Calpain 1 Binding Capacities of the N1-Line Region of Titin Are Significantly Enhanced by Physiological Concentrations of Calcium†

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ABSTRACT: Calpain 1, an ubiquitous well-known calcium-dependent intracellular protease, was recently shown to bind tightly to the proximal end of the I-band titin segment in a calcium-dependent manner [Raynaud et al. (2005) *FEBS J. 272*, 2578−2590]. In the present work we identified the titin Ig-domain of concern by this interaction and the role of calcium in this interaction using a recombinant fragment of titin spanning the I2−I6 region and its subfragments. The heterodimeric form of calpain 1 binds to this titin fragment with a very high affinity ($K_d = 5.1 \pm 0.2 \times 10^{-7}$ M) at much lower calcium levels than those saturating the high-affinity binding sites of the peptidase ($K_d = 25\ \mu$M). Investigation of this interaction with I2−I6 subfragments clearly showed that the dimeric form of calpain 1 binds exclusively to the Ig-domain I4 of titin with an affinity similar to that of the whole I2−I6 segment. As for the I2−I6 fragment, this interaction is calcium regulated. Calcium was shown to bind tightly to titin ($K_d = 1.9 \times 10^{-7}$ M), causing an oligomerization of the titin segment. At physiological calcium concentration (10^{-6} to 10^{-8} M), the prevailing form of the titin fragment is a trimer, suggesting that calpain 1 binds to this titin structure. From the present findings, it was concluded that calcium binding to titin increased the amount of bound calpain 1 (up to 40% of the total calpain 1) and that this bound calpain 1 might constitute a reservoir for this peptidase. In this context, we proposed a schematic diagram of this series of calcium-dependent events with the inherent unanswered questions. These events are probably under a complex regulation involving undoubtedly different yet unidentified proteins.

The calpain family is a group of intracellular cysteine proteases requiring calcium ions for activity. Although their physiological function is still not fully understood, several sets of evidence support their implication in a variety of calcium-regulated cellular processes such as signal transduction, cell proliferation, cell cycle progression, differentiation, apoptosis, membrane fusion, and platelet activation (*2*−*4*). Deregulation of their activity also contributes to various pathological conditions such as neuronal degeneration, Alzheimer's disease, metastasis, and cataract (*4, 5*).

Calpain 1 (microcalpain or μ-calpain) and calpain 2 (millicalpain or m-calpain) are the best characterized calpains and show the widest tissue distribution among members of this family. These calcium-dependent cysteine proteases play important roles in a large set of intracellular events (*2, 6, 7*), particularly in the selective proteolysis of factors involved in the cell cycle (*8*), during apoptosis in association with caspases (*9*), or in the cleavage of membrane−cytoskeleton complexes during cell motility phases (*10*). Calpain 1 (active in vitro at 50 μM Ca^{2+} ions) and calpain 2 (active in vitro at 500 μM Ca^{2+} ions) are heterodimeric structures composed of a different 80 kDa subunit and a common 30 kDa subunit (*11*). In skeletal muscle tissue, they coexist with calpain 3, a monomeric calpain homologous to the 80 kDa calpain subunit (*12*), and with calpain 10, which is deprived of the calcium binding domain or domain IV (*13*).

Cellular localization of ubiquitous calpains in the skeletal muscle fiber has long been highly controversial (*14*−*16*). Moreover, Goll and Geesink (*17*) indicated that a significant part of calpain 1 is tightly bound to myofibrils but the carrier protein has not been identified. Recent ultrastructural investigations revealed that calpain 1 is located at the middle of the I-band and close to the Z-line of freshly prepared skeletal muscle myofibers (*1*). Calpain 1 was found to be strongly associated to particular structures of the myofibrils including the N2-line and the N1-line, two regions also known to bind calcium (*18, 19*). The carrier protein was further shown to be titin, but the exact calpain 1 binding domains are not yet identified. The calpain 1/titin interaction was shown to be

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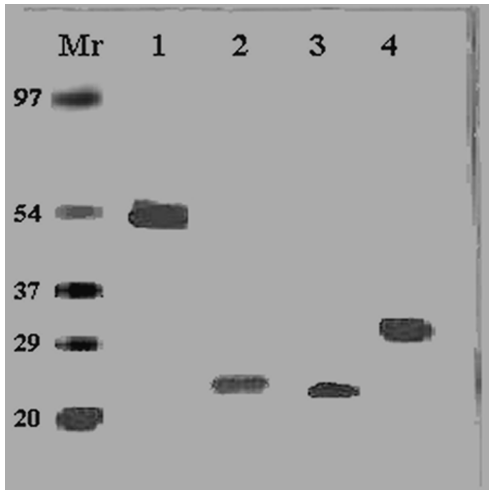


FIGURE 1: SDS−PAGE analysis of the purified titin recombinant fragments including I2−I6 (lane 1), I2−I3 (lane 2), I3−I4 (lane 3), and I5−I6 (lane 4). M_r indicates the molecular mass of the markers used (Amersham-Pharmacia low M_r kit).

calcium regulated, but the role of calcium in this interaction is still unclear. Both proteins are indeed able to bind calcium. Calpains 1 and 2 exhibited relatively low affinity toward this cation since K_d values were assumed to be 25 and 325 μM, respectively (*20*). Regarding titin, several sets of evidence suggested that some region of this giant protein can bind calcium, but all studies carried out so far were focused on the PEVK[1] region of titin which was shown to bind this cation with a dissociation constant of about 9×10^{-7} M (*21*). We recently showed that the linker between Ig-domains Z9 and I1, a sequence rich in Glu residues, binds quite irreversibly calcium with a dissociation constant of about 4.9×10^{-11} M (*22*). Apart from the N2- and N1-line region, the PEVK-rich elastic segment, and the Z9-I1 linker segment, we still do not know whether other regions of titin are able to bind calcium. In addition, we have no clear view about the role of calcium in the physiological function of the different titin segments of concern.

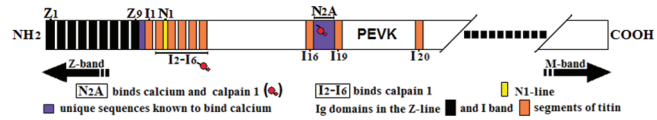
The objective of the present study was therefore to characterize the interaction of calpain 1 with the proximal region of the titin I-band segment which presents several specificities including the presence of the N1-line and the ability to bind calcium and calpain 1 with high affinity (Scheme 1). For this purpose, the recombinant I2−I6 titin segment located in the N1-line region of the protein together with its subfragments was used to identify the exact binding site of calpain 1 and the role of calcium ions in this interaction, a bivalent cation known to enhance the amount of bound calpain.

MATERIALS AND METHODS

Sources of Calpain 1. Porcine calpain 1 was purchased from Calbiochem (CN Biosciences, Notthingam, U.K.).

[1] Abbreviations: ΔF, amplitude of fluorescence changes; DTT, dithiothreitol; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodimide; EGTA, ethylene glycol tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; Ig-domain, immunoglobulin-like domain; I1 to I6, proximal I-band Ig-domains; K_d, dissociation constant; NHS, *N*-hydroxysuccinimide; PAGE, polyacrylamide gel electrophoresis; PEVK, titin-enriched region in PEVK amino acid residues; SDS, sodium dodecyl sulfate.

Scheme 1: Schematic Representation of the Z- and I-Band Region of Titin (Skeletal Isoform)[a]



[a] Calcium and calpain 1 binding sites and N1-line position are indicated in regard to titin organization or below.

Bovine calpain 1 was purified from *Sternomandibularis* muscle as in ref *34*.

Cloning, Expression, and Purification of Titin Fragments. The cDNA for human titin fragment spanning I2 to I6 was donated by I. Richard (Genethon, Evry). Titin fragments corresponding to regions I2−I6, I2−I3, I3−I4, and I5−I6 were obtained by PCR amplification and then cloned into pET 101 TOPO expression vector (Invitrogen) according to the manufacturer's instructions. Primers used are depicted in Table 1. The DNA sequences of positive clones were verified.

Plasmids encoding titin fragments were transformed into competent *Escherichia coli* BL21(DE3) (Invitrogen). The colonies obtained were used to inoculate Luria−Bertani medium containing 100 μg/mL ampicillin. Overnight cultures were then diluted 1:40 in fresh medium, grown to an OD_{600nm} of 0.6 at 37 °C. Expression was induced by adding 1 mM IPTG for 4 h at 37 °C. The cells were collected, resuspended in 50 mL of lysis buffer (50 mM potassium phosphate, pH 7.4, 500 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol, 1% Triton X-100, 40 mM imidazole, 1 mg/mL lyzozyme), and sonicated. The soluble fraction was applied to an 8 mL Ni-Sepharose column (Amersham). After washing with 40 mM imidazole, proteins were eluted successively with 100 and 250 mM imidazole in buffer containing 50 mM potassium phosphate, pH 7.4, 500 mM NaCl, and 5 mM β-mercaptoethanol. Fractions containing recombinant protein as determined by SDS−PAGE and Western blot were pooled and dialyzed against experiment buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, 4 mM EGTA).

Inclusion bodies containing recombinant fragment I5−I6 were solubilized with denaturing buffer including 50 mM potassium phosphate, pH 7.4, 500 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol, 1% Triton X-100, 40 mM imidazole (buffer B), and 6 M guanidine hydrochloride. The extract was then loaded on a Ni-Sepharose column (1×8 cm) equilibrated in 8 M urea in buffer B. The recombinant fragment was eluted with the same imidazole concentration that was used for the more soluble titin fragments. Satisfactory refolding of the purified fragment was then achieved by successive dialysis steps according to Seckler and Jaenicke (*23*) and Rudolph and Lilie (*24*).

Antibodies. Rat polyclonal antibovine calpain 1 was obtained by injection of the native heterodimeric protein (*1*). The monoclonal antibody directed against the C-terminal polyhistidine tag (Invitrogen R932-25) was purchased from Invitrogen (Cergy Pontoise, France).

Electrophoresis and Western Blot Analysis. SDS−PAGE was performed under reducing conditions on a 12.5% slab gel as described by Laemmli (*25*). Molecular masses were estimated by using the Pharmacia low M_r calibration kit. Proteins were revealed using either Coomassie brilliant blue

Table 1: Primers Used for Cloning the Different Titin Fragments Spanning Domains I2−I6, I2−I3, I3−I4, and I5−I6

titin fragments	forward primer	reverse primer
I2−I6	5′-CACCATGCCAAGCAATTGATCACTTTCACAC-3′	5′-CTTCAACTTTGAGTTTGGCAGATGTTTTGGAGG-3′
I2−I3	5′-CACCATGCCAAGCAATTGATCACTTTCACAC-3′	5′-GTTTCATCTTTAATTTACAGGTTGTCTTTTTCCCGTCGA-3′
I3−I4	5′-CACCATGCAGTTGTTGAGTTTGTGAAAGAACTTC-3′	5′-TATAGACAGAGACACGCCCACTGGTGG-3′
I5−I6	5′-CACCATGGTGTGGACGTGATAACACCTC-3′	5′-CTTCAACTTTGAGTTTGGCAGATGTTTTGGAGG-3′

R250 or silver staining. Electrophoresis in nondenaturing conditions was carried out as above on 10% slab gel, but in nonreducing conditions and in the absence of SDS. Immunoblot analyses were carried out as described previously (*26*).

Fluorescent Assays. Assays were conducted using a LS 50B Perkin-Elmer spectrofluorometer by measuring the fluorescence changes of fluorescein isothiocyanate labeled calpain 1 (FITC-calpain 1). Increasing amounts of titin fragments were added to the FITC-calpain 1 (25 μg/mL) in 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM DTT, 4 mM EGTA, 0,6 μM E64, and 100 mM NaCl, and fluorescence was recorded using excitation and emission wavelengths of 495 and 520 nm, respectively.

Binding Assays. Binding assays were carried out with both bovine or porcine calpain 1 and titin fragments (I2−I6, I2−I3, I3−I4, I5−I6) by using solid-phase enzyme-linked immunosorbent assay (ELISA). Bovine calpain 1 was purified from skeletal muscle as in ref *34* and tested in the first experiment to verify whether the origin of the calpain may affect the results. As this was not the case and for facility reasons, only porcine calpain 1 had been subsequently used. Titin fragments (1 μg/mL) in 50 mM Tris-HCl buffer, pH 7.5, 1 mM DTT, 4 mM EGTA, and 100 mM NaCl containing either 12 μM free calcium or without calcium ([Ca] $\leq 2.4 \times 10^{-11}$ M) were immobilized on plastic microtitration plates (Maxisorp, Nalgen Nunc International, Denmark). The plate was then saturated with 5% skimmed milk in PBS buffer, pH 7.4. Incubation with increasing amounts of calpain 1 was performed in 50 mM Tris-HCl buffer, pH 7.5, 1 mM DTT, 0.6 μM E64, 4 mM EGTA, and 100 mM NaCl containing either 500 μM calcium free or no calcium free. Binding assays were monitored at 492 nm using peroxidase-labeled anti-rabbit IgG antibodies (1:2000). Absorbance values were plotted after substraction of nonspecific absorption based upon wells saturated with skimmed milk alone.

Apparent dissociation constant (K_d) determination from ELISA and fluorospectroscopy assays was performed as previously described (*27*). For sigmoidal response of the measured parameter, data were fit to the one-, two-, or three-site models of the nonlinear Hill equation depicted below for calcium titration of titin fragments.

Calcium Titration of the Recombinant Titin Fragments. To avoid calcium contamination, all buffers were prepared with deionized water and previously run three times through a Chelex-100 column (1 × 8 cm) for 1 h each at a low rate of 0.3 mL/min, according to the cleaning procedure reported for aluminum contamination of aqueous solution (*28*). 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. After Chelex-100 treatment of buffer A, no calcium was detected with Quin-2 (*29*). Its concentration in the buffer was therefore assumed to be in the nanomolar range as compared to the control for which the calcium concentration was similarly estimated to be in the micromolar 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 Co., London, U.K.) 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.

The titin recombinant fragments (250 μg/mL) were incubated overnight at 4 °C, under mild continuous stirring, with a few milligrams of Chelex-100 resin prehydrated 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 each recombinant titin fragment was carried out as described previously by Johnson and Tikunova (*30*). Increasing amounts of a 2 M calcium stock solution in buffer A were added to each recombinant fragment (1 μM) and changes in the intrinsic tryptophan fuorescence recorded using a Perkin-Elmer LS50 spectrofuorometer (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-, two-, or three-site models of the nonlinear Hill equation:

$$y = y_{max1}/(1 + 10^{n_1(K_{d1} - pCa)}) + y_{max2}/(1 + 10^{n_2(K_{d2} - pCa)}) + y_{max3}/(1 + 10^{n_3(K_{d3} - pCa)})$$

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

Molecular Mass of the Calcium-Induced Oligomers of the I2−I6 Titin Fragment. The nature of the calcium-induced I2−I6 aggregates was determined by gel filtration of a protein sample preincubated with defined calcium concentrations. After overnight treatment of the I2−I6 recombinant fragment with a few milligrams of the Chelex-100 resin in 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl, 1 mM dithiothreitol (DTT), and 4 mM EGTA (buffer A), the resin was sedimented by centrifugation at 500*g* for 5 min and the supernatant collected. Calcium concentration in the I2−I6 fragment solution was adjusted to the desired value by addition of an aliquot of a 2 M calcium stock solution in buffer A and incubated at room temperature for 30 min under gentle stirring. A sample of each mixture (250 μL) was then

loaded on a Superose 12 HR 10/30 column previously equilibrated in buffer A. Proteins were eluted at a flow rate of 0.3 mL/min and fractions of 0.3 mL 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).

Affinity Chromatography Detection of the Calpain−Titin Complex on a Ni-Sepharose Column. As EGTA cannot be used with Ni-Sepharose, the calcium level was adjusted by pretreatment of all protein solutions and buffers overnight with Chelex 100 as described above. Experiments were performed at room temperature using 25 μg of calpain 1 mixed with 10 μg of I2−I6 in binding buffer A in which EGTA was omitted. The presence of EGTA could indeed strip nickel ion from the chelating group coupled to agarose and was therefore omitted in the binding buffer used in the present experiment. After an incubation of 60 min at room temperature of calpain 1 with the I2−I6 fragment, 50 μL of a Ni-Sepharose suspension (dynamic binding capacity 40 μg of His-tagged protein/μL of medium) (Amersham Biosciences) was added and the mixture kept at room temperature for 60 min before sedimentation of the gel by centrifugation at 500*g* for 5 min. The pelleted gel was washed three times with 4 volumes of the incubation buffer and gently mixed for 5 min. Bound proteins were eluted from the gel by addition of 2 volumes of buffer A containing 250 mM imidazole. The medium was sedimented by centrifugation and the supernatant collected. The elution step was repeated three times, and each supernatant collected was saved for SDS−PAGE and immunoblotting analysis.

Protein Determination. Protein concentrations were measured by using Bradford's method (Bio-Rad protein assay) with rabbit immunoglobulin as the standard (*31*).

RESULTS

Expression and Purification of the Recombinant Titin Fragments. For cloning of the I2−I6 fragment, primers were defined according to the sequence of each domain provided in the I-band genomic sequence of the whole titin gene (Web site: http://www.embl-heidelberg.de/ExternalInfo/Titin/genomic/I-Band.html). Cloning of the titin region comprising exons 29 to 32 encoding domains I2 to I6 was carried out using primers 1 (forward primer 5′-CACCATGCCAAGCAATTGATCACTTTCACAC-3′) and 2 (reverse primer 5′-CTTCAACTTTGAGTTTGGCAGATGTTTTGGAGG-3′). The cloned cDNA was sequenced and translated to protein and the deduced protein sequence analyzed for the occurrence of all expected Ig-like domains using the ScanProsite tool. All domains were well identified according to the sequences available in the Swiss-Prot database (features of ID Q8WZ42) except the C-terminal end of the polypeptide comprising the last 89 amino acids. From the different analysis performed, we concluded that this error resulted from the identification of the linker between I5 and I6 as an Ig-domain designed I6 (for more details, see the Supporting Information provided with this paper). Hence, our fragment contains in fact full-length I2 to I5 domains and the I5−I6 linker. For a purpose of clarity and a simplification of the denomination, this fragment was subsequently referred to as I2−I6 and the designed I5−I6 subfragment contains only the I5-domain plus the I5−I6 linker.

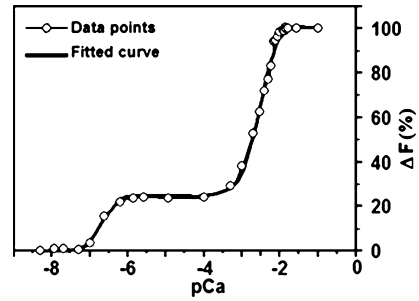


FIGURE 2: Calcium titration of the I2−I6 titin fragment using the intrinsic tryptophan fluorescence as the marker of the induced conformational changes. Incubations were carried out in 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl, 1 mM dithiothreitol (DTT), and 4 mM EGTA. Various amounts of calcium were added to the I2−I6 titin fragment (1 μM), and fluorescence was measured. The data points were fitted to the two-site models of the nonlinear Hill equation and K_d values determined: $K_{d_1} = 1.9 \pm 0.09 \times 10^{-7}$ M; $K_{d_2} = 3.3 \pm 0.8 \times 10^{-3}$ M.

All recombinant fragments were expressed and purified by chromatography on a Ni-chelating Sepharose column as described in the Materials and Methods. Purified fragments appeared homogeneous as assessed by SDS gel electrophoresis (Figure 1). The molecular masses determined by SDS−PAGE under reducing conditions were about 50 kDa for the I2−I6 fragment and 23, 22, and 30 kDa for tandems I2−I3, I3−I4, and I5−I6, respectively.

Calcium Titration of the Titin I2−I6 Recombinant Fragment and Ca^{2+}-Induced Polymerization. Previous findings strongly suggested that calpain 1 binds to the N1-line region of titin in a calcium-dependent manner (*1*). To assess whether calcium effect occurs through its binding to either calpain 1 or titin or both, calcium titration of the I2−I6 titin fragment was carried out by measuring changes in intrinsic tryptophan fuorescence as previously described (*22*).

Using Ca^{2+} concentrations ranging from 10^{-11} to 0.1 M, calcium titration of the I2−I6 titin recombinant fragment provided a biphasic curve (Figure 2). The first half-maximal binding was observed between Ca^{2+} concentrations of about 10^{-7} and 10^{-6} M followed by a plateau between 10^{-6} and 10^{-4} M and a second half-maximal binding between 10^{-4} and 0.1 M.

The fluorescence data fitted well to the two-site models of the nonlinear Hill equation providing two K_d values of $1.9 \pm 0.09 \times 10^{-7}$ M and $3.3 \pm 0.8 \times 10^{-3}$ M (mean values ± SD for three independent determinations). The estimated Hill coefficient values indicate a significant cooperativity between titin molecules in calcium binding for the first high-affinity binding site ($n = 2.3$) and no cooperativity for the low-affinity binding site. It is worthy to note that the K_d values of titin for calcium are much lower than that reported for calpains. Calpains indeed were assumed to exhibit K_d values of approximately 25 μM for calpain 1 and 325 μM for calpain 2 (*20*), suggesting that, in physiological conditions, titin will be saturated with calcium much earlier than calpain 1. Assuming that all Ig-domains are equivalent, the present results would suggest either the presence of two different binding sites/Ig-domains or a Ca^{2+}-induced ordered oligomeric organization of titin with higher affinity for this cation as previously observed for the Z9-I1 recombinant titin fragment (*22*). Within this titin fragment, Ig-domains are indeed structurally closely related and showed neither Glu-

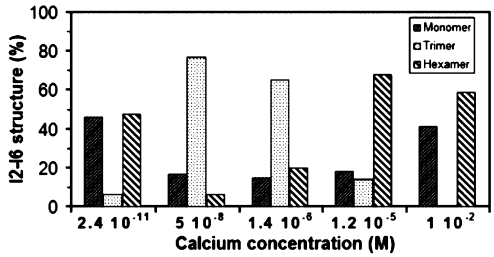


FIGURE 3: Identification by gel filtration on a Superose 12 column of the different I2−I6 titin oligomers induced by calcium. For each calcium concentration the percentage of each structure is indicated.

rich regions nor EF-hand structures, suggesting that calcium binding takes place through a cooperative interaction of the cation with Ig-domains from different strands as previously suggested for the Z9-I1 titin fragment (*22*). Such feature has been reported for the PEVK region of titin which is prone to be more flexible in the presence of calcium (*32*).

Here, only the second hypothesis was therefore tested, and the identity of the various polymeric forms was assessed through the M_r determination of titin mixtures incubated with various amounts of calcium corresponding to the major transition points noticed from the titration curve. The Ca^{2+} concentrations selected were 10 mM, 1.2×10^{-5} M, 1.4×10^{-6} M, 5×10^{-8} M, and 2.4×10^{-11} M.

According to the data of Figure 3, only three types of I2−I6 organization including monomers, trimers, and hexamers were found at all calcium levels tested with M_r of about 60, 180, and 360 kDa as assessed by gel filtration on a Superose 12 column. For a given calcium concentration, the percentage of each type of I2−I6 oligomer was plotted. At very low Ca^{2+} level (2.4×10^{-11} M), the majority of the I2−I6 titin fragment is either in a monomeric (46%) or in an hexameric (48%) form. Upon raising the calcium concentration to the first transition point (5.0×10^{-8} M), the major form detected is the trimer, and this is also true for the next calcium concentration, i.e., 1.4×10^{-6} M, corresponding the end of this transition. At higher calcium levels, the trimer decreases significantly, and the major forms detected were the hexamer complex and the monomeric form. The trimer is totally absent at the highest calcium concentration tested (0.01 M). From these findings, it was concluded that, in aqueous phase, calcium governed the spatial organization of the titin I2−I6 fragment.

Does the I2−I6 Titin Fragment Contain the Calpain 1 Binding Site? This was the first question to be addressed before detailed investigation of the interaction process, and this was done by affinity chromatography of the complex using Ni-Sepharose gel. The recombinant I2−I6 titin fragment was thus tagged with histidine at its N-terminal end, allowing its purification by affinity chromatography on a Ni-Sepharose column. This property was used to test the binding of calpain 1 to I2−I6. If they bind to each other, the complex might be retained on the column and eluted with imidazole as this was done for the elution of the I2−I6 fragment alone. It is worthy to note that, as EGTA cannot be used to control the calcium concentration, the present experiment was carried out with protein solutions and buffers pretreated with Chelex 100. As shown in Figure 4a, when calpain alone was loaded on the column, the whole protein was recovered in the unretained fraction (Figure 4a, lanes 1−4), and nothing was

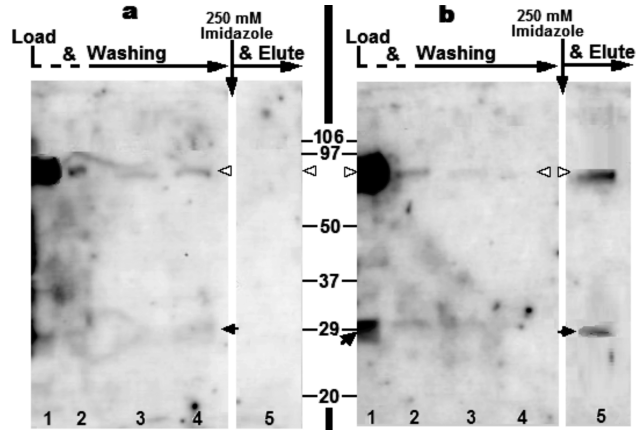


FIGURE 4: Affinity chromatography fractionation of the calpain 1/I2−I6 complex using Ni-Sepharose gel in the absence of calcium as assessed by Western blot using the rat polyclonal anti-calpain 1 antibody. Fractionation was performed in batch. 50 μL of the gel suspension was added to calpain 1 (25 μg/mL) alone (a) and a mixture of calpain 1 (25 μg/mL) and I2−I6 titin fragment (10 μg/mL) preincubated for 60 min at room temperature (b) in a total volume of 200 μL. After 60 min at room temperature, the gel was decanted by centrifugation for 5 min at 500*g*. The gel was washed three to four times with 2 volumes of the initial buffer, and each supernatant was saved and analyzed by SDS−PAGE. Bound proteins were then eluted with the same buffer containing 250 mM imidazole. The resin was washed again several times with the same buffer and sedimented, and the supernantants were saved for SDS−PAGE analysis. Lanes: 1, unretained proteins revealed with the rat calpain 1 antiserum; 2−4, washing supernantants; 5, fraction eluted with 250 mM imidazole and polled altogether.

obtained upon elution with 250 mM imidazole (Figure 4a, lane 5) as revealed by Western blot with the calpain 1 antibody raised against the dimeric form. By contrast, when a preincubated mixture containing I2−I6 and an excess of calpain 1 was run on the same column, the excess of calpain 1 was recovered in the unretained fraction (Figure 4b, lanes 1−4) whereas the I2−I6 bound calpain 1 was eluted with 250 mM imidazole. Interestingly, upon dissociation with 250 mM imidazole of the tagged I2−I6 recombinant fragment preincubated with calpain 1 from the column, we noted the coelution of both calpain subunits (30 and 80 kDa) as revealed by Western blot (Figure 4b, lane 5). This result clearly demonstrates that the calpain 1 dimer is not dissociated when bound to titin and stressed that titin binds calpain 1 in its native form. Cosedimentation (with both anti-His-tag and anti-calpain 1 antibodies) and chemical cross-linking (with the zero-length bifunctional reactant EDC/NHS) experiments confirmed these findings (not shown).

Calpain 1 Binding to I2−I6 at Different Calcium Concentrations. Binding of calpain 1 to the I2−I6 titin fragment in liquid phase was followed by measurement of the changes in the fluorescence (ΔF) of FITC- labeled calpain 1 (25 μg/mL = 2.3×10^{-7} M) upon addition of increasing amounts of the titin fragment. The experiment was done in the presence of three different calcium concentrations corresponding to the previously established transition points. All profiles shown in Figure 5 indicate that the plot of ΔF versus I2−I6 titin fragment concentration is polyphasic.

At the lowest calcium level (2.4×10^{-11} M), a triphasic increase in the ΔF was observed upon addition of increasing levels of the titin fragment. Fluorescence of calpain 1-FITC starts to rise at a I2−I6 level of about 1×10^{-8} M up to

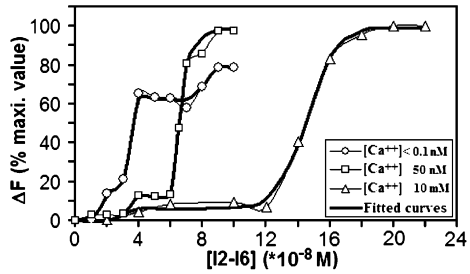


FIGURE 5: Interaction of the I2−I6 titin fragment with FITC-conjugated calpain 1 as assessed by fluorescent assay. Various amounts of I2−I6 titin fragment were added to calpain 1 (25 μg/mL) in 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM DTT, 4 mM EGTA, 0.6 μM E64, and 100 mM NaCl, and changes in the peptidase fluorescence were recorded. Incubations were carried out in the presence of different concentrations of calcium as indicated. For the calcium concentration noted <0.1 nM, the residual concentration was estimated to be 2.4×10^{-11} M. The data points were fitted to the two- or three-site models of the nonlinear Hill equation and K_d values determined (see text for more details and for K_d values).

about 15% of the maximum value. This soft increase was followed by a sharp one up to 65% of the maximum value. A slight decrease in the ΔF value of about 10−12% between I2−I6 concentrations of 4 and 7×10^{-8} M was then noticed, a feature observed in all experiments. This limited drop in the fluorescence of the FITC-labeled calpain 1 suggests a release of the peptidase concomitantly with a reorganization of the I2−I6 complex. The ΔF values increased again thereafter up to 78% of the maximum value obtained at higher calcium concentrations, suggesting that, at low Ca^{2+} levels, the I2−I6 complex cannot reach its optimum calpain 1 binding capacities. Fitting this curve with the three-site models of the nonlinear Hill equation provided K_d values of 18 ± 2 nM for the first transition T1, 33 ± 4 nM for the second transition T2, and 77 ± 9 nM for the last transition T3 (mean values ± SD for three independent determination), respectively.

When incubation was performed in the presence of 50 nM calcium, binding of calpain 1 to titin starts at higher levels of I2−I6 fragment, and two phases can be distinguished. A first rise in ΔF occurs between 3 and 4×10^{-8} M followed by a plateau between 4 and 6×10^{-8} M and a sharp increase up to the maximum value (100%) between 6 and 9×10^{-8} M. It is worthy to note that the ΔF value corresponding to an I2−I6 concentration of 8×10^{-8} M is often out of the fitted curve, a feature observed in most of our experiments with 50 nM calcium. Dissociation constants determined by fitting the curve with the two-site models of the nonlinear Hill equation were 29 ± 4 nM and 65 ± 7 nM, respectively. It must be emphasized that the plateau between 2 and 3 μg/mL corresponds exactly to the second pseudoplateau observed previously at a calcium concentration of 2.4×10^{-11} M, suggesting that similar events affect the I2−I6 complex at these concentrations. This would also suggest a series of the I2−I6 titin complex reorganization occurring at defined concentrations of the fragment, an assumption supported by the concomitant appearance of the different phases and the decrease in the corresponding ΔF values upon increasing the calcium concentration in the mixture. For example, the ΔF value decreased from 78% at the lowest Ca^{2+} concentration to 5−6% at 50 nM calcium. Similarly, the ΔF value increased from 78% to 100% at an I2−I6 concentration of 9×10^{-8} M. In addition, the first phase, between 0.5 and 1 μg/mL, disappeared at 50 nM calcium. Increasing calcium concentration from 50 to about 650 nM (end of this transition) did not change the K_d value but led to a progressive lowering of the plateau value between 4 and 6×10^{-8} M μg/mL of titin and an alignment on the fitting curve of the ΔF value at 4 μg/mL (not shown).

Table 2: Fluorescent Assay of Calpain 1/Titin I2−I6 Interaction: Dissociation Constants (nM) Determined in the Presence of Various Amounts of Calcium

	K_d values for each transition[a] (nM)		
	2.4×10^{-11} M Ca	5×10^{-8} M Ca	1×10^{-2} M Ca
transition 1	18 ± 2	ND[b]	ND
transition 2	33 ± 4	29 ± 4	30 ± 5
transition 3	77 ± 9	65 ± 7	ND
transition 4	ND	ND	140 ± 7

[a] K_d values: mean ± SD for at least three independent experiments. [b] ND: not detected.

Table 3: Solid-Phase Assay (ELISA) of Calpain 1/Titin I2−I6 Interaction: Dissociation Constants and Maximum Absorbance Values Determined in the Presence of Various Amounts of Calcium

	calcium (M)			
	2.4×10^{-11}	5×10^{-8}	12×10^{-6}	1×10^{-3}
K_d (nM)	210	270	320	290
$A_{492nm,max}$	2.45	2.83	3.21	2.95

For calcium concentrations ranging from 1 to 10 mM, a similar biphasic profile was obtained. The first rise in the ΔF to about 3−4% of the maximum value was noted between I2−I6 titin levels of $2-4 \times 10^{-8}$ M. This step was followed by a long plateau ($4-12 \times 10^{-8}$ M) and a sharp increase thereafter above 12×10^{-8} M. Dissociation constants determined by fitting the curve with the two-site models of the nonlinear Hill equation were 30 ± 5 nM and 140 ± 7 nM, respectively. From these findings it can be stressed that calpain 1 binding to the titin I2−I6 fragment is optimal at an I2−I6 concentration of 9×10^{-8} M and a calcium level very likely centered around the first K_d value of 190 nM previously found upon calcium titration of this titin fragment. Moreover, values of the dissociation constants (K_d) summarized in Table 2 indicate that, in the presence of various amount of calcium, transitions always occurred at similar I2−I6 concentrations and shifted toward higher values upon increasing the calcium level in the mixture. The present shift of the K_d values originated very likely from changes in the polymeric organization of the titin fragment, an organization which depends not only upon the calcium level but also upon the titin concentration.

Calpain 1 binding to the I2−I6 segment was also investigated using solid-phase assays (ELISA). In these assays, calpain 1 binding to coated I2−I6 was carried out in the presence of various amounts of calcium ranging from 2.4×10^{-11} M to 10 mM (Table 3). Coating of calpain 1 and addition of increasing amounts of I2−I6 were not successful since the labeling of the titin fragment with the anti-His-tag antibody was highly variable. Only the first assay was performed in the present work. For this assay, dissociation constants and maximum absorbance values are depicted in Table 3. According to these data, the different calcium concentrations tested cause only very few changes in the K_d values which ranged between 210 and 320 nM and that these

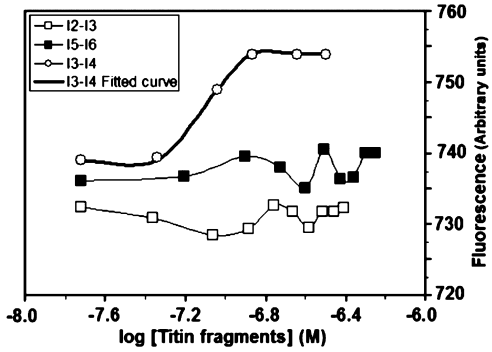


FIGURE 6: Interaction of the I2−I6 titin subfragments with FITC-conjugated calpain 1 as assessed by fluorescent assay. Various amounts of the titin subfragment (Ig-tandem I2−I3, I3−I4, and I5−I6) were added to calpain 1 (25 μg/mL) in 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM DTT, 4 mM EGTA, 0.6 μM E64, and 100 mM NaCl, and changes in the peptidase fluorescence were recorded. The sigmoidal curve obtained with the I3−I4 subfragment was fitted to the one-site models of the nonlinear Hill equation and the K_d value determined: $K_d = 89$ nM.

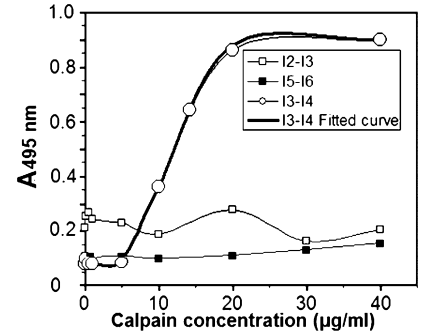


FIGURE 7: Interaction of the I2−I6 titin subfragments with FITC-conjugated calpain 1 as assessed by solid-phase assay (ELISA). Various amounts of calpain 1 were added to coated I2−I3, I3−I4, or I5−I6 titin recombinant fragments (1 μg/mL) in 50 mM Tris-HCl buffer, pH 7.5, 1 mM DTT, 4 mM EGTA, and 100 mM NaCl.

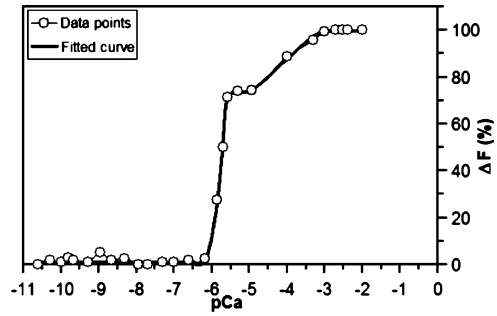


FIGURE 8: Calcium titration of the I3−I4 titin fragment using the intrinsic tryptophan fluorescence as the marker of the induced conformational changes. Incubation were carried out in 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl, 1 mM dithiothreitol (DTT), and 4 mM EGTA. Various amounts of calcium were added to the titin fragment (1 μM), and fluorescence was measured. The data points were fitted to the two-site models of the nonlinear Hill equation and K_d values determined: $K_{d_1} = 1.58 \pm 0.05 \times 10^{-6}$ M; $K_{d_2} = 1.40 \pm 0.07 \times 10^{-4}$ M.

are about 10-fold higher than in liquid-phase assays. Similarly, the maximum absorbance values (A_{492nm}) are only moderately affected by calcium, and all ranged between 2.4 to 3.2 absorbance units. This contrasted with the greater variability in the dissociation constant observed previously in liquid-phase assays. One of the major reasons for these differences is very likely that, when coated into the well, the protein, i.e., the I2−I6 titin fragment, is subjected to important constraints impairing any structural adaptation/modification of the polymeric or nonpolymeric forms.

Identification of the Calpain 1 Binding Site within the I2−I6 Titin Fragment. This was achieved by investigation of calpain 1 binding to different recombinant tandem subdomains of I2−I6 including tandems I2−I3, I3−I4, and I5−I6. The tandem I4−I5 was excluded since, because of its high insolubility, we did not succeed to purify it with a sufficient yield despite the drastic conditions used. Note that this comparative study was carried out with no added calcium and an EGTA concentration of 4 mM indicative of a Ca^{2+} level lower than 2.4×10^{-11} M.

Study of the interaction was first performed by addition of various amounts of each tandem domains to a FITC-conjugated calpain 1 solution and measurement of the changes affecting the fluorescence of the labeled peptidase. As shown in Figure 6, whereas quite no change in the fluorescence was observed upon addition of the tandems I2−I3 and I5−I6, a significant increase in the calpain 1 fluorescence occurred upon addition of increasing amounts of the tandem I3−I4. As the tandem I2−I3 did not affect the calpain 1 fluorescence, it can be concluded that calpain 1 binds to domain I4 with a very low K_d value of 89 ± 1 nM, suggesting an affinity of calpain 1 to the Ig-domain I4 wholly comparable to that observed for the I2−I6 fragment in the absence of calcium and for the last transition T3 (K_d = 77 nM) (Table 2).

Similar results were obtained by solid-phase ELISA analysis of the interaction (Figure 7). Addition of various amounts of calpain 1 to the coated titin fragments led to an increase in the absorbance only in the presence of the tandem I3−I4. By contrast, no modification of the absorbance value was noted when tandems I2−I3 or I5−I6 were coated in the wells, indicating that these fragments did not bind calpain 1. Calpain 1 tight binding to I3−I4 occurred with a K_d value similar to that observed in the previous experiment (K_d = 98 ± 2 nM). These findings confirmed that, within the I2−I6 titin fragment, calpain 1 binds specifically to I4 and did not interact with the other Ig-domains. It must be also emphasized that calpain 1 binding to I4 is as strong as to the longer I2−I6 fragment.

Influence of Calcium on Calpain 1/I3−I4 Interaction. We previously showed that calcium is an essential modulator of the titin/calpain 1 interaction. Therefore, the first question arising is to know whether the calcium effect will be similar with that observed for titin fragment I2−I6. As for the I2−I6 fragment, calcium titration of I3−I4 was performed using the intrinsic Trp fluorescence as an index of the conformational changes. As shown in Figure 8, a biphasic change in the intrinsic fluorescence wholly comparable with the I2−I6 fragment was obtained. The curve was fitted with the two-site models of the Hill equation to determine the dissociation constant for the two binding sites. Half-maximal binding was found to occur at pCa values of about −5.8 and −3.5 for the first and second transitions, respectively. These correspond to dissociation constants of $1.58 \pm 0.05 \times 10^{-6}$ for the highest affinity binding transition and $1.40 \pm 0.07 \times 10^{-4}$ for the low-affinity binding transition. As compared to the I2−I6 fragment, whereas the highest affinity binding site for I3−I4 showed approximately a 10-fold higher K_d value

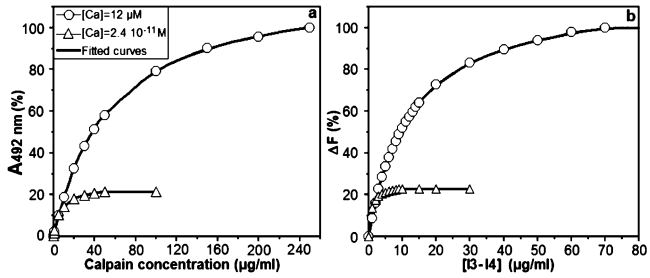


FIGURE 9: Influence of calcium on calpain 1/I3−I4 interaction as assessed by ELISA (a) and fluorescent (b) assays in the absence ([Ca] $\leq 2.4 \times 10^{-11}$ M) or the presence of 12 μM calcium.

($K_d = 1.9 \times 10^{-7}$ for I2−I6), the low-affinity binding exhibited approximately a 20-fold lower dissociation constant ($K_d = 3.3 \times 10^{-3}$ for I2−I6). The affinity for calcium was therefore higher for I2−I6 at lowest calcium levels while the reverse is observed at higher calcium levels in the millimolar range. According to the findings obtained by gel filtration for I2−I6, this difference might originate from the nature of the calcium-induced complex formed by each fragment and their compactness as well.

Calpain 1/I3−I4 interaction was tested in the absence of calcium and then in the presence of 12 μM calcium, a concentration corresponding to the middle of the plateau between the two low- and high-affinity binding sites (Figure 9a). In solid-phase assays (ELISA) and in the absence of calcium, addition of increasing calpain 1 led to a rapid saturation of the binding sites with a K_d value of $5.8 \pm 0.2 \times 10^{-8}$ M. By contrast and although with a lower affinity ($K_d = 5.1 \pm 0.2 \times 10^{-7}$ M), in the presence of 12 μM calcium, the I3−I4 fragment showed a higher propensity to bind calpain 1 since about 5-fold more calpain 1 is bound in these conditions (1.39 versus 0.26 absorbance units). It is therefore clear that, as for the I2−I6 titin fragment, the presence of physiological concentration of calcium improved significantly the calpain 1 titin binding capacity even with very short fragment such as I3−I4.

Liquid-phase assays confirmed these findings and clearly demonstrate the significantly higher calpain 1 binding capacity of the I3−I4 titin fragment in the presence of calcium as compared to the results obtained in its absence (ΔF of 12 versus 56) (Figure 9b). The K_d values are closely similar to those obtained using the solid-phase assay, i.e., 3.6×10^{-8} M versus 5.8×10^{-8} M in the absence of calcium and 5.7×10^{-7} M versus 5.1×10^{-7} M in its presence.

DISCUSSION

Ubiquitous calpains 1 and 2 also called μ- and m-calpains with reference to their different calcium requirements are calcium-dependent peptidases widely expressed in mammalian tissues (*2*−*4*). Despite the large set of investigations on these peptidases, their intracellular localization in skeletal muscle has long been controversial (*14*−*16*). Raynaud et al. (*1*) recently localized calpain 1 at the level of the N1- and N2-lines of the sarcomeres, two regions known to bind calcium and further showed that this binding was calcium dependent. These two transversal structures also bind tightly and quite irreversibly calcium ions (*1, 18, 19*). In the present work, we addressed therefore the following questions: (a) what is the exact binding site of calpain 1 in the N1-line region of the titin molecule, and (b) what is the precise role of calcium in this interaction since both proteins are able to bind this cation?

Previous findings stressed that calpain 1 binds very likely to a titin region running from Ig-domains I2 to I6 (*1*). For the reasons indicated in the Supporting Information, the present work was carried out on a titin proximal fragment comprising domains I2 to I5 and the I5−I6 linker. Preliminary experiment using the whole segment confirmed that the calpain 1 binding site was present in the cloned fragment. All subsequent investigations were performed on this segment and a series of cloned subfragments (I2−I3, I3−I4, and I5−I6). The titin segment is composed of 448 amino acids running from ^{2171}KQLITFTQEL... to....TSGKLTVA^{2618} according to the protein sequence provided at the following Web site: http://ca.expasy.org/uniprot/Q8WZ42.

As calcium is the primary effector of the calpain 1/titin interaction, its ability to bind to titin was tested. Calcium was found to bind tightly ($K_d = 1.9 \times 10^{-7}$ M) to the titin I2−I6 fragment causing its oligomerization with a large prevalence of trimers at calcium concentrations ranging from 10^{-6} to 10^{-8} M (up to 80% of the different oligomers). Either above or below this range, mainly hexamers and monomers were obtained. Such calcium-induced conformational changes have been noticed for the PEVK region of titin; these changes affect titin's mechanical properties and more specially its stiffness (*32*). Calcium affinity to the I2−I6 segment is further very close to that reported for the PEVK elastic region of titin ($K_d = 9 \times 10^{-7}$ M) (*21*). The calcium affinity to titin is wholly comparable to the high-affinity binding sites of the skeletal muscle troponin C ($K_d \approx 2.1 \times 10^{-7}$ M). Moreover, intracellular calcium ion concentration is believed to range approximately from 10^{-8} to 10^{-6} M. According to this well-recognized statement, we might consider that, within the cell, the proximal N1-line region of titin would be always under a trimer form. In addition, because of the low calcium concentrations of concern, the suggested regulation of titin oligomerization by calcium is very likely of physiological significance.

How the six titin strands emanating from the end of each myosin filament are organized within the I-band is still enigmatic and far from being clear. Near the end of the thick filament, six titin molecules are associated into a single bundle, the end filament (*35, 36*). Nothing is known about the organization of titin in the middle of the I-band, and near the Z-disk, the molecules are associated with thin filaments (*37, 38*). But where this interaction takes place in the thin filament, what are the titin Ig-domains concerned, and what is the strongness of this interaction are unsolved questions. Moreover, although a ratio of 3:1 has been proposed (*39*), the actin filament/titin strands ratio is still controversial and not yet definitely established. Hence, the state of titin strands in the vicinity of the Z-line is opened, and a pack similar to that found in the end filaments cannot be excluded. Whether strands are packed in dimer or trimer or more should be a fruitful area of future investigation. For a group of six strands, the present findings would suggest that they are packed into trimers, a feature in good agreement with the titin strands/actin filaments ratio of 3:1 previously reported (*39*). Such strand packing can also contribute significantly to the rigidity of this portion of titin which maintains a constant length upon sarcomere stretching (*40*). In the present study, titin aggregation was found to be triggered by physiological levels of calcium. By contrast, interaction of this titin region with actin filaments seemed to

be not calcium dependent (*41*). In a previous work we reported the aggregation of the Z9-I1 titin fragment by very low levels of calcium ($K_d \sim 10^{-11}$ M) (*22*). This led to the question on whether titin enters the Z-band as individual strands or as packs of associated strands. Such a feature has been mentioned for the end filament which is located close to the myosin filament (*35, 36*). Hence it is obvious that quite nothing is known about the titin strands organization within the sarcomere and all experimentally demonstrated hypotheses will be helpful to fill these gaps.

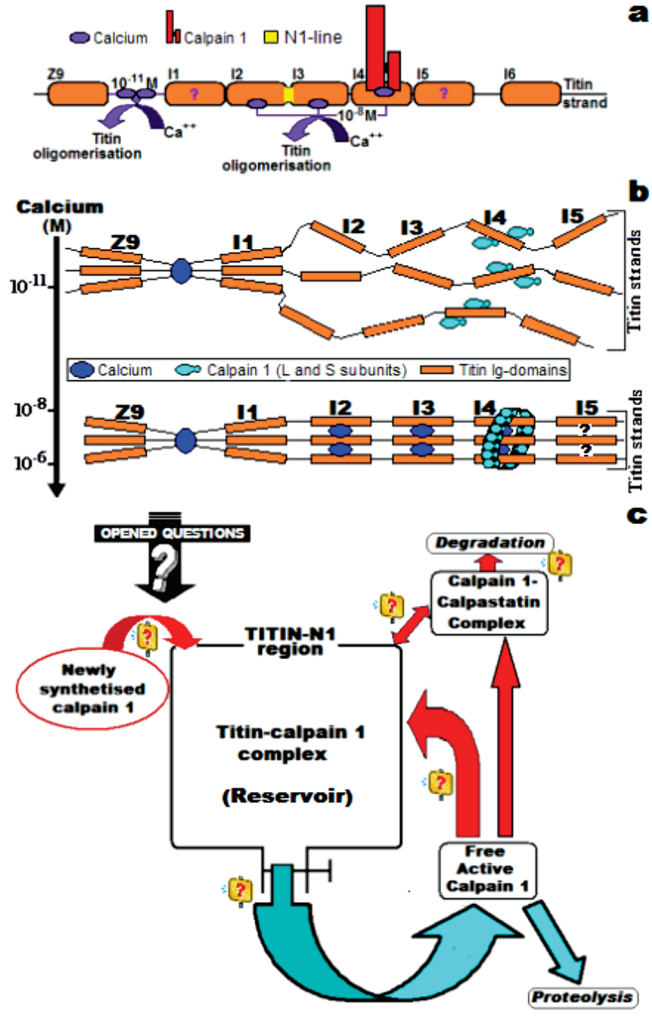
Interaction of calpain 1 with the I2−I6 titin segment was then investigated in the presence of different calcium concentrations selected according to the dissociation constant of calcium binding to this titin fragment. Optimum calpain 1 binding to titin was achieved at calcium concentrations corresponding to the first transition observed upon calcium titration of I2−I6, i.e., approximately 1.9×10^{-7} M. At this Ca^{2+} concentration maximum binding of calpain 1 to titin was observed for a concentration of the fragment of about 4.5 μg/mL, i.e., 6×10^{-8} M. At very low calcium level ([Ca] $\leq 2.4 \times 10^{-11}$ M) the amount of calpain 1 bound to the titin segment did not exceed 20% of the maximum value achieved in the above conditions.

The identification of the binding site within this titin segment was then carried out using different recombinant subfragments including I2−I3, I3−I4, and I5−I6. Results obtained using solid- (ELISA) and liquid- (fluorescence of FITC-labeled calpain 1) phase assays clearly demonstrate that calpain 1 binds undoubtedly to the I4 Ig-domain with an affinity comparable to that observed for the whole I2−I6 fragment (Scheme 2a). Calpain 1 binding is also regulated by calcium in a similar way than for the I2−I6 segment, a cation inducing an oligomerization of the subfragment I3−I4. The present high-affinity binding to titin is good agreement with previous findings indicating that this bound calpain is not extractable even in drastic conditions (*33*).

According to the present findings and to previous ones showing the pseudoirreversible binding of calcium to the linker segment between domains Z9 and I1 (*22*), titin strands would be strongly associated by very low concentrations of calcium ($\leq 10^{-11}$ M) at the level of the Z9-I1 linker. As shown in Scheme 2b, in such conditions, each I2−I5 segment could behave freely, and each of them will bind tightly small amounts of calpain 1. Increasing calcium concentration to about $10^{-8}-10^{-6}$ M induced an ordered oligomerization of the I2−I5 segments, a complex able to bind about 5-fold more calpain 1 at the level of the I4 Ig-domain.

At physiological levels of calcium (about 10^{-8} M) much lower than the level needed for calpain 1 saturation ($K_d \approx 25$ μM), the amount of bound calpain is therefore relatively important, suggesting that a major part of the total enzyme present in the cell is under the bound state. Bound calpain 1 might be considered as a reservoir from which the peptidase is delivered according to the cell requirements. Such assumption agrees well with the proposal that up to 40% of the total calpain 1 is bound to myofibrils (*33*). This hypothesis is depicted in Scheme 2c together with the inherent questions related to several key points of this global scheme and illustrated by question marks. How is the freshly synthesized protein translocated to its titin binding sites (N1- and N2-lines)? Does the calpain 1 primary sequence contain a targeting signal? How is the protein released from its binding sites? Can the releasing process be reversible? The schematic diagram presented in Scheme 2c therefore emphasized the absolute needs for further research on calpain 1, its translocation

Scheme 2: Prospective Analysis of the Outputs of the Present Findings[a]



[a] (a) Definitive localization of calpain 1 in the proximal N1-line region of titin showing the binding of the peptidase with the Ig-domain I4 of titin and not with any other domains among the I2−I6 recombinant fragment used in the present work. This schematic diagram indicates also the calcium binding site (Z9-I1 linker and domains I2 to I4) identified so far and emphasizes the role of calcium in the oligomerization of the titin fragment noted at 10^{-11} and 10^{-8} M calcium. (b) Spatial organization of the proximal segment of titin in the presence of increasing calcium concentration ranging from 10^{-11} to 10^{-6} M. At the lowest calcium concentration, titin strands showed more freedom and bind small amounts of calpain with a high affinity. Increasing calcium levels to $10^{-8}-10^{-6}$ M implies an ordered organization of the titin strands into trimers with a probably higher rigidity and a calpain 1 binding capacity about 5-fold greater. (c) Pseudoirreversible binding of calpain 1 to titin led us to consider that the bound peptidase may constitute a reservoir from which the enzyme would be delivered to the cell on request. Each question mark relates to an unanswered question including the transport of the freshly synthesized enzyme to its titin binding site, the mode of release of calpain 1 from the reservoir and the potential reversibility of this process, and, finally, which is to become to the active free enzyme bound or not to its specific inhibitor.

to titin, its release from its binding site, and the identity of the protein effectors involved in these processes.

SUPPORTING INFORMATION AVAILABLE

Primers used for cloning the I2−I6 titin fragment were defined according to the I-band genomic sequence of the whole titin gene provided at the Web site http://www.emblheidelberg.de/ExternalInfo/Titin/genomic/I-Band.html. After sequencing of the cDNA, the deduced protein sequence was analyzed for its Ig-domain content, and this revealed an error in the identification of Ig-domains in this region of titin within the I-band titin gene. In fact, the linker between I5 and I6 was assigned to Ig-domain 6 and the Ig-domain 6 sequence assigned to Ig-domain 7. The Supporting Information provides the correct Ig-domain distribution in the proximal region of the titin I-band segment. This material is available free of charge via the Internet at http://pubs.acs.org.

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