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Myofibrillar M-band proteins represent constituents of native thick filaments, frayed filaments and bare zone assemblages

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Summary

A-segments, native thick filaments, frayed filaments, bare zone assemblages, as well as completely disassembled and reassembled thick filaments from chicken pectoralis major were investigated for the presence of M-band proteins by the colloidal gold labelling technique. Specific polyclonal antibodies against the three M-band proteins identified to date, MM-creatine kinase, M-protein (165 kDa) and a 185 kDa protein myomesin, were prepared. Incubation with anti-M-protein and anti-myomesin antibodies resulted in heavy labelling of all thick filament types mentioned above, with the exception of the completely disassembled and reassembled thick filaments. In that case no labelling was detected with either antibody. In contrast, MM-creatine kinase which is an integral component of the intact M-band structure was detectable on isolated native thick filaments with lower frequency and to a variable extent. Also, bare zone assemblages were only rarely labelled by anti-MM-creatine kinase antibodies.

This study shows that the 'cuff-like' additional material which had previously been observed in the middle of the bare zone of isolated thick filaments represent remnants of all three M-band proteins, whereas the extra material in intact bare zone assemblages mainly consists of myomesin and M-protein, but not of MM-creatine kinase.

Myomesin and M-line protein may be important for the assembly and structural maintenance of thick filaments as well as for anchoring of additional M-band proteins, e.g. MM-creatine kinase which is bound less tightly to thick filaments and, in accordance with earlier results, seems to represent within the M-band some of the prominent bridge-forming structures.

Introduction

The M-band traverses the centre of the bare zone of skeletal muscle, the crossbridge-free

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area of thick filaments, where the individual myosin molecules are packed in an antiparallel fashion (Huxley, 1963, 1972; Pepe, 1967). In electron micrographs of longitudinal ultrathin cryosections the M-band can be resolved in up to nine (M1–M9) substriations (Sjöström & Squire, 1977). The three to five major striations, depending on the muscle fibre type, are the result of m-bridges connecting each thick filament to its six neighbours (Knappeis & Carlsen, 1968; Luther & Squire, 1978). The midpoints of the m-bridges have been observed in some cases to be connected by structures called m-filaments which run parallel to the thick filaments (Knappeis & Carlsen, 1968; Thornell & Sjöström, 1975). In addition m-filaments are thought to be connected by Y-shaped secondary m-bridges (Luther & Squire, 1978). The M-band shows some variability, depending on fibre type and animal species, in number and widths of the m-bridges (Sjöström *et al.*, 1982; Thornell & Carlsson, 1984).

So far, three proteins have been identified as true M-band components, namely, the muscle-specific isoenzyme of creatine kinase, MM-CK (Turner *et al.*, 1973; Wallimann *et al.*, 1977), the 165 kDa M-protein (Masaki & Takaiti, 1974; Trinick & Lowey, 1977) and a 185 kDa protein which was recently discovered by monoclonal antibody techniques and is named myomesin (Grove *et al.*, 1984). MM-CK was recently shown to be a component of the primary m-bridges M4 and M4′ and possibly also of the M1 m-bridge (Strehler *et al.*, 1983; Wallimann *et al.*, 1983).

Since antibodies reacting with both M-protein and myomesin were shown to label the entire M-band between the M6 and M6′ substriations (Strehler *et al.*, 1983), these authors attributed the two proteins to the m-filaments and possibly to the M6 and M6′ m-bridges or to the ensheathments around the thick filaments. Such a direct attachment of protein(s) to thick filaments along the length of the M-band would fit well with published structural data (Crowther & Luther, 1984). *In vitro* studies, however, of the interaction of M-protein and MM-CK with purified myosin and its subfragments and with each other have shown that such interactions are either weak or absent (Woodhead & Lowey, 1983).

In isolated native thick filaments a thickening in the centre of the bare zones with additional material has frequently been observed. This material is thought to be related to remnant M-band proteins presumably binding rather strongly to native thick filaments, since extra material remains attached when native thick filaments are sequentially disassembled into intact bare zone assemblages (Trinick & Cooper, 1980; Niederman & Peters, 1982). Upon close examination, this extra material can also be seen in the middle region of filaments that were frayed into three subfilaments from their tips towards the bare zone which remains intact (Maw & Rowe, 1980; Trinick, 1981). Therefore, we attempted to find out (a) whether this additional material in the bare zone was related to the presence of M-band proteins and (b) which of the three known M-band proteins were still attached to the bare zones under different conditions.

For this purpose we prepared polyclonal antibodies specific for the 165 kDa M-protein, the 185 kDa myomesin and for MM-CK and studied, by using the colloidal gold labelling method (Roth, 1983), the attachment of these three M-band proteins to isolated

A-segments, native thick filaments, bare zone assemblages and frayed filaments as well as completely disassembled and reassembled native thick filaments. M-protein and myomesin were found to be attached to isolated native bare zone regions under all the conditions tested and to contribute to the frequently observed extra material within that region. A preliminary report of the data has been presented in abstract form (Bähler *et al.*, 1984).

Materials and methods

Myofibril preparation
Myofibrils were prepared from fresh chicken pectoralis muscle in a solution containing 0.1 M KCl, 1 mM EGTA, 5 mM EDTA, 1 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulphonyl fluoride, pH 7.0, according to Kundrat & Pepe (1971) as modified by Wallimann *et al.* (1977).

Immunofluorescence
Indirect immunofluorescence localization was performed as described earlier (Wallimann *et al.*, 1977). Fluorescein-conjugated goat anti-rabbit IgG was purchased from Cappel Laboratories, Cochranville, U.S.A.

Preparation of antibodies
The preparation and characterization of polyclonal antibodies against MM-CK was described earlier (Perriard *et al.*, 1978). Antibodies initially prepared against M-protein (Strehler *et al.*, 1979, 1980), which turned out later to contain anti-myomesin antibodies also, were used as starting material from which antibodies specific for each of the two high M_r M-band proteins, 165 kDa M-protein and 185 kDa myomesin, were affinity purified according to the method of Olmsted (1981). Preparative 5% polyacrylamide–sodium dodecyl sulphate (SDS) slab gels (Laemmli, 1970) of freshly prepared chicken pectoralis major myofibrils were run. The protein bands were transferred electrophoretically to nitrocellulose paper (Towbin *et al.*, 1979). To increase blotting efficiency the filter paper on the cathode side of the gel was soaked with 10% SDS. After electrophoresis, protein binding sites on the nitrocellulose were blocked with 10% horse serum in phosphate-buffered saline (PBS) consisting of 0.15 M NaCl, 10 mM phosphate, pH 7.4, the paper incubated with antibodies and thoroughly washed in PBS. To visualize the position of the protein-antibody bands, small strips cut off from both ends as well as from the middle of the blots were incubated with fluorescein-conjugated second antibodies. After aligning the stained strips with the rest of the nitrocellulose paper, those regions on the paper corresponding to the position of M-protein and myomesin were cut out separately. The antibodies were eluted for 2–3 min with 1 M propionic acid, the eluates immediately neutralized with an equal volume of 1 M Tris–HCl at pH 10 and dialysed against PBS. Since there was a small amount of degradation product of myomesin within the M-protein strips, antibodies against M-protein were additionally cross-adsorbed with myomesin–nitrocellulose strips. Antibodies were stored at −20° C after adding bovine serum albumin to a final concentration of 1 mg ml^{-1}.

Filament preparations
Native thick filaments were prepared exactly according to the procedure of Niederman & Peters (1982). All procedures were carried out at 4° C. Strips from chicken pectoralis muscle were first tied on to wooden tooth sticks and placed in a Ringer solution consisting of 0.1 M NaCl, 2 mM KCl,

2 mM $MgCl_2$, 1 mM EGTA, 0.1% (w v^{-1}) glucose, 6 mM potassium phosphate, 2 mM dithiothreitol (DTT), pH 7.0, for four days. Then thick and thin filaments were separated by placing 4–5 muscle strips in a relaxing solution (0.1 M KCl, 10 mM $MgCl_2$, 1 mM EGTA, 5 mM ATP, 6 mM potassium phosphate, 2 mM dithiothreitol, pH 7.0) to which 0.05 mg ml^{-1} of phenylmethylsulphonyl fluoride was added, teased apart with needles and allowed to stand for 15 min. The buffer was replaced and the preparation blended twice for 10 s at speed 4 in the 50 ml Sorvall Omnimixer attachment filled to the top. Residual myofibrils removed by centrifugation at 1000*g* for 5 min were used for indirect immunofluorescence staining. The supernatant contained A-segments as well as thick and thin filaments. Quantity and quality of the A-segments varied from preparation to preparation. Thick filaments were then enriched by centrifugation of the supernatant at 40 000*g* for 30 min and the pellet was used as starting material for all further experiments.

Bare zone assemblages were formed from thick filament preparations under the same conditions (either in 0.2 M KCl, 3 mM $MgCl_2$, 0.25 mM EGTA, 1.5 mM ATP, 9 mM potassium phosphate, 1 mM dithiothreitol, pH 7.0; or in 0.1 M KCl, 1 mM $MgCl_2$, 20 mM potassium phosphate, 1 mM dithiothreitol, pH 8.0) as described by Niederman & Peters (1982). The protein concentration was adjusted so that a 1:10 dilution gave an $A_{320\,nm}$ of about 0.5 or that the undiluted solution contained 2.5 mg ml^{-1} protein as determined by Biuret (Gornall *et al.*, 1949). Repolymerized bare zone assemblages (Niederman & Peters, 1982) were obtained from native thick filaments that were first dialysed against 0.2 M salt or pH 8.0 buffer to give bare zone assemblages (see above) to which the stoichiometric amount of free myosin still contained in the dialysis bag was allowed to repolymerize simply by changing the dialysis buffer to a lower ionic strength (0.1 M KCl) or pH (7.0). For complete disassembly thick filaments were dialysed against the relaxing solution containing 0.6 M KCl and subsequently reassembled within the same bag by dialysis against relaxing solution. Fraying of native thick filaments was accomplished by diluting 10 µl of the thick filament solution (2–4 mg ml^{-1}) rapidly into an Eppendorf tube containing 0.5 ml of a 2 mM imidazole–HCl buffer with 0.5 mM ATP at pH 7.3–7.8 or 0.5 ml of a 2 mM Hepes buffer at pH 7.4. In order to absorb thick filaments while they were fraying carbon-coated glow-discharged EM grids had been immersed in the above solutions prior to the addition of the thick filament solution.

Preparation of colloidal gold

Polydisperse colloidal gold was prepared using the borohydride method of Tschopp *et al.* (1982). Protein A–gold complexes were made as described by Roth (1983). Alternatively, we used goat anti-rabbit IgG gold complexes (GarG, 5 nm) purchased from Janssen Life Sciences Products Division, Belgium.

Gold labelling and electron microscopy

The whole labelling procedure was performed at room temperature. Samples were applied onto glow discharged carbon coated grids and the unadsorbed material was washed off with sample buffer. The grids were placed into a drop of 1% bovine serum albumin in PBS for about 2 min and then incubated for about 20 min with antibodies serially diluted in the same buffer. Excess antibody was removed by thoroughly washing with PBS and the grids incubated for about 20 min either with goat anti-rabbit IgG gold complexes (diluted 40 fold) or with protein A–gold complexes. The grids were thoroughly washed again with PBS. Occasionally, samples were fixed with 3% formaldehyde or 2.5% glutaraldehyde in PBS after this step or were prefixed directly after adsorption onto the grids with the same fixatives in sample buffer. But routinely one worked without sample fixation. Subsequently the grids were rinsed with 0.1 M ammonium acetate, the specimens negatively stained with 1% (w v^{-1}) aqueous uranyl acetate and examined in a Siemens 102 or Jeol 100 C electron microscope.

Results

Characterization of polyclonal antibodies against M-protein and myomesin

Polyclonal antibodies originally thought to be raised solely against the 165 kDa M-protein (Strehler *et al.*, 1979, 1980) are shown here to recognize two protein bands on an immunoblot of freshly prepared myofibrils (Fig. 1d), namely the 165 kDa M-protein and the 185 kDa myomesin. The higher M_r protein, myomesin, has recently been identified by monoclonal antibodies as a new M-band component being different from M-protein (Grove *et al.*, 1984). These data are confirmed here by polyclonal antibodies (see Materials and methods) specific for each of the two M-band proteins (Fig. 1b,c).

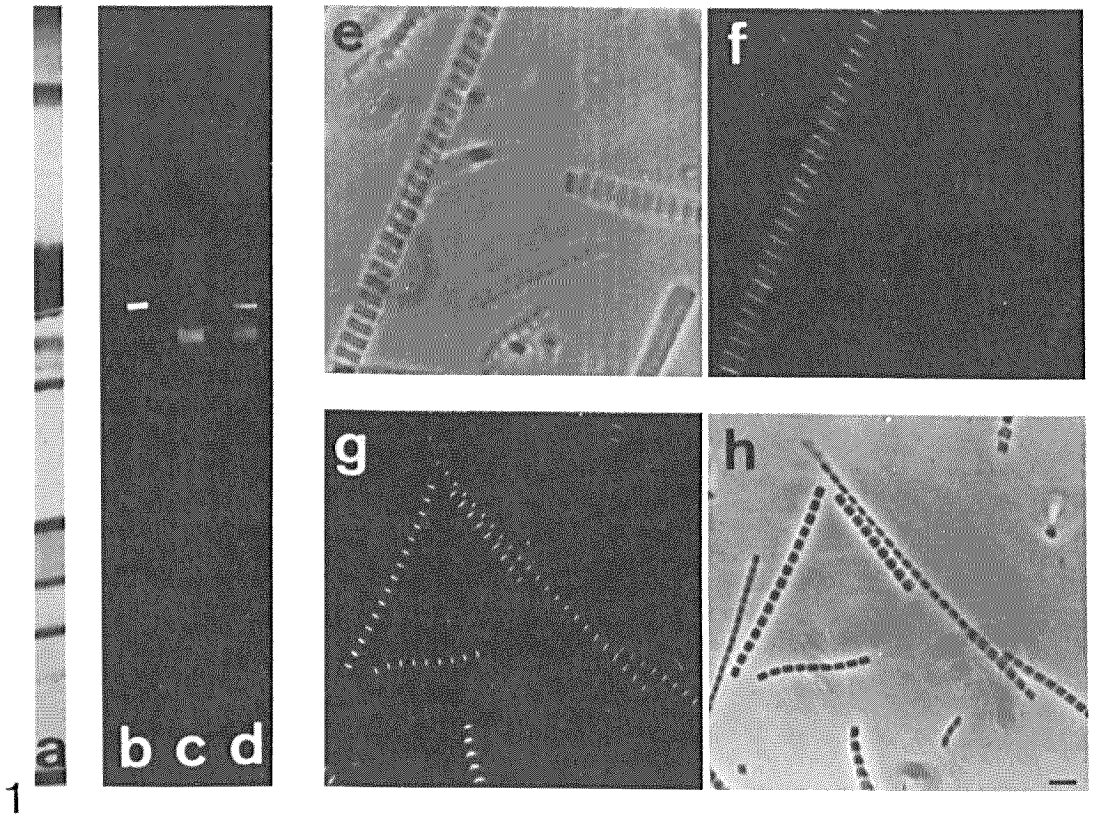


Fig. 1. Characterization of polyclonal, monospecific antibodies against M-protein and myomesin, purified by elution from antibody–M-band protein complexes electrophoretically blotted to nitrocellulose. (a) Sodium dodecylsulphate 5% (w v^{-1}) PAGE of chicken pectoralis major myofibrils. Stained for protein with Coomassie blue. Myomesin and M-protein migrate slightly faster than myosin heavy chain, the most prominent band. (b) Protein bands after electrophoretical transfer to nitrocellulose and incubation with rabbit anti-chicken myomesin. (c) Anti-M-protein. (d) Initial mixture of anti-M-protein–myomesin antibodies; second antibody: fluorescein labelled goat anti-rabbit IgG. (e, f) Isolated myofibrils stained by the indirect immunofluorescence technique with the monospecific antibody against myomesin. (g, h) Against M-protein. Paired photographs are phase contrast (e, h); and fluorescence (g, f). Scale bar: 5 μm.

They were eluted from M-band proteins blotted to nitrocellulose. The two antibodies clearly recognized two immunologically distinct protein bands. It could also be demonstrated by Cleveland digests (Cleveland *et al.*, 1977) of rabbit M-protein and myomesin that the two proteins are distinctly different from each other (not shown). In addition, both antigens were demonstrated by the indirect immunofluorescence technique to be localized in the M-band (Fig. 1e–h). Thus, the antibodies after affinity purification with denatured proteins are able to recognize and bind to the two M-band proteins in their native conformations.

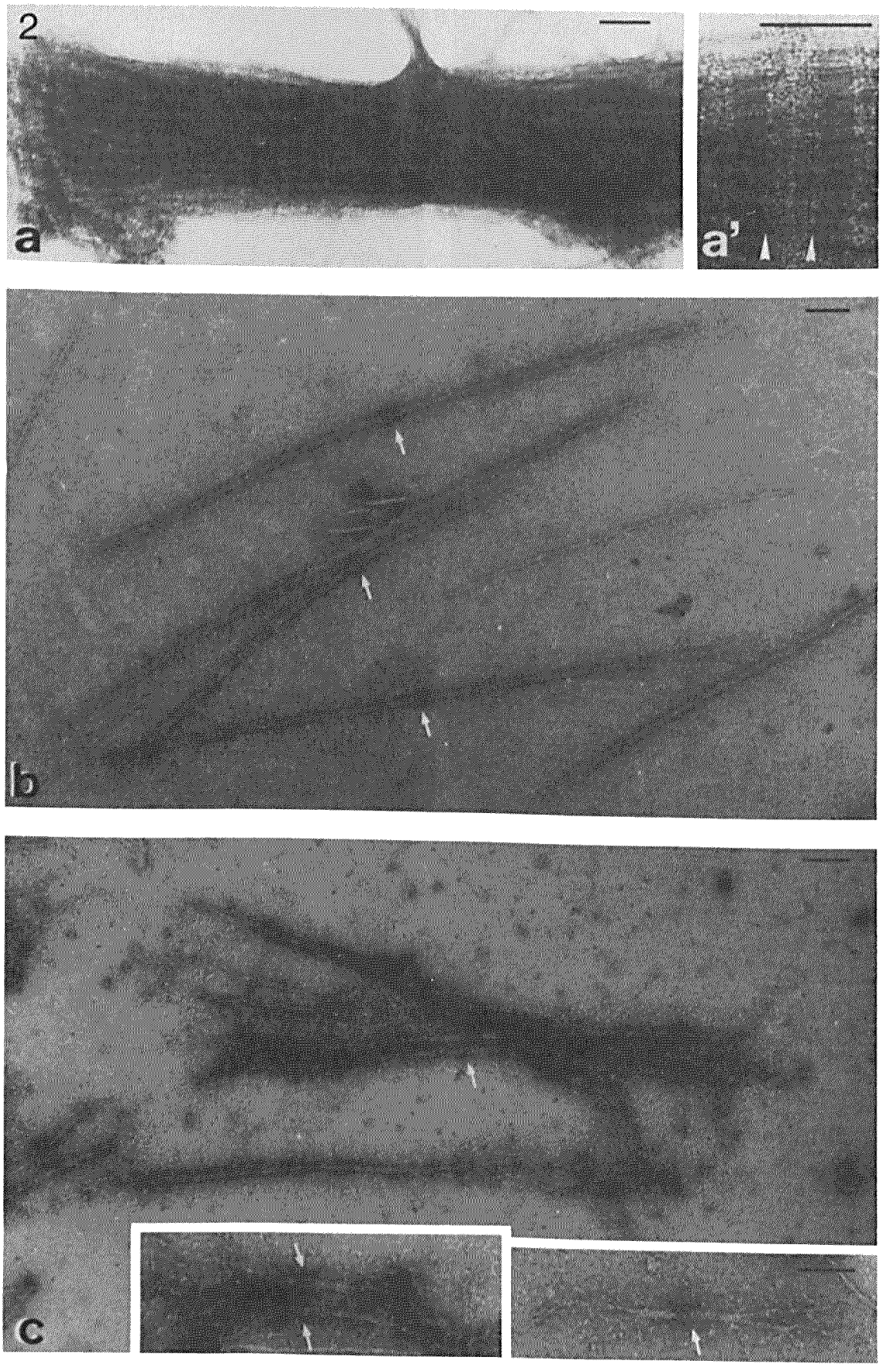
Appearance of M-band in isolated A-segments

Isolated A-segments stained by negative contrast (Fig. 2a) displayed within the M-band a number of clearly resolved substriations. The most prominent and easily recognizable band was in the middle of the M-band corresponding, according to the terminology of Sjöström & Squire (1977), to the M1 M-line, although somewhat weaker in contrast. Lines M4 and M4′ to which MM-CK contributes, were also routinely observed, especially at higher magnification (Fig. 2a, outer right hand side). The lines M2, M2′ and M3, M3′ could also be frequently resolved. Lines M6 and M6′ were either very faint or not visible.

Filament preparations

Native thick filaments very often showed, in the middle of the defined bare zone, some cuff-like extra material thought to be composed of M-band proteins. The cuff-like M-band material persisted about the bare zone even after fraying of native thick filaments by fast dilution into buffer of very low ionic strength (Fig. 2c, indicated by arrows). Thick filaments from chicken, like rabbit and rat, also fray into three subfilaments, supporting the idea of a three stranded structure of chicken thick filaments. However, at very low ionic strength rapid aggregation of filaments was noticed as has been reported by Trinick (1981). In addition, a strong tendency for concomitant disassembly of frayed thick filaments from chicken, even at pH 7.4, was observed which finally led to bare zone assemblages with frayed ends (Fig. 2c, insets). But most

Fig. 2. Negatively stained preparations of isolated A-segments, native thick filaments and frayed filaments. (a) Isolated A-segment, showing the 11 known nonmyosin protein stripes in each half of the A-band and the prominent M1 subline in the middle of the M-band. (a′) Enlargement of the M-band region. Arrowheads point to the M4 and M4′ m-bridges flanking the central, most prominent substriation M1. Substriations M2 and M2′ as well as M3, M3′ are clearly resolved. Substriations M6 and M6′ beyond M4 and M4′ are only faint. (b) Native thick filaments with a defined bare zone and cuff-like extra material in the M-region (arrows). (c) Native thick filaments partially frayed into three subfilaments, leaving intact only the bare zone region. Cuff-like extra material (arrows) also visible. Insets show disassembled native thick filaments (frayed bare zone assemblages) which were produced under the same conditions. All samples after adsorption on glow discharged carbon films and negative staining with 1% uranyl acetate. Scale bars: 0.1 μm.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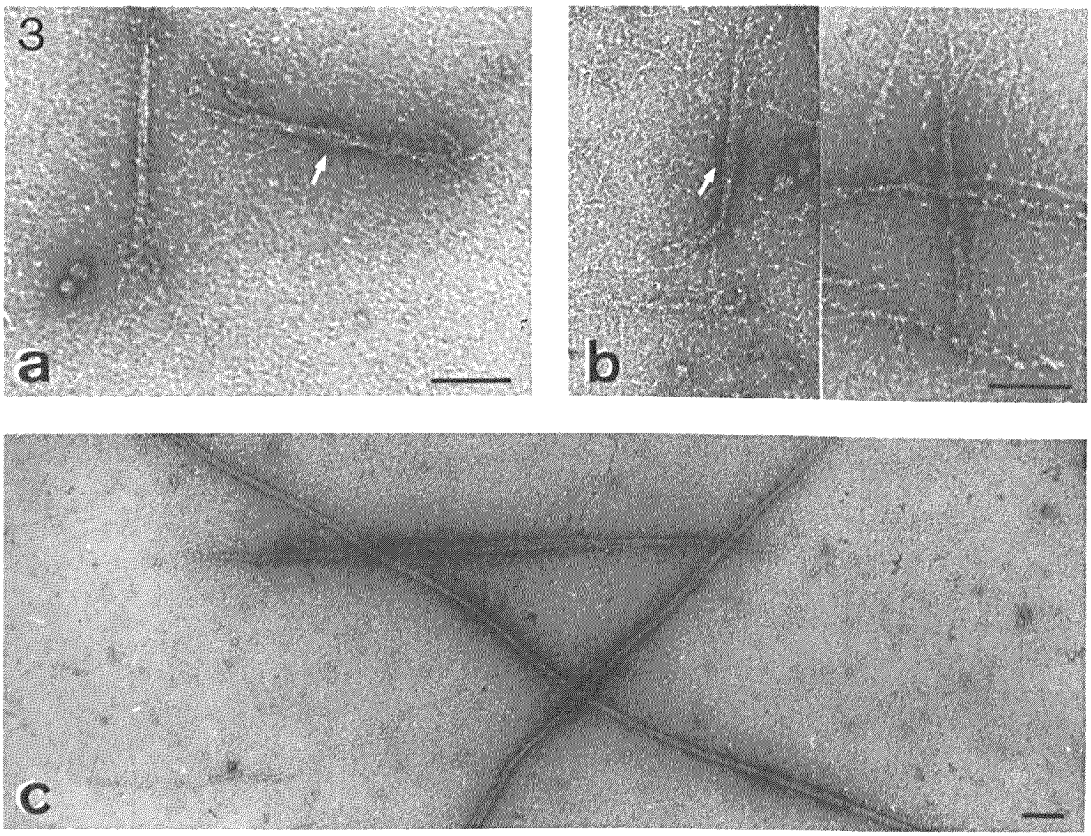
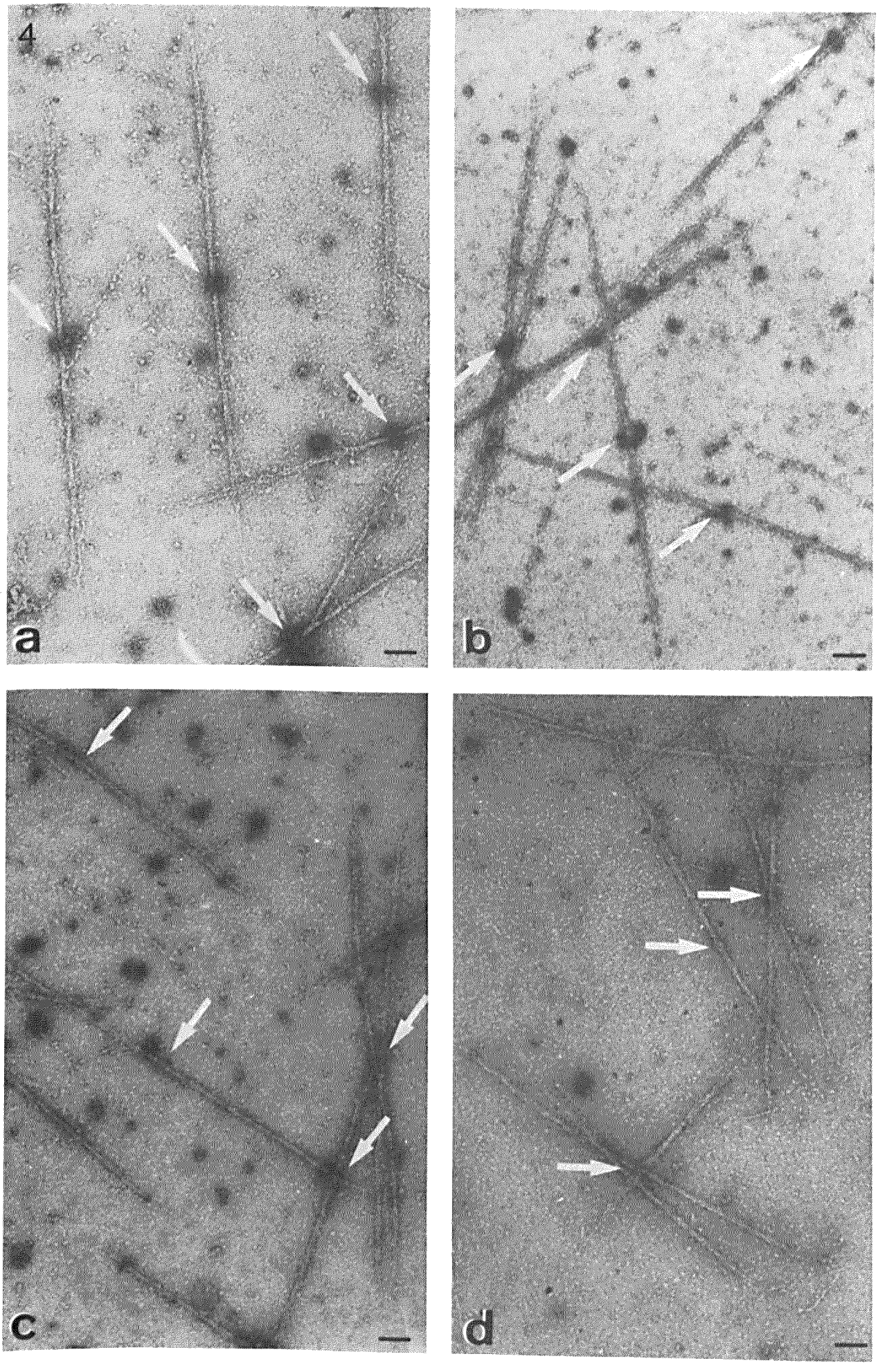


Fig. 3. Negatively stained bare zone assemblages and completely dissolved and subsequently reassembled filaments. (a) Bare zone assemblages formed by raising the KCl concentration to 0.2 M. (b) Bare zone assemblages formed at pH 8.0 (see Materials and methods). Arrows indicate cuff-like extra material in the middle of the bare zones. (c) Native thick filaments completely dissolved and subsequently reformed (synthetic filaments). Note the absence of a defined bare zone region. Scale bars: 0.1 μm.

importantly, the M-band regions still displaying the extra cuff-like material (Fig. 2c, arrows) were most resistant to further depolymerization.

Furthermore, bare zone assemblages produced by dialysing thick filaments against the 0.2 M KCl solution also often showed extra M-band material (Fig. 3a). Bare zone assemblages produced by dialysis of thick filaments against the pH 8.0 buffer also exhibited a similar appearance (Fig. 3b), but were rarely resolved due to their tendency to aggregate with contaminating thin filaments. The characteristics of repolymerized bare zone assemblages (bare zone assemblages to which myosin was allowed to

Fig. 4. Indirect gold labelling for M-band proteins of isolated native thick filaments (negative staining). Native thick filaments were incubated with (a) anti-M-protein IgG; (b) anti-myomesin IgG; (c) anti-MM-CK IgG; (d) preimmune IgG, followed by goat anti-rabbit IgG–gold. Arrows indicate bare zone regions. Scale bars: 0.1 μm.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polymerize at both ends under physiological conditions) will be described in detail elsewhere (Bähler, in preparation). Finally, native filaments which had been completely dissolved and again reassembled lacked an apparent bare zone and also no M-band material attached to their centre parts could be detected (Fig. 3c).

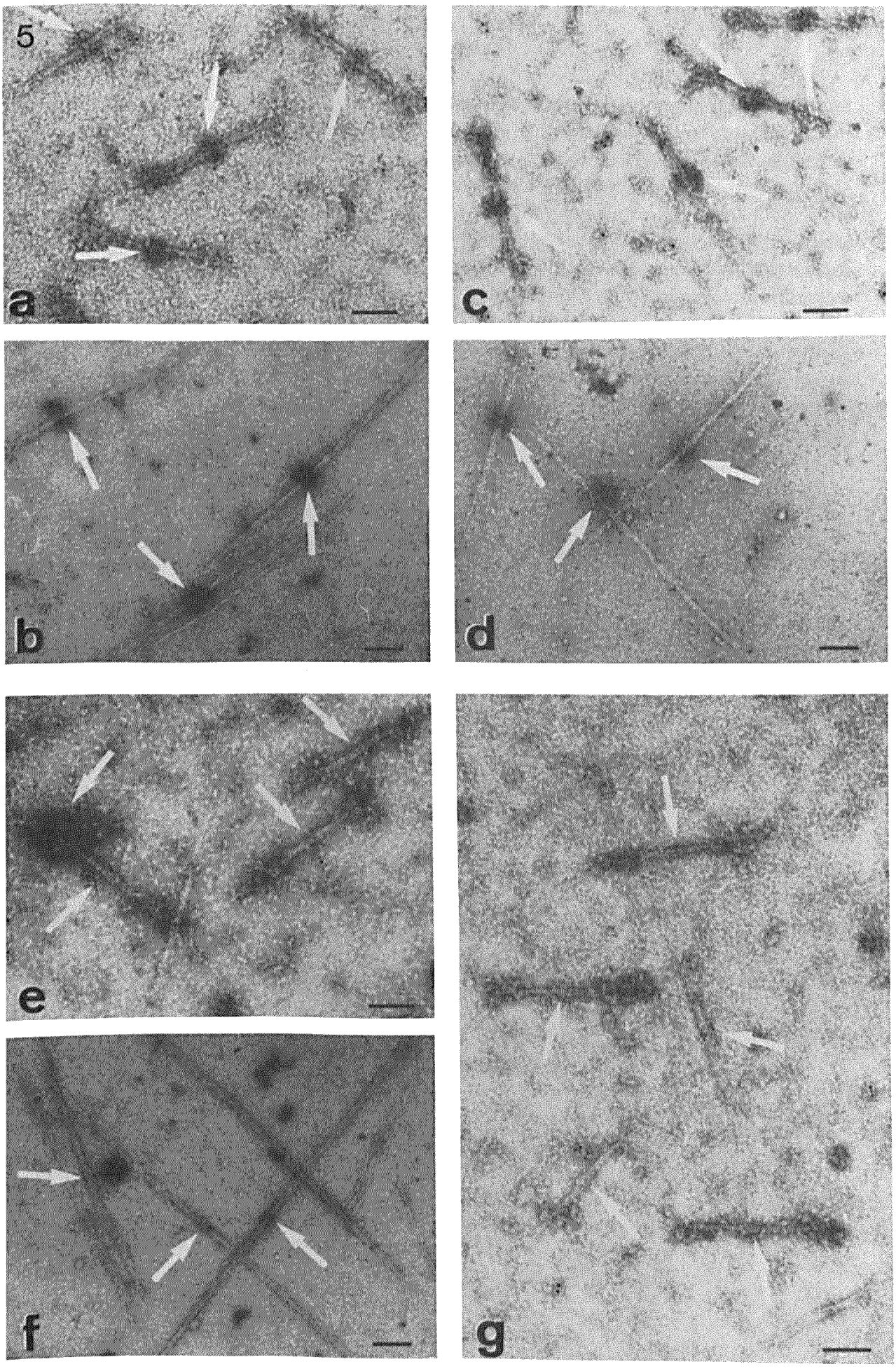
Labelling of native thick filaments with anti-M-band protein antibodies

Incubation of native thick filaments on glow discharged carbon-coated grids with antibodies against M-protein followed by protein A–gold resulted in heavy labelling of the middle part of the bare zones of almost every thick filament (Fig. 4a). The labelling was not confined to a specific location within the M-band region, thus rendering impossible a precise substructural localization of M-protein. The same results were obtained when thick filaments were labelled for myomesin, in that almost every filament within the bare zone was heavily decorated with gold particles (Fig. 4b). Somewhat different results were obtained when thick filaments were labelled for MM-CK. The labelling was less intensive (Fig. 4c) and even though there was some variation in the number of labelled filaments, usually less than 50%, one could never obtain labelling of all filaments as was seen with anti-M-protein and anti-myomesin antibodies. In addition, the labelled filaments were generally decorated with fewer gold particles compared to filaments labelled for M-protein and myomesin. Nevertheless, all myofibrils still present in crude thick filament preparations exhibited strong fluorescence in the M-band when labelled by indirect immunofluorescence technique with antibodies against MM-CK (not shown). No decoration by gold particles was found in the middle of the bare zones of native thick filaments after incubation first with preimmune IgG followed by gold complexed second antibody (Fig. 4d). There was always some unspecific labelling with protein A–gold in the background due to the high affinity of the gold cluster complexes for glow discharged carbon films.

Labelling of bare zone assemblages and repolymerized bare zone assemblages with anti-M-band protein antibodies

M-protein and myomesin were also found to be constituents of bare zone assemblages (produced by increasing the salt concentration from 0.1 M to 0.2 M KCl) as is demonstrated by the antibody labelling patterns (Fig. 5a,c). No difference between labelling with anti-M-protein and anti-myomesin antibodies of native thick filaments

Fig. 5. Indirect gold labelling for M-band proteins of bare zone assemblages and repolymerized bare zone assemblages (negative staining). (a) Bare zone assemblages formed at 0.2 M KCl incubated with anti-M-protein IgG; (c) anti-myomesin IgG; (e) anti-MM-CK IgG; (g) preimmune IgG, followed by goat anti-rabbit IgG–gold. Arrows indicate bare zone regions. Bare zone assemblages formed at pH 8.0 to which myosin was allowed to repolymerize by changing pH, referred to as repolymerized bare zone assemblages (Niederman & Peters, 1982), incubated with (b) anti-M-protein; (d) anti-myomesin; (f) anti-MM-CK IgG; followed by goat anti-rabbit IgG–gold. Arrows indicate bare zone regions. Scale bars: 0.1 μm.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and bare zone assemblages was observed. Identical results were also obtained when monomeric myosin was allowed to polymerize onto pre-existing bare zone assemblages to give so-called repolymerized bare zone assemblages (Niederman & Peters, 1982; not shown). Bare zone assemblages which had been formed by increasing the pH value were incubated with primary antibodies followed by goat anti-rabbit IgG gold complex after repolymerization, since otherwise aggregation occurred. Nevertheless, these repolymerized bare zone assemblages were also strongly labelled by anti-M-protein as well as anti-myomesin antibodies (Fig. 5b,d). Bare zone assemblages produced by either of the two conditions were labelled only to a low extent when incubated with antibodies against MM-CK (Fig. 5e,f). Controls performed with preimmune IgG showed no

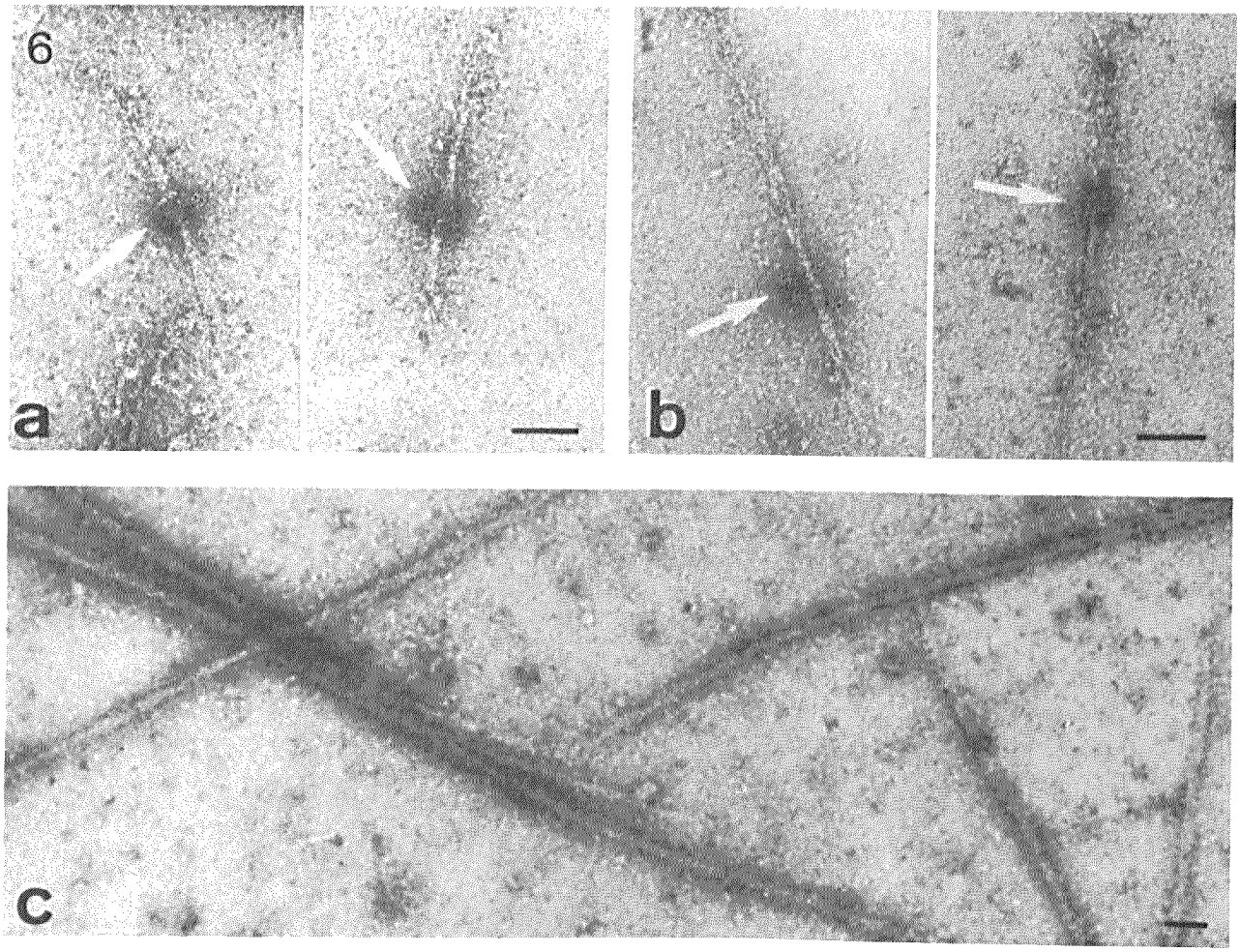

Fig. 6. Indirect gold labelling for M-protein and myomesin of 'frayed' bare zone assemblages and disassembled–reassembled filaments (negative staining). (a) 'Frayed' bare zone assemblages formed in 2 mM Hepes, pH 7.4, incubated with anti-M-protein; (b) anti-myomesin IgG; followed by goat anti-rabbit IgG–gold. (c) Native thick filaments completely disassembled and subsequently reassembled after incubation with both anti-M-protein and anti-myomesin IgG; followed by protein A–gold. Note the lack of specific labelling. Scale bars: 0.1 μm.

labelling (Fig. 5g). Also bare zone assemblages with fraying ends (produced under fraying conditions) were heavily labelled with anti-M-protein as well as anti-myomesin antibodies (Fig. 6a,b). In conclusion, M-protein and myomesin were attached to every bare zone region derived from native thick filaments.

Labelling of disassembled–reassembled filaments with antibodies against M-protein and myomesin
After disassembly and subsequent reassembly of native thick filaments, no significant labelling was detected by a mixture of anti-M-protein and anti-myomesin antibodies (Fig. 6c). In most cases, the number of gold particles in the background drastically increased, presumably due to solubilized M-protein and myomesin that was adsorbed on to the carbon film, and was subsequently decorated by the antibodies. It seems therefore that, once removed from myosin, M-protein as well as myomesin does not rebind to synthetically formed thick filaments under the conditions used.

Discussion

The 165 kDa M-protein is known to be firmly bound to the myofibril *in situ* and can only be quantitatively extracted by dissolving the myofibril by high salt buffers (Trinick & Lowey, 1977). M-protein has been found as a persistent contaminant in myosin preparations (Component B; Starr & Offer, 1971, 1982), suggesting a possible interaction with myosin. The same is probably true also for the 185 kDa myomesin. Based on antibody labelling of ultrathin frozen sections with an antibody recognizing both M-protein and myomesin direct attachment to thick filaments of the two M-band proteins along the entire M-band from M6 to M6′ was proposed as one possibility by Strehler *et al.* (1983). At this time M-protein and myomesin were considered the same protein. The other possibility proposed by the same authors was that these proteins constitute the so-called m-filaments. But, since the antibodies used by them are shown here to recognize both proteins, a combination of the two possibilities may also be possible. Due to its molecular size and shape the 165 kDa M-protein has also been suggested to represent a component of m-filaments (Woodhead & Lowey, 1982). However, as far as the existence of m-filaments is concerned, very recent three-dimensional reconstructions of the M-band from single cross-sections of fish skeletal muscle although with a limited resolution of 7 nm only, revealed in fact ridges running along the thick filament from M1 to M6 and M6′, with no clear indication for the existence of separate m-filaments (Crowther & Luther, 1984; Luther & Crowther, 1984). It is at these ridges where the primary m-bridges seem to be inserted (Crowther & Luther, 1984). Our results indicate in correspondence with the reported structural work an attachment (direct or indirect) of the 165 kDa M-protein and the 185 kDa myomesin to the thick filament since isolated A-segments labelled with antibodies against M-protein or against myomesin both did not decorate the M1 and, neither the M4 and M4′ primary m-bridges (unpublished observation). In addition, the m-filaments, if they existed, had to be held in their position in part by M4 and M4′ primary m-bridges consisting of

MM-CK (Strehler *et al.*, 1983; Wallimann *et al.*, 1983); in this case labelling frequencies of native thick filaments after incubation with anti-MM-CK, anti-M-protein and anti-myomesin antibodies would have been expected to be very similar if M-protein and/or myomesin were to represent m-filaments. This was clearly not the case in our experiments where labelling with anti-M-protein as well as anti-myomesin was shown to occur with greater frequency and with higher intensity compared to labelling with anti-MM-CK antibodies. However the attachment to the thick filament may not be direct for there could equally well be more as yet unidentified smaller bridging structures which hold M-protein and myomesin close to the thick filament without direct binding to myosin. The notion that the two high M_r M-band proteins interact directly with myosin is considerably weakened by the lack of any *in vitro* evidence for such an interaction (Woodhead & Lowey, 1983).

M-protein and myomesin adhere to the bare zone derived from native thick filaments until the complete dissolution of this most stable part of the thick filament. Hence, a role of M-protein and myomesin for the stabilization of the bare zone region may be postulated and the two proteins may be responsible for anchoring other M-band proteins, e.g. MM-CK to the M-band. Evidence for such a role are results reported by Niederman & Peters (1982) who had shown that the stable bare zone assemblages act as nuclei for filament assembly. However, the fact that the bare zone assemblages were resistant to further depolymerization and still had M-band proteins present, probably does not necessarily imply that the M-band material has a protective role. M-band material may remain present because the myosin arrangement in the bare zone is relatively resistant to further depolymerization, perhaps, because of the intrinsic stability of the anti-parallel packing of the myosin tails themselves in the native assembly.

Purified myosin itself forms short filaments at 0.2 M KCl or pH 8.0, representing the conditions used for the formation of native bare zone assemblages, although the filaments look slightly different depending on the condition (Kaminer & Bell, 1966). In addition, synthetic thick filaments formed from purified myosin at approximately physiological conditions also appear to be thicker than native thick filaments (Emes & Rowe, 1978; Persechini & Rowe, 1984) and, by raising the pH, they tend to get shorter and thinner (Kaminer & Bell, 1966). Thus the presence of the two M-band proteins, M-protein and myomesin, may be important for the proper initial packing of myosin to the correct width or in the maintenance of the bare zone structure. This may be supported by the fact that neither M-protein nor myomesin are bound or rebind to synthetically formed thick filaments. Also, such a function of these proteins is supported by the early appearance of myomesin and M-protein during myogenesis in cultured postmitotic cells as has been shown before (Eppenberger *et al.*, 1981).

The third M-band protein known to date, MM-CK, is an integral component of the intact M-band, but according to the findings reported here, no absolute necessity exists for its presence in isolated native thick filaments. This is in agreement with earlier studies which have demonstrated that MM-CK is not as firmly bound to the M-band as the other two known M-band proteins (Strehler *et al.*, 1980, 1983). In addition, MM-CK is

rather easily and selectively extracted from myofibrils either by low salt treatment or incubation with an excess of monovalent anti-MM-CK Fab' fragments (Wallimann *et al.*, 1978, 1983). There is a good possibility that the structural role of MM-CK in the M-band concerns more the proper interconnection of thick filaments within the whole A-segment (m-bridges) than a direct interaction with the myosin array within a single thick filament. This idea is supported by structural data (Luther *et al.*, 1981) and the 'floating A-segments' of phorbol ester treated cultured muscle cells, which still contain MM-CK (Doetschman & Eppenberger, 1984). Recently, however, Arps & Harrington (1982) reported that isolated native thick filaments prepared from rabbit muscle contain bound MM-CK which could be removed by low ionic strength extraction, though it was not mentioned how much and where MM-CK was bound to the isolated native thick filaments. Interestingly, the same authors found that approximately 25–30% of the MM-CK extracted from isolated native thick filaments could rebind to the extracted filaments, whereas approximately 80% of extracted MM-CK could rebind to extracted rabbit myofibrils (Arps & Harrington, 1982). This was in contrast to synthetically formed thick filaments from purified myosin to which MM-CK obviously did not bind. From all these experiments it seems likely that the binding of MM-CK to the M-band needs additional components which exist together with myosin in proper M-band substructures. The failure of MM-CK to remain fully attached to isolated native thick filaments may indicate a partial disruption of these substructural domains during thick filament preparation.

As already described for MM-CK, M-protein as well as myomesin, once separated from myosin, does not reattach to myosin or thick filaments in quantities that can be detected by our immuno-gold labelling technique. This is in accordance with Woodhead & Lowey (1983) and Strehler *et al.* (1982) who found no significant interaction of M-band proteins with myosin and with each other.

Therefore, future interaction studies of M-protein and myomesin with myosin must be designed to start with (bipolar?) arrays of a few myosin molecules, since M-protein and myomesin probably only interact with an intermediary arrangement of such myosin molecules during filament formation.

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