Localization of Titin using Polyclonal and Monoclonal Antibodies

J. TRINICK, A. WHITING, J. WARDALE AND R. JEACOCKE

Muscle Biology Division
AFRC Institute of Food Research - Bristol Laboratory,
Langford, Bristol, BS18 7DY, U.K.

Introduction

Titin is an unusual protein which has been implicated in elastic connections thought to connect the ends of thick filaments to the Z-band in muscle. It is the third most abundant protein of muscle, forming approximately 8% of the mass of myofibrillar protein; but it was discovered only relatively recently by Wang et al. (1979), because its huge molecular weight of $\sim 10^6$ Da precludes entry into polyacrylamide gels of the type in general use. Initially it was possible to isolate titin only in denaturing solvents, but recently it has been purified in its native state (Trinick et al., 1984; Wang et al. 1984). Independently Maruyama et al (1976) have studied the properties of a preparation called connectin which was also isolated under denaturing conditions. Subsequently, connectin was shown to contain titin (Maruyama et al., 1981) and the high molecular weight (HMW) component of connectin (equivalent to titin) has also now been isolated in its native state (Maruyama et al, 1984). Localisation of titin has been achieved by labelling muscle fibres or myofibrils with antibodies. Most of this work has been carried out by light microscopy using antibodies to which a fluorophore was attached. The results indicated that titin was located mainly at the junction of the A & I-bands, but weaker labelling suggested that it may extend through the A-band to near the M-line. Interestingly, when titin antibodies were used to label myofibrils that had had their thick and thin filaments extracted, the fluorescent intensity was strongest on either side of the Z-band, suggesting that the antibodies were attached to a structure that had sprung back on extraction (Wang et al., 1979).

Recently Maruyama et al. (1985) have reported electron microscope data from thin sections of single muscle fibres labelled with polyclonal antibodies. Disordered labelling was observed across the I-band, in addition to more ordered binding at the ends of the A-band and inside the A-band. The labelling throughout the I-band, which had not previously been reported, led these workers to conclude that titin bridges the whole of the distance between the ends of thick filaments and the Z-band.

In this paper we report further light and electron microscope data from myofibrils labelled with antibodies to titin. Two polyclonal sera and 8 different types of monoclonal antibody were used. The results suggest that titin is present in the region between the edge of the M-line and a point about 1000Å beyond the end of the A-band. The results are compatible with the idea that the whole of this distance may be bridged by a single titin molecule. A more extensive description of these and other results was recently submitted to Journal of Molecular Biology.

Materials and Methods

(a) Preparation of antibodies

(i) Polyclonal sera: Two different polyclonal anti-titin sera were raised by immunizing goats with purified titin, either in its denatured form (prepared as described by Wang et al., 1979) or in its native state (Trinick et al., 1984). Details of the immunization procedure and treatment of the sera are given in Trinick and Lowey (1976).

(ii) Preparation of monoclonal antibodies: BALB/C strain mice were immunised against native rabbit titin and used in a fusion with the mouse myeloma line P3-X63-Ag8.653, essentially as described by Galfre et al. (1977). Antibodies screening by ELISA (performed as described by Voller and Bidwell (1975)) was carried out from the sixth day after fusion and positive hybridomas were cloned by limited dilution.

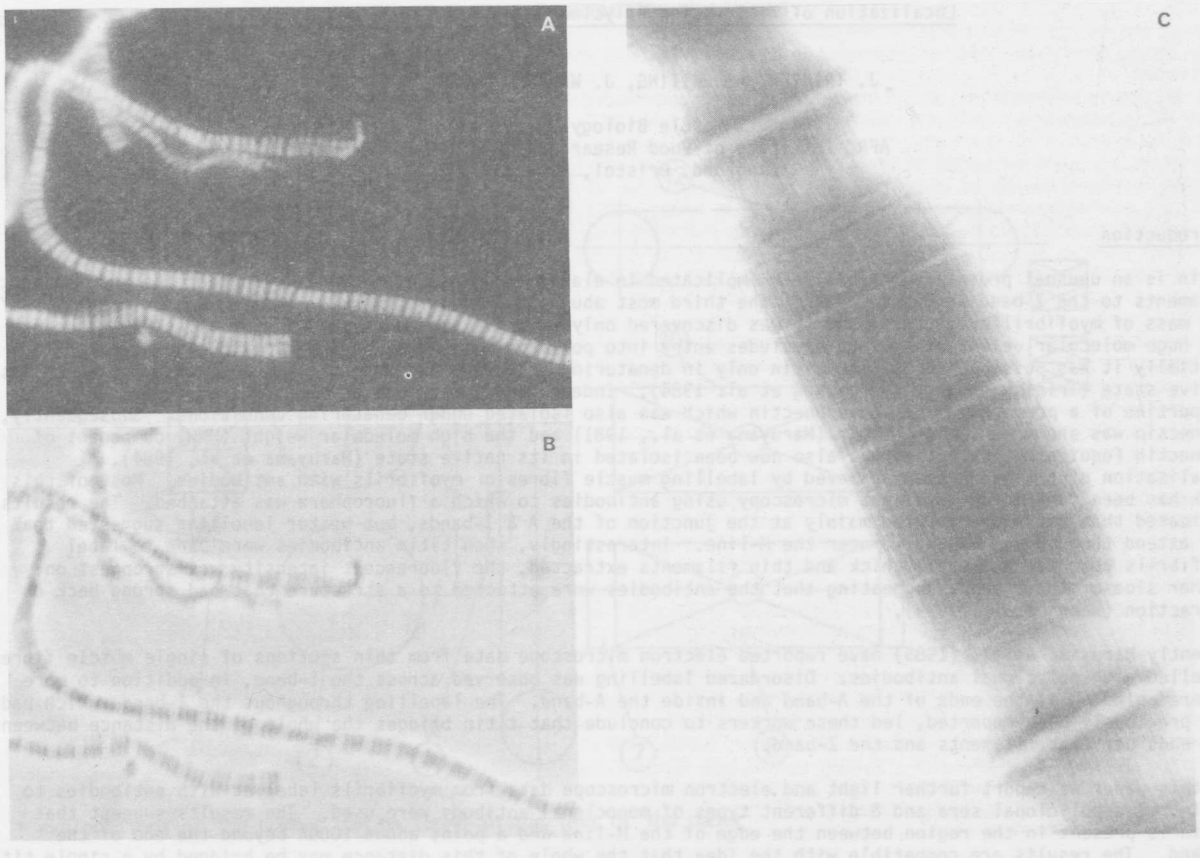
The specificity of both monoclonal and polyclonal antibodies was established by immunoblotting. The polyclonal sera showed reactions with bands other than titin; however, since these sera were used principally to demonstrate the absence of titin at particular locations, this did not affect their usefulness.

(b) Preparation of rigor myofibrils

(i) 24 h myofibrils: Rigor myofibrils were prepared from muscle 24 h post-mortem as described by Knight and Trinick (1982).

(ii) 1 h myofibrils: In order to minimise proteolytic damage, myofibrils were also prepared from muscle that had been induced into rigor as soon as possible post-mortem as follows. The psoas muscles were removed from a rabbit immediately after death and allowed to cool on ice for 4 min. Thin strips about 1 mm in diameter were then split off, stretched to the required sarcomere length and immersed for 30 min at room temperature in a rigor inducing solution containing; 120 mM KCl, 10 mM KH_2PO_4, 2 mM $MgCl_2$, 0.4 mM fluorodinitrobenzene, 1 mM iodoacetamide, 5 µg/ml rotenone A, 5 µg/ml antimycin A and 0.5% v/v Triton X-100 (pH 7.0 at 20 °C); this was called Buffer A. The mechanical properties of the muscle in response to this treatment were monitored as described by Jeacocke (1984). The poisoned strips were rinsed briefly in Buffer A containing no Triton X-100 before being homogenized into myofibrils, again in the absence of Triton X-100 (approx 5 vols buffer to 1 vol muscle). The myofibrils were then washed by 3 cycles of centrifugation (5 min, 2000 g) followed by resuspension in Buffer A without either Triton X-100 or $MgCl_2$, but containing 5 mM EGTA and 2 mM EDTA.

(c) Electron and light microscopy: Conditions for reaction of antibodies with myofibrils and light and electron



microscope procedures are described in Cooper and Trinick (1983).

Results and Discussion

(a) Light microscopy of myofibrils labelled with polyclonal antibodies.

Although previous data based on light microcopy using fluorescent antibodies have indicated that titin does not extend throughout the I-band, the doubt has remained that post-mortem proteolysis of the myofibrils used in these experiments could have caused the titin to be severed and spring back to the A-I junction. This possibility seemed real because to preserve the structure of myofibrils, it is generally necessary to allow muscle to go into rigor before homogenization into myofibrils (see Locker et al. (1976) for discussion of this point), and this process usually takes several hours.

We have therefore sought to reduce the likelihood of proteolysis, by labelling with antibodies myofibrils that had been induced into rigor very soon post-mortem. Since rigor results from depletion of ATP, it was possible to speed this process by blocking the 3 routes by which fresh ATP is synthesised. These routes are glycolysis, creatine phosphate breakdown and oxidative phosphorylation, each of which was blocked by a separate poison. Iodoacetamide blocks glycolysis at glyceraldehyde-3-phosphate dehydrogenase (Lunsgaard, 1930), rotenone and antimycin block oxidative phosphorylation (Potter and Reif, 1952), and FDNB is an inhibitor of creatine phosphokinase activity (Cain et al., 1962)). When incubated in Ringer solution containing these agents rigor was induced in approximately 15 min, as judged by mechanical stiffness measurements.

Using this procedure, it was possible to obtain myofibrils approximately 1 h post mortem. These were reacted with the fluorescent IgG fractions prepared from immune sera to either native or denatured titin (Fig 1). The strongest fluoresence in such myofibrils comes from a broad band between 0.6 μm and 1.0 μm from the M-line. This band is broader than one would expect were labelling to occur at only one axial position and the labelling is so strong as to be visible in the phase contrast image. There is another band of labelling at the M-line and weak fluoresence can be observed throughout the A-band. No fluorescent intensity was observed in the I-band, other than from part of the broad stripe centered on the A/I-junction referred to above. The labelling pattern obtained using the rapidly prepared myofbrils was very similar to that with myofibrils obtained 24 h post-mortem.

Fig 1 (a and b). Fluorescence/phase contrast light micrographs of freshly prepared myofibrils labelled with the IgG fraction of an anti-serum to native titin. Magnification 2100 X.

Fig 1 (c). Negatively stained myofibril labelled with a monoclonal antibody that binds to a site approximately 0.5μm from the M-line centre. Note, the particular preparation seen here had rabbit anti-goat IgG added to it to enhance the visibility of the labelling stripe. The stripe is therefore somewhat broader than would otherwise be the case. Magnification 57600 X.

These results support the view that titin does not extend throughout the I-band, although it does extend somewhat into it (1000 A). On this basis, if titin does form part of a system of elastic connections extending to the Z-line, there must be another protein or proteins that bridge the remainder of the distance to the Z-line. There are 2 caveats to this conclusion. We cannot rule out that proteolysis allowing springing back of connecting filaments to the ends of the thick filaments could have occurred even in the brief period between death of the animal and the microscopy. However it seems inherently unlikely that all the connecting filaments would have been severed in this period, particularly in view of the fact that significant numbers of gap filaments bridging the space between the ends of the thin and thick filaments in overstretched muscle were observed in beef sternomandibularis muscle fixed 1 h post mortem (Locker and Leet, 1975). We also cannot rule out the possibility that titin is present throughout the I-band, but that antibodies do not recognize it because of deformation of epitopes by stretching. However, this too seems unlikely in view of the fact that the polyclonal sera should have contained antibodies to epitopes located all over the surface of titin. It is conceivable that epitope deformation could account for the disordered binding of antibody to the I-band observed by Maruyama et al. (1985) in electron micrographs of thin sections of muscle, but non-specific binding seems a more plausible explanation of this result.

(b) Electron microscopy of negatively stained myofibrils reacted with monoclonal anti-titin antibodies.

Figure 1c shows the type of labelling pattern obtained when myofibrils were reacted with a particular type of monoclonal antibody and viewed in the electron microscope after negative staining. Using this technique a marker for the antibody, such as ferritin or colloidal gold, is not required, because the ordered structure of the myofibril produces transverse stain-excluding stripes of labelling when reacted with antibody. In the case of each of eight different types of monoclonal antibody to titin the labelling pattern consists of a single such transverse stripe in each half sarcomere. This particular antibody labelled an epitope 0.5 μm from the centre of the M-band. Other antibody types labelled various epitopes between 1000 A and 8500 A from the M-line centre (ie, from near the edge of the bare zone to about 1000 A beyond where the thick filament is usually thought to terminate (Craig and Offer, 1976).

The width of the monoclonal antibody stripes was quite narrow (~150 A), approximately the size of a single IgG molecule (Valentine and Green, 1967), and a similar width was measured for the other stripes in the cross-bridge regions of the thick filament. The stripes located near the bare zone and the A-I/junction were, however, significantly wider than this, about 400 A. How this greater width arises is not clear. The positions of the stripes inside the A-band did not vary significantly in myofibrils of different sarcomere length, suggesting that the titin in the A-band is attached to thick filaments.

Taken together the above data suggest that titin is located predominently in the A-band, but extends about 0.1 μm into the I-band. The fact that antibodies can bind over much of this distance suggests further that titin is probably not a core protein in thick filaments, but that it is located somewhere external to the thick filament shaft. We cannot, however, completely rule out the alternative possibility that titin does form a core, but that parts of it are sufficiently exposed to be available to antibody. The observed labelling with the polyclonal antibodies is strongest at the A/I-junction, suggesting that antigenic deteminants are more exposed in this region. This result is not unexpected since titin is thought to form end-filaments, small structures that extend approximately 1000 A beyond the ends of thick filaments (Trinick, 1981).

The finding that there is only one site of binding for each type of monoclonal antibody in each half A-band, but that such sites are located throughout and just beyond the A-band, indicates that each titin molecule may bridge a distance of 8500 A or more. This distance is compatible with the maximum lengths of purified titin strands visualized by rotary shadowing (Trinick et al., 1984). The symmetry of the labelling patterns about the M-line indicates that the titin molecules reverse their polarity at the M-line.

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References

Cain, D.F., Infante, A.A. and Davies, R.E. (1962). Nature 196, 214-217.

Craig, R. and Offer, G. (1976). J. Mol. Biol. 102, 325-332.

Galfre, G., Howe, S., Milstein, C., Butcher, G., Howard, J. (1977). Nature (Lond.) 266, 550-552.

Jeacocke, R.E. (1984). Meat Sci. 11, 237-251.

Knight, P.J. and Trinick, J.A. (1982). Methods Enzymol. 85B, 9-12

Locker and Leet (1975). J. Ultrastruct. Res. 52, 64-72.

Locker, R.H., Daines, G.J. and Leet, N.G. (1976). J. Ultrastruct. Res. 55, 173-181.

Lunsgaard, E. (1930). Biochem. Z. 220, 8.

Maruyama, K., Kimura, S., Ohashi, K. and Kuwano, Y. (1981). J. Biochem. (Tokyo), 89, 701-709.

Maruyama, K., Kimura, S., Yoshidomi, H., Sawada, H. and Kikuchi, M., 1984. J. Biochem. 95:1423-1433.

Maruyama, K., Matsubara, S., Natori, R., Nonomura, Y., Kimura, S., Ohashi, K., Murakami, F., Handa, S. and Eguchi, G. (1977). J. Biochem. (Tokyo), 82, 317-337.

Maruyama, K., Yoshioka, T., Higuehi, H., Ohashi, K., Kimura, S. and Natori, R. (1985). J. Cell Biol. 101, 2167-2172.

Potter, V.R. and Reif, A.E. (1952). J. Biol. Chem. 194, 287-297.

Trinick, J. (1981). J. Mol. Biol. 151, 309-314.

Trinick, J. and Lowey, S. (1977). J. Mol. Biol. 113, 343-368.

Trinick, J., Knight, P., and Whiting, A., 1984. J. Mol. Biol. 180:331-356.

Valentine, R.C. and Green, N.M. (1967). J. Mol. Biol. 27, 615-617.

Voller, A., Bidwell, D.E. (1975). Br. J. Exp. Pathol. 56, 338-339.

Wang, K., McClure, J. and Tu, A. (1979). Proc. Nat. Acad. Sci., U.S.A. 76, 3698-3702.

Wang, K., Ramirez-Mitchell, R. and Palter, D. Proc. Natl. Acad. Sci. USA. 81:3685-3689.