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Differential regulation of the calpain–calpastatin complex by the L-domain of calpastatin

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ABSTRACT

Here we demonstrate that the presence of the L-domain in calpastatins induces biphasic interaction with calpain. Competition experiments revealed that the L-domain is involved in positioning the first inhibitory unit in close and correct proximity to the calpain active site cleft, both in the closed and in the open conformation. At high concentrations of calpastatin, the multiple EF-hand structures in domains IV and VI of calpain can bind calpastatin, maintaining the active site accessible to substrate. Based on these observations, we hypothesize that two distinct calpain–calpastatin complexes may occur in which calpain can be either fully inhibited (I) or fully active (II). In complex II the accessible calpain active site can be occupied by an additional calpastatin molecule, now a cleavable substrate. The consequent proteolysis promotes the accumulation of calpastatin free inhibitory units which are able of improving the capacity of the cell to inhibit calpain. This process operates under conditions of prolonged [Ca^{2+}] alteration, as seen for instance in Familial Amyotrophic Lateral Sclerosis (FALS) in which calpastatin levels are increased. Our findings show that the L-domain of calpastatin plays a crucial role in determining the formation of complexes with calpain in which calpain can be either inhibited or still active. Moreover, the presence of multiple inhibitory domains in native full-length calpastatin molecules provides a reservoir of potential inhibitory units to be used to counteract aberrant calpain activity.

1. Introduction

Calpains are proteins that belong to a family of Ca^{2+}-dependent cysteine proteases and are ubiquitously distributed and localized in the cytosol along with their endogenous inhibitor calpastatin [1–4]. These proteases have been widely studied as they regulate the function of specific proteins by selective cleavage in response to calcium signaling [5–9].

Abnormal activity of calpain has been associated with several pathological conditions characterized by alterations in Ca^{2+} homeostasis [10–15]. Because efficient $[Ca^{2+}]_i$ control is crucial to the physiological function of the calpain/calpastatin system, special efforts have been directed at characterizing the mechanisms that control calpain activity and the conditions that promote aberrant calpain function [16–20]. Occurrence of the transition to abnormal function in the presence of persistent $[Ca^{2+}]_i$ alteration, indicates that calpain proteolysis is not sufficiently regulated by calpastatin inhibition. Furthermore, co-localization in the same cellular compartment of calpain and of a large excess of calpastatin, makes it difficult to explain how calpain-mediated proteolysis can normally occur, pointing to the existence of mechanisms that allow calpain to escape from inhibition by calpastatin.

It is known that, following $[Ca^{2+}]_i$ increase, calpastatin diffuses in the cytosol to control the amount of calpain that translocates to the plasma membranes. Because calpastatin remains confined in the cytosol, the membrane-associated calpain can undergo activation. Therefore, membranes are generally considered the preferential site of calpain activity [1–4,21,22].

Another mechanism that controls the amount of inhibitor available for interaction with calpain involves phosphorylation of calpastatin at the L-domain [23–26]. This process affects the inhibitory efficiency and selectivity of calpastatin, and can promote changes in its localization [23,24,26,27] by modulating calpain activity under conditions of elevated $[Ca^{2+}]_i$ [23,27].

Calpain can also digest calpastatin and thus liberate inhibitory units that are still active [28]. These fragments may be of great importance in cells with altered calcium homeostasis as they promote amplification of the cells' inhibitory capacity [12,20].

Finally, calpastatin can be extensively degraded by calpain. Under these conditions the cell is deprived of the capability to inhibit calpain [28].

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Formation of the calpain–calpastatin complex has been extensively studied at the structural level using a highly conserved recombinant region of the inhibitory units found in full-length natural calpastatin [29–31]. The inhibitory domain of calpastatin has thus been demonstrated to bind both to the catalytic cleft and, to other sites in the EF-hand regions of calpain domains IV and VI, if with lower affinity and only in the presence of Ca^{2+} [29–33]. Specifically, three conserved sub-regions of the inhibitory domain (named A, B, and C [29]) are able to interact with calpain through different binding sites. However, whether these calpain sites can be occupied by more than one molecule of calpastatin remains to be established [34].

Although the structural basis of calpain inhibition has been clarified, further study is still required because natural calpastatins contain not only four inhibitory units, but also the L- and/or XL-domains, which lack inhibitory activity [3,35].

The L-domain has been found to be involved in the calpain–calpastatin interaction in the absence of Ca^{2+}, which indicates that this interaction does not require opening of the calpain catalytic cleft [25].

As for full-length calpastatin, whether each inhibitory domain can associate separately with a calpain molecule to form complexes having different calpain:calpastatin stoichiometries has yet to be determined [34]. Furthermore, as it has been demonstrated that, in the presence of calcium, calpain can loosely aggregate [36], the number of calpastatin molecules associated with these structures may be greater than one. The fact that calpastatin forms having one or four inhibitory domains display approximately the same inhibitory efficiency [37] points to a 1:1 stoichiometry.

Here we provide novel insights into the calpain–calpastatin complex, based both on functional interactions and on competitive experiments carried out to evaluate the role of the XL–L domains in the kinetics of the enzyme–inhibitor complex formation. We found that the calpastatin forms containing the XL–L- and L-domains lose inhibitory efficiency at concentrations that would be expected to promote complete calpain inhibition. We also established that interaction between the inhibitory domain of calpastatin and the open substrate cleft of calpain can occur either directly, or via the L-domain. The L-domain seems to play a key role in directing proper positioning of the first inhibitory domain in the active site of calpain at high concentrations, however, the calpastatin forms containing the XL–L domains are able to interact, through the inhibitory domain, with an additional calpain site. When this association occurs, correct binding of other calpastatin molecules to the substrate cleft is prevented. Because this type of interaction takes place in the presence of Ca^{2+} and does not promote calpain inhibition, the still-active calpain molecules are able to degrade calpastatin, thus producing free inhibitory units.

2. Materials and methods

2.1. Materials

Leupeptin, aprotinin, 4-(2-amino-ethyl)benzensulfonyl fluoride (AEBSF), and fluorescamine were purchased from Sigma Chem. Co.

2.2. Purification of the ubiquitous calpain forms

Human erythrocyte calpain was purified to homogeneity as described in [38] and μ-calpain and m-calpain were purified from rat skeletal muscle following the procedure described in [39,40]. The three purified proteases showed similar specific activities: erythrocyte calpain 2100 units/mg, μ-calpain 2000 units/mg, m-calpain 1920 units/mg. One unit of calpain activity is defined as the amount required to cause the release of 1 μmol/h of free α-amino groups under the specified conditions.

2.3. Assay of calpain and calpastatin activity

Calpain activity was assayed using human acid-denaturated globin as substrate, in the absence or in the presence of increasing amounts of purified recombinant calpastatins, by measuring the amount of free amino groups liberated following fluorescamine-based quantitative determination [40].

2.4. Preparation of recombinant calpastatins

RNCAST300, RNCAST600, and RNCAST110 (calpastatin L-domain GenBank Y13590) proteins were prepared as described in [25,41]. The protein constructs corresponding to the region of calpastatins cast468, cast48, cast46, cast8, cast4, and castZERO (GenBank DQ186624–DQ186629) containing the XL/L domains and only the first inhibitory unit (from exon 1xa to exon 12) were produced as described in [42]. To obtain recombinant full-length calpastatins (Type I (GenBank AB026997), Type II (GenBank NM_009817.3), and Type III (GenBank AK029293)), approximately 5 μg total RNA extracted from mouse brain was reverse transcribed to cDNA with a random hexamer primer (ThermoScript RT-PCR system Invitrogen). To obtain the three forms of calpastatin by PCR, the cDNA was amplified in the presence of the specific forward primers: Type I: Sn EcoRI 5′-AAGAATTCAAAATGTCCCAG CCCGG, Type II: Sn EcoRI 5′-AAGAATTCATGGCATTCGCAAGTTGGTG, Type III: Sn EcoRI 5′-AAGAATTCATGAGTACCACAGAGACTAAGGCAATT and the same reverse primer: Asn SalI 5′-AAAGTCGACGCTGAATTTCT ATT-CAGATACCCA. PCR conditions were: a denaturation step for 1 min at 98 °C; then 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 2 min for 35 cycles. A 5-min extension step at 72 °C was performed after the last cycle of PCR. Calpastatin amplicons were digested with EcoRI and SalI and ligated into pGEX-6P1 GST Expression Vector (GE Healthcare). GST–fusion constructs were transformed into Library efficiency DH5α chemically competent *Escherichia coli* (Invitrogen) according to the manufacturer's instructions. Recombinant protein expression was induced by 4 h of incubation at 37 °C in the presence of 1 mM isopropyl-D-thiogalactopyranoside (IPTG, Sigma). The resulting GST–calpastatin constructs were purified by GST–agarose (Sigma) and on-column digestion with PreScission protease (GE Healthcare).

2.5. Preparation of recombinant small subunit of calpain (30 kD)

For the preparation of recombinant human calpain small subunit (30 kD) (GenBank NM_001749.2) cDNA template was generated using 5 μg of human lymphocyte total RNA. The transcript for the calpain 30 kD subunit was obtained by PCR using the following primers: Sn BamH1 5′-TGGGATCCATGTTCCTGGTTAACTCGTTCTTGAAG, Asn XhoI 5′-AAAC TCGAGGCTCCAGTTCAGGAATACATAGTCAGC. PCR conditions were: a denaturation step for 1 min at 98 °C; then 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min for 35 cycles. A 5-min extension step at 72 °C was performed after the last cycle of PCR. After digestion by BamH1 and XhoI, the PCR product was cloned into pGEX-6P1 and a recombinant small subunit of calpain was produced. To obtain the purified protein the same protocol described above for the purification of recombinant full-length calpastatin was used.

Before cloning, the coding sequence of the clones containing the different calpastatin constructs and calpain small subunit, was confirmed by sequencing with a CEQ 2000XL DNA analysis system (Beckman Coulter).

2.6. Determination of calpain and calpastatin concentrations

To prevent data errors in the calculation of IC_{50}, the concentration of each of the recombinant calpastatin species and of the native calpain forms was established precisely. SDS-PAGE and Coomassie Blue staining were performed to check for the presence of contaminating proteins during the purification steps. Finally, the concentration of each purified

protein was determined by alkaline hydrolysis [43]. A known amount of bovine serum albumin was used as standard protein. The amount of the amino groups liberated following alkaline hydrolysis was calculated by fluorescamine-based quantitative determination.

2.7. Immunoprecipitation

Immunoprecipitation was carried out by incubating RNCAST600 (0.5 μg) with increasing amounts of RNCAST110 (molar ratio RNCAST600/RNCAST110, 1/1, 1/2, 1/4) in the presence of purified human erythrocyte calpain (1 μg). The proteins were diluted in 50 mM sodium borate buffer pH 7.5, containing 1 mM EDTA (IP buffer) and incubated with anti-calpain mAb 56.3 (0.5 μg) [44] for 2 h at room temperature (50 μl, final volume). Protein G–Sepharose (30 μl) diluted 1:1 in IP buffer was then added to the samples, and the mixtures were rotated end-over-end for 2 h at 4 °C. Sepharose beads were collected and washed three times with IP buffer (0.1 ml each wash). The beads were then suspended in SDS-PAGE loading buffer (50 μl) and heated for 5 min at 100 °C. The beads were discarded by centrifugation, and the clear solutions were submitted to 15% SDS-PAGE followed by Western blotting. The immunoprecipitated calpastatin species were immunodetected with anti-calpastatin mAb 35.23 [37].

2.8. Curve fitting and quantitative analysis of the two-site interaction model for the calpain–calpastatin complex

Curve fitting was performed using the nonlinear regression facility (curve fit) of GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA, USA). Multiple data sets generated several fractional inhibition versus calpastatin concentration curves. The data were collected by measuring the activity of a fixed amount of calpain (2 nM) in the presence of different concentrations of calpastatin. Fractional inhibition (indicated in the Y axis of Fig. 1–4) was calculated by measuring the difference between the activity of calpain in the absence of inhibitor minus the residual activity of the enzyme in the presence of calpastatin (numerator), divided by the activity of calpain in the absence of inhibitor (denominator). Thereby, when the ratio is 1, calpain residual activity is zero. Calpain activity was measured as the amount of $-NH_2$ groups released from the digested globin substrate. The calpastatin concentration required to promote 50% inhibition of calpain activity (fractional inhibition = 0.5) is defined as IC_{50}.

2.9. Detection of calpastatin Type I, Type II and Type III protein levels in skeletal muscle of control and FALS mice

Frozen skeletal muscle samples from control and transgenic mice (100 days old) were kindly provided by Prof. G. Bonanno (Department of Pharmacology, University of Genova, Italy). Aliquots (1 mg tissue) were rapidly minced and resuspended in 3 volumes (w/v) of chilled 50 mM sodium borate buffer, pH 7.5, containing 0.5 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mg/ml leupeptin, 10 μg/ml aprotinin, and 2 mM AEBSF. Minced tissue was homogenized 4 times for 15 s with a Waring blender and lysed by sonication (4 bursts for 15 s with a 30 s cooling period between bursts). The particulate material was discarded by centrifugation at 50,000 ×*g* for 10 min at 4 °C. The supernatant was heated for 3 min at 100 °C and centrifuged at 50,000 ×*g* for

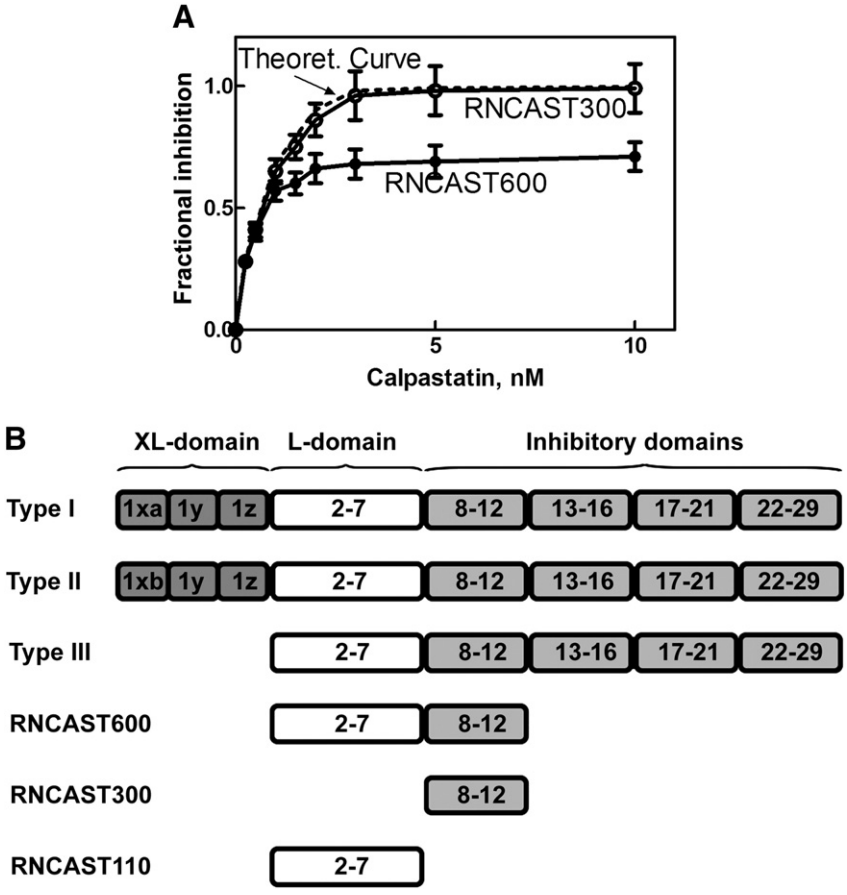

Fig. 1. Kinetics of calpain inhibition by RNCAST600 and RNCAST300. (A) Purified human erythrocyte calpain (2 nM) was incubated for 10 min at 30 °C in the presence of increasing amounts of recombinant RNCAST600 or RNCAST300. Calpain activity was measured as described in the Materials and methods section. Fractional inhibition is calculated as the difference between the activity of calpain in the absence of inhibitor minus the residual activity measured in the presence of calpastatin (numerator), divided by the activity of calpain in the absence of inhibitor (denominator) (see also the Materials and methods section). The data reported are the arithmetic mean ± SD of five different assays. (B) Schematic representation of the multiple recombinant calpastatin forms used in this paper. The numbers inside the rectangles indicate the exons included in each domain.

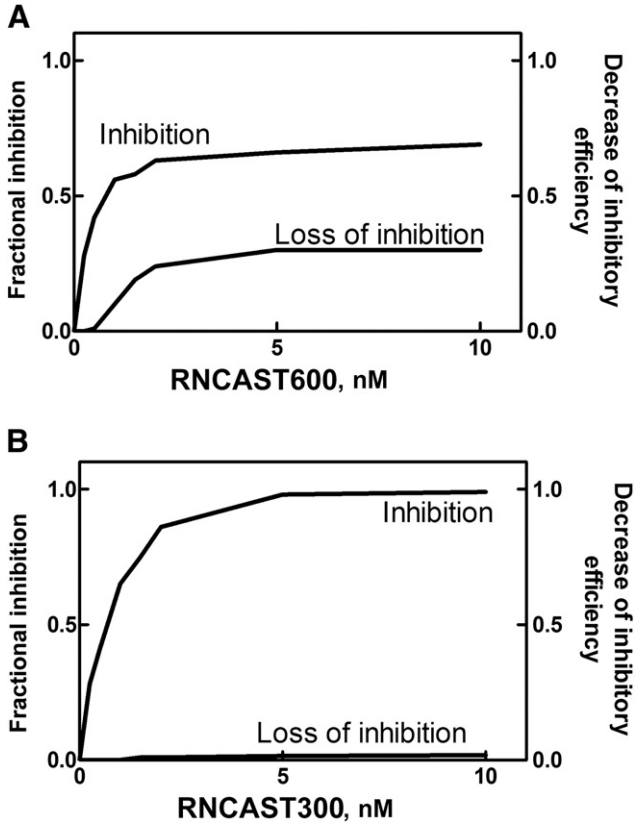

Fig. 2. Evaluation of inhibitory efficiency (second phase) of RNCAST600 and RNCAST300. To obtain the kinetics of the second phase curve (*loss of inhibition*), a representative inhibition curve of RNCAST600 (A) and RNCAST300 (B), both reported in Fig. 1, has been elaborated by calculating the difference between the inhibition values of the theoretical curve minus the mean of the actual values obtained with both calpastatins.

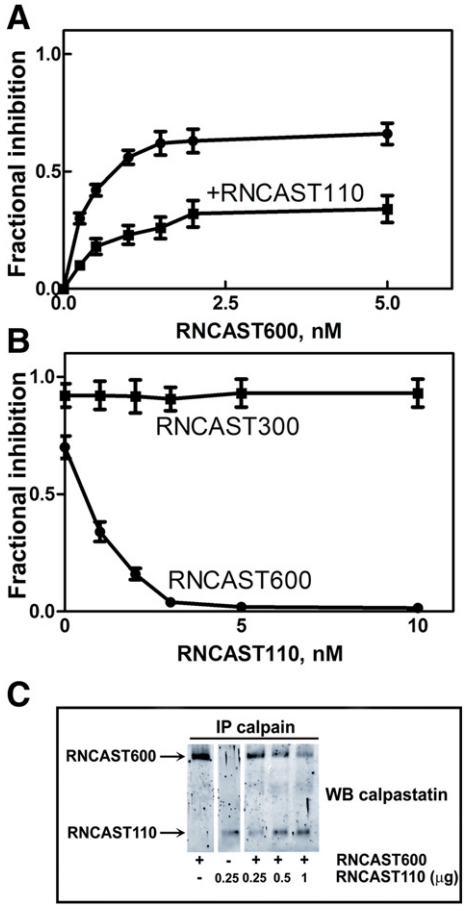

Fig. 3. Effect of RNCAST110 (L-domain) on calpain inhibition by RNCAST600 and RNCAST300, and on the association of RNCAST600 with calpain. (A) Purified erythrocyte calpain (2 nM) was incubated with increasing amounts of RNCAST600 in the absence or in the presence of purified L-domain (2 nM) (+RNCAST110). Calpain activity was measured as described in the Materials and methods section. (B) Purified erythrocyte calpain (2 nM) was incubated with RNCAST600 (3 nM) or RNCAST300 (3 nM) in the presence of increasing amounts of purified L-domain (RNCAST110). Fractional inhibition was calculated as indicated in Fig. 1. The data reported in each curve are the arithmetic mean ± SD of six different assays. (C) RNCAST600 (0.5 μg) or RNCAST110 (0.25 μg) was incubated with purified human erythrocyte calpain (1 μg) (lanes 1 and 2). RNCAST600 (0.5 μg) was also incubated with the indicated amounts of RNCAST110 in the presence of purified human erythrocyte calpain (1 μg). The proteins were immunoprecipitated with anti-calpain mAb 56.3 and the immunoprecipitated material was submitted to Western blotting. Calpastatins were immunodetected with anti-calpastatin mAb 35.23.

10 min at 4 °C. An aliquot of the clear heated extract (75 μg) was submitted to 9% SDS-PAGE [45] and Western blotting [46]. The different forms of calpastatin were detected with monoclonal antibody 35.23 [37], and anti-calpastatin Domain IV mAb (Calbiochem). To distinguish between calpastatin Type I and Type II two polyclonal antisera were kindly provided by Prof. T. Parr (University of Nottingham, UK). The first one detects exon 1xa of calpastatin Type I, and the other exon 1xb of calpastatin Type II [47].

The immune-reactive material was developed with an ECL detection system (GE Healthcare), detected with a Bio-Rad Chemi Doc XRS apparatus, and quantified using the Quantity One 4.6.1 software (Bio-Rad).

2.10. Digestion of purified calpastatin Type III by calpain

Purified human erythrocyte calpain (4 pmol) was incubated at 25 °C in 0.04 ml of 50 mM sodium borate buffer, pH 7.5, containing 0.1 mM $CaCl_2$ for 10 min in the presence of increasing amounts of recombinant calpastatin Type III in calpastatin to calpain molar ratios ranging from 0.25 to 10. Reactions were stopped by addition of a 6× Laemmli loading solution, heated, and submitted to SDS-PAGE (12%) and Western blotting. Calpastatin digestion was detected with anti-calpastatin Domain IV mAb (Calbiochem). The amount of 15 kD species was detected and quantified as described above with the Bio-Rad Chemi Doc XRS apparatus, and quantified using the Quantity One 4.6.1 software (Bio-Rad).

3. Results

3.1. Kinetics of calpain inhibition by RNCAST600 and RNCAST300

As the calpain–calpastatin association has been studied at the structural level using a single inhibitory domain [29–33], much remains to be elucidated. In order to obtain data that can be more specifically related to physiological conditions, we studied the inhibition kinetics of calpain promoted by multiple calpastatin forms that contain not only the inhibitory domain but also the N-terminal region, namely the XL–L and L-domains.

We began by analyzing the inhibitory pattern of RNCAST600 [25], which includes the first inhibitory unit and the N-terminal L-domain. As shown in Fig. 1A, the kinetics of calpain inhibition was characterized by biphasic behavior. Specifically, at low RNCAST600 concentrations the kinetics fit with the curve of a competitive inhibitor having the same K_i (evaluated as the inhibitor concentration promoting 50% inhibition, IC_{50}). At higher concentrations of RNCAST600, the degree of inhibition,

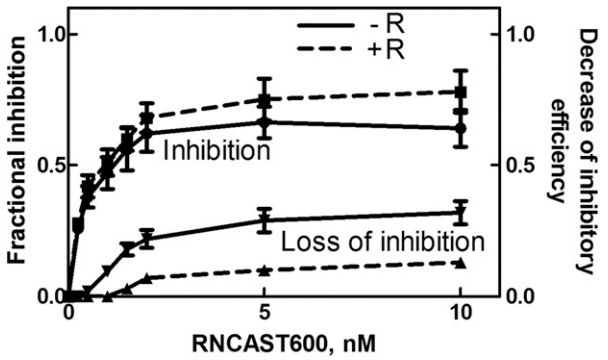

Fig. 4. Effect of calpain small subunit (30 kD) on calpain inhibition by RNCAST600. Purified erythrocyte calpain (2 nM) was incubated with increasing amounts of RNCAST600 in the absence or in the presence of recombinant calpain regulatory subunit (5 nM) (+R, *dotted curves*). Fractional inhibition was calculated as indicated in the legend to Fig. 1. The loss of inhibition curve was determined as described in the legend to Fig. 2. The data shown are the arithmetic mean ± SD of six different experiments.

and therefore the efficiency of calpastatin, were significantly reduced and the second part of the curve diverged from the theoretical one (Fig. 1A, dotted line). Even in the presence of a large excess of RNCAST600, calpain retained 30–35% activity. Conversely, RNCAST300, which contains the same inhibitory domain as RNCAST600, but lacks the L-domain (see Fig. 1B for the structure of the various calpastatin forms used in this paper), showed the classical kinetics, which is superimposable to the theoretical curve and corresponds to the formation of an enzyme/competitive inhibitor complex (Fig. 1A), even at high concentrations. Thus, this calpastatin form inhibits calpain almost completely, as is the case with synthetic inhibitors such as leupeptin [48].

Taken together these data suggest that the L-domain present in the calpastatin molecule affects its inhibitory efficiency by modifying the association of this calpastatin form with the catalytic cleft of calpain. The unusual kinetics shown by RNCAST600 may be interpreted on the basis of previous findings, which indicated that the inhibitory domain of calpastatin can associate with calpain at multiple sites located both at the catalytic and at the EF-hand domains [29–32].

As RNCAST600 differs from RNCAST300 exclusively for the presence of the L-domain, it is reasonable to hypothesize that the biphasic kinetics results from a direct interaction between the L-domain and calpain. In this respect we previously reported that the L-domain associates with a specific site on the calpain catalytic domain and that this association occurs independently of the presence of Ca^{2+} [25]. This observation implies that calpastatin can bind the inactive form of calpain. Therefore, the inhibition kinetics of RNCAST600 may be due to the interaction between calpastatin and two distinct binding sites on calpain. The first interaction determines the inhibition of proteolytic activity and occurs at the catalytic cleft, whereas the second, which promotes the decrease of inhibitory efficiency, possibly involves the penta EF-hands.

To distinguish between these binding properties, we separated the kinetics of calpain inhibition by RNCAST600 reported in Fig. 1 in two different curves (Fig. 2A): the first curve shows the first inhibitory phase, while the other describes the second phase and is characterized by the decrease of inhibitory efficiency. We calculated the loss of inhibitory efficiency by evaluating the difference between the theoretical and the actual RNCAST600 curve shown in Fig. 1. On the basis of this extrapolation, it clearly appears that the inhibitory effect of RNCAST600 was significantly reduced at calpastatin concentrations greater than those promoting about 50% inhibition and was poorly increased at concentrations more than five-fold higher. Indeed, after this initial phase at which inhibition was the only detectable effect, the second phase took place and calpain inhibition reached a plateau at concentrations of RNCAST600 higher than 2 nM (Fig. 2A). The IC_{50} for the first inhibitory phase was approximately 1 nM. Much more calpastatin was required to detect the loss of inhibitory efficiency observed during the second phase, and maximal decrease of efficiency occurred at roughly 4–6 nM.

As expected, in the presence of RNCAST300, which is capable of promoting full calpain inhibition, the second phase was almost undetectable (Fig. 2B). These different kinetic properties may be attributed to the presence of the L-domain in RNCAST600. Alternatively, as suggested by structural studies, they may be explained, by the existence of multiple calpastatin binding sites on calpain [29–34]. Those structural studies, however, did not include the L domain in the calpastatin constructs that they used, and therefore did not take its role into account.

Overall, the presence of more than one binding site on RNCAST600 suggests that the L-domain may promote the formation of an alternative calpain/calpastatin complex in which the calpain retains catalytic activity.

3.2. Effect of the L-domain on calpain inhibition by RNCAST600 and RNCAST300

In order to characterize the role of the L-domain within the biphasic interaction between calpastatin and calpain, we tested the effect of the isolated L-domain (RNCAST110) on calpain inhibition by RNCAST600.

As shown in Fig. 3A, the addition of 2 nM RNCAST110 to increasing concentrations of RNCAST600 decreased calpain inhibition by approximately 50%, and the biphasic kinetics was still observed. When a fixed concentration of RNCAST600 was incubated with increasing amounts of RNCAST110, calpain inhibition was progressively reduced (Fig. 3B). Conversely, the presence of RNCAST110 had no effect on the RNCAST300 inhibition pattern (Fig. 3B). These kinetic data suggest that RNCAST110 and the L-domain present in RNCAST600 compete for a calpain binding site which is distinct from the catalytic cleft.

With the aim of further exploring this molecular interaction, we also carried out immunoprecipitation experiments. As shown in Fig. 3C, both RNCAST110 and RNCAST600 can be separately immunoprecipitated with calpain. However, when we added increasing amounts of RNCAST110 to a fixed concentration of RNCAST600, the RNCAST600 bound to calpain was reduced proportionally to the amount of RNCAST110 used. Since RNCAST110 has no effect on RNCAST300 inhibitory efficiency, we postulate that the two binding sites, although both localized in calpain domain II, are functionally independent.

As observed with RNCAST600, all the calpastatin forms containing the L-domain and at least one inhibitory unit (see also Table 2) showed decreased calpain inhibition in the presence of RNCAST110 (data not shown).

3.3. Effect of different exon compositions of the XL–L- and L-domains on the inhibitory efficiency of different calpastatin forms

We sought further support for our multiple binding site hypothesis by attempting to establish whether calpastatin forms that differ in their exon composition display calpain inhibitory kinetics similar to that of RNCAST600.

We tested the inhibitory efficiency of eleven recombinant calpastatin forms in the presence of human erythrocyte calpain. IC_{50} values were calculated by determining the amount of calpastatin required to promote 50% inhibition of calpain activity, and the decrease in inhibitory efficiency detectable at high calpastatin concentrations during the second phase, was measured as reported in Fig. 2A. We separately analyzed these two parameters according to the exons composing the XL–L domains and the multiple inhibitory units present in full-length calpastatins. The data reported in Table 1 show that IC_{50} was not directly related to the presence/absence of a single exon in the XL–L domains. Selective exon composition, however, seemed to reflect higher or lower affinity for the catalytic cleft. The presence of either exons 1xa, or 1xb did not significantly affect IC_{50} values, nor the

Table 1
Effect of calpastatin domain composition on the biphasic inhibition of erythrocyte calpain.

Calpastatin form	XL–L exon composition	Inhibitory domains	IC_{50} (nM)	Loss of inhibitory efficiency (nM)
RNCAST300	None	1	1.0 ± 0.2	nd
RNCAST600	2, 4, 5, 6, 7, 8	1	1.3 ± 0.2	5 ± 1
Cast468	1xa, 1y, 2, 4, 5, 6, 7, 8	1	3.4 ± 0.2	5 ± 1
Cast46	1xa, 1y, 2, 4, 5, 6, 7	1	0.7 ± 0.1	9 ± 1
Cast48	1xa, 1y, 2, 4, 5, 7, 8	1	1.1 ± 0.2	10 ± 1
Cast8	1xa, 1y, 2, 5, 7, 8	1	3.1 ± 0.2	4 ± 1
Cast4	1xa, 1y, 2, 4, 5, 7	1	0.4 ± 0.1	13 ± 1
castZERO	1xa, 1y, 2, 5, 7	1	0.8 ± 0.1	10 ± 1
Type I	1xa, 1y, 1z, 2, 4, 5, 6, 7, 8	4	1.2 ± 0.3	7 ± 1
Type II	1xb, 1y, 1z, 2, 4, 5, 6, 7, 8	4	1.6 ± 0.3	7 ± 1
Type III	2, 4, 5, 6, 7, 8	4	1.3 ± 0.3	7 ± 1

Recombinant calpastatins were produced in *E. coli* and purified to homogeneity as described in the Materials and methods section. Human erythrocyte calpain (2 nM) was incubated in the presence of increasing amounts of each calpastatin form and both the inhibition and loss of inhibition curves were separately generated as described in Fig. 2. The values indicating the loss of inhibitory efficiency correspond to the concentration of calpastatin required for the beginning of the second non inhibiting phase. Each value is the arithmetic mean ± SD of four independent calpain assays.

number of inhibitory units. Indeed, full-length calpastatins that contain 4 repetitive domains showed IC_{50} values that were similar to the forms having a single inhibitory unit. Thus, the non-inhibitory domain seems to play a role in positioning the first inhibitory unit in the proximity of the calpain active site cleft, regardless of the fact that the catalytic pocket is in the closed or open conformation. Both calpain sites may be occupied by the same calpastatin molecule and the first inhibitory domain may be inserted in the catalytic cleft. As the additional inhibitory units present in full-length calpastatins are ineffective, their role remains elusive.

We then found that the second phase of calpain inhibition (see Table 1), which is characterized by lower inhibitory efficiency, occurred in all the calpastatins containing the XL–L- or the L-domain. As mentioned above, RNCAST300, which is constituted of a single inhibitory unit, lacked the second phase. Specifically, RNCAST600 and cast 468, both of which contain the same L-domain and a single inhibitory unit, differ only by the presence of the XL-domain in cast468. As the second inhibition phase occurred at similar concentrations, we hypothesize that the XL-domain is not involved in the decrease in inhibitory efficiency and that the presence of exon 1xa instead of exon 1xb in the XL-domain (compare calpastatin Type I with Type II), has no impact on the decrease in efficiency observed during the second phase.

Regarding the L domain (exons 2–8), we previously established in rat brain that full-length calpastatin Type I undergoes alternative splicing at exons 4, 6, and 8 [42]. We produced these splicing forms as recombinant proteins containing only the first inhibitory unit. Table 1 shows that in cast4 (the alternative splice variant containing only exon 4) unlike in cast8 (containing only exon 8) the decrease in inhibitory efficiency occurs at a much higher calpastatin concentration. Cast48 (which contains both exon 4 and exon 8), showed a loss in inhibitory efficiency that seems to reflect the functional contribution of each exon alone. The concomitant presence of exon 6 (cast468) seems to be synergistically related to the presence of exon 8. Indeed, cast468 showed very high affinity for the second calpain site, which does not promote calpain inhibition, while in cast46 the loss of inhibitory efficiency was similar to that of cast48.

We conclude that the presence of exon 4 seems to improve the inhibitory efficiency observed when either exon 8 or exon 6 is expressed. Unexpectedly, the second phase of the calpastatin splicing form lacking exons 4, 6, and 8 (castZERO) in the L-domain, was similar to that of cast 46 and cast 48. This finding suggests that these exons may be involved both in the occurrence of the second phase and in other regulatory functions mediated by post-translational modifications [23,27].

3.4. Effect of calpain small subunit (30 kD) on calpain inhibition by RNCAST600

With the aim of gaining further insights into the second phase of RNCAST600 inhibition, we also explored the possible involvement of calpain EF-hand regions in the decrease in inhibitory efficiency observed at high calpastatin concentrations. Calpain is known to bind the isolated calpastatin inhibitory unit [29–34] to both the large and the small subunits in a Ca^{2+}-dependent manner, as each of the subunits contains five EF-hands [1–3]. Therefore we expressed recombinant 30 kD calpain small subunit and studied its effect on the formation of the calpain-RNCAST600 complex.

As shown in Fig. 4, calpain inhibition by RNCAST600 is increased in the presence of the isolated 30 kD subunit, whereas the loss of inhibitory efficiency (second phase) is significantly delayed and reduced. Based on these observations we infer that the EF-hand regions are directly involved in the formation of a calpain–calpastatin complex, which under these experimental conditions leaves the calpain active site accessible to the inhibitor. Thus the "loss of inhibitory efficiency" observed only at high calpastatin concentrations may be determined by the fact that the inhibitor binds to the small subunit of a given calpain molecule because the affinity of calpastatin for the 30 kD subunit is lower than its affinity for the active site. The result of this interaction is that no more calpastatin can reach the catalytic cleft.

Under our experimental conditions the excess of free 30 kD subunits determines the competitive removal of calpastatin from the small subunit of the native calpain. Consequently, as more calpastatin molecules reach the catalytic cleft calpain inhibition is increased.

In conclusion, binding of calpastatin to one calpain site excludes interaction of another inhibitor molecule with the other site. This explains the persistency of residual calpain activity even in the presence of an excess of calpastatin.

As shown in Table 2 the effect of the 30 kD subunit was observed in all the calpastatin forms containing the L-domain. Exceptions are RNCAST300, which lacks the L-domain, and calpastatin Type III, which is deprived of the XL-domain and contains four inhibitory units. This observation further confirms the role of the L-domain in the formation of a functional enzyme–inhibitor complex.

3.5. Different specificity and inhibitory efficiency of the various calpastatin forms assayed in the presence of ubiquitous members of the calpain family

We incubated recombinant full-length calpastatins [35], in the presence of μ- and m-calpains purified from rat skeletal muscle. As shown in Table 3, Type I efficiently inhibited both calpain isozymes whereas Type II was more effective on μ-calpain than m-calpain. Type III, which differs from Types I and II as it lacks the XL-domain, displayed similar efficiency on both calpains. For purpose of comparison, Table 3 also shows the effect of these calpastatins on erythrocyte calpain, which belongs to the μ-calpain family. These findings may indicate that the lower inhibitory efficiency observed for Type II calpastatin on m-calpain results from a different type of interaction between Type II and the catalytic subunit of m-calpain, as calpains share the same regulatory subunit [3].

3.6. Expression of the different calpastatin forms in skeletal muscle of SOD1 transgenic mice

We explored whether different calpastatin forms undergo differential regulation of their expression level depending on the activation/activity of the Ca^{2+}-dependent proteolytic system. To this purpose we measured the protein levels of calpastatin Types I, II, and III in skeletal muscle from SOD1 transgenic mice. These animals are generally utilized as a model for studying Familial Amyotrophic Lateral Sclerosis (FALS) and display altered calcium homeostasis [12].

As reported in Table 4, we observed that the amounts of Type I and Type II were increased in FALS mice vs. control animals, whereas the

Table 2
Changes in the inhibitory efficiency of different calpastatin species incubated with erythrocyte calpain, in the presence of isolated L-domain or 30 kD regulatory subunit.

Calpastatin form	Domain composition	Inhibitory effect of the L-domain (RNCAST110)	Inhibitory effect of the regulatory subunit (30 kD)
RNCAST300	1	nd	nd
RNCAST600	L-1	↓	↑
Type I	XL–L–1–2–3–4	↓	↑
Type II	XL–L-1–2–3–4	↓	↑
Type III	L-1–2–3–4	↓	↓

Human erythrocyte calpain (2 nM) was incubated in the presence of increasing amounts of each calpastatin form in the absence or in the presence of RNCAST110 (2 nM) or calpain regulatory subunit (30 kD) (5 nM). To establish if the addition of exogenous free L-domain (RNCAST110) or 30 kD regulatory subunit promotes an increase (↑) or a decrease (↓) in calpain inhibition by the indicated calpastatins, fractional inhibition versus calpastatin concentration curves have been generated. For each calpastatin form the curves plotted in the absence and in the presence of the free domains were compared (see Fig. 3A and 4). Experiments were carried out as reported in the legend to Fig. 3A and 4.

level of Type III, the most represented isoform in control mice, was not modified.

Along with these three forms, FALS animal skeletal muscle contains a fourth calpastatin species with a mass of about 15 kD. This form, which is not produced by a specific mRNA, derives from calpain-mediated digestion of full-length calpastatins. This process causes the liberation of free inhibitory domains that retain inhibitory activity [12,20,28,49].

As shown in Fig. 5, we detected accumulation of 15 kD fragments in vitro when a large excess of full-length calpastatin was exposed to calpain proteolysis. Therefore, under this condition, following association with the second site, native calpastatin, prevented complete inhibition of calpain.

Taken together, these data provide new insights into the role of the 15 kD form. This form shows high inhibitory efficiency, and is able to control calpain activation. Our observations indicate that the second site is involved in the recognition of calpastatin as a substrate.

4. Discussion

Interaction of calpain with calpastatin is considered to be the most important and specific regulatory mechanism of protease activity. A detailed structural analysis of a complex formed between calpain and one of the four calpastatin inhibitory domains has provided important information on the binding sites involved [29–34].

It has been previously reported that in addition to calpain catalytic cleft, an isolated calpastatin inhibitory domain can bind to multiple lower affinity sites that are localized in the EF-hand domains IV and VI of calpain, thereby stabilizing the inhibitory complex [30,33,34].

To explore the role of the other domains in full-length calpastatins we analyzed the interaction between calpain and different calpastatin constructs that contain the XL–L or L- N-terminal domains and one or four inhibitory units. We have established that the L-domain is responsible for the decrease in inhibitory efficiency at high calpastatin concentrations. As a result, even in the presence of an excess of calpastatin, calpain is never fully inhibited. Our observations indicate that the L-domain recognizes a site at calpain domain II not coincident with the catalytic cleft and which possibly delivers the downstream inhibitory unit to the substrate cleft. Furthermore, the calpastatin forms containing the L-domain associate with additional sites at the EF-hand regions of calpain, via their inhibitory domain, although with lower affinity and in the presence of Ca^{2+}. These sites have been previously identified by structural studies carried out with recombinant calpastatin constructs lacking the L-domain [29–34].

Thus, the presence of the L-domain could increase the affinity of the downstream inhibitory unit for the active cleft and when this complex is formed, calpain is inhibited. At higher calpastatin concentrations the association of the inhibitor with calpain occurs at the other sites in the EF-hands of the protease. We hypothesize that in the presence of Ca^{2+}, the interaction of the calpastatin forms containing the L-domain with the EF hands, could create a steric hindrance that hampers the binding of another calpastatin molecule at the catalytic site. In these conditions calpain retains activity and can digest substrates including another calpastatin molecule. The data herewith reported, besides suggesting a mechanism by which calpastatin can reduce its inhibitory efficiency, also provide an explanation for the fact that full-length calpastatins show an inhibitory efficiency similar to that of the forms having only the first inhibitory domain.

Yet we want to stress that our findings are not in disagreement with previous studies reporting that calpastatin can simultaneously bind four calpain molecules, one for each inhibitory unit [34]. It should be noted that the calpastatin construct used by Hanna et al. is just lacking the L-domain which is essential in our experiments to detect the biphasic kinetics of calpain inhibition. Moreover, our data are based on functional measurements of calpain inhibition rather than on the structural characterization of the calpain/calpastatin interaction. In the model here presented the association of calpastatin with calpain does not necessarily lead to the inhibition of the protease.

Interestingly, the calpain molecules that are not inhibited by calpastatin digest the inhibitor to free 15 kD inhibitory units. This process can also be directed to calpastatin species that have lost the L-domain but still contain more than one repetitive unit. The binding of these calpastatins to the EF-hand structures promotes the association with calpain active site, of other calpastatin molecules that are first digested to 15 kD inhibitory units, and then foreshortened to inactive fragments [28].

Table 3
Inhibitory efficiency (IC_{50}, nM) of different calpastatin forms on ubiquitous calpains.

Calpastatin form	μ-Calpain	Erythrocyte calpain	m-calpain
RNCAST300	0.9 ± 0.1	1.0 ± 0.2	1.9 ± 0.3
RNCAST600	1.0 ± 0.2	1.3 ± 0.2	1.4 ± 0.3
Type I	1.1 ± 0.2	1.2 ± 0.3	1.5 ± 0.2
Type II	1.3 ± 0.2	1.6 ± 0.3	3.5 ± 0.2
Type III	1.2 ± 0.1	1.3 ± 0.3	1.3 ± 0.2

Recombinant calpastatins were produced in *E. coli* and purified to homogeneity as described in the Materials and methods section. Ubiquitous μ-calpain and m-calpain were purified from rat skeletal muscle as described in the Materials and methods section and in [3]. Both enzymes (2 nM) were separately incubated in the presence of increasing amounts of each calpastatin form and residual calpain activity has been measured. Inhibition curves have been generated to calculate IC_{50} and each value is reported as the arithmetic mean ± SD of five different calpain assays. For comparison the IC_{50} measured in the presence of erythrocyte calpain and already indicated in Table 1 is also reported.

Table 4
Protein level of the different calpastatin forms in skeletal muscle of control and FALS mice.

	Calpastatin level (arbitrary units)	
Calpastatin form	Control	FALS
Type I	35 ± 8	65 ± 10
Type II	135 ± 16	276 ± 18
Type III	525 ± 48	405 ± 34
15 kD	10 ± 4	235 ± 20

Clear heated extracts were obtained from skeletal muscle of control and FALS mice as described in the Materials and methods section. Aliquots (75 μg) were submitted to SDS-PAGE and Western blotting. The different calpastatin forms were detected using the specific antibodies (see the Materials and methods section). The amount of each calpastatin reactive band was quantified as described in the Materials and methods section, using Quantity One 4.6.1 software (Bio-Rad).

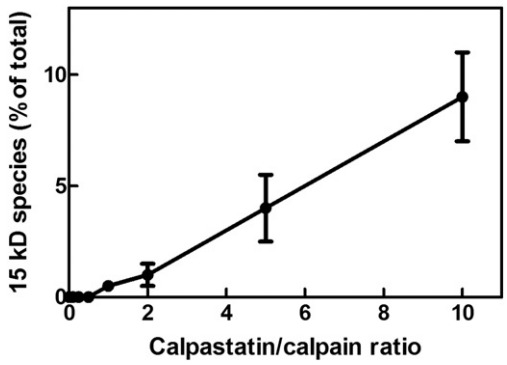

Fig. 5. Production of 15 kD species by calpain-mediated digestion in conditions of high calpastatin concentrations. Purified human erythrocyte calpain (4 pmol) was incubated in the presence of increasing amounts of recombinant Type III calpastatin (up to 40 pmol) at the indicated calpastatin/calpain molar ratios. The 15 kD calpastatin species was detected by Western blotting and quantified by densitometric analysis using the Chemi Doc XRS system (Bio-Rad) as described in the Materials and methods section and in [12]. The values reported are the arithmetic mean ± SD of five different experiments.

Previously we observed that in erythrocytes from hypertensive rats the inhibitor was nearly absent and the calpastatin fragments were almost undetectable [51]. Now we can hypothesize that the low levels of calpastatin in red blood cells from hypertensive patients [50] and the absence of inhibitor in genetically hypertensive rats [51] may be due to non-conservative degradation of calpastatin. Hence, while the presence of the L-domain could limit the digestion of calpastatin by favoring the correct positioning of the inhibitory unit in calpain active site, the absence of this domain induces calpastatin digestion at a higher rate as observed in erythrocytes from hypertensive rats.

Instead, we detected high levels of free calpastatin inhibitory domains in tissues actively synthesizing new calpastatin molecules and in conditions of elevated and prolonged alterations in Ca^{2+} homeostasis, such as in hypertension and amyotrophic lateral sclerosis [12,49]. The maintenance of calpain activity also in the presence of high levels of calpastatin could be important to overcome these dangerous conditions because new calpastatin fragments, more efficient than the inhibitor species originally expressed in the cells, are produced [12,20,49].

Thereby, the alternative binding of calpastatin to calpain, which is determined by the presence of the L-domain, can be visualized as a mechanism responsible for the transition of calpastatin from an inhibitor to a calpain substrate. It may indicate that within physiological and transient variations in $[Ca^{2+}]_i$, calpastatin can regulate calpain activity by a classical enzyme–inhibitor interaction. Conversely, a large and persistent increase in $[Ca^{2+}]_i$ promotes fragmentation of the inhibitor to overcome the severe cell conditions caused by abnormal calpain activation [12,20,49]. Indeed, these calpastatin fragments provide a very efficient inhibitory device that, at difference with native calpastatins, cannot be antagonized by any other inhibitory or non-inhibitory calpastatin species.

In conclusion, our findings demonstrate that the L-domain, which in itself lacks inhibitory capacity, plays a key role in regulating the inhibitory efficiency of calpastatin. The existence of this functional role is supported by our experimental results, which show that all the calpastatin forms naturally expressed and containing the L-domain are characterized by a biphasic calpain inhibition curve. The observation that a massive accumulation of the 15 kD species in skeletal muscle of FALS animals occurs along with Ca^{2+} increase and over-activation of calpain, provides additional support to the model herewith proposed (Fig. 6).

(A) Binding of the L-domain to calpain domain II positions the first inhibitory unit in close and correct proximity to the calpain active site cleft. In the presence of Ca^{2+} calpain is inhibited. An excess of free L-domains induces a decrease in calpain inhibition because the exogenous L-domains interfere with the correct association of calpastatin with the catalytic cleft. (B) The L-domain containing-calpastatin associates with the EF-hand structures both in domains IV and VI of calpain through its inhibitory unit and in the presence of Ca^{2+}. In this complex, the catalytic site cannot be occupied by the inhibitory unit of a full-length calpastatin and calpain is active. Exogenous 30 kD calpain subunits

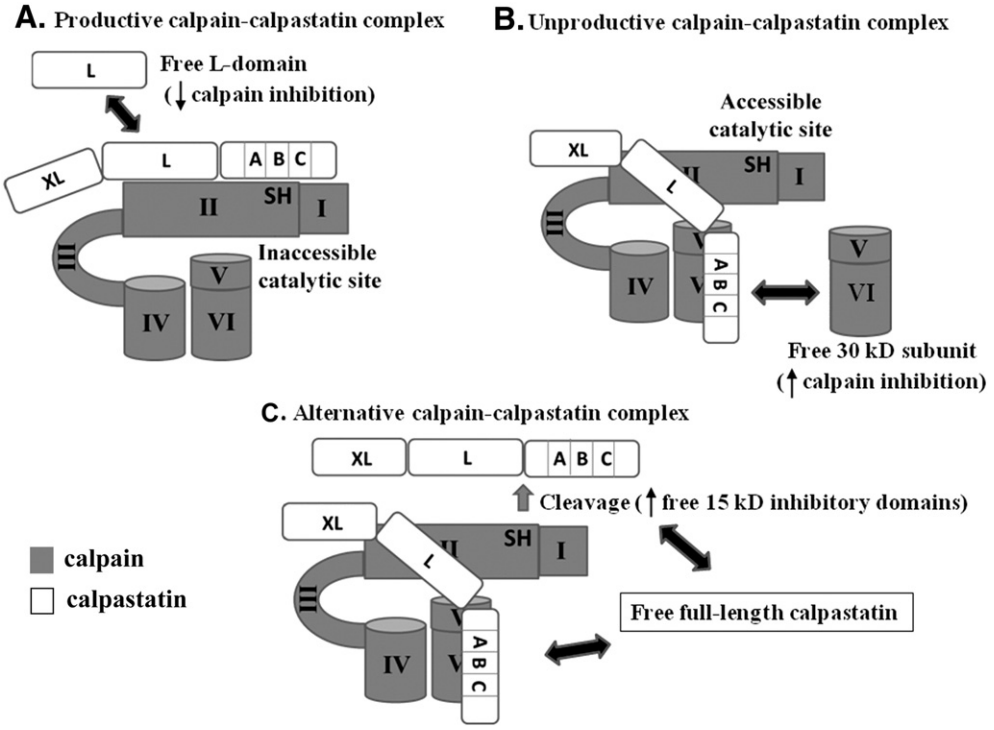

Fig. 6. Model for the formation of alternative complexes between calpain and L-domain-containing calpastatins. Calpain heterodimer (gray) is schematized and both the catalytic (domains I–IV) and the regulatory (domains V–VI) subunits are shown. The functional thiol group of the catalytic cleft is also indicated. A calpastatin molecule (white) containing the XL–L domains and one inhibitory unit (subdomains A, B, C are evidenced) is also shown.

compete with the regulatory subunit of native calpain for binding to calpastatin. As a result, the amount of the calpastatin interacting with the EF-hands of calpain is reduced, and more inhibitor can correctly associate with the catalytic cleft, thus leading to increased calpain inhibition. (C) Since binding of calpastatin to the EF-hand sites leaves the active site accessible, another molecule of inhibitor can be inserted in the catalytic cleft and undergo limited proteolysis with the production of free, still-active 15 kD calpastatin fragments. Under these conditions calpain is not inhibited and calpastatin behaves as a substrate.

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