of CK with its respective interaction partners in a physiological context: ATPases with high energy turnover hydrolyse large amounts of ATP to ADP + H^+, which would successively lead to an acidification of the microenvironment around those ATPases. This acidic surrounding would enhance the tendency of MM-CK to associate with those ATPases or spatially closely located (structural) proteins. The associated MM-CK could then ensure a proper energy supply for the ATPases under high workload by replenishing the ATP from the large PCR pool. Since CK uses a H^+ for this reaction to regenerate ATP, it would, secondly, prevent a further acidification of the myofibrillar compartment. This could, as a consequence, lead to reduced activity or even inhibition of the ATPases. The collaterally exposed MM-CK-specific lysine pair K104/115 and to a lesser extent K8/24 seem to be the key residues on MM-CK for this interaction, at least for the interaction in the M-band. These very lysine residues thus might act as pH sensors by changing to a positively charged, protonated, state at pH 7 and below. In general, this would favour an interaction of MM-CK with negatively charged sites and could be the molecular basis for an interaction of CK with other ATP consuming sites like the Ca^{2+}-ATPases (SRCA and PMCA), or other energy consuming processes.[29–31]

Materials and Methods

cDNA cloning and sequencing

Human (h) and chicken (c) myomesin (My) as well as human M-protein (Mp) cDNA constructs for two-hybrid analysis were amplified from their respective cDNAs[32,33] by PCR.[34] The following primer sequences were used for subcloning:

cMy6 forward 5′ TTT(ACGCGT)GACGATAAACTTG ATATTCCC;

cMy8 reverse 5′ TTT(GTCGAC)CTCAGCTACAACG GGATCAGT;

hMy7 forward 5′ TTT(ACGCGT)GCA CCACCATCT CCACC;

hMy8 reverse TTT(GTCGAC)AACCTCTTTCCTTC CTGG ACGGGT;

cMp6 forward 5′ TTT(ACGCGT)CCATCAGCCCCTG GTCGGGTG;

cMp8 reverse (GTCGAC)TGCTTCAACAAACACTG GTTC.

Nucleotides in parentheses indicate restriction enzyme sites introduced for directional cloning of resulting PCR fragments. The chicken M-protein fragment cMp6-8 was isolated by RT-PCR from a chicken mRNA preparation using the primer

TTTACGCGTGACGATAAACTTGATATTCC

for reverse transcription and the same primer as well as

TTTGTCGACCTCAGCTACAACGGGATCAG

for PCR. Primer sequences were designed on the basis of the published chicken M-protein cDNA sequence.[35]

Creatine kinase (CK) mutants were constructed as described elsewhere.[14] Recombinant titin cDNA fragments hTz1-z3, hTz7-z8, hTa1-a4 and hTm1-m3 (encompassing Ig-like domains located in the Z-disc, A-band and M-band, respectively) were a kind gift from Dr M. Gautel (King's College, London). All cloning procedures followed standard protocols.[36] The identity of the derived constructs was verified by DNA sequencing.

Yeast two-hybrid screens and two-hybrid protein interaction analysis

Different fragments of human myomesin and M-protein as well as titin cDNAs were cloned into a pLexA bait vector.[37] The plasmid was transformed into the *Saccharomyces cerevisiae* L40 reporter strain using a modified lithium acetate protocol.[38] Subsequently, the bait-bearing strain was co-transformed first with a human skeletal muscle and secondly with a human cardiac cDNA library in the pAct2 Gal4 activation domain vector (both libraries were obtained from Clontech, Heidelberg, Germany). Selection for *HIS3* reporter gene activation was performed on selection agar plates without histidine, leucine or tryptophan (SD-LWH) essentially as described.[37] Colonies appearing after four to five days at 30 °C were assayed in filter assays for β-galactosidase activity, and library plasmids from positive clones were isolated following the Matchmaker protocol (Clontech) and their inserts sequenced.

Forced yeast two-hybrid analysis was performed by cotransformation of bait and prey vectors into L40 yeast cells and initial growth on selection medium (SD-LW). Subsequently, the activation of the HIS3 reporter gene was analysed both by plating the cells on double-selection medium (SD-LWH) and by filter assays for β-galactosidase.

Protein expression and purification

Myomesin and M-protein cDNA constructs were amplified as described above and cloned into a modified pET vector.[5] Expression of soluble His-tagged polypeptides was then induced in *Escherichia coli* BL21[DE3]-pLysS (Stratagene, Heidelberg, Germany) using 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Purification was carried out on a Ni-NTA agarose column (Qiagen, Hilden, Germany) according to standard protocols. Purified proteins were analysed by SDS–PAGE and Western blotting.

Native creatine kinase and the mutants thereof were expressed in *E. coli* BL21[DE3]pLysS and purified as described.[14]

Purification of native chicken myomesin from tissue

Chicken pectoralis muscle was chopped into small pieces and homogenised in a Waring blender (2 × 30 seconds) in ice-cold LSB buffer (100 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM β-mercaptoethanol, 10 mM Tris–HCl (pH 6.8), containing 2 mM NaP_2O_7). This buffer and all subsequent buffers contained a protease inhibitor cocktail (Complete, Roche, Rotkreuz, Switzerland) according to the manufacturer's instructions. Myofibrils were harvested (15 minutes, 3000*g*), washed three times in LSB, and resuspended in extraction solution (0.6 M KCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM β-mercaptoethanol, 10 mM imidazole, pH 7.0) for 40 minutes. The supernatant obtained after centrifugation (one hour at 20,000*g*) was dialysed extensively against buffer A (50 mM Tris–HCl (pH 7.9), 2 mM EGTA, 1 mM β-mercaptoethanol) and subsequently clarified by centrifugation (one hour at 100,000*g*). A saturated ammonium sulphate solution was slowly added to 40% saturation. After stirring (30 minutes) and centrifugation (15 minutes at 15,000*g*) the pellet was collected, redissolved and dialysed against GF buffer (25 mM Hepes (pH 7.0), 1 mM EDTA, 500 mM NaCl) and fractionated over a size-exclusion column (Superose 12, Pharmacia). The myomesin-containing fractions were pooled and dialysed against 20 mM Hepes (pH 6.8) for immobilisation on a BiaCore sensor chip.

Biosensor assays

Purified intact myomesin. myomesin and M-protein fragments were coupled to carboxymethyldextrane (CMD)-coated biosensor chips (CM5, BiaCore, Uppsala, Sweden) following the manufacturer's instructions. Briefly, CMD cuvettes were activated by EDC/NHS and fragments were coupled at 0.05–0.1 mg/ml in 10 mM Hepes (pH 6.8). Protein densities of 2000–4000 response units were aimed at preventing steric inhibition. Protein interactions with creatine kinase (wild-type) and mutants thereof were monitored on a Biosensor (BiaCore, Uppsala, Sweden) in binding buffer (10 mM imidazole (pH 6.8), 80 mM sodium propionate, if not stated otherwise in the text) with sampling rates of one second. Regeneration of the sensor cuvette was achieved with binding buffer containing 0.1% (w/v) SDS followed by an eight minute washing/refolding phase with binding buffer. The observed binding curves were fit assuming single-phase kinetics, and single-phase dissociation/association. The kinetic parameters were calculated from these fits using the BiaEvaluation software (v2.1, BiaCore, Uppsala, Sweden).

Acknowledgements

We are grateful for the many helpful discussions with all members of the Wallimann and Fürst groups. In addition, we thank Dr Peter van der Ven for help with cloning of the bait constructs. Dr J.-C. Perriard is thanked for his donation of the chicken myomesin cDNA, and Dr M. Gautel for providing the titin clones. We are especially grateful to Dr H. M. Eppenberger (Prof. Emeritus) for his continued interest in this work, which started in his laboratory some 30 years ago, now solving the puzzle of how an ambiquitous enzyme, like CK, can form a specifically defined subcellular microcompartment at the sarcomeric M-band, and identifying the binding-partners involved. This work was supported by an EMBO short-term fellowship, ETH graduate training grants, SNF grant (31-62024.00) and BONUS-29 program of the ETH, the German Parent Organization "Benni & Co", the "Deutsche Gesellschaft für Muskelkrankheiten", the "Swiss Foundation for muscle research" (to T.W.), as well as by a grant from the Deutsche Forschungsgemeinschaft (Fu 339-2 to D.F.).

References

1. Luther, P. & Squire, J. (1978). Three-dimensional structure of the vertebrate muscle M-region. *J. Mol. Biol.* **125**, 313–324.
2. Fürst, D. O., Obermann, W. M. & van der Ven, P. F.

(1999). Structure and assembly of the sarcomeric M band. *Rev. Physiol. Biochem. Pharmacol.* **138**, 163–202.
3. Grove, B. K., Kurer, V., Lehner, C., Doetschman, T. C., Perriard, J. C. & Eppenberger, H. M. (1984). A new 185,000-dalton skeletal muscle protein detected by monoclonal antibodies. *J. Cell Biol.* **98**, 518–524.
4. Trinick, J. & Lowey, S. (1977). M-protein from chicken pectoralis muscle: isolation and characterization. *J. Mol. Biol.* **113**, 343–368.
5. Obermann, W. M., Gautel, M., Steiner, F., van der Ven, P. F., Weber, K. & Fürst, D. O. (1996). The structure of the sarcomeric M band: localization of defined domains of myomesin, M-protein, and the 250 kD carboxy-terminal region of titin by immunoelectron microscopy. *J. Cell Biol.* **134**, 1441–1453.
6. Obermann, W. M., Gautel, M., Weber, K. & Fürst, D. O. (1997). Molecular structure of the sarcomeric M band: mapping of titin and myosin binding domains in myomesin and the identification of a potential regulatory phosphorylation site in myomesin. *EMBO J.* **16**, 211–220.
7. Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K. & Eppenberger, H. M. (1992). Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. *Biochem. J.* **281**, 21–40.
8. Eppenberger, H. M., Dawson, D. M. & Kaplan, N. O. (1967). The comparative enzymology of creatine kinases. I. Isolation and characterization from chicken and rabbit tissues. *J. Biol. Chem.* **242**, 204–209.
9. Schlegel, J., Zurbriggen, B., Wegmann, G., Wyss, M., Eppenberger, H. M. & Wallimann, T. (1988). Native mitochondrial creatine kinase forms octameric structures. I. Isolation of two interconvertible mitochondrial creatine kinase forms, dimeric and octameric mitochondrial creatine kinase: characterization, localization, and structure-function relationships. *J. Biol. Chem.* **263**, 16942–16953.
10. Schlegel, J., Wyss, M., Eppenberger, H. M. & Wallimann, T. (1990). Functional studies with the octameric and dimeric form of mitochondrial creatine kinase. Differential PH-dependent association of the two oligomeric forms with the inner mitochondrial membrane. *J. Biol. Chem.* **265**, 9221–9227.
11. Trask, R. V. & Billadello, J. J. (1990). Tissue-specific distribution and developmental regulation of M and B creatine kinase mRNAs. *Biochim. Biophys. Acta*, **1049**, 182–188.
12. Wallimann, T., Doetschman, T. C. & Eppenberger, H. M. (1983). Novel staining pattern of skeletal muscle M-lines upon incubation with antibodies against MM-creatine kinase. *J. Cell Biol.* **96**, 1772–1779.
13. Stolz, M., Kraft, T. & Wallimann, T. (1998). The isoenzyme-diagnostic regions of muscle-type creatine kinase, the M-260 and M-300 box, are not responsible for its binding to the myofibrillar M-band. *Eur. J. Cell Biol.* **77**, 1–9.
14. Hornemann, T., Stolz, M. & Wallimann, T. (2000). Isoenzyme-specific interaction of muscle-type creatine kinase with the sarcomeric M-line is mediated by NH(2)-terminal lysine charge-clamps. *J. Cell Biol.* **149**, 1225–1234.
15. Wallimann, T., Turner, D. C. & Eppenberger, H. M. (1977). Localization of creatine kinase isoenzymes in myofibrils. I. Chicken skeletal muscle. *J. Cell Biol.* **75**, 297–317.
16. Kraft, T., Messerli, M., Rothen-Rutishauser, B., Perriard, J. C., Wallimann, T. & Brenner, B. (1995). Equilibration and exchange of fluorescently labeled molecules in skinned skeletal muscle fibers visualized by confocal microscopy. *Biophys. J.* **69**, 1246–1258.
17. Pan, J. W., Hamm, J. R., Rothman, D. L. & Shulman, R. G. (1988). Intracellular pH in human skeletal muscle by 1H NMR. *Proc. Natl Acad. Sci. USA*, **85**, 7836–7839.
18. Stolz, M. & Wallimann, T. (1998). Myofibrillar interaction of cytosolic creatine kinase (CK) isoenzymes: allocation of N-terminal binding epitope in MM–CK and BB–CK. *J. Cell Sci.* **111**, 1207–1216.
19. Turner, D. C., Wallimann, T. & Eppenberger, H. M. (1973). A protein that binds specifically to the M-line of skeletal muscle is identified as the muscle form of creatine kinase. *Proc. Natl Acad. Sci. USA*, **70**, 702–705.
20. Strehler, E. E., Carlsson, E., Eppenberger, H. M. & Thornell, L. E. (1983). Ultrastructural localization of M-band proteins in chicken breast muscle as revealed by combined immunocytochemistry and ultramicrotomy. *J. Mol. Biol.* **166**, 141–158.
21. Wallimann, T., Schlösser, T. & Eppenberger, H. M. (1984). Function of M-line-bound creatine kinase as intramyofibrillar ATP regenerator at the receiving end of the phosphorylcreatine shuttle in muscle. *J. Biol. Chem.* **259**, 5238–5246.
22. Ventura-Clapier, R., Veksler, V. & Hoerter, J. A. (1994). Myofibrillar creatine kinase and cardiac contraction. *Mol. Cell Biochem.* **133-134**, 125–144.
23. Foucault, G., Vacher, M., Merkulova, T., Keller, A. & Arrio-Dupont, M. (1999). Presence of enolase in the M-band of skeletal muscle and possible indirect interaction with the cytosolic muscle isoform of creatine kinase. *Biochem. J.* **338**, 115–121.
24. Ponticos, M., Lu, Q. L., Morgan, J. E., Hardie, D. G., Partridge, T. A. & Carling, D. (1998). Dual regulation of the AMP-activated protein kinase provides a novel mechanism for the control of creatine kinase in skeletal muscle. *EMBO J.* **17**, 1688–1699.
25. Suzuki, K., Sorimachi, H., Yoshizawa, T., Kinbara, K. & Ishiura, S. (1995). Calpain: novel family members, activation, and physiologic function. *Biol. Chem. Hoppe Seyler* **376**, 523–529.
26. Labeit, S. & Kolmerer, B. (1995). Titins: giant proteins in charge of muscle ultrastructure and elasticity. *Science*, **270**, 293–296.
27. Wallimann, T. & Eppenberger, H. M. (1985). Localization and function of M-line-bound creatine kinase. M-band model and creatine phosphate shuttle. *Cell Muscle Motil.* **6**, 239–285.
28. Grove, B. K., Cerny, L., Perriard, J. C. & Eppenberger, H. M. (1985). Myomesin and M-protein: expression of two M-band proteins in pectoral muscle and heart during development. *J. Cell Biol.* **101**, 1413–1421.
29. Saks, V. A., Lipina, N. V., Sharov, V. G., Smirnov, V. N., Chazov, E. & Grosse, R. (1977). The localization of the MM isozyme of creatine phosphokinase on the surface membrane of myocardial cells and its functional coupling to ouabain-inhibited (NA^+, K^+)-ATPase. *Biochim. Biophys. Acta*, **465**, 550–558.
30. Sharov, V. G., Saks, V. A., Smirnov, V. N. & Chazov, E. I. (1977). An electron microscopic histochemical investigation of the localization of creatine phosphokinase in heart cells. *Biochim. Biophys. Acta*, **468**, 495–501.

31. Rossi, A. M., Eppenberger, H. M., Volpe, P., Cotrufo, R. & Wallimann, T. (1990). Muscle-type MM creatine kinase is specifically bound to sarcoplasmic reticulum and can support $Ca2^{+}$ uptake and regulate local ATP/ADP ratios. *J. Biol. Chem.* **265**, 5258–5266.
32. Vinkemeier, U., Obermann, W., Weber, K. & Fürst, D. O. (1993). The globular head domain of titin extends into the center of the sarcomeric M band. cDNA cloning, epitope mapping and immunoelectron microscopy of two titin-associated proteins. *J. Cell Sci.* **106**, 319–330.
33. Bantle, S., Keller, S., Haussmann, I., Auerbach, D., Perriard, E., Mühlebach, S. & Perriard, J. C. (1996). Tissue-specific isoforms of chicken myomesin are generated by alternative splicing. *J. Biol. Chem.* **271**, 19042–19052.
34. Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. & Arnheim, N. (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*, **230**, 1350–1354.
35. Noguchi, J., Yanagisawa, M., Imamura, M., Kasuya, Y., Sakurai, T., Tanaka, T. & Masaki, T. (1992). Complete primary structure and tissue expression of chicken pectoralis M-protein. *J. Biol. Chem.* **267**, 20302–20310.
36. Ausubel, F. M., Brent, R., Kingston, E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1994).*Current Protocols in Molecular Biology*, Wiley, New York.
37. Stenmark, H., Vitale, G., Ullrich, O. & Zerial, M. (1995). Rabaptin-5 is a direct effector of the small GTPase Rab5 in endocytic membrane fusion. *Cell*, **83**, 423–432.
38. Vojtek, A. B., Hollenberg, S. M. & Cooper, J. A. (1993). Mammalian Ras interacts interacts directly with the serine/threonine kinase Raf. *Cell*, **74**, 205–214.
39. Kraft, T., Hornemann, T., Stolz, M., Nier, V. & Wallimann, T. (2000). *J. Muscle Res. Cell Motil.* **21**, 691–703.

Edited by J. Karn

(Received 24 June 2003; accepted 15 July 2003)

Note added in proof: In the meantime, a comparative analysis of the M-band binding motives of creatine kinases throughout the animal kingdom show a very high evolutionary conservation of the major M-band charge clamp, the K104/K115 lysine pair, and a high conservation of K115/K104 in cytosolic CK of all vertebrates, lower-vertebrates and even down to the protochordates, indicating an early evolutionary appearance of this myofibrillar binding region(s) concomitant to the appearance of sarcomeric muscle (Uda, K., Suzuki, T., & W. R. Ellington, *Int. J. Biochem. Cell Biol.*, (2003) in press).