Mapping of a Myosin-binding Domain and a Regulatory Phosphorylation Site in M-Protein, a Structural Protein of the Sarcomeric M Band

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The myofibrils of cross-striated muscle fibers contain in their M bands cytoskeletal proteins whose main function seems to be the stabilization of the three-dimensional arrangement of thick filaments. We identified two immunoglobulin domains (Mp2–Mp3) of M-protein as a site binding to the central region of light meromyosin. This binding is regulated in vitro by phosphorylation of a single serine residue (Ser76) in the immediately adjacent amino-terminal domain Mp1. M-protein phosphorylation by cAMP-dependent kinase A inhibits binding to myosin LMM. Transient transfection studies of cultured cells revealed that the myosin-binding site seems involved in the targeting of M-protein to its location in the myofibril. Using the same method, a second myofibril-binding site was uncovered in domains Mp9–Mp13. These results support the view that specific phosphorylation events could be also important for the control of sarcomeric M band formation and remodeling.

INTRODUCTION

Thick and thin myofilaments, the major structural and functional entities of cross-striated myofibrils, are kept in their remarkably stable and regular arrangement by a highly complex cytoskeleton (see, for example, review by Small et al., 1992). While thin filaments are organized by the Z-discs, the hexagonal thick filament lattice seems cross-linked in the central bare zone by bridging structures, the so-called M-bridges and M-filaments (Luther and Squire, 1978).

Until now, only a small group of proteins has been shown to be true M band constituents. MM creatine kinase is thought to be the component that makes up the M4-M4' bridges (Wallimann et al., 1983; Wallimann and Eppenberger, 1985). It is not yet clear whether, in addition to its obvious role in energy metabolism, MM creatine kinase is also involved in the linkage of thick filaments. Three structural proteins were revealed as true M band constituents: M-protein (Masaki and Takaiti, 1974), myomesin (Grove et al., 1984), and titin (Nave et al., 1989; Vinkemeier et al., 1993). Immunofluorescence studies revealed striking differences in embryonic expression patterns of these proteins: while both titin and myomesin are expressed from the earliest stages of myocyte differentiation (Grove et al., 1985; Fürst et al., 1989; Carlsson et al., 1990), M-protein shows a stage- and tissue-specific distinct redistribution. First, M-protein is transiently expressed in all fetal cross-striated muscle fibers. It is subsequently suppressed in skeletal muscle fibers around birth and finally is reexpressed in adult type-II fibers (Grove et al., 1985, 1987; Carlsson et al., 1990). Recently, the use of a panel of sequence-assigned antibodies in immunoelectron microscopy allowed the proposal of a structural model that describes the layout of titin, myomesin, and M-protein in the sarcomeric M band. Both titin and myomesin are arranged largely in an antiparallel and staggered manner, while M-protein seems to bridge the thick filaments essentially in a perpendicular direction (Obermann et al., 1996).

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Although M-protein has been known for a long time and protocols for its purification did exist, it has been remarkably difficult to describe, at the molecular level, its binding to other myofibrillar proteins. Studies performed independently by three different groups concluded “that such interactions are either weak or absent in vitro” (Mani and Kay, 1978; Woodhead and Lowey, 1983; Bähler et al., 1985). We addressed this question by producing a panel of recombinant M-protein and myosin rod fragments. Purified recombinant proteins were subsequently used to search for respective binding sites. These experiments identified M-protein domains Mp2 to Mp3 as myosin-binding site and showed that this interaction can be regulated by phosphorylation.

**MATERIALS AND METHODS**

**Expression of M-Protein Fragments in Escherichia coli**

Partial M-protein sequences were amplified by polymerase chain reaction (PCR) (Saiki et al., 1985) using the full-length human cDNA (Vinkemeier et al., 1993) as a template. PCR products were cloned into a pET23a vector derivative. This provided the recombinant protein fragments with a carboxy-terminally located His6 sequence, followed by an EEF-tag that can be detected with a monoclonal antibody. Subsequent to growth of E. coli BL21(DE3)pLysS cells (Studier et al., 1990) at OD<sub>600</sub> = 1.0 in LB medium supplemented with 2% glucose, 100 mM KCl, 0.2% Tween-20, 5 mM 2-mercaptoethanol containing 5 μM E64 and 1 mM PMSF as protease inhibitors. After centrifugation at 16,000 x g, soluble recombinant proteins were enriched by metal chelate affinity chromatography. Briefly, protein solutions were applied onto Ni-NTA-agarose columns (Qiagen, Hilden, Germany), which were washed with buffer A and subsequently with buffer B (same as buffer A, except pH 6.0). Finally, the recombinant proteins were eluted with 500 mM imidazole in buffer B. After dialysis against buffer C (50 mM Tris-HCl, pH 7.9, 100 mM KCl, 2 mM MgCl₂, 0.2% Triton X-100, 5 mM 2-mercaptoethanol) containing 5 μM E64 and 1 mM PMSF as protease inhibitors. After centrifugation at 16,000 x g, soluble recombinant proteins were enriched by metal chelate affinity chromatography. Briefly, protein solutions were applied onto Ni-NTA-agarose columns (Qiagen, Hilden, Germany), which were washed with buffer A and subsequently with buffer B (same as buffer A, except pH 6.0). Finally, the recombinant proteins were eluted with 500 mM imidazole in buffer B. After dialysis against buffer C (50 mM Tris-HCl, pH 7.9, 100 mM KCl, 2 mM MgCl₂, 0.2% Triton X-100, 5 mM 2-mercaptoethanol) containing 5 μM E64 and 1 mM PMSF as protease inhibitors. After centrifugation at 16,000 x g, soluble recombinant proteins were enriched by metal chelate affinity chromatography. Briefly, protein solutions were applied onto Ni-NTA-agarose columns (Qiagen, Hilden, Germany), which were washed with buffer A and subsequently with buffer B (same as buffer A, except pH 6.0). Finally, the recombinant proteins were eluted with 500 mM imidazole in buffer B. After dialysis against buffer C (50 mM Tris-HCl, pH 7.9, 100 mM KCl, 2 mM MgCl₂, 0.2% Triton X-100, 5 mM 2-mercaptoethanol) containing 5 μM E64 and 1 mM PMSF as protease inhibitors. After centrifugation at 16,000 x g, soluble recombinant proteins were enriched by metal chelate affinity chromatography. Briefly, protein solutions were applied onto Ni-NTA-agarose columns (Qiagen, Hilden, Germany), which were washed with buffer A and subsequently with buffer B (same as buffer A, except pH 6.0). Finally, the recombinant proteins were eluted with 500 mM imidazole in buffer B. After dialysis against buffer C (50 mM Tris-HCl, pH 7.9, 100 mM KCl, 2 mM MgCl₂, 0.2% Triton X-100, 5 mM 2-mercaptoethanol) containing 5 μM E64 and 1 mM PMSF as protease inhibitors. After centrifugation at 16,000 x g, soluble recombinant proteins were enriched by metal chelate affinity chromatography.

**Phosphorylation of Proteins and Synthetic Peptides**

Purified M-protein as well as its proteolytic trypsin fragment and recombinant M-protein fragments were added at a final concentration of 0.2 μg/ml to 20 μl of assay buffer (50 mM MES, pH 6.9, 100 mM KCl, 2 mM MgCl₂). Phosphorylation reactions were at 30°C for 30 min with 1 U protein kinase A from porcine heart (Sigma Chemical, St. Louis, MO) or 1 μl of muscle extract and 1 μCi [γ-<sup>32</sup>P]-ATP (3000 Ci/mmol, Amersham, Arlington Heights, IL). After addition of sample buffer (Laemmli, 1970) and heating to 65°C for 10 min, poly peptides were analyzed by 4–12% or 6–20% SDS-PAGE. Gels were dried, and autoradiography was performed at ~80°C with intensifying screens. For a quantification of the phosphorylation, bands from individual assays were cut out of the gels and homogenized. Gel fragments were suspended in “Ready Safe” (Beckman, Fullerton, CA) and measured in a Beckman LS 8100 liquid scintillation counter. Means of the incorporated counts were calculated from three independent experiments.

Synthetic peptides were phosphorylated for kinetic experiments essentially as described previously (Kemp et al., 1977). Briefly, the reaction mixture (total volume, 70 μl) contained the respective peptide at concentrations ranging from 0.1 to 1.5 mM in the following solution: 10 μCi [γ-<sup>32</sup>P]-ATP (0.5 mM), 62.5 mM MES, pH 6.9, 12.5 mM magnesium acetate, 0.25 mM EGTA, and either protein kinase A (catalytic subunit, Boehringer, Mannheim, Germany) or sarcomeric muscle extracts prepared as described previously (Obermann et al., 1995). Subsequent to incubation at 37°C for 1, 2, 5, 10, and 15 min, 8-μl aliquots were removed from the reaction and phosphorylated peptides were separated from [γ-<sup>32</sup>P]-ATP by the phosphocellulose binding technique (Casnellie, 1991). Apparent K<sub>m</sub>...
and Vmax values were determined by fitting the data of a double-reciprocal Lineweaver-Burk plot to the Michaelis-Menten equation using the method of least squares.

Phosphoamino Acid Analysis

32P-labeled protein was recovered from sample buffer by the method of Wessel and Flügge (1984), dried, and hydrolyzed in 6 M HCl at 110°C for 2 h. After lyophilization the hydrolysate was dissolved in 10 μl H2O and applied to a Polygram CEL400 Uni layer plate (Merck, Darmstadt, Germany). Electrophoretic separation of phosphoamino acids was in 10% acetic acid, 1% pyridin, pH 3.5, at 800 V for 1 h. Radioactively labeled phosphoamino acids were identified by visualization of standard phosphoamino acids with ninhydrin and autoradiography.

Myosin-binding Assays

M-protein was treated with endoproteinase Asp-N (see above), and the digest was subjected to gel filtration on a Superose 12 HR10/30 column. One microliter of each fraction was spotted onto nitrocel lulose membranes (BA 85, Schleicher & Schüll, Dassel, Germany). After air drying, the strips were blocked with overlay buffer (1% BSA, 0.2% Tween 20, 100 mM KCl, 20 mM imidazol-HCl pH 7.0, 0.1 mM DTT) for 30 min. Purified LMM and recombinant LMM-30 were biotinylated (NHS-LC-Biotinylation kit, Pierce, Rockford Chemical, Rockford, IL) following the instructions of the manufacturer, diluted to 0.1 mg/ml in overlay buffer, and used to treat the blocked strips for 60 min. After three washes with overlay buffer, the strips were incubated for 30 min with an avidin-biotin-peroxidase complex (ABC Peroxidase staining kit, Pierce). After a final washing cycle, binding of LMM to M-protein fragments was visualized by reaction in 5 ml of 100 mM Tris-HCl, pH 7.5, supplemented with 100 μl diaminobenzidine (40 mg/ml stock), 25 μl NiCl2 (80 mg/ml stock), and 1.5 μl 30% H2O2. All steps were carried out at room temperature.

In the case of recombinant M-protein fragments, both α-chymotryptic myosin subfragments (myosin rod and LMM) and recombinant LMM fragments (LMM75, LMM59, LMM50, LMM50–75, LMM30) were dialyzed to 50 mM KCl, 5 mM Na-phosphate, pH 7.0, to allow the formation of filaments. Approximately 1 μg of each suspension was spotted onto nitrocellulose membranes. All subsequent steps were as described above except that individual strips were incubated for 60 min with the respective M-protein fragments was visualized by reaction in 5 ml of 100 mM Tris-HCl, pH 7.5, supplemented with 100 μl diaminobenzidine (40 mg/ml stock), 25 μl NiCl2 (80 mg/ml stock), and 1.5 μl 30% H2O2. All steps were carried out at room temperature.

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Construction of the pCMV5-T7 Vector and of Recombinant Constructs

pCMV5 vector was a generous gift from Dr. V. Gerke (MPI, Göttingen). Its polycloning site was excised by digestion with restriction enzymes EcoRI and SmaI and was replaced by a new cassette containing, from 5′ to 3′, the following restriction enzyme sites: BamHI, MluI, HindIII, Sall. This polylinker was followed by a sequence encoding the 12-amino acids long T7-tag MASMFTGQQMQGR (Lutz-Freyermuth et al., 1990; Tsai et al., 1992) and an in-frame stop codon. The T7-tag peptide is recognized by a specific monoclonal antibody that was purchased from Novagen (Heidelberg, Germany).

Desired M-protein fragments were amplified by PCR (Saiki et al., 1985) using sense primers containing an MluI site and antisense primers containing a Sall site adjacent to a matching sequence of 30 bp derived from the human cDNA (Vinkemeier et al., 1995). Resulting PCR fragments were double digested with MluI and Sall and ligated to the correspondingly cut pCMV5-T7 vector following standard protocols (Sambrook et al., 1989). Plasmids were propagated in JM 109 bacteria, and the DNA was purified on Qiagen columns (Qiagen, Hilden, Germany).

Cell Culture and Transfections

BHK-21/C13 and C2C12 cells were cultured in DMEM supplemented with 2 mM L-glutamine, antibiotics (100 U/ml penicillin and 1 μg/ml streptomycin), and 10% FCS (BHK) or 15% FCS (C2C12), all from Life Technologies (Eggenstein, Germany). Approximately 8 h before transfection, cells were seeded on glass coverslips at a density that resulted in 40–50% confluency at the time of transfection. The cells were transfected with the recombinant pCMV5-T7 constructs using a modification of the calcium phosphate precipitation method (Sambrook et al., 1989). Briefly, after incubation with the transfection mixture for 16 h, the cells were washed with PBS and glycerol shocked for 2 min using 15% glycerol in culture medium. Fresh medium was subsequently added. At different time intervals after transfection, cells were fixed for 10 min in 3% paraformaldehyde in PBS and permeabilized for 10 min in 0.5% Triton X-100 in PBS. After being rinsed with PBS, the specimens were used in immunofluorescence assays or stored in PBS at 4°C until use. Alternatively, the culture medium was replaced by a low-nutrition medium (DMEM, L-glutamine and antibiotics as above, and 0.4% Ultroser G [Life Technologies]), to induce differentiation in both cell types. Between 1 and 6 d after this medium change, cells were fixed and permeabilized as described above.

Immunohistochemistry

Fixed and permeabilized transfected cells were rinsed with PBS/0.05% Tween 20 (PBS1) and incubated with an antibody directed against the T7 tag (immunoglobulin G2b [IgG2b]; Novagen). In some experiments the cells were double-stained using standard procedures (Van der Ven et al., 1993) with the T7 tag antibody and an M-protein domain-specific antibody (MpAA259, IgA; Obermann et al., 1996), a titin antibody (9D10, IgM; Wang and Greaser, 1985), or tetramethylrhodamine-5-isothiocyanate-conjugated phalloidin (Sigma, Deisenhofen, Germany) to identify F-actin. The appropriate Ig subtype-specific fluorescein isothiocyanate- or Texas Red-conjugated secondary antibodies were purchased from Southern Biotechnology Associates (Birmingham, AL). They were diluted in PBS/T according to the recommendations of the manufacturer.

Miscellaneous Procedures

Protein concentrations were determined with the Bio-Rad dye reagent (Bio-Rad, Richmond, CA). Synthetic peptides were obtained by Fmoc chemistry on a Pep Synthesizer TM9050 (Millipore, Bedford, MA). Microsequencing was done as reported (Obermann, et al., 1995). All DNA cloning steps followed standard protocols (Ausubel et al., 1995, Sambrook et al., 1989). SDS-PAGE and immunoblotting were as described previously (Fürst et al., 1988).

RESULTS

M-protein Domains Mp1 to Mp2 Interact with Myosin Filaments in Vitro

To locate the myosin-binding site of M-protein, we used limited proteolysis of the purified bovine protein to produce defined subfragments. Most revealing was cleavage with endoproteinase AspN, which provided a ~45-kDa fragment and a ~110-kDa fragment (arrows in Figure 2A). Microsequencing located their amino termini to Glu136 and Glu496 of the translated human cDNA sequence. Thus, the 45-kDa fragment comprises essentially domains Mp2 to Mp4, and the 110-kDa fragment covers the carboxy-terminally situated remainder of the molecule (for domain designations see sketch in Figure 1). The two fragments were...
M-protein Is Phosphorylated by PKA in the Amino-terminal Domain Mp1

M-protein bears a unique amino-terminal domain (Mp1; 151 residues) and 12 repeat domains of either the Ig cII and fibronectin type III class (Vinkemeier, et al., 1993). Detailed computer analysis of the translated human cDNA sequence predicted two target sites for cAMP-dependent PKA: Ser39 in the motif KKRAS and Ser76 in the sequence KRVS. To analyze whether one or both of these serine residues can be phosphorylated in the native protein, we incubated purified bovine M-protein with PKA in the presence of [γ-32P]ATP. Figure 4 documents the phosphorylation of M-protein by PKA (Figure 4A, lane 1, and Figure 4B, lane 1). For a preliminary mapping of the phosphorylation site(s), we subjected M-protein to limited proteolysis by trypsin and isolated a stable fragment comprising domains Mp6–Mp13 (established by microsequencing). Since this fragment cannot be phosphorylated by PKA (Figure 4B), the phosphorylation site(s) must lie in Mp1–Mp5. Furthermore, we used a digest of M-protein with endoproteinase AspN. This yielded two fragments comprising domains Mp2–Mp4 and Mp5–Mp13, respectively (see Figure 2A). Since neither fragment can be phosphorylated, we concluded that the phosphorylation site must be located in the unique amino terminus and possibly involves the predicted sites in domain Mp1 (Figure 4, A and B). Phosphoamino acid analysis of radiolabeled purified M-protein demon-
Stratified that phosphorylation occurs exclusively on serine (Figure 4C).

For a detailed kinetic characterization of the potential PKA phosphorylation sites, we synthesized two peptides: LDEYASKKRASTQASSQ, corresponding to residues 29 – 45 of the human sequence, and RVCAKRVSTQEDEEQE, comprising residues 69 – 84 (Vinkemeier, et al., 1993). Both peptides were efficiently phosphorylated by PKA. The kinetics of the reaction were compared with the kinetics of the liver pyruvate kinase peptide LRRASLG (“kemptide”; Kemp et al., 1977) and a peptide from a region in myomesin, which is also phosphorylated by PKA (EKARLKSRSAP-WTGQ; Obermann et al., 1997). Table 1 shows that $k_M$ values of both M-protein peptides were $\sim 1000$ mM, which is approximately twice the $k_M$ of the myomesin peptide. The $V_{\text{max}}$ was not determined in absolute numbers, since the concentration of PKA in the commercially available preparations could not be measured. Therefore, the numbers in Table 1 give relative units. Thus, the $V_{\text{max}}$ for the phosphorylation of the first M-protein peptide (LDEYASKKRASTQASSQ) is $\sim 30\%$ of that of the myomesin peptide reaction, while the $V_{\text{max}}$ for the second M-protein peptide (RVCAKRVSTQEDEEQE) is $\sim 50\%$.

To examine whether both sites are similarly recognized in the native M-protein molecule, several recombinant constructs were made in which either one (Ser39, Ser76) or both serine residues were mutated to alanine. Since the amino-terminal domain Mp1 alone remained entirely insoluble after expression in E. coli, larger constructs comprising domains Mp1–Mp5 were used. All recombinant proteins expressed in E. coli...
were purified in the native state as described in MATERIALS AND METHODS. Phosphorylation was assayed by incubation with \[^{32}P\]ATP in the presence of PKA, followed by SDS-PAGE and autoradiography. Figure 5 shows that both normal Mp1–5 and the mutant Mp1–5(Ser39Ala) were readily phosphorylated to comparable levels, while the mutant Mp1–5(Ser76Ala) and the double-mutant Mp1–5(Ser39, Ser76Ala) were essentially nonreactive. To determine the stoichiometry of the phosphorylation, the amount of phosphate incorporation into M-protein and the recombinant protein fragments was compared by scintillation counting. Again, only M-protein, “wild-type” Mp1–5, and the mutant Mp1–5(Ser39Ala) were phosphorylated at the same level (0.97, 0.95, and 0.96 mol of phosphate per mol of protein, respectively), while both the other mutants were essentially negative (our unpublished results). Thus, it seems that in the native M-protein and in the fragment Mp1–5, serine 76 is the only substrate for PKA.

Interaction of M-Protein with Myosin Is Modulated by PKA Phosphorylation

Because the experiments described above delineate a myosin-binding site located in proximity of a target site for PKA (serine 76), we analyzed whether PKA phosphorylation of M-protein influences the binding to myosin. In the binding assays, a series of proteolytic and recombinant myosin rod fragments were overlaid with purified bovine M-protein before or after PKA phosphorylation. Figure 6a shows that M-protein bound to the myosin rod derivatives exactly as described above for the proteolytic and recombinant fragments (Figures 2 and 3). On the other hand, phosphorylation of M-protein by PKA almost completely abolished the binding (Figure 6b).

Different myosin derivatives were also used to map the M-protein–binding site on the rod portion of myosin at greater precision. While proteolytic myosin rod, and proteolytic and recombinant LMM, as well as LMM 59 and LMM 50, were recognized by M-protein, LMM 50–75 and LMM 30 failed to bind M-protein (Figure 6a). We therefore conclude that the M-protein–binding site on the myosin heavy chain is confined to the 169 amino acids between residues 1506 and 1674 located in the central part of LMM (numbers refer to

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Table 1. Phosphorylation of synthetic peptides by PKA

<table>
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<tr>
<th>Peptide sequence</th>
<th>Apparent $K_M$</th>
<th>$V_{max}$</th>
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<tr>
<td>LRRASLG</td>
<td>16</td>
<td>20.2 mmol min$^{-1}$ mg$^{-1}$</td>
</tr>
<tr>
<td>LRKASLG</td>
<td>1400</td>
<td>17.1 mmol min$^{-1}$ mg$^{-1}$</td>
</tr>
<tr>
<td>LRRASLG</td>
<td>260</td>
<td>16.9 mmol min$^{-1}$ mg$^{-1}$</td>
</tr>
<tr>
<td>EKARLKRPSAPWTGQ</td>
<td>14</td>
<td>266.4 cpm/min</td>
</tr>
<tr>
<td>LDEYASKKRASTQASSQ</td>
<td>993</td>
<td>42.0 cpm/min</td>
</tr>
<tr>
<td>RVCAKRSTQEDEQE</td>
<td>1027</td>
<td>66.5 cpm/min</td>
</tr>
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</table>

*The upper part of the table lists apparent $K_M$ and $V_{max}$ values for a well characterized peptide substrate (kemptide) for PKA (Kemp et al., 1977) and two derivatives. The lower part summarizes our measurements using kemptide, a peptide from myomesin (see Obermann et al., 1996) and the two peptides from M-protein described in this study. Phosphorylation reactions were carried out as described in MATERIALS AND METHODS. Peptide sequences are given in column 1.
the amino acid sequence of rabbit fast skeletal muscle myosin heavy chain; see Maeda et al., 1987).

**Transfection of Cultured Cells**

To identify the regions of M-protein required for the incorporation of the molecule into the sarcomere, cultured cells were transfected with a series of constructs in the eukaryotic expression vector pCMV5-T7. The pCMV vector results in strong, constitutive expression of cDNAs in most eukaryotic cells under control of the cytomegalovirus promoter (Andersson et al., 1989). We replaced the original site for cloning foreign DNAs by introducing, immediately adjacent to the start codon, a new cassette containing recognition sites for the restriction enzymes BamHI, MluI, HindIII, SalI, and a sequence encoding the T7-tag, a 12-amino acid residue epitope (Lutz-Freyermuth et al., 1990; Tsai et al., 1992). Upon transfection, this results in the expression of a recombinant protein bearing at its carboxy terminus the T7-tag, which can be detected with a T7-tag–specific monoclonal antibody.

The effect of overexpression of specific portions of M-protein was studied mainly in BHK-21/C13 cells. These cells, which can be routinely transfected with high efficiency (5–25%, depending on the transfected construct), express already under certain culture conditions several sarcomeric proteins that assemble into myofibril-like structures (MLS). These resemble early myofibrils in “classical” muscle cells (Schaart et al., 1991; Van der Loop et al., 1996). Before the distribution and the effect of the M-protein constructs were studied, their correct expression was verified. At 24–40 h after transfection, cells were harvested in SDS-sample buffer and analyzed by Western blotting. In each case the T7 tag-specific antibody recognized a protein of the expected size. In addition, the correct expression was confirmed with domain-assigned antibodies for most of the constructs (our unpublished results).

Transfected cells were induced to differentiate and were inspected by immunofluorescence between 1 and 6 d later. To allow for a comparison with the internal sarcomeric cytoskeletal structures, the localization of T7-tagged proteins was usually compared with that of an epitope of titin located close to the A/I
junction. Figure 7 (E–H) shows that the construct comprising domains Mp2–Mp3 is localized primarily along MLS. In some cases, the recombinant protein was also revealed in dispersed cytoplasmic aggregates. In such cells, MLS were usually destroyed and the recombinant protein was found to colocalize with...
titin in these aggregates (Figure 7, E and F). In contrast, transfection with single domains (Mp2 containing the interdomain linker or Mp3) consistently resulted in a diffuse distribution of the expressed recombinant protein and a normal appearance of MLS (Figure 7, A–D; see also Table 2).

When cells were transfected with a construct comprising the unique amino-terminal domain Mp1 in addition to domains Mp2 and Mp3, the recombinant protein was recovered essentially in the nucleus (our unpublished results). A diffuse cytoplasmic distribution without obvious association with MLS was detected upon transfection with constructs encoding domains Mp4–Mp8 or Mp9–Mp13. In these cases we did not observe any destructive effects on MLS or a negative effect on the ability of the cells to differentiate (see Table 2).

To extend the results of the transfection studies with BHK cells, we also transfected C2C12 mouse myoblasts. Similar to the situation in BHK cells, an obvious colocalization of Mp2–Mp3 was found with nascent or mature myofibrils (Figure 8, A–D), while other constructs (e.g., Mp4–Mp8) showed a diffuse cytoplasmic localization (Table 2). Unlike in BHK cells, myofibril structure seemed not affected (Figure 8, B, D, and E). Surprisingly, Mp9–Mp13 was also distributed along filaments in a regular and discontinuous punctate pattern (Figure 8E). Inclusion of the head domain Mp1 into the construct Mp2–Mp3 typically resulted in a nuclear localization of the recombinant protein. In the few cases in which a cytoplasmic localization was also observed, the protein colocalized with stress fiber-like structures or myofibrils (Table 2). Both cell lines (BHK and C2C12) were also transfected with Mp1–Mp3 and the constructs in which either Ser39 or Ser76 was mutated to Ala (see above). In general, transfection efficiencies were very low in these experiments, and all resulting recombinant proteins showed almost exclusively nuclear localization (our unpublished results).

### DISCUSSION

M-protein is a prominent structural protein of sarcomeric M bands in vertebrate fast and cardiac muscles (Perriard, 1993). Together with myomesin and titin, it forms the structures that cross-link myosin thick filaments in the central bare zone. We therefore started to search for binding sites between these proteins and their possible regulation.

Both limited proteolysis of purified M-protein and a series of recombinant constructs identified a myosin-binding site in domains Mp2–Mp3. Consistent with earlier observations (Mani and Kay, 1978; Woodhead and Lowey, 1983; Bährer et al., 1985) we found this interaction to be rather weak. It was best documented in solid-phase overlay assays (Figures 2, 3, and 6). The location of the myosin-binding site revealed an interesting difference between M-protein and myomesin, the second major structural protein of the M band. Although both proteins share the same domain organization and exhibit ~50% identity over the repeat domains, their binding properties seem very different. Likewise, their structural organization within the sarcomeric M band inferred by immunoelectron microscopy is different (Obermann et al., 1996). In myomesin the amino-terminal domain My1 seems to harbor the myosin-binding site (Obermann et al., 1997), while the subsequent pair of Ig domains Mp2–Mp3 shows myosin binding in the case of M-protein (Figure 3). In further experiments it was not possible to attribute the myosin-binding site to a single domain of M-protein. This observation resembles the situation found for the interactions of both myomesin and C-protein with titin. In both cases, constructs consisting of at least three domains were necessary to reveal binding of titin (Freiburg and Gautel, 1996; Obermann et al., 1997). Thus, it seems that the interactions of titin with other cytoskeletal proteins of the Ig superfamily binding arises by a combination of weak but

### Table 2. Summary of the immunofluorescence patterns of cell transfection studies

<table>
<thead>
<tr>
<th>Construct</th>
<th>Association with MLS/myofibrils</th>
<th>Disruption of MLS/myofibrils</th>
<th>Nuclear localization</th>
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<tr>
<td>BHK-21/C13</td>
<td>C2C12</td>
<td>BHK-21/C13</td>
<td>C2C12</td>
</tr>
<tr>
<td>Mp2</td>
<td>−</td>
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*BHK-21/C13 cells and C2C12 cells were transiently transfected with the M-protein constructs listed in column 1. Columns 2-7 give the respective localization and/or the effect of each construct as analyzed by immunofluorescence microscopy. For details see RESULTS.*
cooperative interactions. It seems possible that a similar situation also holds for the interaction of M-protein and myosin.

Since the myosin-binding site of M-protein (Mp2 plus Mp3) is adjacent to the unique amino-terminal domain Mp1 of the molecule, we investigated this

Figure 8. Expression of recombinant M-protein fragments in differentiating C2C12 cells. C2C12 cells transfected with constructs encoding T7-tagged Mp2-Mp3 (A–D) or Mp9–Mp13 (E) were allowed to differentiate for 2 (E) or 6 (A to D) d, respectively. Subsequently they were stained with T7-tag antibody (A, C, and E) and tetramethylrhodamine-5-isothiocyanate-labeled phalloidin (B) or MpAA259 (D) followed by secondary antibody. Note that most of the expressed Mp2–Mp3 polypeptide associates with myofibrils (arrowheads in A–D). Transfection with Mp9–Mp13 leads to expression of a polypeptide that binds to myofibrils in a periodic manner (arrowheads in E). For details see RESULTS. Magnification, 1050×.
molecular region in more detail. Computer analysis of the translated human M-protein cDNA sequence revealed two potential target sites for cAMP-dependent protein kinase A (Vinkemeier et al., 1993) in the peptides KRAS* (residues 35–39) and KRVS* (residues 73–76). Indeed, purified M-protein can be specifically phosphorylated by PKA as well as by muscle extracts. Phosphorylation is serine specific and involves the amino-terminal domain Mp1 (Figure 4). Kinetic characterization of synthetic peptides encompassing both motifs demonstrated a $K_M$ value in the range of 2× of that of a recently characterized PKA phosphorylation site in myomesin (Table 1; Obermann et al., 1997). The $V_{\text{max}}$ value is ~ 50% of the same substrate. These values are within the range of other target sequences for PKA characterized previously (Pearson and Kemp, 1991). Analyses using recombinant M-protein fragments, in which either one or both of these serine residues were mutated to alanine, revealed that only serine residue 76 can be phosphorylated by PKA (Figure 5). This indicates that the three-dimensional structure of domain Mp1 in the native M-protein molecule allows reactivity with PKA exclusively for Ser76. This result is made more significant by the finding that this site is well conserved between chicken (Noguchi et al., 1992) and human sequences (Vinkemeier et al., 1993).

The functional importance of this phosphorylation site is indicated by the finding that it seems to control the interaction of M-protein with myosin. Thus, while domains Mp2–Mp3 bind constitutively to myosin LMM, phosphorylation of Ser76 in the adjacent domain Mp1 modulates this binding. The phosphorylated fragment essentially lacks myosin binding. This may indicate complex changes of the three-dimensional structure of a larger molecular region of M-protein as the result of Ser76 phosphorylation.

Earlier biochemical data indicate that M-protein also binds to titin (Nave et al., 1989). In our binding assays we have not yet been able to identify a titin-binding site in M-protein (our unpublished results). This is most likely due to the fact that our constructs did not cover the entire carboxy-terminal region of titin. In particular, the 500-residue insertion between Ig domains m3 and m4 (Gautel et al., 1993) was not included in the assay, since it could not be produced as recombinant protein (Gautel, personal communication). Our recent immunoelectron microscopy revealed that a construct comprising domains Mp2–Mp3 localized primarily to MLS in transfected cells (Figure 7 and Table 2). Thus it seems that the myosin-binding site of M-protein is involved in the specific targeting in the myofibril. Similar observations were made when C2C12 mouse myoblast cells were transfected. While the construct Mp2–Mp3 was again found in nascent myofibrils, the fragment Mp4–Mp8 was distributed diffusely in the cytoplasm (Figure 8 and Table 2).

Interestingly, the construct Mp9–Mp13 was also recovered in myofibrils (Figure 8 and Table 2). This indicates that in the carboxy-terminal third of M-protein, another myofibrillar binding site, possibly a titin-binding site (see above), can be expected.

The myosin-binding site in M-protein provides the second example of a protein–protein interaction in the sarcomeric M band whose binding status is controlled by PKA phosphorylation. For myomesin, phosphorylation was shown to regulate the binding to titin (Obermann et al., 1997). In both cases phosphorylation could provide a mechanism that controls the events during sarcomeric M band assembly, regeneration, and turnover in a spacial and temporal order. The binding of M-protein to myosin could be a mechanism necessary to provide a stronger thick filament association in myofibrils of fast and cardiac muscle fibers, since only these fibers of the adult animal express M-protein.

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