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

Wolfgang M.J. Obermann,* Mathias Gautel,‡ Frank Steiner,* Peter F.M. van der Ven,* Klaus Weber,* and Dieter O. Fürst*§

*Max-Planck-Institute for Biophysical Chemistry, Department of Biochemistry, D-37077 Göttingen, Germany; ‡European Molecular Biology Laboratory, Biological Structures and Biocomputing Program, D-69012 Heidelberg, Germany; and §University of Potsdam, Department of Cell Biology, D-14471 Potsdam, Germany

Abstract. The M band of vertebrate cross-striated myofibrils has remained an enigmatic structure. In addition to myosin thick filaments, two major structural proteins, myomesin and M-protein, have been localized to the M band. Also, titin is expected to be anchored in this structure. To begin to understand the molecular layout of these three proteins, a panel of 16 polyclonal and monoclonal antibodies directed against unique epitopes of defined sequence was assembled, and immunoelectron microscopy was used to locate the position of the epitopes at the sarcomere level. The results allow the localization and orientation of defined domains of titin, myomesin, and M-protein at high resolution. The 250-kD carboxy-terminal region of titin clearly enters the M band with the kinase domain situated ~52 nm from the central M1-line. The positions of three additional epitopes are compatible with the view that the titin molecule reaches ~60 nm into the opposite sarcomere half. Myomesin also seems to bridge the central M1-line and is oriented parallel to the long axis of the myofibril. The neighboring molecules are oriented in an antiparallel and staggered fashion. The amino-terminal portion of the protein, known to contain a myosin binding site, seems to adopt a specific three-dimensional arrangement. While myomesin is present in both slow and fast fibers, M-protein is restricted to fast fibers. It appears to be organized in a fundamentally different manner: the central portion of the polypeptide is around the M1-line, while the terminal epitopes seem to be arranged along thick filaments. This orientation fits the conspicuously stronger M1-lines in fast twitch fibers. Obvious implications of this model are discussed.

THE stable and ordered arrangement of thick and thin filaments in sarcomeric muscle myofibrils arises from a complex cytoskeletal framework (for review see Small et al., 1992). Its major structures, the M bands and Z discs, seem to be interconnected by the giant protein titin (also called connectin). In the M band, the thick, hexagonal filament lattice appears to be stabilized by additional components. Some aspects of M band substructure have been deduced from negatively stained longitudinal cryosections (Sjöström and Squire, 1977). Thus, the M band appears as a zone of higher density, about 75 nm wide, which depending on fiber type and animal species consists of three to five major cross striations. These striations are thought to reflect bridging structures, called M-filaments and secondary M-bridges (Luther and Squire, 1978).

Only a very limited number of proteins localized within the M band region have been identified and characterized by antibody labeling of myofibrils. The ATP-regenerating enzyme MM-creatine kinase seems to compose the prominent M4/M4′ striations of the M band (for review see Wallimann and Eppenberger, 1985). M-protein was characterized rather early (Masaki and Takaiti, 1974; Trinick and Lowey, 1977), and polyclonal antibodies were used to describe its localization (Strehler et al., 1983). Since these antibodies also reacted with myomesin, a later identified M band protein, the exact disposition of M-protein in the M band remained unknown. Myomesin, discovered by mAbs as a contaminant of conventional M-protein preparations (Grove et al., 1984), has only recently been characterized on the molecular level (Obermann et al., 1995). Specific antibodies have established a differential fiber-type distri-

Address all correspondence to Prof. Dr. Dieter O. Fürst, University of Potsdam, Department of Cell Biology, Lennéstr. 7a, D-14471 Potsdam, Germany. Tel.: 49 331 967 2873. Fax: 49 331 967 2861.

bution and distinct developmental expression pattern for myomesin and M-protein. Accordingly, myomesin seems intimately associated with myosin since it was found in all skeletal and cardiac muscle fibers from the earliest stages of myofibrillogenesis. M-protein, on the other hand, was only detected in fast skeletal and cardiac muscle fibers and appears much later in development (Grove et al., 1985, 1987; Carlsson et al., 1990).

Individual titin molecules have been shown by immunoelectron microscopy to span the entire half sarcomeres from the Z disc to the edge of the M band (Fürst et al., 1988, 1989). Isolated titin molecules revealed a tight association of titin with both myomesin and M-protein (Nave et al., 1989; Vinkemeier et al., 1993).

cDNA cloning has defined a new family of proteins that are primarily composed of two repeating motifs, the immunoglobulin (Ig)-like domains and the fibronectin type III (Fn)-like domains. Titin (Labeit and Kolmerer, 1995), myomesin, and M-protein (Noguchi et al., 1992; Vinkemeier et al., 1993), as well as C-protein (Einheber and Fischman, 1989; Fürst et al., 1992; Weber et al., 1993) and H-protein (Vaughan et al., 1993), have all been ascribed to this group of intracellular proteins of the immunoglobulin superfamily (for review see Fürst and Gautel, 1995). The modular organization of myomesin, M-protein and the M band portion of titin is demonstrated in Fig. 1. The availability of primary sequences has made it possible to approach the problem of M band structure by immunoelectron microscopy using a panel of antibodies directed against defined epitopes of three major cytoskeletal M band proteins. The results allow us to propose a model describing the molecular layout of three major structural M band proteins.

Materials and Methods

Constructs of Titin, Myomesin, and M-protein Used for Antibody Production and Epitope Mapping

Original λ phage isolates containing cDNAs coding for human myomesin, human M-protein (Vinkemeier et al., 1993), and the 250-kDa M band region of human cardiac titin (Gautel et al., 1993) were used as templates for PCR amplification of the domains employed in domain mapping and immunizations (see below). PCR products were cloned into a modified pET23a vector (Qiagen, Hilden, Germany), providing the resulting proteins with an additional EEF-tag at their carboxy termini. Since this tag is recognized by mAb YL 1/2 (Wehland et al., 1984), expression in *Escherichia coli* BL21(DE3)pLysS was monitored by immunoblot analysis. Purification of the soluble recombinant proteins on Ni-NTA agarose columns due to the oligohistidine tag followed standard protocols (Qiagen).

Production and Characterization of Antibodies

To facilitate the nomenclature of 16 antibodies without sacrificing the name of the previously reported 5 antibodies, we have adopted the following scheme. The first one or two letters indicate the target protein: T (titin), My (myomesin), and Mp (M-protein). After the actual antibody name, polyclonal antibodies are highlighted by ra or rt, depending on whether they were raised in rabbits or rats. Murine mAbs do not carry a further signature. Thus, the myomesin specific mAbs B4 (Grove et al., 1984), kindly provided by Dr. H. Eppenberger, and BB78 (Vinkemeier et al., 1993) are now MyB4 and MyBB78. Similarly the M-protein–specific mAbs AA241 and AA259 (Vinkemeier et al., 1993) and AA280 are called MpAA241, MpAA259, and MpAA280. The new antibodies were obtained as follows: domain specific antibodies (see Fig. 1) were raised by standard immunization schemes against certain bacterially expressed domains, which were purified to homogeneity. This provided rabbit antibodies Tm8ra (m8 domain in the titin carboxy-terminal portion), My673ra (eighth domain of myomesin), and Mp660ra (second domain of M-protein). The entire amino-terminal regions of myomesin or M-protein were used to obtain My190Nrt and Mp669ra. Purification of the IgG fractions was by ammonium sulfate precipitation followed by MonoQ and/or protein G chromatography as described (Nave et al., 1991). In addition, three domain specific mAbs were obtained from female Balb/c mice that had been immunized by a standard immunization scheme (Fürst et al., 1988, 1989) with one of the following domains from the 250-kDa M band region of titin (Gautel et al., 1993; Fig. 1): kinase domain (T40), insert 4 between m5 and m6 domains (T41), and m9 domain (T51). These antibodies were used as hybridoma culture supernatants.

Peptide specific antibodies were also used. Synthetic peptides containing an additional carboxy-terminal cysteine were obtained by Fmoc chemistry on a Pep Synthesizer TM9050 (Millipore Corp., Bedford, MA). Peptides purified by reverse phase HPLC were characterized by automatic sequence analysis and/or mass spectrometry. Peptides were coupled via their extra cysteine with m-maleimidobenzoyl-*N*-hydroxy-sulfosuccinimide ester (Sulfo-MBS; Pierce, Rockford, IL) to chicken egg ovalbumin (grade VI; Sigma Immunochemicals, Deisenhofen, Germany), and antibodies were elicited in rabbits by a standard immunization protocol (Nave et al., 1991). The human myomesin peptides KQSTASKQSTASKQSTAS (from the amino-terminal region My1) and EKARLKSRPSAPWTGQ (loop between domains My4 and My5) provided polyclonal antibodies MyW6ra and MyW17ra. The human M-protein peptide GEKEIAIYQDDLEGDA (loop between domains Mp4 and Mp5) yielded antibody MpW4ra. For location of the peptides, see Fig. 1. The IgG fractions of these antibodies were obtained as described above.

In general, the new antibodies were first monitored by indirect immunofluorescence microscopy on frozen muscle sections and subsequently by Western blotting on total muscle extracts (Fürst et al., 1988) to document their specific reaction with either titin, myomesin, or M-protein. Subsequently, their reaction was tested in Western blots on the original domain used as antigen in comparison to a mixture of other domains. In the case of the peptide antibodies, domains covering the peptide sequence were used as positive controls while other domains served as negative controls. Two antibodies, T40 and Tm8ra, did not react with their antigens in Western blots. Here antibody specificity was demonstrated by dot blots using the native titin domain preparations spotted onto nitrocellulose (Obermann et al., 1995).

Immunoelectron Microscopy

Muscle fibers were prepared from rat psoas and bovine sternomandibularis muscle. Detergent extraction, incubation with first and second antibodies, fixation, and Epon embedding followed our standard protocol (Fürst et al., 1988). In the case of murine monoclonal antibodies, which were used as 10-fold concentrated hybridoma supernatants, the second antibody was an affinity purified sheep anti–mouse antibody as described before (Fürst et al., 1988). Rabbit and rat polyclonal antibodies were used at an IgG concentration of 1 mg/ml. Second antibodies (purchased from Rockland, Inc., Gilbertsville, PA) were also used at a concentration of 1 mg/ml. Ultrathin sections, positively stained with uranyl acetate and Reynold's lead citrate, were viewed in an electron microscope (model CM12; Phillips Electronic Instrs., Co., Mahwah, NJ) operated at 80 kV. The distances of the antibody decoration lines, i.e., of the underlying epitopes, from the center of the sarcomere were measured both directly in the microscope (using the built-in image-measuring function) and on micrographs using a micrometer scale. The middle of the respective decoration line was always referred to in the measurements. To obtain a sufficient statistical estimate, at least 50 sarcomeres from two or more independent embeddings were analyzed.

Miscellaneous Procedures

Bovine skeletal muscle M-protein was isolated using the procedure described for chicken skeletal muscle protein (Eppenberger and Strehler, 1982). The purified protein in buffer C (50 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM DTT) was subjected to limited proteolysis using either trypsin (Sigma Immunochemicals) or endoproteinase AspN (Boehringer Mannheim Corp., Indianapolis, IN) at a substrate to enzyme ratio of 200:1 (wt/wt) at room temperature. After SDS-PAGE and blotting to polyvinylidene fluoride (PVDF) membranes, the major fragments were characterized by their amino-terminal sequences as described in detail for myomesin (Obermann et al., 1995). All DNA cloning steps followed standard protocols (Ausubel et al., 1987; Sambrook et al., 1989).

Results

Production and Epitope Mapping of Antibodies

Fig. 1 summarizes the domain organization of the M band proteins myomesin and M-protein (Vinkemeier et al., 1993) and gives the corresponding information for the 250-kD carboxy-terminal region of titin (Gautel et al., 1993). To obtain a better understanding of the sarcomeric M band by immunoelectron microscopy, we aimed at a panel of antibodies directed against specific epitopes along the defined modular structure of the three proteins. We have mapped the epitopes of the murine mAbs to myomesin (MyB4, MyBB78) and M-protein (MpAA280, MpAA241, MpAA259) and raised additional domain specific antibodies to both proteins in rabbits. Since antibodies to the 250-kD carboxy-terminal region of titin were not available, we have raised four domain specific antibodies: three mAbs and a rabbit antibody. Fig. 1 summarizes the epitopes of the 16 antibodies used and Figs. 2–6 provide the documentation for their epitope specificity.

Myomesin Antibodies and the Location of their Epitopes

Two monoclonal antibodies were available. MyBB78 was raised against the 190-kD protein bound to the M band end of purified titin, which was later identified as myomesin (Nave et al., 1989; Vinkemeier et al., 1993). From the results on human cDNA clones from a λgt11 expression library, the epitope of MyBB78 was expected in domains My12-My13 (for domain nomenclature see Fig. 1). MyB4 was originally used to identify myomesin as an M band protein (Grove et al., 1984). To precisely map the epitopes of these two mAbs, a series of overlapping and individual constructs covering the entire human myomesin polypeptide was expressed in bacteria, purified, and used in immunoblotting. Two of the single domains were also used to produce rat or rabbit polyclonal antibodies. My190Nrt is directed against My1; My673ra was raised with domain My8. Finally, two peptide-specific antibodies were raised in rabbits: MyW6ra is directed against the conspicuous KQSTAS motif, which is found in eight tandem repeats in domain My1 of human myomesin (Vinkemeier et al., 1993). MyW17ra was elicited with the central portion of the loop sequence connecting domains My4 and My5 (Fig. 1; for sequences see Materials and Methods).

SDS-PAGE and corresponding immunoblots showed that all four new myomesin antibodies recognize only myomesin in a total bovine muscle extract obtained by SDS solubilization (results not shown). Fig. 2 provides the antibody reactivity pattern using immunoblots of bacterially expressed overlapping constructs of human myomesin. It confirms that the two polyclonal sera raised against a single domain (My190Nrt and My673ra) react only with the original antigen (Fig. 2 *B*, lanes *1–4*). Epitope mapping also showed that mAb MyBB78 detects exclusively domain My12 (Fig. 2 *B*, lanes *7* and *8*), while mAb MyB4 is specific for domain My11 (Fig. 2 *B*, lanes *5* and *6*). Fig. 1 summarizes the positions of the epitopes along the myomesin molecule.

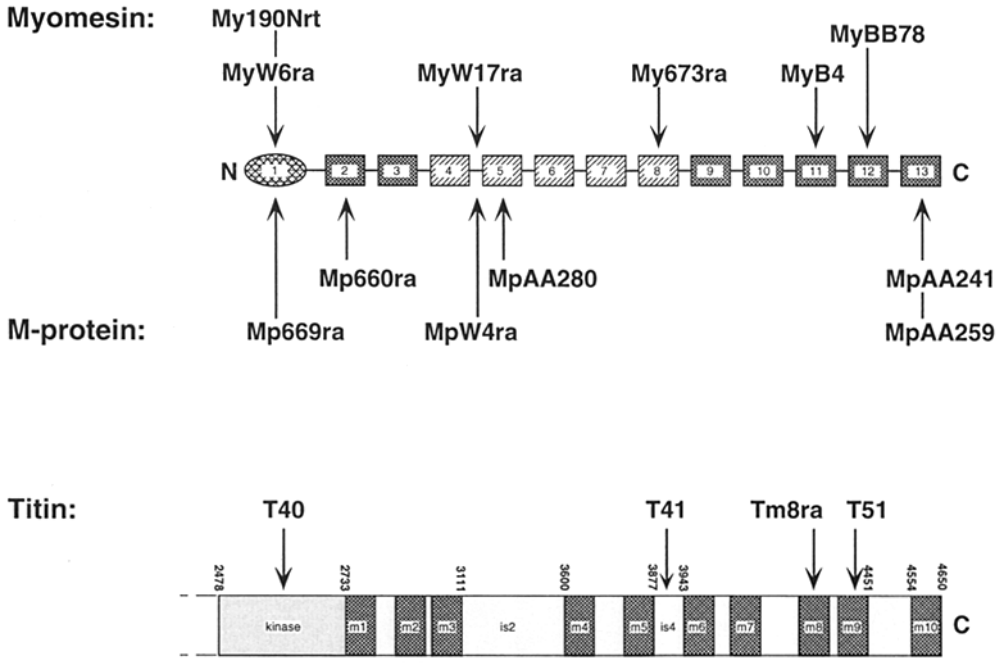

Figure 1. Schematic representation of the molecular architecture of myomesin, M-protein, and the carboxy-terminal 250-kD region of titin and the positions of the epitopes for the antibodies used in this study. The presentation emphasizes the modular construction of the proteins from repetitive immunoglobulin cII (*cross-hatched rectangles*) and fibronectin type III repeats (*rectangles with shades*) interspersed by unique sequence stretches of varying length (*is*, insertion). In titin, the 10 domains (m1–m10) following the kinase domain are all Ig-domains. For ease of orientation, the model of myomesin and M-protein was drawn only once, since the order of domains is identical in both proteins (Vinkemeier et al., 1993). Domains were numbered in consecutive order, starting with the unique amino-terminal region as 1. Myomesin epitopes are given above and M-protein epitopes below the sketch. For titin, only the 250-kD carboxy-terminal M-band portion is shown (Labeit et al., 1992; Gautel et al., 1993). Numbers on titin give the amino acid residues in the 250-kD carboxy-terminal region described by Labeit et al. (1992). N and C mark the amino- and carboxy-terminal ends of all three proteins. The additional amino-terminal head domains of myomesin (*My1*) and M-protein (*Mp1*) are marked. Arrows indicate the positions of the epitopes and the names of the individual antibodies described in Results.

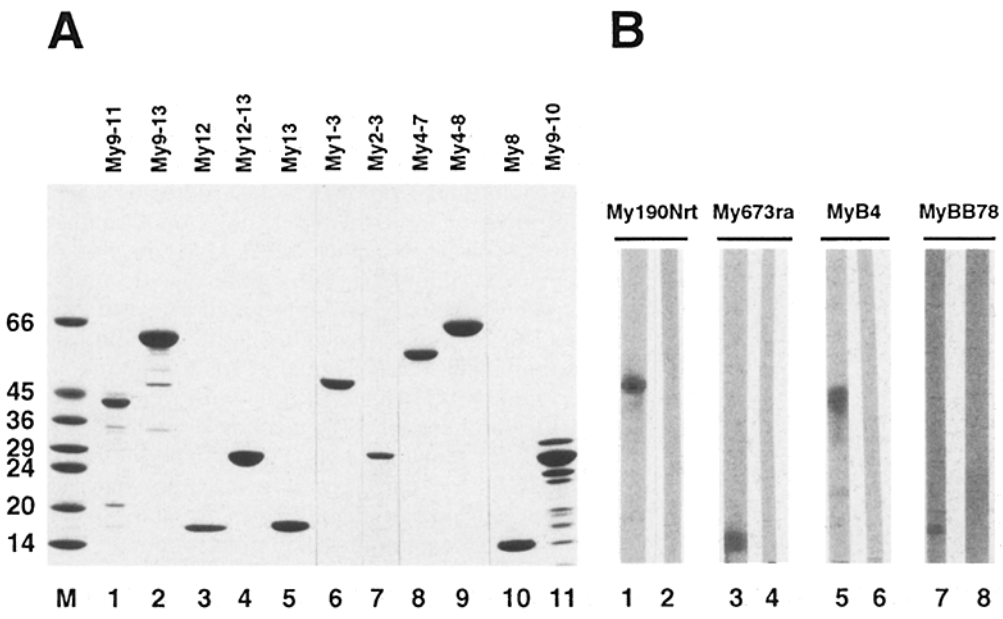

Figure 2. Determination of epitope positions of antibodies specific for myomesin. (*A*) The recombinant constructs of human myomesin in 6 to 20% SDS-PAGE: My9–11 (lane *1*), My9–13 (*2*), My12 (*3*), My12–13 (*4*), My13 (*5*), My1–3 (*6*), My2–3 (*7*), My4–7 (*8*), My4–8 (*9*), My8 (*10*), and My9–10 (*11*). M is a molecular mass standard (in kD). For location of these domains in the myomesin molecule see Fig. 1. (*B*) The reactivity of myomesin antibodies in immunoblots with various combinations of the recombinant fragments: My190Nrt reacts with the construct My1-3 (lane *1*) but not with a mixture of My2–3, My4–8, and My9–13 (lane *2*). My673ra decorates My8 (lane *3*) but not the mixture of My1–3, My4–7, and My9–13 (lane *4*). mAb MyB4 stains My9-11 (lane *5*) but not the mixture of My1–3, My4–8, My9–10, and My12–13 (lane *6*). mAb MyBB78 reacts with My12 (lane *7*) but not with the mixture of My1–3, My4–8, My9–11, and My13 (lane *8*). For nomenclature of domains, see Fig. 1.

M-protein Antibodies and Epitope Location

Earlier work on M-protein associated with the M band end of purified bovine titin provided the three mAbs MpAA241, MpAA259, and MpAA280 (Nave et al., 1989; Vinkemeier et al., 1993). From the results on human cDNA clones from a λgt11 expression library, the first two mAbs were thought to react with the carboxy-terminal region of M-protein (Vinkemeier et al., 1993). The epitopes of these antibodies were now accurately mapped using bacterially expressed overlapping constructs covering the entire human M-protein molecule (Fig. 3). Both mAbs, MpAA241 and MpAA259, reacted exclusively with domain Mp13. Since MpAA280 did not react with human M-protein, another approach for epitope mapping was taken. Limited proteolysis of purified bovine myomesin provided a set of stable fragments (Obermann et al., 1995). Fig. 4 *A* shows a similar analysis for bovine M-protein by

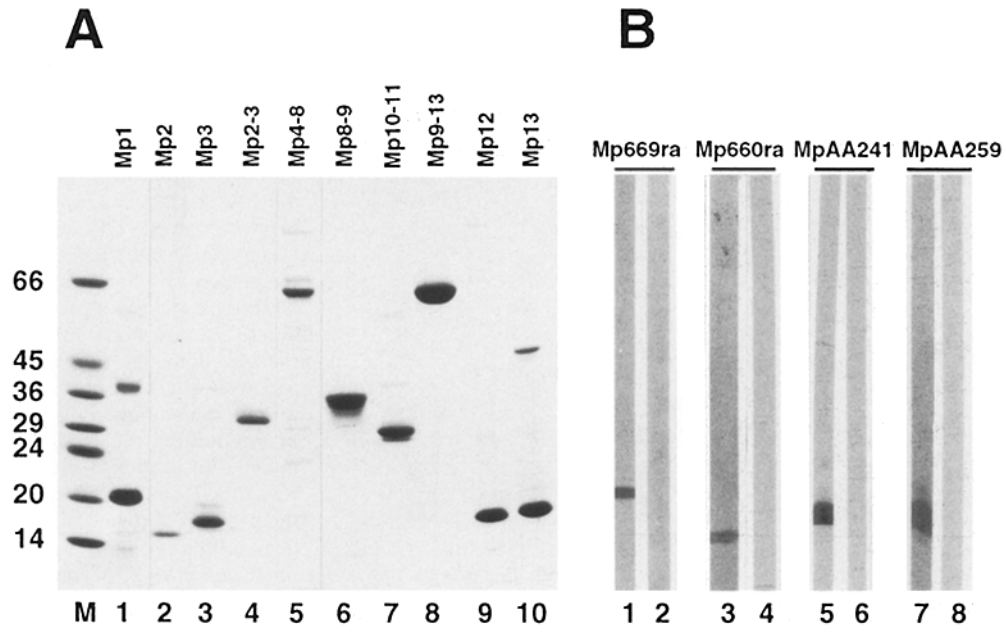

Figure 3. Determination of epitope positions of antibodies specific for M-protein. (*A*) All constructs of M-protein used on 6–20% SDS-PAGE: Mp1 (lane *1*), Mp2 (*2*), Mp3 (*3*), Mp2-3 (*4*), Mp4-8 (*5*), Mp8-9 (*6*), Mp10-11 (*7*), Mp9-13 (*8*), Mp12 (*9*), and Mp13 (*10*). Lane *M* shows a molecular mass standard (in kD). For location of these domains in the M-protein molecule, see Fig. 1. (*B*) The reactivity of M-protein antibodies in immunoblots with various combinations of the recombinant fragments: Mp669ra stains Mp1 (lane *1*) but not the mixture of Mp2–3, Mp4–8, and Mp9–13 (lane *2*). Mp660ra reacts with Mp2 (lane *3*) but not with the mixture of Mp1, Mp3, Mp4–8, and Mp9–13 (lane *4*). Both mAbs MpAA241 and MpAA259 decorate Mp13 (lanes *5* and *7*) but not the mixture of Mp1, Mp2–3, Mp4–8, Mp8–9, Mp10–11, and Mp12 (lanes *6* and *8*). For nomenclature of domains, see Fig. 1.

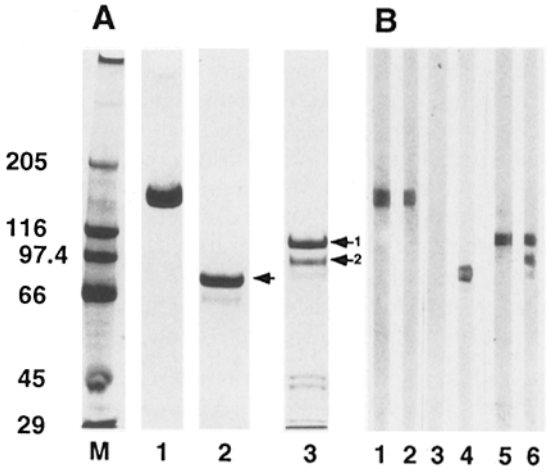

Figure 4. Localization of the epitope position of mAb MpAA280 specific for M-protein. (*A*) On 4–12% SDS-PAGE purified bovine skeletal muscle M-protein (lane *1*), M-protein after limited treatment with trypsin (lane *2*) and after limited treatment with endoproteinase AspN (lane *3*). Amino-terminal sequences obtained on the fragments marked by arrows could be perfectly aligned with the translated human cDNA sequence. The trypsin fragment (lane *2*) starts with Val704. Fragment 1 of the endoproteinase AspN digest starts with Glu496 while band 2 starts with Asp604 in the human sequence. (*B*) The corresponding immunoblots: antibody MpAA280 on purified M-protein (lane *1*), on M-protein after trypsin digestion (lane *2*), and after endoproteinase AspN cleavage (lane *3*); lanes *4–6* are the blots using MpW5ra on the identical set of samples. Since mAb MpAA280 recognizes only total M-protein (lane *1*) and the larger band of the endoproteinase AspN digest (lane *5*) but not the other two proteolytic fragments (lanes *3* and *5*), its epitope must be located in domain Mp5 (for location of this domain in the M-protein molecule, see Fig. 1). Antibody MpW5ra, which is not used further in this study, serves as a control. It recognizes all four protein bands (lane *2*, *4*, and *6*). M is a molecular mass standard (in kD).

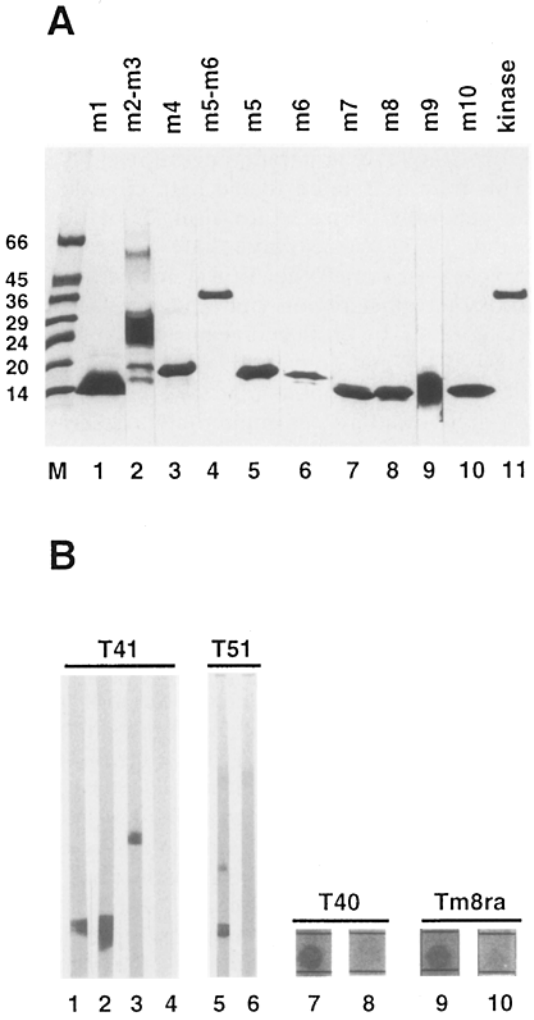

Figure 5. Determination of epitope positions of antibodies specific for titin. (*A*) All constructs of titin used on a 6–20% SDS–polyacrylamide gel. The name of the respective construct is indicated above each lane: m1 (*1*), m2-m3 (*2*) m4 (*3*), m5–m6 (*4*), m5 (*5*), m6 (*6*), m7 (*7*), m8 (*8*), m9 (*9*), m10 (*10*) and the kinase domain (*11*). In M, a molecular mass standard is shown (in kD). (*B*) The reactivity of mAbs T41 and T51 in immunoblots with titin constructs: lanes 1–4, mAb T41 on m5 (lane *1*) and m6 (lane *2*), both containing is4, and on the two-domain construct m5–m6 (lane *3*). Lane *4* documents the negative reaction with a mixture of domains m1, m2–m3, m4, m7, m8, m9, m10, and the kinase domain. mAb T51 recognizes exclusively Ig-domain m9 (lane *5*) and is negative on a mixture of all other domains (lane *6*). Gel lanes and blots were compiled from different gels. Lanes *7–10* show the reactivity of two titin antibodies in dot blots on M band titin recombinant fragments: mAb T40 stains the kinase domain (lane *7*) but not the mixture of all Ig-domains (lane *8*). Tm8ra decorates Ig-domain m8 (lane *9*) but not a mixture of all other constructs (lane *10*).

SDS-PAGE. Mild treatment with endoproteinase AspN generated two large fragments of molecular masses 110 and 97 kD, respectively, while trypsin generated a smaller fragment of about 87 kD. Amino-terminal sequencing showed that these three fragments start with Glu496, Asp607, and Val704, respectively. In corresponding immunoblots, mAb MpAA280 reacted exclusively with the largest fragment (Fig. 4 *B*). Thus, its epitope involves domain Mp5 of bovine M-protein (Fig. 1).

Two rabbit antibodies were raised using two of the purified recombinant domains of human M-protein. Antibody Mp669ra is directed against Mp1, while Mp660ra was obtained with Mp2 (Fig. 1). In addition, a peptide-specific rabbit antibody was raised. MpW4ra is directed against a peptide from the loop connecting domains Mp4 and Mp5 (Fig. 1; for sequence see Materials and Methods). All three new M-protein antibodies recognized only M-protein in immunoblots of a total muscle extract obtained by SDS solubilization (not shown). Fig. 3 confirms that the two domain-specific antibodies, Mp669ra and Mp660ra, reacted exclusively with the single domain used for immunization. This was also the case for the peptide antibody MpW4ra (not shown).

Titin Antibodies

The carboxy-terminal portion of titin is composed of a complex array of a kinase domain followed by 10 Ig modules interspersed by various unique insertions (Gautel et al., 1993; Fig. 1). Four representative regions within this 250-kD region were selected for antibody production: the kinase domain, the insert is4 (linker sequence located be-

tween domains m5 and m6), and domains m8 and m9. The corresponding sequences were expressed as recombinant proteins in *E. coli* and purified in a native state on Ni-NTA-agarose columns using their carboxy-terminal $(His)_6$ tag. Although a standard immunization protocol was used, generally a surprisingly weak immunological response was observed. This may be related to the high cross species conservation generally observed for titin (Labeit et al., 1992; Fritz et al., 1993). We also note that we were unable to raise mAbs against some other titin domains from the 250-kD carboxy-terminal region (m5 and m10). Murine mAbs could, however, be produced against the kinase domain (T40), is4 (T41), and domain m9 (T51). Domain m8 yielded antibodies only in rabbits (Tm8ra). The titin-specific reaction of T41 and T51 in immunoblots is shown in Fig. 5. Since T40 and Tm8ra did not react with denatured polypeptides, their reaction with native proteins was monitored in dot blots (Fig. 5 *B*). Table I summarizes the immunoglobulin subclass and the cross species reactivity patterns of the new titin mAbs.

Immunoelectron Microscopy

The preembedding immunolabeling procedure used here was used several times in the past for the electron microscopic localization of structural proteins in the sarcomere (Craig and Offer, 1976; Strehler et al., 1983; Dennis et al., 1984; Fürst et al., 1988). Briefly, fiber bundles of rat psoas muscle were tied on plastic plates at varying lengths, Triton extracted, and immersed in antibody solutions before conventional plastic embedding. Ultrathin sections were then analyzed in the electron microscope. Rat psoas muscle was selected because it contains both slow type and fast type muscle fibers in roughly equal amounts, thus allowing for a direct comparison of the labeling patterns in both fiber types. Only in the case of mAb MpAA280, which seems specific for bovine M-protein (Table I), *sternomandibularis* muscle of freshly slaughtered cows was used.

The immunolabeling experiments for all 16 antibodies are shown in Figs. 6–8. The decoration patterns were characteristic for the respective antibody and occured as doublets in distinct, centrally symmetric positions of the sarcomeric M band. Only in two cases (with mAbs MyB4 and MpAA280) was it difficult to make out a doublet of decoration lines. Our previous observations, however, indicated that the width of a single line with mAbs is around 10 nm (Fürst et al., 1988, 1989; Nave et al., 1989). Therefore, it was also possible to place them to the positions indicated in Table II. As with all titin A band antibodies analyzed previously (Fürst et al., 1988, 1989), no variation in staining patterns with respect to varying sarcomere length was observed with any of the antibodies used in this study. Also, no difference was observed in the labeling of slow and fast fibers with any of the four distinct antibodies to titin and the six distinct antibodies to myomesin. On the other hand we confirmed that M-protein is found in fast fibers only (see Introduction). All six distinct antibodies to M-protein left the slow fibers undecorated.

An Ultrastructural Model of the Sarcomeric M Band Deduced from Immunoelectron Microscopy

Earlier studies had implied that titin must extend into the central M band, although a direct proof by specific antibodies was still lacking (Nave et al., 1989; Vinkemeier et al., 1993). The availability of cDNA information for the 250-kD carboxy-terminal region of human titin (Labeit et al., 1992; Gautel et al., 1993; Fig. 1) enabled us to produce four domain-assigned antibodies suitable for ultrastructural analysis (see above). All of them decorated within the M band, and the pair of decoration lines is centrally symmetric to the M1-line (center of the M band; Fig. 6; Table II).

The catalytic kinase domain was positioned at 52 nm from the M band center by mAb T40 (Fig. 6 *a*). It therefore defines the junction between the M band and the preceeding P zone regions of titin (Sjöström and Squire, 1977). The insertion between domains m5 and m6 of titin (Fig. 1), which contains four KSP-motifs, possibly involved in developmentally regulated phosphorylation events (Gautel et al., 1993), was localized at a distance of 32 nm from the M band center by mAb T41 (Fig. 6 *b*). Two further epitopes situated closer to the carboxy terminus, domains m8 and m9 (see Fig. 1), were localized at 48 and 52 nm from the M1-line by antibodies Tm8ra and mAb T51, respectively (Figs. 6, *c* and *d*).

The approximate colocalization of the titin kinase domain and domains m8 and m9 at around 50 nm from the M1-line, in comparison with the KSP-motifs at 32 nm, is compatible with the view that the carboxy-terminal seven domains of titin cross the M band center (see discussion). This would place the carboxy-terminal end of titin almost 60 nm into the opposite sarcomere half and would imply that the structural elements in the titin end are arranged in a consecutive order parallel to the thick filament axis. The dimensions of the smaller interdomain insertions can at present not be deduced from our data and the analysis of their primary structure offers no obvious clues. The immu-

Table I. Properties of mAbs

Clone number Immunoglobulin type	T40 IgM	T41 IgG1	T51 IgG1	MyBB78 IgG2a	MpAA280 IgG1	MpAA241 IgG1	MpAA259 IgA
Human skeletal muscle	+	+	+	+	−	+	+
Rat skeletal muscle	+	+	+	+	−	+	+
Mouse skeletal muscle	+	+	+	+	−	+	+
Bovine skeletal muscle	+	+	+	+	+	+	+
Chicken skeletal muscle	−	ND	ND	+	−	+	+
Alligator skeletal muscle	−	ND	ND	−	−	−	−

Summary of the reactions of the mAbs on various muscles assayed by immunofluorescence microscopy on frozen sections. The reactivity of mAb B4 was documented earlier for chicken and rat skeletal and cardiac muscles (Grove et al., 1984, 1985).

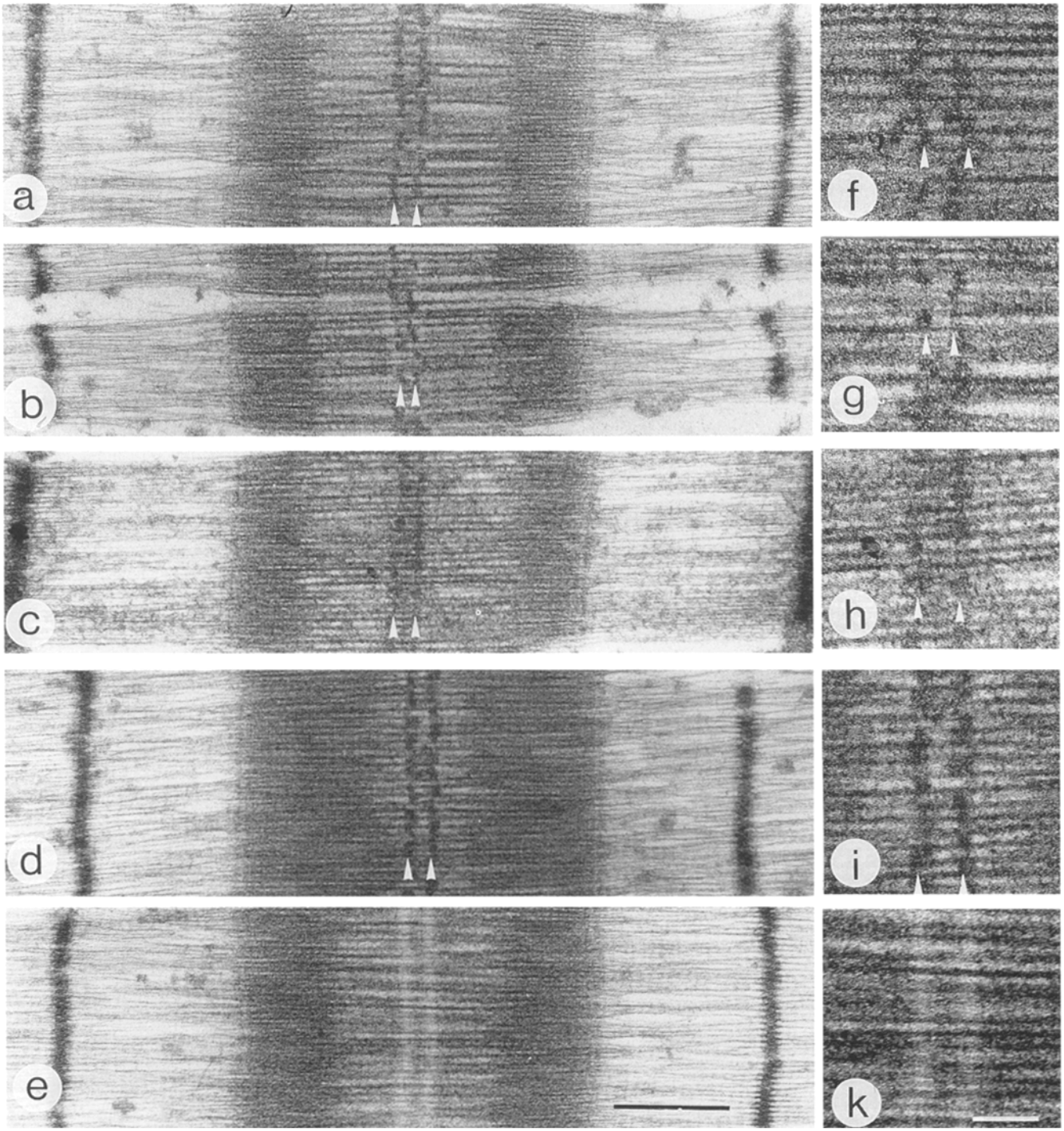

Figure 6. Immunoelectron microscopic localization of defined titin epitopes in the M band. Extracted fiber bundles of rat psoas muscle were incubated with epitope-specific mAbs followed by the corresponding second antibody and subsequently processed for electron microscopy (see Materials and Methods). Positions of decoration lines are indicated by white arrowheads. The following titin specific antibodies were used: T40 (*a* and *f*), T41 (*b* and *g*), Tm8ra (*c* and *h*), and T51 (*d* and *i*); (*e* and *k*) is a control without primary antibody. While in the left column whole sarcomeres are shown, in the right column higher magnification views of the respective M band regions are given. The measured distances from the central M1-line are summarized in Table II. Bars: (*a–e*) 500 nm; (*f–k*) 150 nm.

noelectron microscopical data show no fiber-type differences of the positions of the four titin epitopes, indicating that the basic arrangement of titin filaments is not subject to isoform variations.

Myomesin and M-protein

No difference was observed in the labeling of slow and fast fibers with any of the six antibodies to myomesin. On the

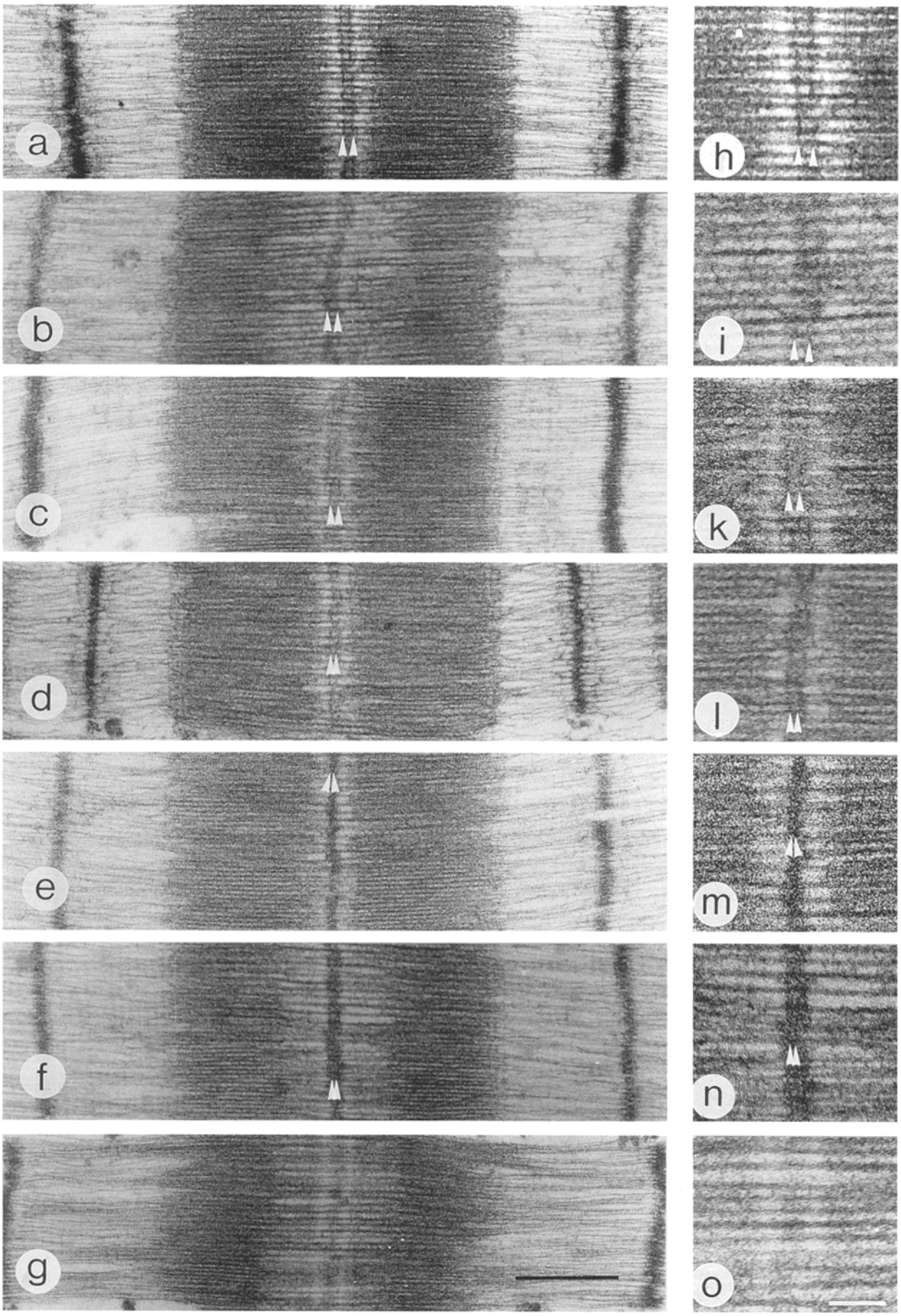
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Table II. Epitopes and Sarcomere Decoration Positions of Antibodies Relative to the Central M1-line

Protein	Ab. short code	Antibody name	Epitope position (compare Fig. 1)	Distance form M1-line (nm)
Myomesin	A	My190Nrt	My1	27.1 ± 1.56
	B	MyW6ra	My2	26.67 ± 1.89
	C	MyW17ra	My4-5 (loop)	19.9 ± 1.52
	D	My673ra	My8	13.2 ± 1.87
	E	MyB4	My11	3.1 ± 2.39
	F	MyBB78	My12	10.1 ± 1.45
M-protein	G	Mp669ra	Mp1	14.89 ± 2.92
	H	Mp660ra	Mp2	15.3 ± 2.98
	I	MpW4ra	Mp4-5 (loop)	5.27 ± 2.09
	K	MpAA280	Mp5	1.87 ± 1.81
	L	MpAA241	Mp13	14.89 ± 1.29
	M	MpAA259	Mp13	17.81 ± 2.72
Titin	N	T40	kinase	51.82 ± 2.76
	O	T41	m5-m6 (is4)	31.9 ± 2.45
	P	Tm8ra	m8	47.89 ± 2.42
	R	T51	m9	51.78 ± 3.08

Sarcomere decoration positions of antibodies with known epitopes relative to the central M1-line of the M band. Epitope positions along the modular structures of myomesin, M-protein, and titin are from Figs. 2–6 as summarized in Fig. 1. The order of antibodies is from the amino- to the carboxy-terminal end of each protein. Each antibody is also given a single letter name (column 2) to facilitate the orientation in Fig. 9. Distances in nm (last column) indicate the center of antibody decoration lines. Mean values and standard deviations of multiple measurements from at least two independent embeddings are given (see Materials and Methods).

other hand, all six distinct antibodies to M-protein left the slow fibers undecorated. These results show that M-protein is limited to fast fibers as previously proposed (see Introduction) and document that myomesin is a general M band component in all cross-striated muscle fibers.

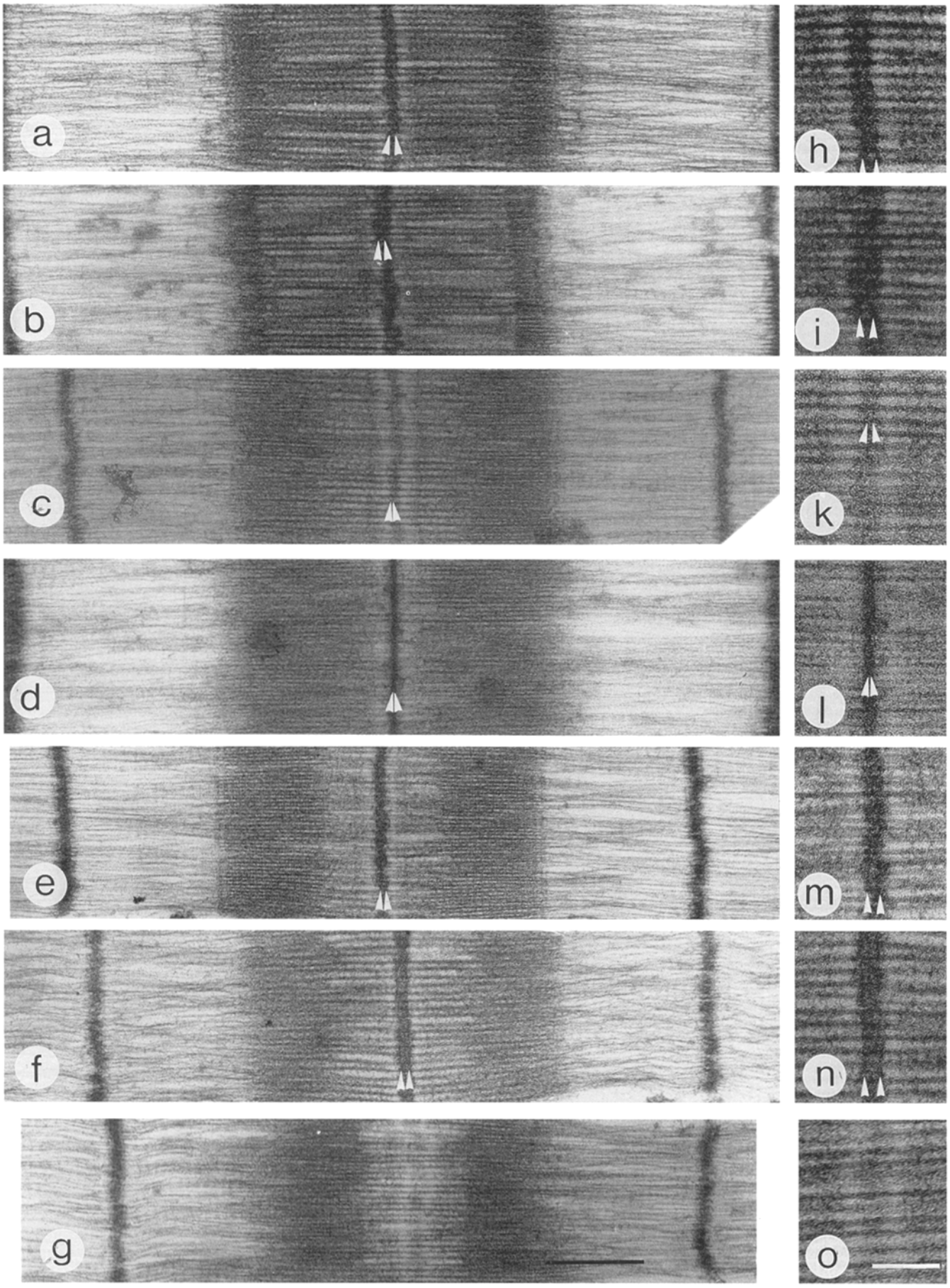
The most amino- and carboxy-terminally situated epitopes of myomesin occurred at distinct distances from the M1-line. Domain My1 was revealed at a distance of about 27 nm from the M1-line by the two antibodies My190Nrt and MyW6ra (Fig. 7, *a* and *b*; Table II). In contrast, domain My12 is located by antibody MyBB78 at approximately 10 nm (Fig. 7 *f*). Given a contour length of myomesin molecules of close to 50 nm (Obermann et al., 1995), the immunoelectron microscopical results are compatible with a model in which the molecules bridge the M1-line and show an antiparallel and staggered arrangement (see Discussion). The decoration patterns of the remaining three antibodies (MyW17ra, My673ra, and MyB4), which bind at more interior positions of the myomesin molecule (Fig. 1; Table II), are in line with this view (Fig. 7, *c–e*; Table II). Thus, domains My9 and My10, for which antibodies are not available (see Discussion), are expected at the M1-line (Fig. 9). The observation that domains My1 and My2 show the same localization invites some speculation in Fig. 9, since this region of the myomesin molecule harbors a myosin binding site (Obermann et al., 1995).

Although myomesin and M-protein share the same modular organization (Fig. 1; Vinkemeier et al., 1993), the immunoelectron microscopical results indicate a distinct arrangement in the M band. The different epitopes of M-protein seem more concentrated in the central part of the M band. Thus, domain Mp1 labels at 15 nm from the M1-line (antibody Mp669ra; Fig. 9 *a*) and the carboxy-terminal domain Mp13 labels at practically the same distance, (i.e., 18 nm for mAbs MpAA241 and 15 nm for MpAA259) (Fig. 8, *e* and *f*; Table II). Distinct decoration positions of the latter two mAbs, though specific for the same domain, can be explained by the dimensions of these Ig-domains, which are around 3 × 4 nm (Pfuhl and Pastore, 1995). Thus, most likely these mAbs bind to different faces of the same barrel-like domain. The position of the epitopes situated in the more central part of the M-protein molecule cannot be resolved (Fig. 8; Table II). These results lead to the speculation that the central portion of M-protein could be organized perpendicular to the myofibrillar axis while the terminal domains face outward (Fig. 9). Formally, the possibility exists that M-protein molecules could, like myomesin, also be arranged in an antiparallel fashion; the molecular length of almost 50 nm, however, makes this possibility very unlikely.

Discussion

The structural models of the sarcomeric M band discussed to date are based on high resolution electron microscopy and image analysis (Luther and Squire, 1978; see also Introduction). Only few protein components of the M band have been identified so far. They include the muscle-specific isoform of creatine kinase (MM-CK) and three structural proteins, titin, myomesin, and M-protein. Various attempts to determine the exact disposition of these polypeptides were of rather limited success, hence the correlation to distinct ultrastructural features remained largely unknown. To obtain a correlation of M band architecture and the molecular layout of its principal structural proteins, we have combined epitope mapping of previously characterized mAbs with immunolabeling by a whole panel of new, sequence-assigned antibodies directed against titin, myomesin, and M-protein. Generally, this approach yielded ultrastructural information with a resolution down to a single Ig- or Fn-domain. Since the width of antibody decoration bands is approximately 10 nm, this value would be the actual limit of resolution. If, however, the center of these dark bands is considered, the described nominal resolution is achieved. The combined immunoelectron microscopical localization of defined epitopes of titin, myomesin, and M-protein now yielded detailed knowledge about the spatial order of certain portions of these proteins and thus allowed us to propose the model depicted in Fig. 9. This is the first time that detailed molecular maps can be drawn that describe the layout of the three structural M band proteins.

Figure 7. Immunoelectron microscopic localization of myomesin epitopes. Treatment of extracted fiber bundles of rat psoas muscle was as in Fig. 6 and in Materials and Methods. Positions of decoration lines are indicated by white arrowheads. The following antibodies were used: My190Nrt (*a* and *h*), MyW6ra (*b* and *i*), MyW17ra (*c* and *k*), My673ra (*d* and *l*), mAb MyB4 (*e* and *m*), and mAb MyBB78 (*f* and *n*); (*g* and *o*) is a control without primary antibody. Micrographs in *a–g* show whole sarcomeres, *h–o* give higher magnification views. The measured distances from the central M1-line are summarized in Table II. Bars: (*a–g*) 500 nm; (*h–o*) 200 nm.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Especially revealing were the data for titin. The cDNA sequence of its carboxy-terminal region had demonstrated the presence of an MLCK-like kinase domain followed by 10 Ig-domains. Unlike the C- and D-zone regions of A band titin (Labeit et al., 1992; Labeit and Kolmerer, 1995), the M band region contains diverse sequence insertions between its Ig-domains of up to 500 amino acids (Gautel et al., 1993). The approach of mapping distinct domains ultrastructurally now reveals information on the spatial arrangement of this large molecular region of almost 250 kD. The position of the titin kinase domain at the edge of the M band near the M6-line is separated from the head domains of both myomesin and M-protein by 25 and 37 nm, respectively. Thus, although both head domains contain putative phosphorylation sites (Vinkemeier et al., 1993), they are therefore unlikely to represent substrates for the titin kinase. Similarly, the first row of myosin heads in the P1-line (Sjöström and Squire, 1977) is separated by 29 nm from the kinase domain. The titin domain pattern upstream from the kinase region shows an unusual group of seven domains in a pattern conserved between *Caenorhabditus elegans* twitchin and vertebrate titin (Labeit et al., 1992). These seven domains have a predicted dimension of about 28 nm and are therefore likely to span the 29-nm gap between the kinase and the first row of cross-bridges. The first row of myosin heads, therefore, most likely is at the level of the most carboxy-terminally situated titin 11-domain super-repeat (Labeit et al., 1992). Because of their distance to the titin kinase, both the M band proteins and the myosin-associated light chains are very likely not substrates for the titin kinase. The titin kinase could, however, be envisioned to contact thin filament ends in contracted sarcomeres or to interact with a soluble substrate, e.g., dissociated light chains.

The extended conformation for the 490-residue-long insertion is2 between Ig-domains m3 and m4 of titin, which is the most likely explanation of our data, implies a substantial overlap for this region of about 35 nm. In such a model, neighboring antiparallel titin ends would show an overlap of about 35 nm for this insertion (Fig. 9). A largely extended nature of the insertion is reflected in secondary structure analysis of this region, which predicts long stretches of α-helical potential (Gautel et al., 1993) and regions of coiled-coil forming potential (Gautel, M., unpublished observations). The insertion is2 may therefore be involved in titin–titin interactions and may participate in the formation of the dimers or higher multimeres that can be observed in electron micrographs as multiple strands attached at their M band ends (Nave et al., 1989). Since we can now assume that the total overlap of titin polypeptides entering the M band is almost 120 nm, the interaction between neighboring, antiparallel titin molecules may be substantial. Formally, a "U-turn model", in which the insertion is2 forms a U-shaped loop and the most carboxy-terminal seven Ig-domains would have an orientation opposite to the rest of the molecule, is compatible with our data. Several attempts to obtain more antibodies that would fill the "gap" between the kinase-domain and Ig-domains m5/m6 were unsuccessful, either because the proteins used for immunization were poorly antigenic or the resulting antibodies yielded no convincing results in immunoelectron microscopy experiments. We believe, however, that several arguments make the proposed model more plausible (Fig. 9). The principles of opposite polarity together with antiparallel and staggered overlap of proteins in the M band has also been shown to be true for myosin (Offer, 1987) as well as myomesin (this study). The described overlap of titin molecules would not only provide for titin–titin interactions but would also allow for multiple interactions of titin with myomesin. This particular three-dimensional arrangement of proteins would enable the sarcomeric M band to withstand higher mechanical strength.

The spatial arrangement of titin domains m8 and m9 also imposes certain constraints both on the orientation of these domains as well as the interdomain insertions. Since the dimension of titin Ig-domains is 4 nm in their long axis (Pfuhl and Pastore, 1995), the positions of m8 and m9 (48 and 52 nm, respectively) imply that both domains should be oriented with the axis of their β-barrels parallel to the thick filament axis. This further implicates their β-strands, and therefore the lateral surfaces of the domains as interaction sites for their potential ligands, rather than the interstrand loops. Similarly, the titin Ig-domains in the C-zone of the thick filaments were found to share exposed, conserved residues mostly in their predicted β-strands (Freiburg and Gautel, 1996). The general attachment of Ig-domains onto thick filaments and their respective ligands therefore appears to be side-on.

The identical antibody decoration lines in different fiber types for titin as well as myomesin clearly suggest that the molecular arrangements as well as the interactions between both proteins must follow the same rules and can be expected to be conserved between fiber types. The M band region of titin can be expressed in differentially spliced isoforms in the insertion is7 between Ig-domains m9 and m10. This finding was considered as a mechanism to regulate the presence or absence of the M1-line in some different muscle types and species (Kolmerer et al., 1996). However, our ultrastructural localization of m9 and the general layout of titin in the M band places this insertion far outside the central M band proximal to the M6-line. Thus, it seems that differential splicing of titin is most likely not involved in the differential appearance of the M1-line. The functional significance of the splice-variants between domains m9 and m10 therefore awaits experimental elucidation, but they seem not directly involved in alterations of central M band ultrastructure.

Equally interesting were the data for myomesin: They

Figure 8. Immunoelectron microscopic localization of M-protein epitopes. Treatment of fibers was as in Fig. 7 and in Materials and Methods. Positions of decoration lines are indicated by white arrowheads. The following antibodies were used: Mp669ra (*a* and *h*), Mp660ra (*b* and *i*), MpW4ra (*c* and *k*), mAb MpAA280 (*d* and *l*), mAb MpAA241 (*e* and *m*), and mAb MpAA259 (*f* and *n*); (*g* and *o*) is a control without primary antibody. Except for MpAA280, which was used on bovine *sternomandibularis* muscle (see text), all other antibodies were analyzed on rat psoas muscle. Micrographs in *a–g* show whole sarcomeres, *h–o* give higher magnification views. The measured distances from the central M1-line are summarized in Table II. Bars: (*a–g*) 500 nm; (*h–o*) 150 nm.

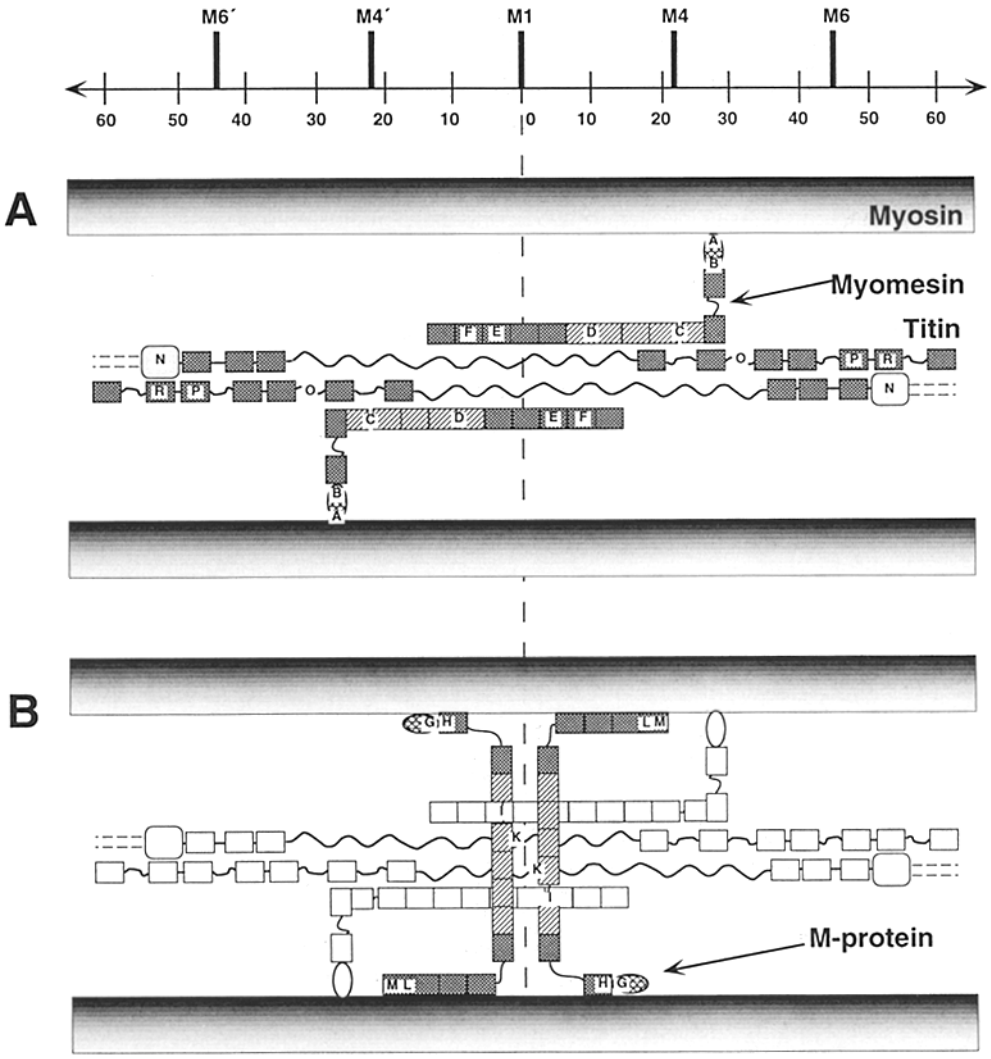

Figure 9. Schematic presentation of the arrangement of titin, myomesin, and M-protein in the M band compatible with the immunoelectron microscopical results. At the top of the diagram, the positions of the most prominent M band striations as defined by Sjöström and Squire (1977) and a scale bar are indicated. Numbers give the distances in nm from the center of the A band, the M1-line. *A* summarizes the layout of titin and myomesin deduced from labeling data. Crosshatched and shaded boxes reflect Ig- and Fn-domains, respectively (see also Fig. 1). Antibody decoration positions found are indicated by letters corresponding to the antibody code in Table II. Only two molecules of each kind are shown in order not to overburden the figure. *B* highlights, in addition to the structures shown in *A*, the proposed location of M-protein around the M1-line. For details see text.

describe the molecules as largely parallel to thick filaments and arranged in an antiparallel and staggered fashion. Approximately the amino-terminal one-third of the molecule is in one sarcomere half, the rest is in the opposite half. Strehler et al. (1983) and Wallimann and Eppenberger (1985) discussed myomesin as a protein that could run parallel to myosin, but at that time their immunolocalization data were largely based on polyspecific antibodies and therefore could not prove this point. The data presented here reveal that their hypothesis is indeed essentially correct. Only the amino-terminal region seems to adopt a special conformation, since the most amino-terminally situated antibody is found only 7 nm distant from the loop between domains My4 and My5 (see results). Interestingly, it is this part of myomesin which harbors its myosin binding site (Obermann et al., 1995). It is tempting to assume that this complex structure offers excellent means to regulate myomesin–myosin binding, especially via the unique sequence in the amino-terminal ~100 amino acids (see sketch in Fig. 9 *A*). Since it also binds to titin (Nave et al., 1989), it is attractive to envision myomesin as a crosslinker that connects thick filaments with titin in the M band. If one tries to bring these findings into accord with the ultrastructural model of Luther and Squire (1978), one is struck by the so called M-filaments, i.e., structures running parallel to thick filaments between M4 and M4′ bridges. These could indeed correspond to myomesin molecules forming a tight complex with two titin polypeptides.

Since myomesin is found in all fiber types, it can be assumed to constitute the principal titin-anchoring protein in the M band. In contrast, M-protein is only revealed in fast twitch fibers. Carlsson et al. (1990) suggested from immunolabeling data with polyclonal antibodies that M-protein could constitute the M1-lines. This would agree with our localization of the central portion of M-protein using mAb MpAA280 (see Figs. 8 and 9). Unfortunately, we did not succeed in raising antibodies against neighboring domains that would be suited for immunoelectron microscopy, but we believe that the data as a whole justify this view. Our results further imply that both amino and carboxy termini are organized along the thick filament. The molecular length of M-protein (almost 50 nm; Obermann et al., 1995) fits this model well and makes a parallel orientation with the terminal domains facing outward in opposite directions less likely. Thus the terminal regions can be expected to be responsible for M-protein–thick filament interaction. In this context it is interesting to speculate that the unique amino-terminal sequence of M-protein might offer means

to regulate such a binding, maybe via phosphorylation of the two putative target sites for cAMP-dependent protein kinase A (Vinkemeier et al., 1993).

The proposed constellation of M-protein molecules, being largely arranged perpendicular to the long axis of myofibrils, could provide for a stronger connection between thick filaments. This might be necessary to meet the stronger strain that is imposed on fast twitch fibers upon their contraction cycles. Good support for this view also comes from the finding that fast twitch fibres have conspicuously stronger M1-lines than their slow twitch counterparts (Pask et al., 1994).

The M band model presented puts certain constraints on possible protein–protein interactions. Necessarily only such domains that are found in spatial proximity can also be able to interact in vivo. The results obtained so far open the road for a number of biochemical in vitro studies to test the predictions of the model.

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