Myofibrillar tightly bound calcium in skeletal muscle fibers: a possible role of this cation in titin strands aggregation

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Abstract In muscle cells, part of the calcium is tightly bound to the N1- and N2-line of the sarcomere but its physiological significance was unknown. In the present work we reported the ability of a recombinant titin fragment spanning titin domains Z9 to I1 to tightly bind calcium ions with a K_d of 0.049 ± 0.004 nM. We further showed that calcium induced a spontaneous aggregation of the titin fragment and that the major aggregate is a tetramer. The implication of these findings on the organization of the six titin strands that emanate from the end of the thick filament within the I-band is discussed.

Key words: Skeletal muscle; Titin; Recombinant fragment; N-line; Calcium; Titin polymerization

1. Introduction

Single titin molecules, a giant protein (3.0–3.7 MDa; 1 μm long) [1] also known as connectin [2], span half of the sarcomere from M-line to Z-line [3], and pairs of molecules can therefore specify events over the entire sarcomere. Recent findings suggested that there are six titin molecules per thick filament, a number in good adequation with the three-fold symmetry of thick filaments and the two-fold symmetry of thin filaments [4,5]. Emerging from the tips of the thick filaments are stalk-like structures called end-filaments, whose size, location and distinctive 4 nm cross suggest they are an aggregate of a part of the titin molecule striations [6,7], but how the titin molecules pack together to form them remains to be determined. From each myosin filament, it was assumed that two pairs of titin strands move out and interact with the actin filaments close to the Z-band [5] suggesting that they are relatively free within the I-band. The two other strands span the I-band to the Z-line where they might interact with actin filaments of opposite polarity from the next half sarcomere [4]. For a better efficiency of the titin molecules in controlling passive elasticity of sarcomeres, a strict coordination of the shortening or strengthening of the elastic region of each titin strand would have to be performed with exquisite precision. This could be achieved through a regulated aggregation at defined region of the titin spanning from the A-band to the Z-band before they interact with actin filaments.

As emphasized for the end-filaments we still do not know how the titin strands can form such aggregates. Can this property be regulated by calcium located at the N1- and N2-line levels of the sarcomere [8,9]? Such assumption is supported by the ability of titin to bind calcium [10,17]. The fact that calcium bound to the N1- and N2-lines of the sarcomere can not be displaced even with relatively high concentrations of ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) in the range of 10–20 mM (personal observation) supports this hypothesis. Furthermore this unpublished observation suggests an unusually very high binding affinity and very likely much higher than that reported for the elastic portion of titin [10].

In the present work we address questions about how the titin strands can aggregate and the biological function of the N1- and N2-line bound calcium in this context was tested. We analyzed the ability of a recombinant titin fragment, spanning domains Z9 to I1 of titin, to bind calcium and to polymerize in the presence of this cation. Domains Z9 and I1 which are close to the N1-line insertion located between domains I2 and I3, are linked by a segment containing a coiled-coil sequence [11] as well as a negatively charge region enriched in Glu residues. The N1-line region is further known to bind calcium as evidenced in pyroantimonate treated fibers [8,9].

2. Materials and methods

2.1. Materials

Superose 12 HR 10/30 gel filtration column was purchased from Amersham Pharmacia Biotech. (Orsay, France). Chelex-100 resin was obtained from Sigma Chemical Co. (St Quentin Fallavier, France). Quin-2 was supplied by Molecular Probe (Interchim, Montluçon, France).

2.2. Recombinant fragment preparation

Recombinant titin fragment spanning Z9 to I1 domains of titin and located in the N1-line region of the protein was expressed in *Escherichia coli* using the pET expression systems as described previously [12].

2.3. Calcium titration of the recombinant Z9–I1 titin fragment

2.3.1. Preparation of calcium buffer. To avoid calcium contamination, all buffers were prepared with deionized water previously run three times through a Chelex-100 column (1×8 cm), for 1 h each at a flow rate of 0.3 ml/min, according to the cleaning procedure reported

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for aluminum contamination of aqueous solution [20]. All subsequent experiments were carried out in 50 mM Tris–HCl buffer pH 7.5 containing 100 mM NaCl, 1 mM dithiothreitol (DTT) and 4 mM EGTA (buffer A). Similarly, buffer A was also run three times through the Chelex-100 column, for 1 h each at a flow rate of 0.3 ml/min. After Chelex-100 treatment of buffer A, no calcium was detected with Quin-2 [21]. Its concentration in the buffer was therefore assumed to be in the nM range as compared to the control for which the calcium concentration was similarly estimated to be in the μM range. The free calcium concentration was then calculated from the contaminating calcium (1 nM), plus the amount of calcium added, using the computer Biosoft Eqcalwin program from Mike Haygall (Biosoft Company, London, UK) with the following logarithmic association constants for metals and H^+ to EGTA: H^+ to $EGTA^{4-}$, 9.53; H^+ to $HEGTA^{3-}$, 8.88; H^+ to H_2EGTA^{2-}, 2.65; H^+ to H_3EGTA^{1-}, 2.00; Ca^{2+} to $EGTA^{4-}$, 11.0; Ca^{2+} to $HEGTA^{3-}$, 5.33. Using a Roebling automatic micro-osmometer (Bioblock, Illkirch, France), the osmotic pressure of buffer A was estimated to be 292 mOsm, a value corresponding to physiological conditions.

2.3.2. Calcium titration of the Z9–I1 fragment. The titin recombinant fragment (250 μg/ml) was incubated overnight at 4°C, under mild continuous stirring, with a few mg of Chelex-100 resin pre-hydrated in buffer A. The solid phase was sedimented by centrifugation at 1000×*g* for 5 min and the polypeptide recovered in the supernatant used for calcium titration. Calcium titration of this recombinant titin fragment was carried out as described previously in [13]. Increasing concentrations of calcium were added, from a 2 M stock solution in buffer A, to the recombinant fragment (1 μM) and changes in the intrinsic tryptophan fluorescence recorded using a Perkin Elmer LS 50 spectrofluorometer (λ_{Exc} 305 nm, λ_{Em} 358 nm). Calibration of the free calcium concentration was carried out by titration of Quin-2 (1 μM in buffer A) using excitation and emission wavelengths of 330 and 495 nm, respectively. The fluorescence data were fit to the one-site model of the non-linear Hill equation:

$$y = y_{max}/1 + 10^{(n*(K_d - p\mathrm{Ca}))}$$

where y = normalized fractional maximal fluorescence y_{max}; y_{max} = maximal percentage enhancement observed in that phase of the titration; n = Hill coefficient; K_d = free Ca^{2+} concentration producing half-maximal enhancement in the indicated phase of titration; pCa = negative logarithm of the free Ca^{2+} concentration producing y% of maximal enhancement. The curves were fit with a least squares non-linear regression (Microsoft Excel Solver, 1997).

2.4. Polymerization of the titin fragment in the presence of calcium

After overnight treatment of the Z9–I1 recombinant fragment with Chelex-100 resin (see below), the mixture was run on a Superose 12 HR 10/30 column pre-equilibrated in buffer A containing 1 mM EGTA instead of 4 mM. The monomeric form eluted at a M_r of about 53 000 Da was collected and used for the calcium-induced polymerization study. The fragment (250 μg/ml) was incubated at room temperature without calcium (control) or with 0.1 and 1 μM calcium for 30 min (free calcium concentrations: 3.35×10^{-11} and 3.35×10^{-10}, respectively). An aliquot of each mixture (250 μl) was then loaded on a Superose 12 HR 10/30 column previously equilibrated with the same buffer. Proteins were eluted at a flow rate of 0.3 ml/min and fractions of 0.3 ml were collected. Calibration of the column was performed using ferritin (418 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (BSA; 67 kDa) and bovine α-lactoglobulin (30 kDa).

2.5. Depolymerization of the Z9–I1 complex in the presence of Chelex-100 resin

The recombinant peptide was incubated overnight at 4°C in buffer A with a few mg of Chelex resin previously hydrated in the same buffer and the supernatant recovered as described above. The control sample was similarly incubated but in the absence of Chelex-100 resin. An aliquot (250 μl) of the samples was then loaded on a Superose 12 HR 10/30 column previously equilibrated with the same buffer. Proteins were eluted at a flow rate of 0.3 ml/min and fractions of 0.3 ml were collected.

2.6. Protein determination

Protein concentrations were measured by using Bradford's method (Bio-Rad protein assay) with rabbit immunoglobulin as the standard [14].

3. Results

3.1. Structure, properties and location in titin of the Z9–I1 recombinant fragment

The recombinant fragment used in the present experiment spans domains Z9 to I1 of titin molecules (Fig. 1A) and connects the Z-line to the I-band. The polypeptide comprises 331 amino acid residues (from residue 1841 to 2172 in human cardiac titin [1]) and, on the basis of its primary sequence (Fig. 1B), its M_r was predicted to be 41 000 Da with the His tag inserted at its N-terminal end (not shown). The immunoglobulin-like domains Z9 (residues 1–90 in Fig. 1B) and

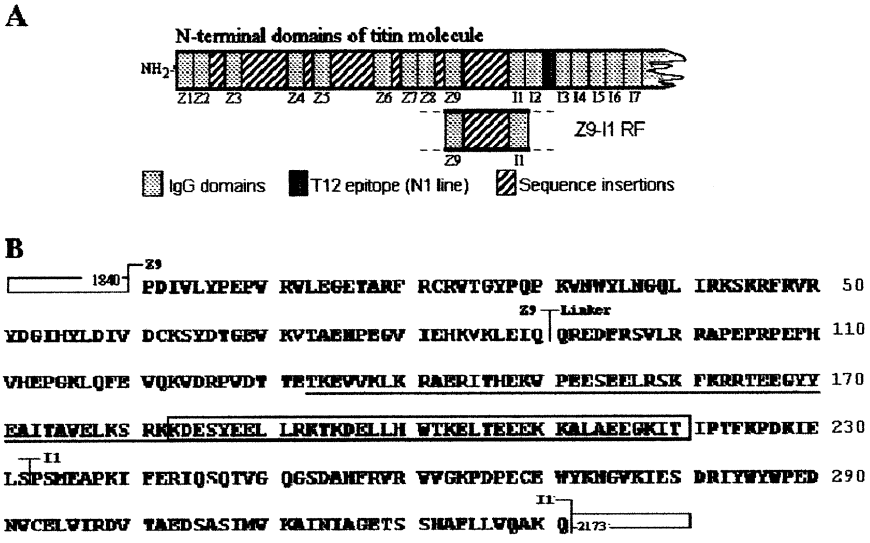

Fig. 1. Main characteristics of the Z9–I1 titin recombinant fragment. A: Localization of the fragment at the N-terminal end of the titin molecule. B: Primary sequence of the titin fragment extending from residue 1840 to 2173 in the cardiac titin sequence [1] with the cluster enriched in Glu residues (underlined and boxed sequences). Abbreviations used: IgG, immunoglobulin like; Z9–I1 RF, Z9–I1 recombinant fragment; Z9, Z9 titin domain; I1, I1 titin domain.

I1 (residues 233–331 in Fig. 1B) are linked by a unique insertion sequence (residues 91–232 in Fig. 1B). This insertion was shown to have a helical propensity with a strong amphiphilic character [11]. Analysis of this sequence using a secondary structure predictive program also predicts a large majority of α-helices separated by very short random coil structures within the underlined sequence (residues 132–220). On the other hand, examination of the sequence revealed a particular high content in Glu residues especially within the underlined sequence and to a greater extent within the boxed sequence (residues 182–220 in Fig. 1B). Therefore, the present cluster of highly negative net charge could be a potential calcium binding site. This was substantiated by preliminary assessment of its calcium binding ability using the Quin-2 fluorescence technique described in [15] (data not shown).

3.2. Calcium titration of the titin Z9-I1 recombinant fragment

This fragment was subjected to quantitative measurements of calcium binding using the method described in [13]. Calcium binding to proteins generally produces large structural changes and these structural changes perturb the environment of intrinsic fluorophores such as Trp and Tyr residues. Study of the binding of calcium to the titin fragment was therefore performed using the changes in the intrinsic tryptophan fluorescence.

As shown in Fig. 2, increasing concentration of calcium causes a sharp increase in the Trp fluorescence and half-maximal binding was found to occur at *p*Ca 10.31 ± 0.03 giving a K_d value of $4.92\times10^{-11}\pm0.38\times10^{-11}$ M (mean ± S.D. for three independent experiments). The estimated Hill coefficient was about 4 suggesting a cooperativity in calcium binding.

To calibrate the free calcium concentrations, the chromophoric chelator Quin-2 was titrated in the same conditions (Fig. 2). The half-maximal increase in fluorescence for Quin-2 occurs at *p*Ca 7.21 ± 0.01 (K_d = 61 ± 2 nM) (mean ± S.D. for three independent experiments) with a Hill coefficient of 1.09. This K_d value is essentially identical to the K_d (60 nM) reported for Quin-2 by the supplier.

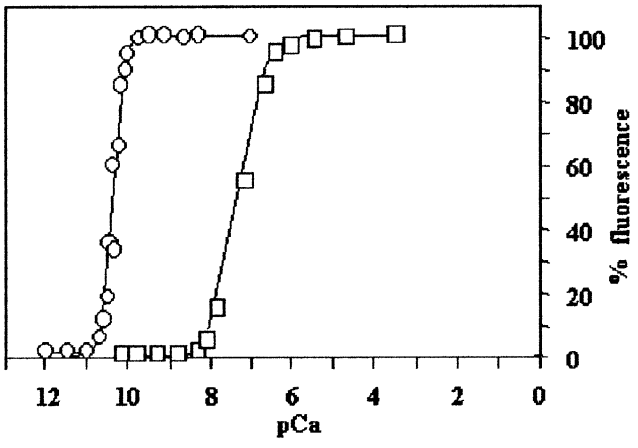

Fig. 2. Calcium titration of Quin-2 (open squares) and of the Z9-I1 titin fragment (open circles). Calcium was added to 1 μM of each compound in 1 ml calcium buffer. 100% increase in the intrinsic tryptophan (Z9–I1) and Quin-2 fluorescence corresponds to 2.2- and 5-fold increases for the titin fragment and Quin-2, respectively. The multistep cleaning of the buffer with Chelex-100 decreases significantly the calcium content and this cation was not more detectable with Quin-2. In the absence of EGTA, the free calcium in the buffer was therefore estimated to be in the nM range (1 nM). This concentration was taken as the basis of the amount of calcium added and of the free calcium determination in the presence of 4 mM EGTA. As an example, in the presence of 4 mM EGTA, 1×10^{-12} M free calcium needs a total calcium of 1.2×10^{-8} M. Assuming that we have 1 nM free calcium in the buffer, a total amount of $\approx1.1\times10^{-8}$ M was added to the mixture.

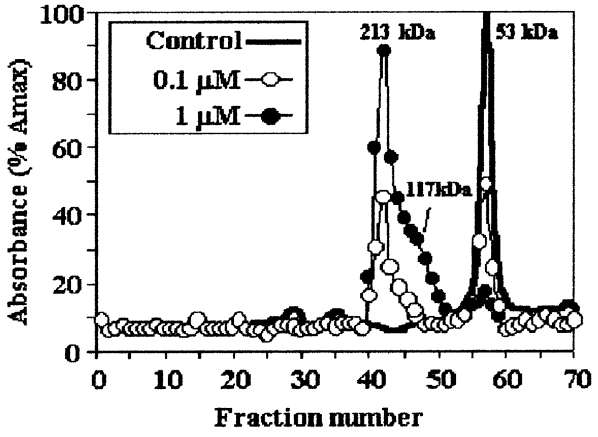

Fig. 3. Elution profile of the recombinant fragment from a Superose 12 HR column after incubation for 30 min at room temperature, without calcium (thick line) or with either 0.1 μM total calcium (open circles) or 1 μM total calcium (closed circles). Elution was performed at 0.3 ml/min and fractions of 0.3 ml were collected.

3.3. Calcium induced polymerization of the Z9–I1 titin fragment

To test this possibility, the fragment was treated with Chelex-100 and the monomeric form purified by gel filtration on a Superose 12 HR 10/30 column. The monomeric form was then incubated with different calcium concentrations and the mixture analyzed by gel filtration on the same column. Gel exclusion chromatography enables a good fractionation of the monomeric (53 kDa) and the aggregated forms of the recombinant Z9–I1 titin fragment (see the control elution profile in Fig. 4).

Upon addition of 0.1 μM calcium (free calcium concentration close to the K_d value obtained above: 3.35×10^{-11} M),

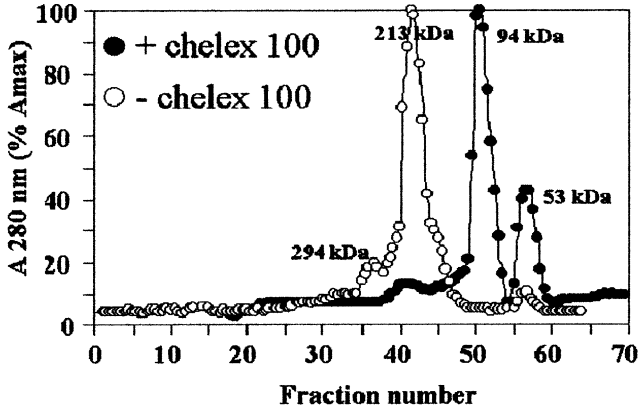

Fig. 4. Elution profile of the recombinant fragment from a Superose 12 HR column after overnight incubation at 4°C with a few mg of Chelex-100 resin (closed circles) or without Chelex-100 resin (open circles). Elution was performed at 0.3 ml/min and fractions of 0.3 ml were collected.

we observed a decrease in the level of the monomeric form and the appearance of an intermediate shoulder with a M_r of about 117 kDa which might possibly correspond to a dimer or a trimer (Fig. 3). Raising the calcium concentration from 0.1 to 1 μM (saturating free calcium concentration of 3.35×10^{-10} as indicated in Fig. 2) led to an almost complete disappearance of the monomeric form and a significant increase in the shoulder coming out just after the tetrameric form. Hence, it can be stressed that calcium induces a polymerization of the Z9–I1 titin fragment. We then tested whether Chelex-100 resin will be able to depolymerize at least partly this aggregate.

3.4. Chelex-100 induced depolymerization of the aggregated Z9–I1 titin fragment

As shown in Fig. 4 for the control sample, the purified titin fragment is originally a mixture comprising the monomeric form (53 kDa), the tetrameric form (213 kDa) and a minor fraction with a M_r of about 394 kDa which might correspond to the hexameric form. It is worthy to note that the major form is the tetramer whereas the hexameric form was always observed as a minor component in the mixture. The presence of the polymerized polypeptide in the mixture is not too much surprising since no caution has been taken with regard to calcium ions in the production and the purification procedures as well as in the composition of the storage buffer which lacked calcium chelators.

Because no effect was observed with Quin-2, Chelex-100 resin, which has a higher affinity for calcium than most of the chromophoric chelators available, we tested the ability of this resin to depolymerize aggregates of the Z9–I1 fragment. As the stock solution of the Z9–I1 fragment mostly comprised the polymerized form, an aliquot was incubated overnight at 4°C with a few mg of Chelex-100 resin. The control sample was similarly treated but in the absence of Chelex-100 resin. For the treated sample, two major peaks were resolved with a M_r of 53 and 94 kDa and a minor one with a M_r of 213 kDa (Fig. 4). Compared to the control sample we observed an almost total disappearance of the tetrameric form (213 kDa), and an increase in the amounts of the 94 and 53 kDa peaks, thus demonstrating a depolymerization of the aggregate by the chelator resin.

4. Discussion and conclusion

In skeletal muscle fibers, most calcium is sequestered in the sarcoplasmic reticulum via a calcium binding protein (CaBP), the calsequestrin and its release in the cytoplasm activates myofibrillar contraction [16]. However, since the seventies, it has been well established that part of the cellular calcium is strongly bound to the myofibrils at the level of the N1- and N2-lines of the sarcomere [8]. The first association of calcium ions with titin/connectin was described several years ago in a hypothesis paper [17] and confirmed by the ability of the PEVK region to bind calcium [15]. However nothing is known about the ability of the N1- and N2-lines to bind calcium. Moreover, although titin/connectin was the best candidate, we did not know precisely to which proteins this calcium bound to and its exact physiological significance.

The present work provides possible answers to these questions. Indeed, in the N1-line region, we demonstrate that calcium binds tightly to a titin fragment spanning the Z9 to I1 domains of the molecule. This recombinant fragment contains a particular insertion linking titin domain Z9 to domain I1 which exhibited a helical propensity with a strong amphiphilic character [11]. Because such amphiphilic α-helices are generally unstable and tend to form aggregates [18], the linker segment was suspected to be responsible for titin polymerization in that region of the molecule. However, from the investigations carried out on a typical α-helix motif (residues 182–220 in Fig. 1B), it was concluded that, in physiological conditions, the peptide did not polymerize [11]. The other important characteristic of the linker segment is its high content in Glu residues suggesting that this linker might be a potential calcium binding site. We demonstrate that this cluster of strong negative net charge was able to bind calcium with an unusually high affinity ($K_d = 4.92 \pm 0.38 \times 10^{-11}$). With such a high affinity, calcium binding would seem quite irreversible, an assumption strengthened by the non-release of this cation upon addition of high concentrations of EGTA to myofibrils ranging from 10 to 20 mM (personal observation). To test this finding, the free calcium concentration was calibrated by titrating Quin-2 in similar conditions. The K_d value of 61 nM obtained for Quin-2 was identical to the value of 60 nM reported by the supplier suggesting that the free calcium concentrations are accurate up to the nM range.

Although the accuracy of the K_d value found for the calcium–titin fragment interaction can be discussed, it is clear that the affinity of this cation to titin is much higher (100–1000-fold) than that observed for Quin-2. Precise control of very low calcium concentrations as here is rather difficult and specialists would say that it is impossible. But we must take in mind that our objective was to evaluate the affinity range of this cation towards titin which is not displaced in current conditions and Quin-2 constitutes a good marker in this context. Besides the accuracy of this K_d value, the most important thing is that we can attest that the affinity of this cation for titin is much higher than for Quin-2.

As assessed by gel filtration, calcium induced the spontaneous polymerization of the recombinant fragment at the expense of the monomer which disappears almost totally at a higher calcium concentration. The major form obtained is the 213 kDa complex which might be the tetramer together with a transient appearance of an intermediate form (possibly a dimer or a trimer). Interestingly, it must be emphasized that the larger aggregate obtained is a tetramer of 213 kDa and not an hexamer as expected from the fact that six titin strands extend through the I-band to the Z-band. When detectable, the hexameric form (294 kDa) was present at a very low level and did not change to a large extent upon addition of calcium. Conversely, treatment of the polypeptide aggregates with a Chelex-100 resin, a high affinity calcium binding polymer, led to a dissociation of the complex into the dimeric and, though to a lesser extent, the monomeric forms. Taken together, these findings suggest that the most probable calcium-induced titin aggregates would be the dimer and/or the tetramer but not the hexamer.

The N1-lines bind calcium but a much greater amount of this bivalent cation is bound to the N2-line region of the sarcomere [9]. Analysis of the titin sequence revealed that the N2A- and N2B-line region contained clusters enriched in Glu residues conferring strong negative net charges to these clusters, which might be potential calcium binding sites of high affinity [1,19]. Hence, we suggest that, similarly to the Z9–I1 fragment, calcium binds to the N2A- and N2B-line

region of titin through such negatively charge clusters. The calcium precipitate band is much wider than in the N1-line region [9]. This observation suggests the presence of a greater number of clusters susceptible to bind calcium within the N2-line area of the titin molecule.

The implication of the calcium-induced aggregation of titin molecules in the N1- and N2-lines is not clear. However, such aggregation would allow a better coordination of the stretching and shortening of each titin strand and would ensure a better force transmission between A-band and Z-line, and probably between adjacent sarcomeres as well. Self-association of the titin molecules was reported at the level of the end-filaments emerging from the tips of the myosin filaments [4]. The present data would support a calcium-dependent association of titin strands to form the end-filaments package. However end-filaments are supposed to contain the PEVK region but we do not know whether they also contained at least part of the N2-line calcium binding region. The last model proposed for the organization of titin strands [4,5] suggests that two pairs of titin strands span the I-band and bind to actin filaments at the level of the Z-line. The third pair was assumed to bind actin filaments of opposite polarity (from the adjacent sarcomere) at the level of the Z-line (see fig. 3a in [5]). How these titin strands are organized within the I-band would have to be clarified in the light of the present findings.

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