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Elastic properties of the titin filament in the Z-line region of vertebrate striated muscle

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Summary

The characteristics of the titin filament in the vicinity of the Z-line were investigated using immunoelectron microscopy. We used monoclonal titin antibodies T-11 and T-12 on single fibres of frog skeletal muscle, and on Z-line-extracted fibres. It is well established that the I-band region of titin is elastic. We find, however, that the elastic properties are not uniform. The T-12 epitope, which binds near the Z-line at the N_1-line level, hardly changes position relative to the Z-line as the sarcomere is stretched. This demonstrates the functional inextensibility of the N_1–Z-line region. After extreme stretch (above 6-μm sarcomere length), this zone finally does elongate; thus, the titin molecule in this region is intrinsically elastic. The functional inextensibility seen at shorter sarcomere lengths may, therefore, be a result of binding of titin to the actin filament in the zone near the Z-line. When the Z-line was extracted, the T-12 epitope remained in the same position as in the unextracted fibres; it did not retract from the Z-line. Failure to retract implies that functional anchoring of titin is not exclusive to the Z-line, but includes some site closer to the A-band. Combined with the results of the above-mentioned stretch experiment, this result implies a likely binding of titin to the thin filament either focally at the N_1 line or all along the entire N_1–Z region. Thus, this region of titin is functionally stiff, but intrinsically elastic.

Introduction

It is now well known that the titin molecule forms a continuous strand between M- and Z-lines (Fürst *et al.*, 1988). The A-band region of this strand is bound to the thick filament and is not stretchable within the physiological sarcomere length range (Wang *et al.*, 1984; Itoh *et al.*, 1988; Whiting *et al.*, 1988; Fürst *et al.*, 1989). Stretch is fully absorbed in the I-band region (Fürst *et al.*, 1988; Itoh *et al.*, 1988; Wang & Wright, 1988; Pierobon-Bormioli *et al.*, 1990). There is some evidence, however, that the elasticity of the I-band region may be grossly different in different regions. The titin epitope T-11, situated in the I-band near the A–I junction, does not move perceptibly from the tip of the thick filament as the sarcomere is stretched (Trombitás *et al.*, 1991); nor does the T-12 epitope, ordinarily situated at the N_1-line, shift from the Z-line as the sarcomere shortens below resting length (Fürst *et al.*, 1988). Furthermore, we have shown that titin filaments severed in the I-band retract only as far as the N_1-line (Trombitás *et al.*, 1990). This result demonstrates that titin has either an inelastic region between the N_1-line and Z-line (Fürst *et al.*, 1989), or that titin associates with the thin filament in this region (Trombitás & Pollack, 1993).

As titin apparently behaves differently in the N_1–Z-line region and near the A–I junction than in the remainder of the I-band, we carried out experiments at a series of sarcomere lengths to elucidate the nature of these differences, particularly the ones in the vicinity of the Z-line. The T-12 epitope remained at the same distance from the Z-line at all physiological sarcomere lengths. This confirms the apparent stiffness of this distal region. Because Z-line extraction did not result in retraction of titin toward the A-band, it appears that the anchoring of the titin filament is not exclusively to the Z-line, but probably also to the thin filament near the Z-line. This explains the apparent stiffness of the N_1–Z region of titin over the physiological sarcomere length range.

Materials and methods

Frog semitendinosus and tibialis anterior muscles were used for these experiments. Single fibres were mechanically skinned by peeling off the sarcolemma in relaxing solution (0.035 mM Ca-proprionate; 6 mM Mg-proprionate; 5 mM K-proprionate; 15 mM K_2EGTA; 117 mM MOPS; 4.4 mM Na_2ATP; 15.6 mM Na_2CP; 57 mM KOH; 20 μg ml^{-1} leupeptin, 5 μg ml^{-1} aprotinin; pCa = 9.2, ionic strength 0.2, pH 7.0). The fibres were

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stretched in small increments to the appropriate sarcomere length. Sarcomere length was set from 2.5 μm to 6.0 μm.

To extract the Z-line, fibres were either treated with low ionic strength solution (5 mM Tris, 10 mM dithiothreitol, 20 μg ml^{-1} leupeptin, 5 μg ml^{-1} aprotinin, pH 7.8) (Morimoto & Harrington 1973; Trombitás & Tigyi-Sebes, 1979) for 24 h, or digested with calcium-activated neutral protease (1 unit per ml; Sigma) in activating solution (in mM: Ca proprionate, 15; Mg proprionate, 6; K_2EGTA, 15; MOPS, 49; Na_2ATP, 4.4; KOH 57; NaCP, 16; pH 7.0) for 30 min at 4° C (Reddy *et al.*, 1975).

Immunolabelling experiments were carried out as reported earlier (Trombitás *et al.*, 1991). The fibres were fixed in a freshly prepared formaldehyde/PBS fixative (3.7% paraformaldehyde, 2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8 mM N_2HPO_4, pH 7.2) for 30 min at 4° C, and washed three times in PBS for 30 min. Non-specific binding sites were blocked using PBS/1% BSA solution for 30 min. Conventionally available monoclonal titin antibodies (T-11; Sigma, T-12, Boehringer) were used for the immunolabelling experiments.

The test fibres were then incubated for 24 h in the primary antibody solution (25 μg ml^{-1} mouse anti-titin from both T-11 and T-12 IgG in PBS/BSA) at 4° C. After washing the preparations three times with PBS/BSA for 30 min, the fibres were treated with secondary antibody solution (50 μg ml^{-1} rabbit anti-mouse IgG (Sigma) in PBS/BSA) for 24 h at 4° C. Control fibres were incubated only in the secondary antibody solution under the same condition as the test fibres. All antibody and rinsing solutions used in this study contained protease inhibitors (20 μg ml^{-1} leupeptin and 5 μg ml^{-1} aprotinin).

The unbound secondary antibody was removed from the fibres by washing in PBS for 30 min per wash, three times. Then the PBS was replaced with MOPS buffer solution (20 mM KMOPS, 5 mM $MgCl_2$, pH 6.8), and the fibres were fixed in 2.5% glutaraldehyde, 0.2% tannic acid, 10 mM $MgCl_2$, 5 mM EGTA and 20 mM KMOPS at pH 6.8 for 30 min at 4° C, according to the method of Reedy and Reedy (1985). Subsequently, the fibres were washed in MOPS buffer and in 100 mM potassium phosphate buffer. Then they were post-fixed in 1% OsO_4, 100 mM potassium phosphate buffer at pH 6.0 for 30 min at 0° C, washed in the same buffer twice for 15 min, and in H_2O in the same way.

Fibres were stained *en bloc* with 2% aqueous uranyl acetate, dehydrated in a graded ethanol series and embedded in Araldite. Ultrathin sections were cut with an LKB Ultratome III, stained with potassium permanganate and lead citrate, and observed and photographed with a Philips 420 electron microscope.

Results

Double immunoelectron microscopy with monoclonal antibodies T-11 and T-12 was used to study the elastic behaviour of titin filaments in the I-band. As reported earlier (Fürst *et al.*, 1988), antibody T-11 labels the I-band 50 nm from the A–I junction whereas T-12 decorates the I-band about 100 nm from the Z-line, at the so-called N_1 line (Fig. 1).

Figure 2 shows a montage of myofibrils fixed at different sarcomere lengths. Sarcomere lengths ranged from 2.5 μm where there was a wide zone of thick/thin filament overlap to 5.2 μm where overlap was zero and broad gaps appeared between thick and thin filaments. The epitope–Z-line separation behaviour of the two antibodies differed: T-11 epitope separation increased proportionally with increasing I-band width whereas the T-12 epitope remained at the same position close to the Z-line.

At the longer sarcomere lengths (Fig. 2c–f), the T-11 epitope began to move perceptibly from the edge of the A-band. This movement contrasts with the absence of movement over the physiological sarcomere length range where the epitope remains (as in Fig. 2, panels a and b) at 50 nm from the edge of the A-band (Trombitás *et al.*, 1991). The movement is apparently induced by the high tension associated with large stretch; it implies that once freed, all or part of the A-band region of titin can elongate.

Meanwhile, at these extended sarcomere lengths, the T-12 epitope position remained unchanged relative to the Z-line (Fig. 2c–f). Note that the T-12 epitope positions remain very regular and strictly parallel to the Z-line. Where the Z-line is skewed or misaligned, the T-12 epitope pattern follows the Z-line skew pattern very faithfully.

When the muscle was stretched to sarcomere lengths beyond 6 μm, the T-12 epitope finally lost its regularity and began to move from the Z-line (Fig. 3a). T-12 antibody deposits may be seen among the thin filaments. T-11 deposits, also seen on this micrograph, lie in the middle of the gap. The possibility that the antibody deposits found among the thin filaments arose from unsuspected movement of some of the T-11 antibodies is ruled out by a control experiment in which T-12 was not used. The thin filament deposits are no longer seen (Fig. 3b). Stretch-induced movement of the T-12 epitope from the Z-line implies that the N_1–Z region of the titin molecule, like the rest of the molecule, is intrinsically elastic. A plausible interpretation, then, is that part or all of this elastic region is ordinarily bound to the neighbouring thin filament until the excessive tension imposed by extreme stretch breaks the bond and frees the strand from the thin filament. This phenomenon is parallel to the one that occurs in the A-band, where high tension ultimately frees at least part of the strand of titin bound to the thick filament (Trombitás *et al.*, 1991; Wang *et al.*, 1991).

The tentative conclusion that titin and the thin filament are bound in the Z-line region was supported by the results of Z-line-extraction experiments. In the control specimen, in which extraction took place in the absence of antibody, the amorphous material of the Z-line was completely extracted (Fig. 4a). The myofibril did not fall apart. Thin filaments from contiguous sarcomeres remained overlapped in the former Z-region, i.e. in the electron-dense residual in the middle of the former Z-line (cf. Morris *et al.*, 1990; Luther, 1991; Szczesna & Lehrer, 1993). The T-12 epitope in the extracted fibre did not retract from the Z-line; it remained at the expected

location (Fig. 4b). The distance from the Z-line was the same as in the unextracted sarcomere (Fig. 4c). This confirms that the distal end of the titin molecule is not anchored exclusively to the amorphous material of the Z-line, although some other binding site on the Z-line is not ruled out.

Discussion

Elastic properties of titin filaments in vertebrate skeletal muscle have now been extensively studied. According to immunoelectron microscopic data, the A-band region is tightly bound to the thick filament (Wang *et al.*, 1984; Fürst *et al.*, 1988; Itoh *et al.*, 1988; Whiting *et al.*, 1988; Pierobon-Bormioli *et al.*, 1990), although intrinsically stretchable (Wang *et al.*, 1984; Itoh *et al.*, 1988; Pierobon-Bormioli *et al.*, 1990). The elastic properties of this region were recently demonstrated in overstretched muscle, where the A-band region pulled free of the thick filament and began to elongate and participate in the build-up of the gap filament (Trombitás *et al.*, 1991). That study also showed that the T-11 epitope did not move away from the A–I junction until the sarcomere lost overlap between the thick and thin filaments, demonstrating that the A-band region of titin does not participate in physiological elongation.

In the I-band, the titin filament has been thought to be functionally free and elastic. However, there have been at least two suggestions that the region near the Z-line might be different. First, using the T-12 antibody which labels the N_1-line region, it was shown that this region did not crumple as the sarcomere shortened from rest length to shorter length (Fürst *et al.*, 1988). Second, using the freeze-break technique, we demonstrated that severed titin filaments that could retract toward the Z-line stopped retracting at the N_1-line level, never reaching the

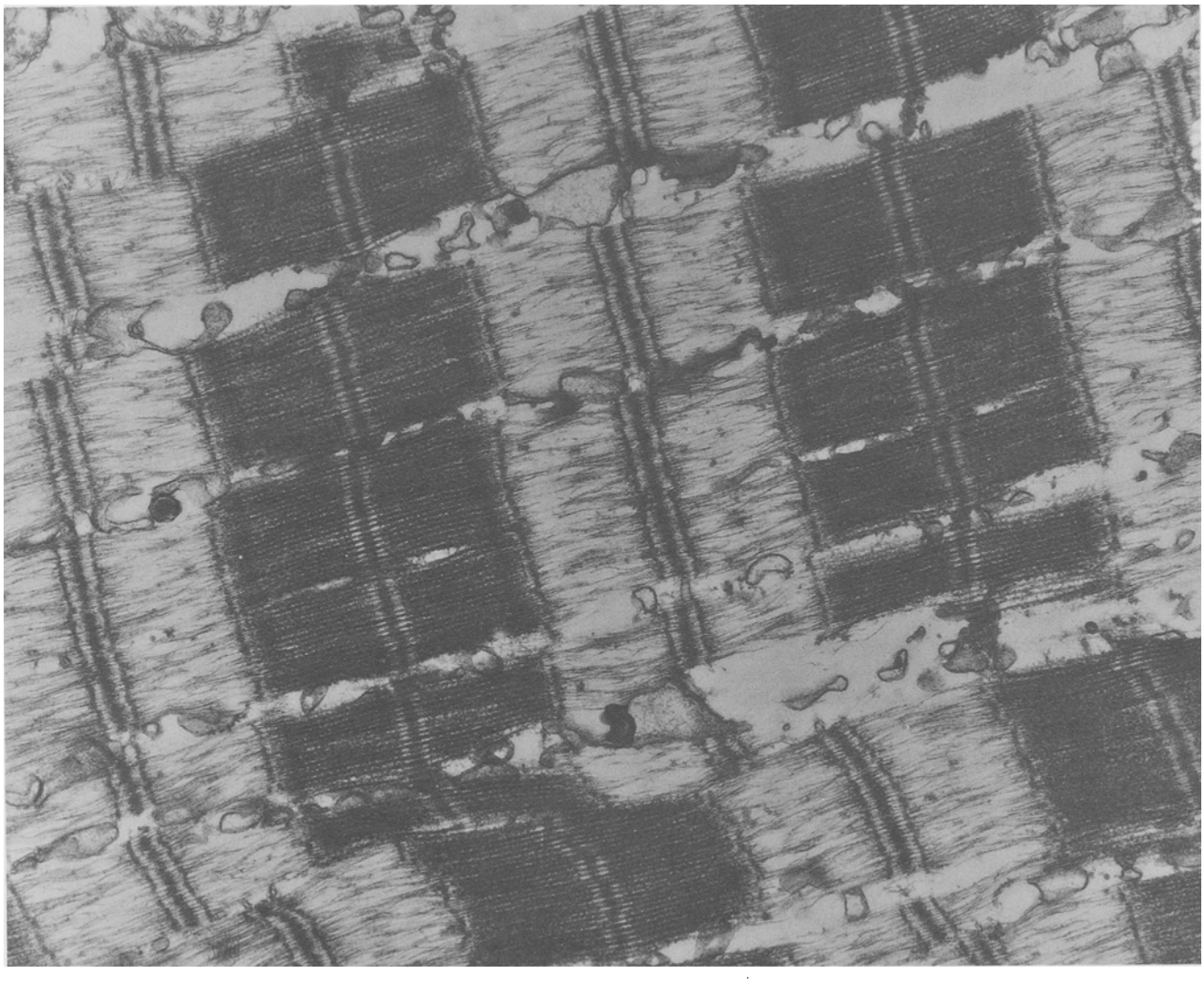

Fig. 1. General view of T-11 and T-12 antibody-decorated fibres. The T-11 epitope labels the titin filaments just beyond the tips of the thick filament; the T-12 epitope labels the titin filament on either side of the Z-line, at the so-called N_1-line. Magnification 25 000.

Z-line (Trombitás *et al.*, 1990). These two findings called our attention to the segment of the titin filament between the N_1-line and Z-line.

As Fig. 2 shows, the titin filament between N_1-line and Z-line did not participate in sarcomere elongation over a wide range of sarcomere stretch. The fact that the

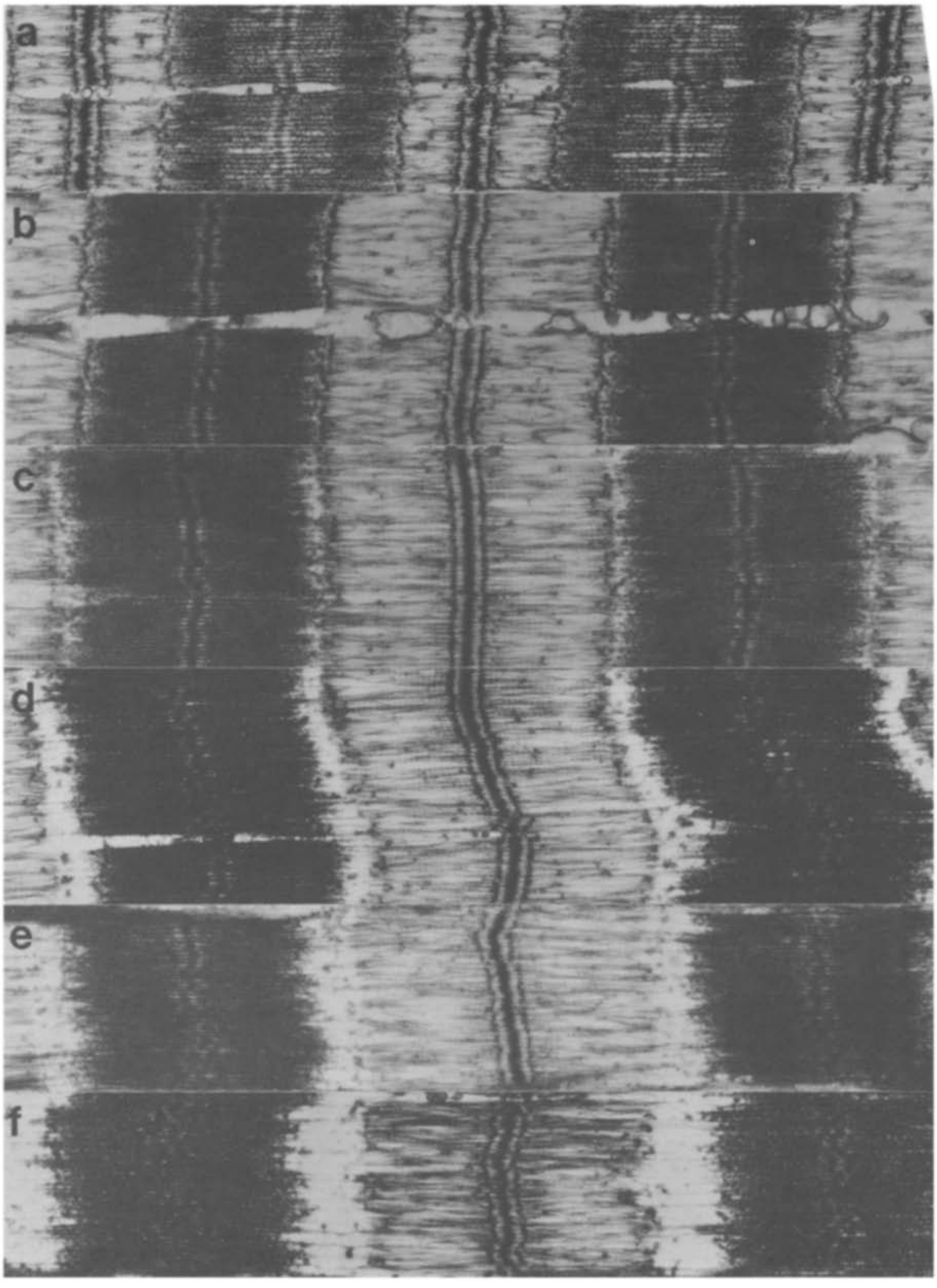

Fig. 2. Montage of myofibrils of different sarcomere length from 2.5 μm (top) to 5.2 μm (bottom). The T-11 epitope moves from the Z-line as I-band width increases; the T-12 epitope remains in the same position relative to the Z-line. Magnification 26 200.

N_1–Z-line region does not participate in elongation can be interpreted potentially in three ways: (1) the titin filament might be rigid in this region, as theorized earlier (Fürst *et al.*, 1988), (2) the region might be elastic but is bound to the thin filament in the N_1-line, or (3) again, the filament may be elastic but it is bound along the entire stretch between N_1-line and Z-line (Trombitás & Pollack 1993).

The first possibility, that the filament is stiff in this region, seems to be contradicted by the observation that the T-12 epitope positions do finally begin to move away from the Z-line if the sarcomere is stretched sufficiently (Fig. 3a). Thus, like the rest of the titin filament, the N_1–Z-line region appears to be intrinsically elastic.

The second possibility, that titin is bound to the thin filament focally at the N_1-line, is contradicted by filament-count measurements made near the Z-line. These counts show apparently separated thin and titin filaments far from the Z-line (Akster *et al.*, 1989) but deny the existence of separate thin and titin filaments in the N_1–Z region (Ullrick *et al.*, 1977; Akster *et al.*, 1989). The filament-count experiments are also consistent with the morphology observable in longitudinal sections: thin filament origins generally appear as stout, robust stubs of larger diameter than the remainder of the filament (e.g. Rowe, 1973). These observations imply that the bond between titin and the thin filament probably extends over the entire N_1–Z region, not at the N_1 line alone. Thus, possibility (3) seems more consistent with the evidence than possibility (2).

The Z-line extraction experiments clarify some of these issues. The fact that extraction of the amorphous material did not elicit titin retraction implies that the anchoring of the titin filament cannot be exclusively to the amorphous material of the Z-line. The result is thus compatible with possibility (3). On the other hand, the observation certainly leaves open the possibility that titin associates with some other component of the Z-line, or with the corresponding strand of titin in the next sarcomere, in either case forming a continuous structural framework.

In conclusion, the A-band zone of titin does not participate in sarcomere elongation at physiological sarcomere length, but it is intrinsically elastic. The passive tension reached when the gap appears in frog muscle is large enough to free titin from the thick filament. Therefore, the bond between the A-band region of titin and the thick filament is moderately strong.

The long region of titin beginning 50 nm from the A–I junction and extending to the N_1-line is free and elastic,

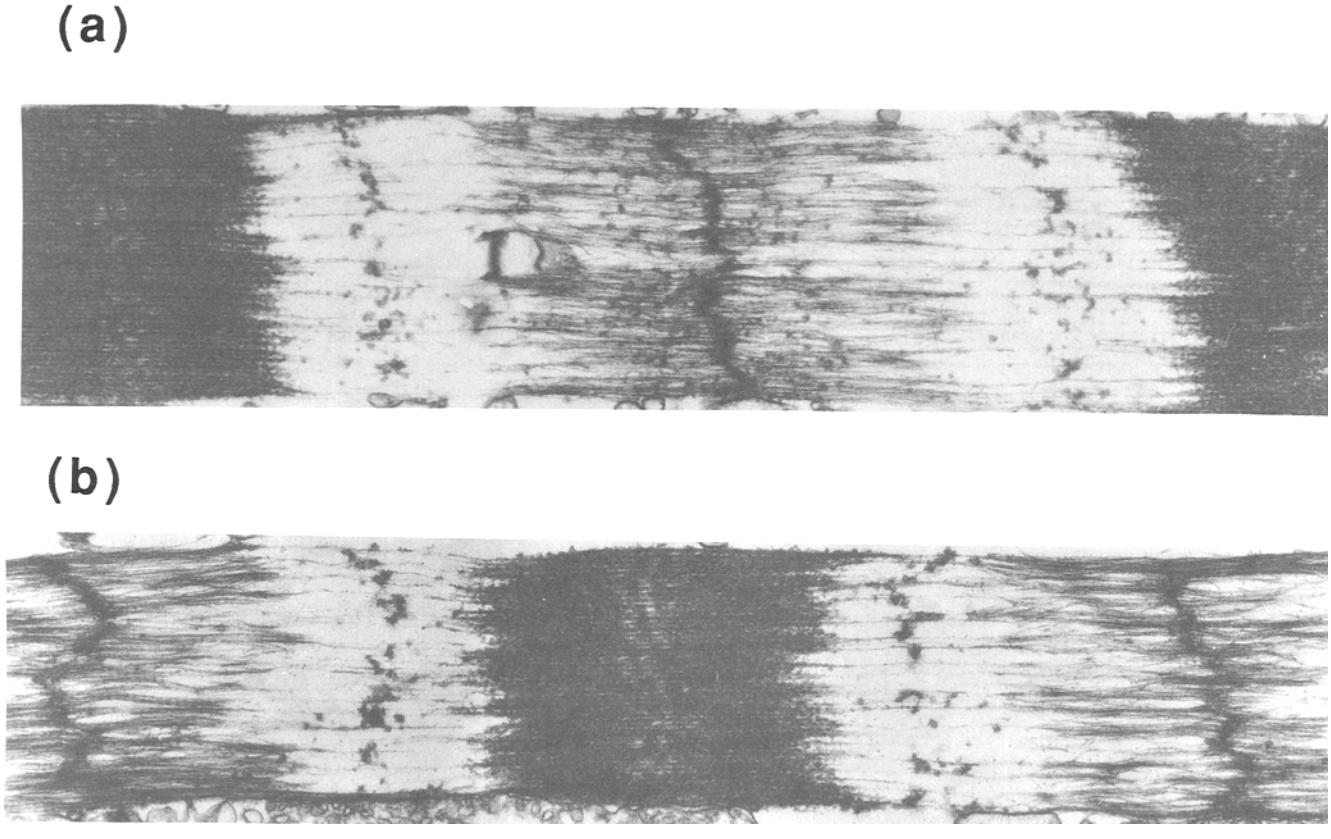

Fig. 3. Highly stretched sarcomere (6 μm). (a) T-11 epitope is approximately in the middle of the gap between thick and thin filaments. The T-12 epitope is no longer recognizable; randomly located antibody deposits demonstrate the unbinding and elongation of the titin N_1–Z-line region. Magnification 30 000. (b) Same as (a) but the T-12 label was omitted. The randomly located antibody deposits seen in (a) are no longer present. Magnification 27 000.

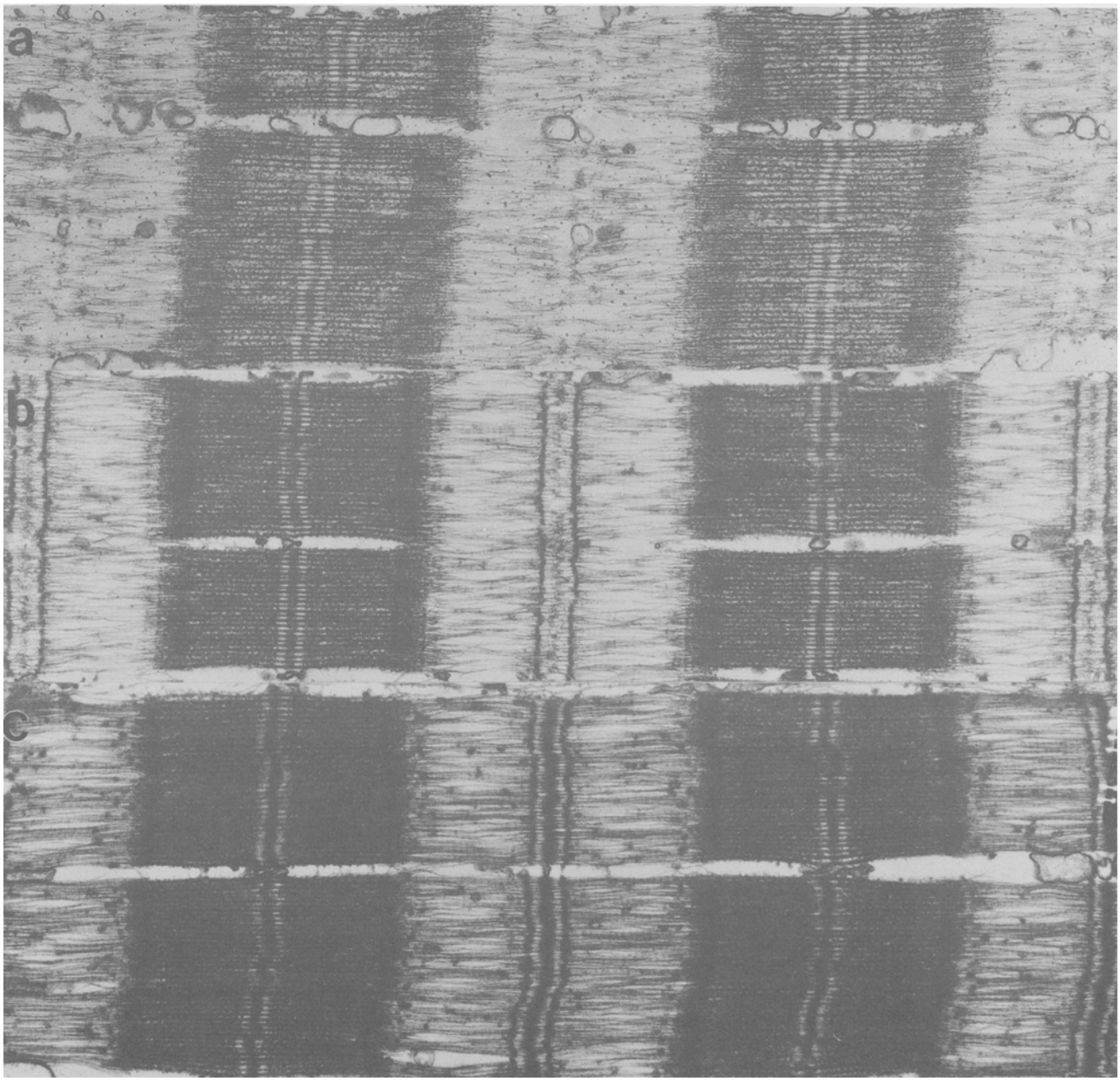

Fig. 4. (a) Control fibre from Z-line extraction experiments. Z-line was extracted by Ca-activated protease. In spite of the stretch, the sarcomere did not fall apart, although the Z-line was extensively extracted. After extraction, the thin filaments appear to remain intact in the former Z-line region. (b) Z-line extraction did not influence the T-12 position. No titin retraction was observed towards the A-band in stretched muscle. (a) The T-12 epitope remained in the same position as in the unextracted fibre. Magnification 25 600.

elongates when the sarcomere is stretched (Itoh *et al.*, 1988; Wang & Wright, 1988; Pierobon-Bormioli *et al.*, 1990), and returns the passively stretched sarcomere to resting length after the muscle is released (Trombitás *et al.*, 1990, 1993). It is the main source of passive elasticity of the sarcomere.

The N_1–Z-line region of titin appears to be functionally bound to the thin filament and because of this association does not participate in physiological sarcomere elongation. Even when the sarcomere is stretched to the point at which the gap filament appears, the passive tension is not large enough to break this bond. Extremely high passive tension (developed above 6 μm sarcomere length) is necessary to free this segment from the thin filament, at which point the segment finally elongates. Thus, like all other regions of titin, this region is intrinsically elastic, although it is oridinarily bound to the thin filament.

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