Journal of Muscle Research and Cell Motility **10**, 446–456 (1989)

The organization of titin (connectin) and nebulin in the sarcomeres: an immunocytolocalization study

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Received February 1989

Summary

Monospecific polyclonal antibodies against two exceptionally large proteins, titin (a-T) and nebulin (a-N) isolated from rabbit skeletal muscles, were raised in guinea pig. Using an immuno-pre-embedding method, we have localized at the ultrastructural level of resolution the reactivity sites in skinned muscle fibres. At resting length a-T and a-N antibodies recognize epitopes which only partially overlap. a-T antibodies decorate mostly the A band with at least four clearly distinguished lines of reaction and one line in the I band, all near the A/I limit; a-N antibodies bind to the same region, but with wider areas of reaction in both A and I bands. To study whether the localization of these reaction sites varies according to the sarcomere length, skinned rabbit psoas fibres were incubated at sarcomere lengths ranging from maximum shortening to overstretching. The results indicate that lines decorated by a-T move away from the Z disc when the sarcomere is lengthened. With respect to the M line, the behaviour was biphasic. When the sarcomere was stretched up to about 2.8 μm, the decorated lines maintain almost the same distance from the M line. When the sarcomere is stretched beyond 2.8 μm, all a-T epitopes move away from the M line and the molecule behaves elastically. At resting length the a-N decoration appears to be localized on three large adjacent bands at the I, A/I and A level. The a-N line of reaction at the edge of the A band moves away from the Z discs as the sarcomere lengthens, while a second line which seems to be localized at the tip of the thin filament moves away from M line when the sarcomere lengthens. In non-overlapping sarcomeres a-N antibodies decorate only the tip of the thin filaments. Our results indicate that titin forms a polar filament connecting the M line to the Z line. In short sarcomeres, the filament seems to have some connections with structures of the A band, since titin epitopes do not move during stretching. These connections are lost at longer sarcomere lengths. On the other hand, our results suggest that nebulin is probably not a constituent of the titin filament.

Introduction

The emerging perspective of striated muscle structure and function must take account of the filamentous network that is observed outside and inside the sarcomere, in addition to the thick and thin filaments.

Two cytoskeletal systems have been described in striated muscle (for review see Wang, 1985). The first consists of intermediate filaments running both transversely and longitudinally. The transverse system is shown to connect adjacent myofibrils at the level of the Z line (Garamvolgyi, 1965) and M line (Granger & Lazarides, 1978; Pierobon-Bormioli, 1981), as well as myofibrils to other cellular components (Pierobon-Bormioli, 1981, 1985, 1988; Tokuyasu *et al.*, 1983; Watkins *et al.*, 1987); it has been suggested that the N line also participates in the cytoskeletal network (Pierobon-Bormioli, 1980, 1985, 1988). The second cytoskeletal endosarcomeric system consists of very thin longitudinal filaments (2–4 nm) and it is thought to represent the structural basis of myofilbrillar elasticity (Fürst *et al.*, 1988; Higuchi & Umazume, 1985; Locker & Leet, 1976; Maruyama *et al.*, 1985; Natori, 1954; Trinick *et al.*, 1984; Wang, 1984) and to control the position of thick filaments during prolonged contractions (Horowitz & Podolsky, 1987).

We have focused our attention on the study of these last constituents, which are still not completely understood even though it seems clear that they are built from very high molecular weight proteins (Maruyama, 1986) named titin (Wang *et al.*, 1979) or connectin (Maruyama *et al.*, 1977) and nebulin (Wang & Williamson, 1980; Wang, 1981); (for reviews see Wang (1984) and Maruyama (1986). From previous studies it is known that these proteins have low solubility properties and because of their large molecular sizes (2600 kDa and about 500 kDa for titin and nebulin respectively) they hardly move in one-dimensional gel electrophoresis unless a very low concentration of polyacrylamide is used (Wang,

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1984). Titin and nebulin contain substantial amounts of bound phosphate, and titin binds Ca^{2+} with relatively high affinity (Wang, 1984). These findings suggest that the physiological properties of the two proteins could be modulated by post-translational modification, such as phosphorylation (Somerville & Wang, 1988). Furthermore it has been demonstrated that titin interacts with AMP deaminase, C protein, myosin and actin (Wang, 1985). The presence of titin in the gap filaments has been shown by immunoelectron microscopy, using specific antibodies, (Maruyama *et al.*, 1987 and Wang, 1984). From other studies (Trinick *et al.*, 1984) on isolated thick filaments, titin molecules are seen as strands emanating from filament ends as well as alongside cross-bridge regions.

A very careful analysis of the distribution of titin epitopes has recently been performed with monoclonal antibodies by using immunoelectron microscopy (Fürst *et al.*, 1988; Itoh *et al.*, 1988). The results provide a linear epitope map, which, starting at the Z line, covers five distinct positions along the I band, the A/I junction and three distinct positions within the A band (Fürst *et al.*, 1988). Other results (Itoh *et al.*, 1988) showed that the domains of titin filaments in the I band region are more extensible than those at both edges of the A band, i.e. in the I band there are extensible titin filament domains, while the domains in the A band are less extensible; these last domains might possibly extend spirally along the surface of thick filaments, showing resistance to stretch, although nothing is known of this aspect.

A second high molecular weight protein that has been suggested to be a component of the endosarcomeric (elastic) cytoskeleton is nebulin (Wang, 1985). This protein was thought to be the defective gene product in Duchenne muscular dystrophy (Wood *et al.*, 1987). However, the nebulin gene has recently been localized on the long arm of chromosome 2, in the region 2q31–q32 (Zeviani *et al.*, 1988), and by immunocytochemical study it has been found that nebulin is present in Duchenne dystrophic muscle (Bonilla *et al.*, 1989).

Early immunofluorescent localization studies have indicated the N2 line, the transverse structure located within the I band, as the predominant structure labelled by anti-nebulin antibodies (Wang & Williamson, 1980). However, the endosarcomeric distribution of an antigen, as determined by immunofluorescent microscopy, can be difficult to identify and to correlate with the underlying ultrastructure. In spite of this, Fürst and co-workers (1988) and Wang and Wright (1988b) by using anti-nebulin antibodies have recently localized nebulin at ultrastructural level. However, the localization of nebulin in the sarcomere and its relationship to titin filaments is still obscure.

In this work we have addressed the problem of the elastic behaviour of titin longitudinal filaments (i.e. are there extensible domains and less extensible domains?) and of the localization of nebulin by immunolabelling, with monospecific polyclonal antibodies against these two proteins, chemically skinned fibres which were fixed at different sarcomere lengths.

Our results show that, in agreement with previous studies, titin epitopes seem to belong to the elastic filament because they change their position according to stretch at sarcomere lengths greater than 2.8 μm. On the other hand, nebulin does not seem to belong to these elastic filaments.

A preliminary account of these observations has appeared elsewhere (Pierobon-Bormioli *et al.*, 1988).

Materials and methods

Purification of titin and nebulin

Titin and nebulin were purified by method II described by Wang (1982) from New Zealand White male adult rabbit back muscle. Briefly, myofibrils were extracted with 0.2 M Tris–HCl/20 mM EDTA/80 mM dithiothreitol/20% SDS/4 mM phenylmethanesulphonyl fluoride, pH 8.0, at 50° C. Titin was precipitated by addition of 0.64 M NaCl to the SDS-solubilized myofibrillar extract. Titin and nebulin were purified by gel filtration on Sephacryl S-500 column. Protein purity was tested by gel electrophoresis. Native titin was purified as described by Trinick and co-workers (1984).

SDS–gel electrophoresis

SDS–polyacrylamide gel electrophoresis was carried out on 4% polyacrylamide slab gels (Wang, 1982). Myofibrils and/or five to eight chemically skinned fibres were washed twice with 5 mM Tris–HCl, pH 8.0, and solubilized in 30 mM Tris–HCl/3 mM EDTA/3% SDS/120 mM dithiothreitol/30% glycerol. After incubation at 50° C for 20 min, about 100 μg were applied to a 4% slab gel and electrophoresed at 25 V for 1–2 h and 40 V overnight. The running buffer was 40 mM Tris–acetate/20 mM sodium acetate/2 mM EDTA/0.1% SDS, pH 7.4. After electrophoresis, the gels were stained with 0.1% Coomassie Blue R, 40% ethanol, 5% acetic acid for 30–60 min, and destained with 40% methanol and 7% acetic acid for 4–6 h.

Preparation of antibodies

Antibodies against titin (a-T) and nebulin (a-N) were raised in guinea pigs by weekly injection of 0.5 and 0.15 mg of purified titin and nebulin, respectively, in complete Freund's adjuvant, for 4 weeks. A booster injection was given after 2 weeks, and the animals were sacrificed after a further week. Guinea pig antibodies were used as total serum. Specificity of antibodies was tested either by the ELISA test or by Western blots.

Western blotting was performed essentially as described by Towbin and co-workers (1979). Proteins were transferred to a nitrocellulose sheet by electrophoresis in 62.5 mM

Tris/175 mM glycine/0.01% SDS for the first hour of the run (at 0.5 A) and the same buffer but without SDS for two additional hours (at 0.5 A). Blot filters were first saturated with 10% low-fat milk (LFM) in 0.9% NaCl for 1 h, and then incubated for 1 h with the antibody diluted 1:1000 with 10% LFM/0.05% Tween-20, in phosphate-buffered saline (PBS: 0.1 M sodium phosphate/0.15 M sodium chloride, pH 7.0). After washing with 0.9% NaCl/0.1% Tween-20 (4 × 5 min), filters were incubated with the second antibody, anti-(guinea pig IgG) conjugated with alkaline phosphatase, diluted 1:1000 in 10% LFM in PBS. Antigen–antibody complexes were visualized by incubating with 5-bromo-4-chloro-3-indolyl phosphate (5 mg/30 ml), Nitro-Blue tetrazolium (1 mg/30 ml)/0.1 M Tris–HCl/0.1 M NaCl/5 mM $MgCl_2$, pH 9.5 (Leary *et al.*, 1983).

Preparation of chemically skinned fibres

The median portion of psoas rabbit muscle was used. Muscle bundles (3–5 mm in diameter, 40–50 mm long) were excised from the bulk of the muscle after being tied to a wooden stick and stretched to 110% of slack length. The muscle bundles were chemically skinned by incubation at 0–4°C for 24 h in 10 ml of a 'skinning solution' containing 5 mM K_2-EGTA, 170 mM K propionate, 2.5 mM K_2Na_2ATP, 2.5 mM Mg propionate and 10 mM imidazole propionate, pH 7.0 (Wood *et al.*, 1978). After 2, 4, 6 and 16 h the skinning solution was replaced with fresh solution. After 24 h the muscle bundles were transferred to a 'storage solution' (skinning solution containing 50% glycerol), and were stored at −20°C. Muscle bundles were used within 3–4 weeks.

For immunocytochemical studies bundles containing 10–15 skinned fibres were tied to a wire steel arc and the sarcomere length was measured by light diffraction using a He–Ne laser lamp (1.5 W). To obtain fibres having sarcomere lengths shorter than the resting length, bundles of skinned fibres were mounted between the clamps and allowed to shorten actively by incubating in a Ca^{2+}-containing solution (pCa 5.0). When the desired sarcomere length was attained, the bundles were subsequently subdivided into thinner strips, mounted on plastic supports and tied at their extremities with a silk thread.

Antibody labelling of the muscle fibres

Chemically skinned fibres were immunostained as described by Maruyama and co-workers (1985). The fibres, previously washed in a rigor solution, were fixed with 10% formalin for 10 min at room temperature. Fibres were washed three times at 10 min intervals in PBS containing 0.1% sodium azide, pH 7.2, and then washed with PBS for 12 h at 0–4°C. Fibres were treated with 1% bovine serum albumin for 24 h at 0–4°C, and then washed with PBS for 24 h. The fibres were incubated with the various antisera diluted three to five times with PBS for 24–36 h at 0–4°C. For the control, non-immune serum diluted five times with PBS was used. After being washed in PBS for 24 h, the fibres were incubated with fluorescein isothiocyanate-labelled anti (rabbit IgG) antibodies diluted with PBS for 24–36 h, then washed with PBS for 24 h. The fibres were then postfixed for 5 min in 10% formalin, washed with PBS for 1 h and then observed by fluorescence microscopy or washed three times with 0.1 M Na cacodylate buffer pH 7.2, postfixed in 1% OsO_4 in 0.1 M Na cacodylate buffer, dehydrated in a graded series of ethanol, treated with propylene oxide and Epon embedded.

Rotary shadowing

The method described by Mould and co-workers in 1985 was carried out by freshly cleaving a piece of mica in small squares and placing a microdrop of the sample solution in the centre of one surface. The other surface was then reapposed on top, causing the drop to spread between the two surfaces. The sample was allowed to adsorb on the mica substrate; the two pieces were separated, dried *in vacuo* at 10^{-5} Torr in a Balzers Unit and shadowed with a platinum wire from an elevation angle of 5°. These replicas were stabilized by coating with carbon, mounted on 300-mesh grids and observed under an electron microscope.

The samples of the protein in 0.5 M KCl/50 mM Tris–HCl at pH 7.9 were diluted tenfold with ammonium acetate (0.1 M, pH 7.6) and then with glycerol to give a final concentration of 50% (v/v) glycerol.

Negative staining

Protein samples in 40 mM Tris–HCl/20 mM sodium acetate/2 mM EDTA/0.1 mM dithiothreitol/0.1% SDS at pH 7.4 were stained with ammonium molybdate or uranyl acetate on grids coated with collodion film and a thin layer of carbon.

Results

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As shown by immunoblot analysis (Fig. 1), monospecific polyclonal antibodies react only with their own antigen and they do not cross-react.

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The site of reaction of a-T antibodies in chemically skinned rabbit muscle fibres is shown in Fig. 2a–f. By comparison with the phase contrast examination of the same field (Fig. 2c), it is evident that the antibody stains the edges of the A band. In fibres stretched to longer sarcomere lengths, the reaction bands progressively space out. The reactivity of α-N antibodies, when compared with the phase contrast inset, is seen at the edges of the I band (Fig. 2g).

ULTRASTRUCTURE OF PURIFIED PROTEINS

Observation of the replicas of purified native titin reveals a string-like structure, as previously described by Trinick and co-workers (1984), The shape is linear or, more often, with a very tortuous and tangled path, with a bifurcation at the end of the filament, suggesting that it is double stranded (Fig. 3a). Also, much smaller globular structures are seen (not shown).

Negative staining was used to study the structure of purified nebulin. As shown in Fig. 3b, nebulin,

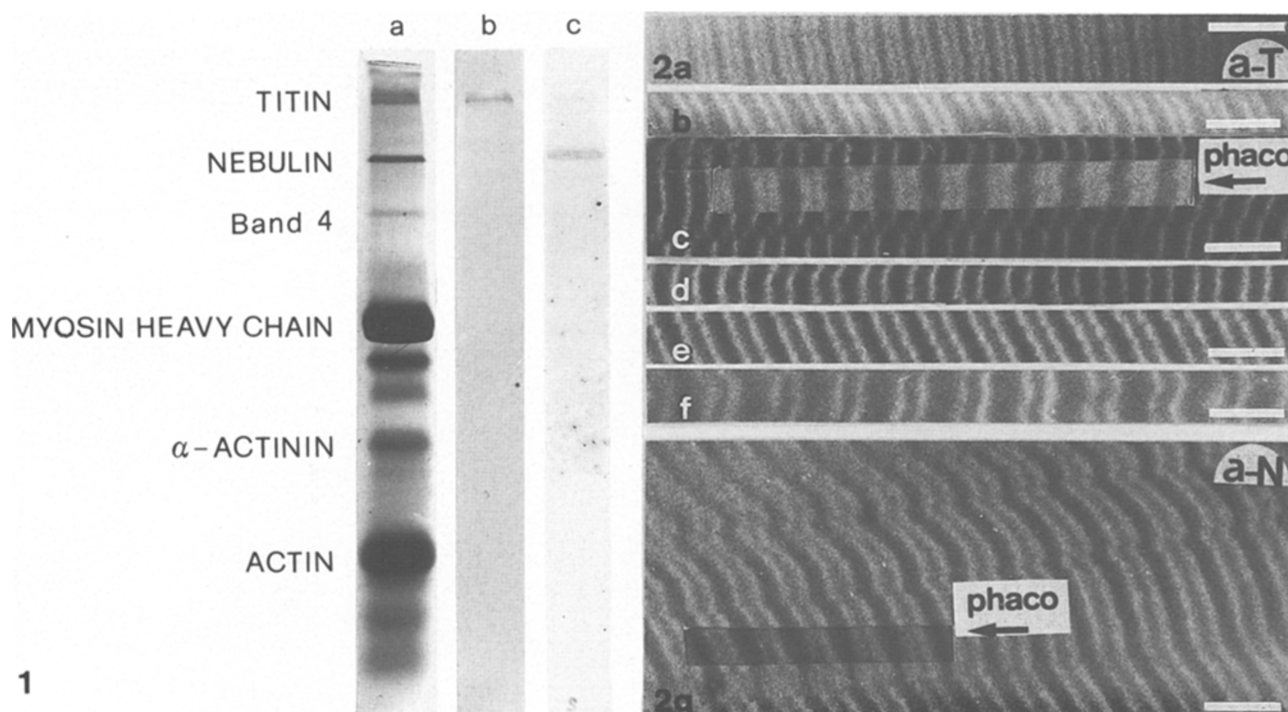

Fig. 1. Immunoblot analysis with monospecific polyclonal antibodies against titin (a-T) and nebulin (a-N). SDS–gel electrophoresis and Western blotting were carried out as described in Materials and Methods on 100 μg of rabbit myofibrillar protein. (a) Coomassie Blue staining; (b) a-T staining; (c) a-N staining.

Fig. 2. Immunofluorescence reactivity of chemically skinned rabbit psoas fibres. (a)–(f) show the behaviour of the reactivity with a-T antiserum at various sarcomere lengths; in (c) the inset (phaco) shows the phase-contrast image of the same field; the strongest reaction is seen at the edges of the A band. (g) shows the immunofluorescence pattern of skinned rabbit psoas fibres treated with a-N antibody; the reaction is prominent at the edges of the I bands. The inset (phaco) shows the phase-contrast image of the same field. Bar = 10 μm.

purified in the presence of SDS, appears to be composed of globular particles. Very often clusters of particles, presumably aggregated during the preparation of the specimen, are observed.

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Titin and nebulin epitopes at resting length

Electron microscopic observations of skinned rabbit muscle fibres treated with a-T antibodies show four stripes of strong reaction, symmetrically arranged in each half of the A band near the A/I limit. Other stripes of weak reaction were seen in the rest of each half of the A band, and an additional stripe in the I band (Fig. 3c). Anti-N antibodies strongly mark a very large band at the A/I level (Fig. 3d), more clearly subdivided in three distinct stripes in shorter sarcomeres (see Fig. 4r). The stripes were located inside the A band, inside the I band and at the A/I limit respectively, and were symmetrically arranged in each half of the sarcomere.

Titin and nebulin epitopes at several different sarcomere lengths

Fibres were incubated with non-immune serum (controls, Fig. 4a–f), anti-T antibodies (Fig. 4g–n) and anti-N antibodies (Fig. 4o–u), at different sarcomere lengths ranging from hypercontraction (2 μm) (Fig. 4a, g and o) to the hyperextension (4.0 μm) (Fig. 4f, n and u).

Fibres incubated with non-immune serum do not show extra lines of electron density at any of the sarcomere lengths examined. When compared with the controls (Fig. 4a), the anti-T guinea pig antibody reaction in hypercontracted fibres (Fig. 4g) is visible as an increased density superimposed symmetrically at the borders of the Z lines. In the range of sarcomere length from about 2.0 μm to 4.0 μm (Fig. 4h and m), when compared with controls (Fig. 4b and e), four major stripes of reaction are clearly seen. These stripes seem to maintain the same distance from the M line and to move away from the Z line when the sarcomere lengthens. The weak reaction, visible at the I band level in Fig. 4h and i, is no longer visible in Fig. 4l and m, except for one stripe, near the A band (Fig. 4l). In overstretched fibres (control Fig. 4f; a-T Fig. 4n) the reaction disappears from the edges of the A band: the major density seen at the level of the I band is partly due to the fact that during incubation with the antibody some shortening of the fibre could have occurred. The gap between thick and thin filaments appears negative (Fig. 4n).

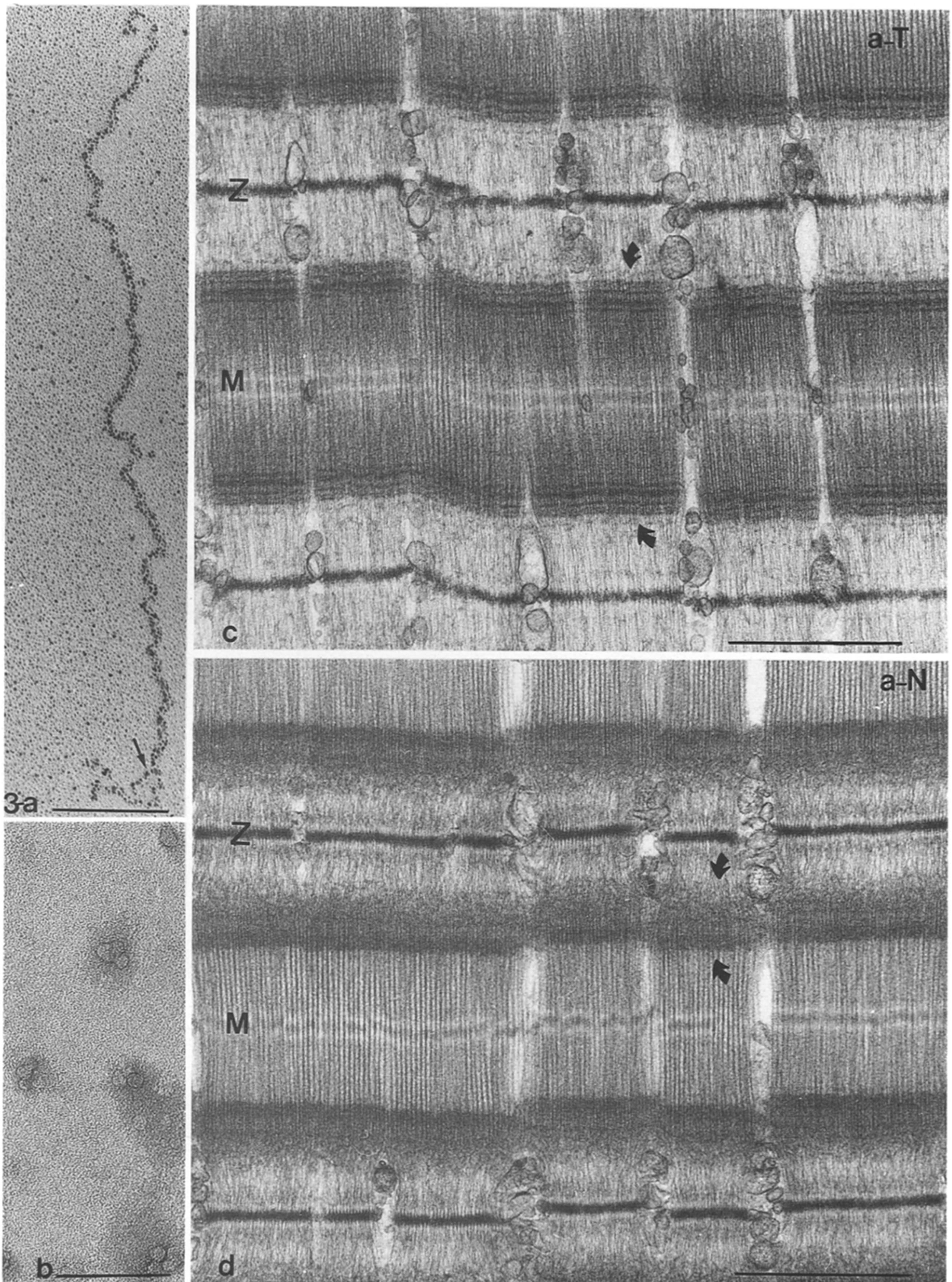


Fig. 3. Ultrastructure of purified titin and nebulin and their localization in the muscle fibre. (a) shows the rotary shadowing of purified titin. This string-like structure shows a double-stranded arrangement at one extremity (arrow) (bar = 100 nm). (b) shows the negative staining of SDS-purified nebulin. Single rounded particles or small clusters of particles are seen in this field (bar = 100 nm). (c) and (d) show immunoelectron microscopical localization of titin and nebulin, respectively, as seen in longitudinal sections of skinned rabbit psoas muscle fibre, immunostained at the resting length. a-T antibody (c) decorates symmetrically several stripes at the edges of the A band (arrows) (bar = 1 μm). a-N reactivity is seen symmetrically at the A/I level as a large band of antibody deposition (arrows). Z = Z line; M = M line (bar = 1 μm).

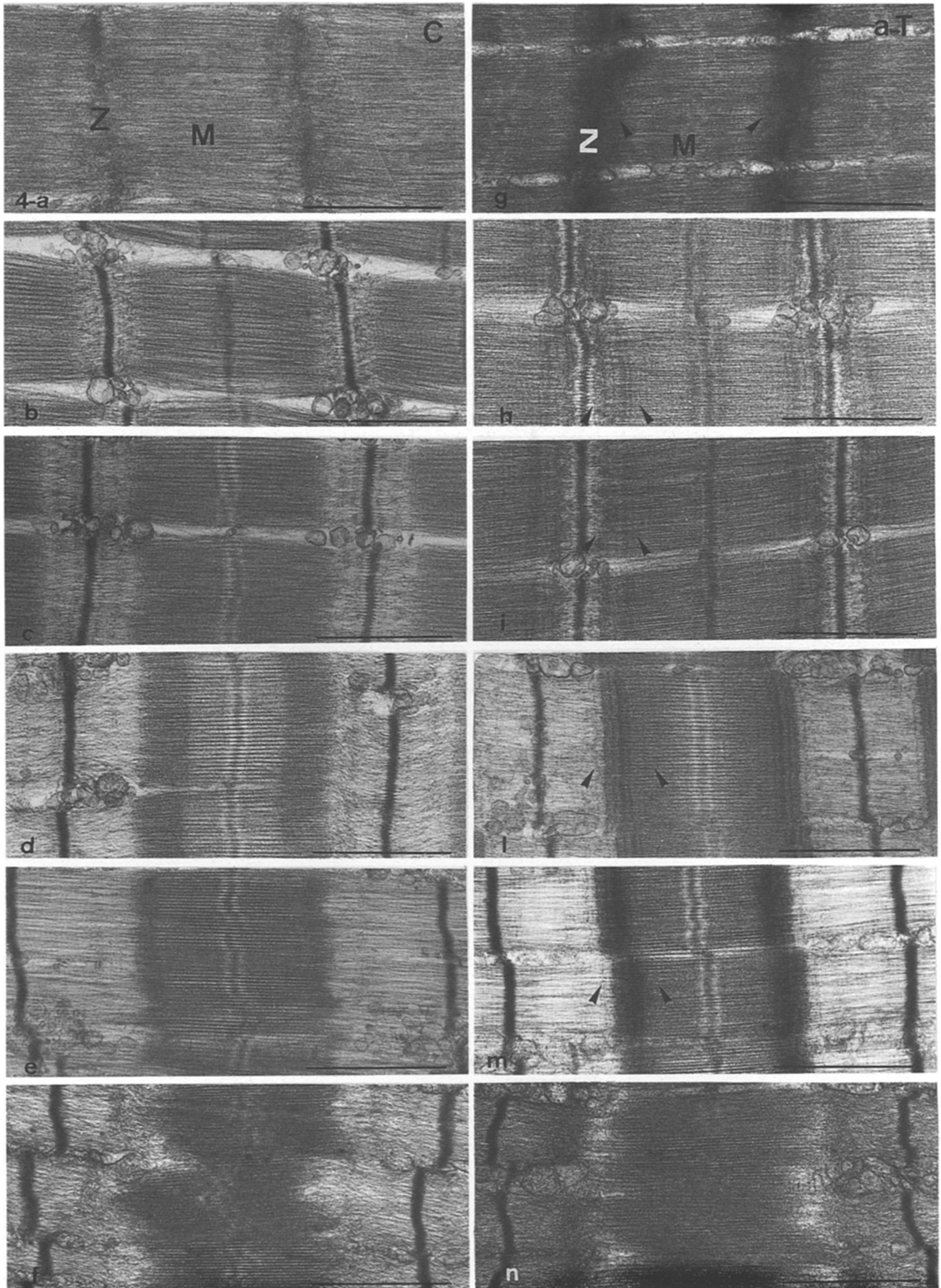
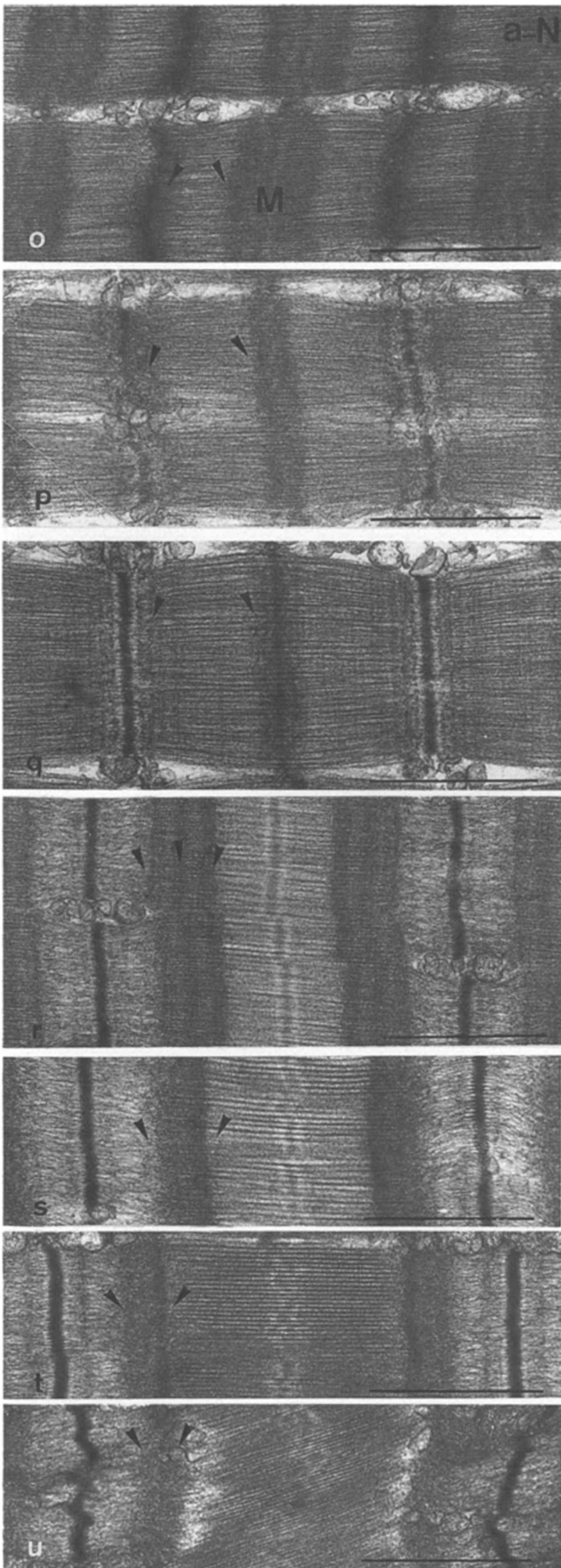


Fig. 4. Immunoelectron microscopy localization of titin and nebulin in longitudinal sections of skinned rabbit psoas fibres at different sarcomere lengths. (a)–(f) control fibres incubated with non-immune guinea pig serum. (g)–(n) a-T antiserum. (o)–(u) (over page) a-N antiserum. Positions of antibody labelling are indicated by arrowheads. Bar = 1 μm.



When compared to controls (Fig. 4a–f), the reaction with anti-N guinea pig antiserum (Fig. 4o–u) is visible, in hypercontracted fibres (Fig. 4o), as lines of density superimposed symmetrically in two distinct regions, one at the border of the Z line and the other at the border of the M line. With elongation of the sarcomeres, the pattern of labelling shows a complex rearrangement. In shortened sarcomeres, several lines of reaction are located at the I/A transition and a strong reaction line is at the M level. When the sarcomere length is increased, this last line moves away from the M line, so that in sarcomeres fixed at resting length, the pattern of reactivity shows three large bands at the A/I level (Fig. 4r). When the sarcomeres are more elongated (see the H zone) (Fig. 4s and t) the reaction progressively disappears from the A band and it remains confined to the I band level in non-overlapping sarcomeres (Fig. 4u). Again the gap region appears negative. Figure 5 shows the reaction pattern of anti-T antibodies in sarcomeres held at a constant length during the whole incubation and embedding time (Fig. 5a–h). Under these very controlled conditions, slight movement of the reactive stripes in relation to the sarcomere length is still evident. Figure 6 summarizes the results of the movement of one titin and one nebulin epitope during stretching by plotting the ratio of the distance of the epitope from the Z line to the M–Z distance, versus the M–Z distance. It is evident that the behaviour of titin is biphasic. At short sarcomere lengths the ratio is decreasing with stretching, indicating that the epitope does not move from the M line. From a sarcomere length of about 2.8 μm upward, the reactive titin epitope moves away from the M line, maintaining the same proportional distance from both M and Z lines. These results indicate that titin is a component of an elastic structure. On the other hand, nebulin shows the opposite behaviour. The ratio increases as the sarcomere length is increased, indicating that the nebulin epitope moves concomitantly with the Z line.

Movements of titin epitopes are not dramatic (Fig. 5). Furthermore it must be pointed out that when the banding patterns of the immunoreaction are compared in sarcomeres at different lengths, some difficulty might arise about their precise localization with respect to the filament and/or the sarcomere length. Preparative procedures in fact cause changes in the length of muscle filaments and the extent of these changes varies consistently in different methods of preparation (Page & Huxley, 1963). We have observed that changes in the I band take place, even after formalin fixation, if the fibre is left free to shorten (for example see Fig. 4s); under these conditions no changes in the A filament length were observed. On the other hand, when muscle fibres were held to a fixed length during the whole

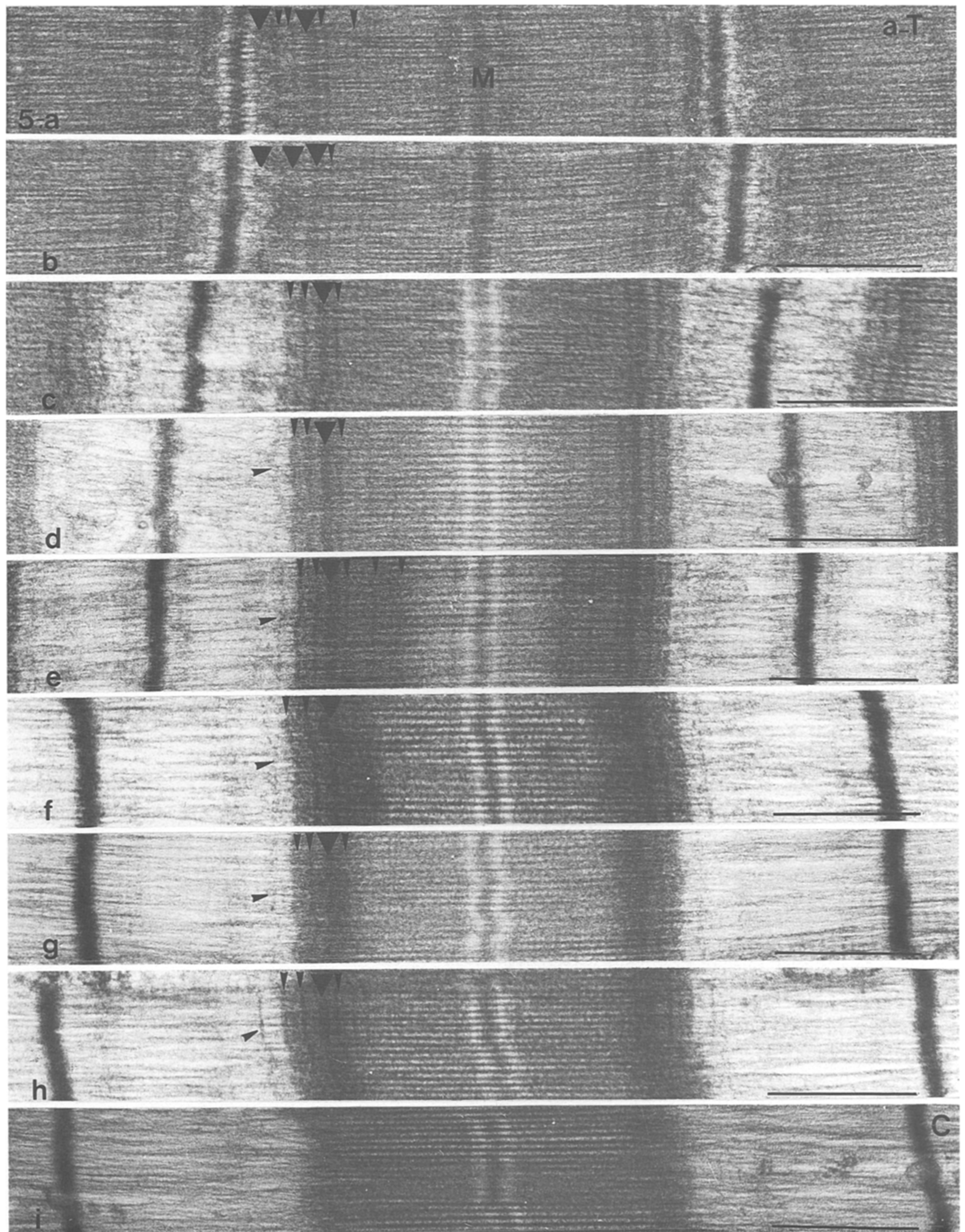


Fig. 5. Immunoelectron microscopic localization of titin in skinned rabbit psoas fibres at different sarcomere lengths. (a)–(h) a-T antiserum. (i) Control. The deposits of reaction product seen at I band level in contracted sarcomere (a, b) are no longer visible in the elongated ones, except for a thin stripe (thin horizontal arrowheads) seen starting from (d). The stripes of reaction product (arrowheads), symmetrically located at the edge of each half of the A band, move away from the Z bands when the sarcomere lengthens, maintaining the same distance from the M band in the range of a short sarcomere length (c, d and e). At longer sarcomere lengths (f, g and h) the reaction bands move away from the M line. Bar = 0.5 μm.

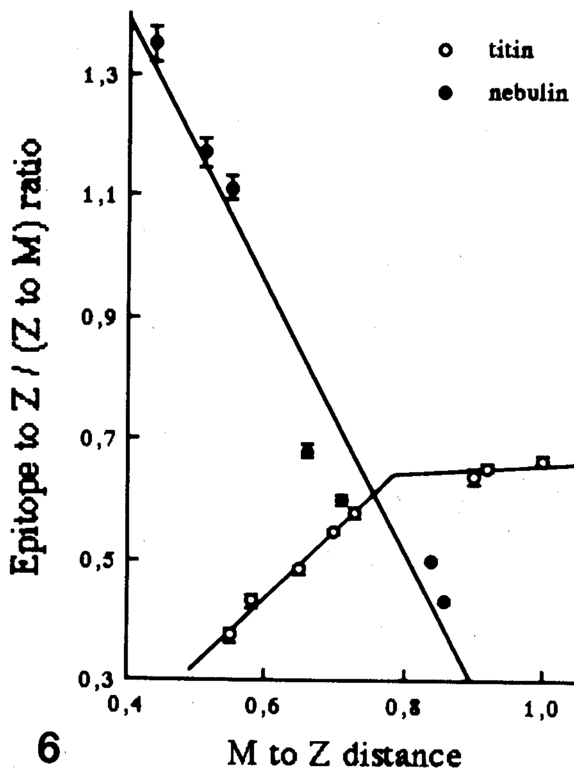


Fig. 6. Changes in the location of immunoreactive sites of titin and nebulin in relation to the sarcomere length. The ratio between the distance of protein immunoreacting site from the Z line and the Z–M distance is plotted as a function of the Z–M distance. Bars indicate standard deviation.

preparation time and the procedure times were rigorously adhered to, reliable and reproducible results could be obtained.

Discussion

Immunoelectron microscopy is the most direct approach to the study of protein localization in a cell. However, several problems may bias the interpretation of results, in particular, the fact that, even by using polyclonal antibodies, one usually obtains the labelling of only a few antigenic sites. This is of relevance in the case of very elongated molecules such as titin. Another problem concerns the techniques that rely on the appearance of bands of electron density, due to the formation of antigen–antibody complexes. Light reaction densities are difficult to recognize in a sarcomere, and so are reactivities that are superimposed on to naturally dense lines such as the Z line and M line. Furthermore, for the formation of a stripe of reaction it is obligatory that the reactive epitopes of different filaments are in register.

For this study, chemically skinned fibre preparations were used instead of intact muscle fibres or purified myofibrils, for two main reasons. First, the chemical skinning makes the sarcolemma highly permeable so that antibody molecules have easy access to internal structures. Furthermore, the structure and function of the chemically skinned fibres are very well preserved (Eastwood *et al.*, 1979; Wood *et al.*, 1978). Therefore, we thought that the structures of the endosarcomeric cytoskeleton should also be preserved. This is an important difference from myofibrils, which are prepared by homogenization of muscle, and it is likely that the mechanical disruption of muscle fibres affects the attachment of these filaments to their anchoring structures. Since this filament is elastic, one can be faced with random accumulation of protein in different locations of the sarcomere (Wang, 1984).

Our results show that a-T antibody binds to several titin epitopes symmetrically with respect to the M band in the two half-sarcomeres. Some epitopes are located in the A band, some at the edge of the A band, and one in the I band. These results are in good agreement with those reported recently for chicken (Fürst *et al.*, 1988) and frog muscle (Itoh *et al.*, 1988). By using a number of different monoclonal anti-titin antibodies these authors were able to show that there is a linear map of epitopes of titin from the A band beyond the N2 line to the Z line. Altogether, these results support the hypothesis that a single molecule of titin spans a half-sarcomere from the M line to the Z line. This is consistent with the estimated length of native titin molecules which is about 1.2 μm (Trinick *et al.*, 1984).

Moderate stretching of skinned fibres does not cause changes in the location of titin epitopes that are at the edge of the A band. However, when the sarcomere is stretched beyond 2.8 μm, these epitopes move, and at higher sarcomere lengths they disappear, probably because titin epitopes that are in register in moderately stretched sarcomere become randomly distributed (Itoh *et al.*, 1988), and are no longer identifiable with this method. These results are in agreement with those obtained with frog muscle fibres, in which, however, the disarrangement of titin epitopes occurs at longer sarcomere lengths (3.5 μm) (Itoh *et al.*, 1988). In conclusion, titin epitopes in the A band show some resistance to motion during moderate stretching. It has been shown that purified native titin binds to C protein and myosin. It is therefore likely that titin interacts in the A band with components of the thick filament and that the strength of this interaction is able to immobilize titin at moderate stretches. The part of the molecule in the I band is free to move even at these low levels of stretching (Itoh *et al.*, 1988; Wang & Wright, 1988a). At higher degrees of stretching, titin is detached from the thick filament and the whole of the molecule behaves elastically.

Our results show that monospecific a-N antibody decorates several stripes in the A band and I band. These results are in partial agreement with those of Wang & Wright (1987a, b, 1988b), who reported that a monospecific anti-nebulin antiserum decorated six to seven pairs of transverse bands within the I band and the A band of each sarcomere. On the other hand, Fürst and co-workers (1988) have shown that only a region close to the N2 line is stained by three different monoclonal antibodies. Therefore, polyclonal serum seems to contain a fraction of antibodies that are reacting with nebulin epitopes that are not recognized by monoclonal antibodies. During both moderate and high stretching, some nebulin bands, i.e. those near the M band, seem to move but maintain a fixed distance to the corresponding Z line. This result indicates that nebulin is rigidly connected to the Z line or to the thin filaments. This is supported by the finding of nebulin epitopes located in non-overlapping sarcomeres. In those sarcomeres, a-N antibody stained the edges of the thin filaments. It has been suggested that nebulin forms a domain of the titin filament that goes from the N2 line to the Z line (Wang, 1985), and more recently it has been suggested that nebulin forms a set of parallel inextensible filaments anchored at the Z line (Wang & Wright, 1988b). Our results indicate that nebulin is probably not a constituent of the titin filament.

Acknowledgements

We thank Miss Stefania Ceoldo and Mr Massimo Fabbri for their contributions. This work was supported by institutional funds from the National Research Council, the MPI and the MDA.

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