Domain II of m-calpain is a Ca^{2+}-dependent cysteine protease

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Received 4 May 2001; revised 6 June 2001; accepted 11 June 2001

First published online 2 July 2001

Edited by Pierre Jolles

Abstract Calpain, a Ca^{2+}-dependent cytosolic cysteine protease, proteolytically modulates specific substrates involved in Ca^{2+}-mediated intracellular events, such as signal transduction, cell cycle, differentiation, and apoptosis. The 3D structure of m-calpain, in the absence of Ca^{2+}, revealed that the two subdomains (domains IIa and IIb) of the protease domain (II) have an 'open' conformation, probably due to interactions with other domains. Although the presence of an EF-hand structure was once predicted in the protease domain, no explicit Ca^{2+}-binding structure was identified in the 3D structure. Therefore, it is predicted that if the protease domain is excised from the calpain molecule, it will have a Ca^{2+}-independent protease activity. In this study, we have characterized a truncated human m-calpain that consists of only the protease domain. Unexpectedly, the proteolytic activity was Ca^{2+}-dependent, very weak, and not effectively inhibited by calpastatin, a calpain inhibitor. Ca^{2+}-dependent modification of the protease domain by the cysteine protease inhibitor, E-64c, was clearly observed as a SDS–PAGE migration change, indicating that the conformational changes of this domain are a result of Ca^{2+} binding. These results suggest that the Ca^{2+} binding to domain II, as well as to domains III, IV, and VI, is critical in the process of complete activation of calpain.

Key words: Calpain; Calpain activation; Ca^{2+} binding; Protease domain; Cysteine protease; EF-hand motif

1. Introduction

Calpain (EC 3.4.22.17) is a cytosolic Ca^{2+}-dependent cysteine protease that regulates cellular substrates by limited proteolysis. Although the physiological functions of calpains are not completely understood, the existence of calpains in a wide variety of living organisms, from human to yeast, strongly suggests their basic and essential functions in intracellular events, such as signal transduction, cell cycle, differentiation, and apoptosis [1–4].

Two mammalian calpains, μ- and m-calpain, are ubiquitously expressed and have been extensively characterized. They form a heterodimer comprising an 80-kDa catalytic subunit and a common 30-kDa regulatory subunit. The 3D structure of m-calpain, in the absence of Ca^{2+}, has recently been determined [5,6]. The 80-kDa and 30-kDa subunits comprise four (I–IV) and two domains (V and VI), respectively. They include a papain-like cysteine protease domain (II), a domain that resembles a Ca^{2+}- and phospholipid-binding C2 domain in 3D structure, but not in primary structure (III) [7], and Ca^{2+}-binding domains containing five EF-hand motifs (IV and VI). The μ- and m-calpains are activated by distinct Ca^{2+} concentrations (micromolar and millimolar levels, respectively), followed by autolysis of the N-terminal α-helix (domain I). The Ca^{2+} concentrations required for the protease activity are much higher than that in the cytosol, suggesting that there should be additional factors, such as phospholipids and activator proteins, to reduce the Ca^{2+} requirement of calpains [8–12].

The 3D structure of the protease domain has significant homology to papain, as predicted from the primary sequence similarities. One of the most intriguing features in the 3D structure of m-calpain is that domain II is divided into two distinct subdomains (IIa and IIb) in the absence of Ca^{2+}. Because of the distance between the two subdomains, the catalytic triad (Cys105 in domain IIa, and His262 and Asn286 in domain IIb; numbers of the residues correspond to the amino acid sequence of human m-calpain 80-kDa subunit (accession number M23254)) is not assembled (see Fig. 4). This composite structure explains the latency of calpain in the absence of Ca^{2+}.

The question arises: is the default structure of domain II of m-calpain 'open' or 'closed'? From the features described below, we developed a working hypothesis: (1) the default of domain II is a 'closed' conformation, i.e. active, as in the case of all other papain-like cysteine proteases; (2) the N-terminal α-helix and domain III force the domain II 'open', i.e. inactive, in the absence of Ca^{2+}; and (3) upon Ca^{2+} binding, the 'constraint' of the domain II is cancelled, and the 'closed' structure is formed by itself.

The 3D structure of m-calpain suggests that several interdomain salt bridges fix the domain II structure 'open'. The N-terminal α-helix is inserted into the hydrophobic pocket of domain VI of the 30-kDa subunit and produces salt bridges between the N-terminal basic residues of the 80-kDa subunit and acidic residues of domain VI. This interaction should anchor domain IIa to domain VI. On the other hand, basic residues in domain IIb interact with acidic residues in domain III, drawing domain IIb to domain III. Since recent studies demonstrated that domain III binds Ca^{2+}, and that mutations

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0014-5793/01/$20.00
PII: S0014-5793(01)02611-4

in the acidic residue(s) of domain III reduce the Ca^{2+} requirement of m-calpain [13,14], it is considered that Ca^{2+} binding to domain III breaks the salt bridges to domains IIb, resulting in a 'closed' conformation of the protease domain.

Although it has long been believed that domains IV and VI regulate the protease activity of calpain, the 3D structure of domain VI in the absence and presence of Ca^{2+} reveals no remarkable structural change upon Ca^{2+} binding [15,16]. Moreover, both domains are distant from domain II in the 3D structure. These facts strongly suggest that domains IV and VI are not involved in Ca^{2+}-dependent activation of the calpain molecule.

Based on amino acid sequence similarity and Ca^{2+}-binding experiments, it was predicted that a 'sixth' EF-hand motif existed in the C-terminal region of domain IIb (His319–Phe331) [17]. The 3D structure of m-calpain reveals that the region does not form an EF-hand structure and no explicit Ca^{2+}-binding structure was found in any part of domain II (see Fig. 4 and Section 4).

Thus, it is hypothesized that the default structure of domain II itself is 'closed'. To test our working hypothesis, and to obtain an understanding of the Ca^{2+}-dependent activation mechanism of m-calpain, we produced and characterized a truncated human m-calpain composed only of domain II. We predicted that domain II should demonstrate Ca^{2+}-independent protease activity. Instead, we found an unexpected property of the domain II: a Ca^{2+}-dependent protease activity.

2. Materials and methods

2.1. Construction of the expression vector

The cDNA fragment corresponding to domain II of human m-calpain was amplified by PCR using *Pfu* polymerase (Stratagene Inc., CA, USA) and a set of primers, 5′-TGTCAGTTACAGATCTCCAGGCGGGAA-3′ and 5′-TGGGCTCCCACGAGAGGGCCATCAAGT-3′. The amplified cDNA was inserted into a *Nde*I site of the pET16b vector (Novagen Inc., WI, USA) to produce a truncated human m-calpain 80-kDa subunit composed of only domain II (residues 19–342) with a N-terminal histidine tag.

2.2. Protein expression

The plasmid constructed was transformed into *Escherichia coli* BL21(DE3) (Novagen), and the transformant was cultured at 37°C in 1 l of LB medium, containing 100 μg/ml ampicillin, to an OD of 0.8–1.0 at 600 nm. Protein expression was induced by the presence of 1.0 mM IPTG for 3 h at 30°C. The harvested cells were suspended in 40 ml of 40 mM imidazole in buffer A (20 mM Tris–HCl, pH 7.5, and 300 mM NaCl), and lysed by a French press (American Instrument Company, MD, USA). The supernatant was recovered by ultracentrifugation (35 000 × *g*, 4°C, 60 min) and filtered through a 0.22-μm pore filter.

The supernatant was then applied to a 7-ml Ni^{2+}-chelating agarose column equilibrated with buffer A. The column was washed with 20 mM imidazole in buffer A, and eluted with 250 mM imidazole in buffer A. The eluted solution was further purified by MonoQ anion-exchange column chromatography with a linear gradient of 0–0.5 M NaCl in 20 mM Tris–HCl (pH 7.5), 5 mM EDTA, and 1 mM dithiothreitol. Purification was confirmed by SDS–PAGE for its homogeneity. The protein concentration was measured by a Bradford method protein assay kit (Bio-Rad Inc., Yokohama, Japan) according to the manufacturer's instruction. The purified protein was stored at 4°C until use.

2.3. Band mobility shift analysis

Recombinant domain II (0.5 μg) was incubated for 14 h at 30°C in the presence or absence of 5 mM Ca^{2+} with or without 1.6 mM E-64c. Samples were electrophoresed on SDS–polyacrylamide gels according to the standard method.

2.4. Caseinolytic assay

Proteolytic activity was measured using casein as a substrate. Recombinant domain II (0.5 μg) was incubated with 3 μg casein in the absence or presence of 5 mM Ca^{2+}, 1.6 mM E-64c, and/or 1 mg/ml *E. coli*-expressed calpastatin domain I (Takara Shuzo, Kyoto, Japan), one of four functional repetitive units of calpastatin. Reactions were stopped by adding SDS sample buffer (100 mM Tris–HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 0.4% (w/v) bromophenol blue, and 2% (v/v) 2-mercaptoethanol). Caseinolytic activity was evaluated by electrophoresis on SDS–polyacrylamide gels with Coomassie brilliant blue staining.

3. Results

3.1. Expression and purification of recombinant domain II of human m-calpain

Domain II (residues 19–342) of the human m-calpain 80-kDa subunit was expressed in *E. coli* BL21(DE3) with an N-terminal histidine tag. The protein was produced in a high yield, soluble form, and purified to homogeneity by Ni^{2+}-affinity and MonoQ column chromatography. The elution fraction from the MonoQ column was between 200 and 300 mM NaCl, which is similar to that of full-length m-calpain. About 12 mg of the protein was purified from a 2-l culture.

3.2. Recombinant domain II of m-calpain shows Ca^{2+}-dependent activity

To investigate whether or not the purified domain II shows Ca^{2+}-independent protease activity, its caseinolytic activity was examined in the absence or presence of 5 mM Ca^{2+}. As shown in Fig. 1, domain II degraded casein in a Ca^{2+}-dependent manner without apparent autolysis. The degradation was incomplete even after a 14-h incubation (Fig. 1a, lane 7). The specific activity for caseinolysis was too low to determine precisely (less than 1/100 of that of full-length m-calpain). Two

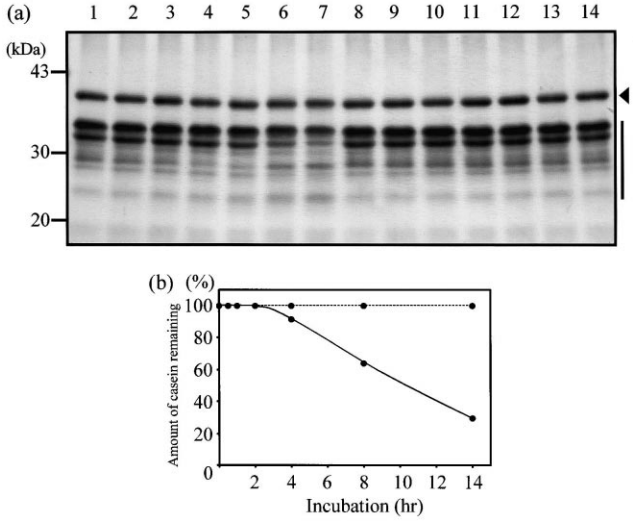

Fig. 1. Caseinolytic activity of the domain II. a: Purified domain II of human m-calpain was incubated with casein in the presence (lanes 1–7) or absence (lanes 8–14) of 5 mM Ca^{2+} for 0 (lanes 1 and 8), 0.5 (lanes 2 and 9), 1 (lanes 3 and 10), 2 (lanes 4 and 11), 4 (lanes 5 and 12), 8 (lanes 6 and 13), or 14 h (lanes 7 and 14). Arrowheads and vertical lines indicate the purified domain II of human m-calpain and heterogeneous casein molecules, respectively. b: The time course of casein degradation is shown in the absence (broken line) or presence (solid line) of 5 mM Ca^{2+}. The casein band intensities were quantified by densitometry.

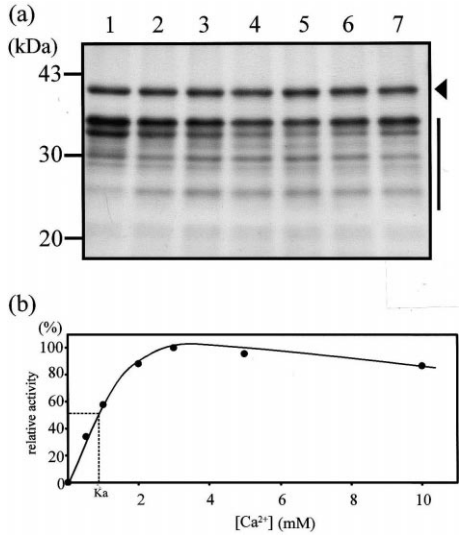

Fig. 2. Ca^{2+} requirement for the activity of domain II. a: Purified domain II was incubated with casein in the absence (lane 1) or presence of 0.5, 1, 2, 3, 5, or 10 mM Ca^{2+} (lanes 2–7). An arrowhead and a vertical line indicate the domain II and casein molecules, respectively. b: The casein band intensities in (a) were quantified by densitometry.

peptidyl fluorescent substrates for μ- and m-calpains, succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide and succinyl-Leu-Tyr-4-methoxy-2-naphthylamide, were not hydrolyzed even after a 14-h incubation at 30°C.

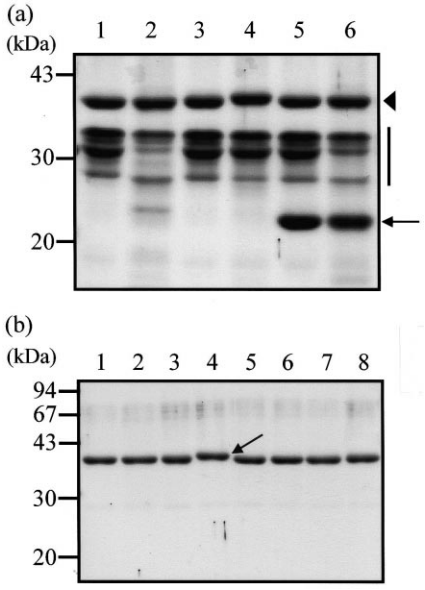

Fig. 3. Effects of E-64c and calpastatin on domain II. a: Caseinolytic activity of the domain II was examined by incubation without inhibitors (lanes 1 and 2), and with E-64c (lanes 3 and 4) or calpastatin domain I (lanes 5 and 6) for 0 (odd lanes) or 14 h (even lanes). An arrowhead, a vertical line, and an arrow indicate the domain II, casein molecules, and calpastatin domain I, respectively. b: Each sample was run on SDS–polyacrylamide gels after incubation for 0 (odd lanes) or 14 h (even lanes) in the presence (lanes 1–4) or absence of Ca^{2+} (lanes 5–8) with (lanes 3, 4, 7, and 8) or without E-64c (lanes 1, 2, 5, and 6). An arrow indicates the domain II that showed a change in migration due to modification by E-64c.

3.3. Ca^{2+} requirement of recombinant domain II

Since the caseinolytic activity of the domain II increased almost linearly until 14 h and was maximal at 30°C, we employed a 14-h incubation at 30°C for further analyses. Under these conditions, the Ca^{2+} dependence of the caseinolytic activity of domain II was examined (Fig. 2a). The Ca^{2+} concentration required for the half-maximal activity (K_a) was calculated to be 0.90 mM, which was even higher than that for full-length m-calpain (approximately 0.4 mM, [18]; Fig. 2b).

3.4. Effect of inhibitors and a mobility shift assay

We examined the effect of calpain inhibitors, E-64c and calpastatin. E-64c is an active site-directed cysteine protease inhibitor with a molecular mass of 357.41, and calpastatin is a ubiquitous protein that inhibits μ- and m-calpains effectively and specifically. As shown in Fig. 3a, domain II was inhibited completely by E-64c (lane 4), and partially by calpastatin

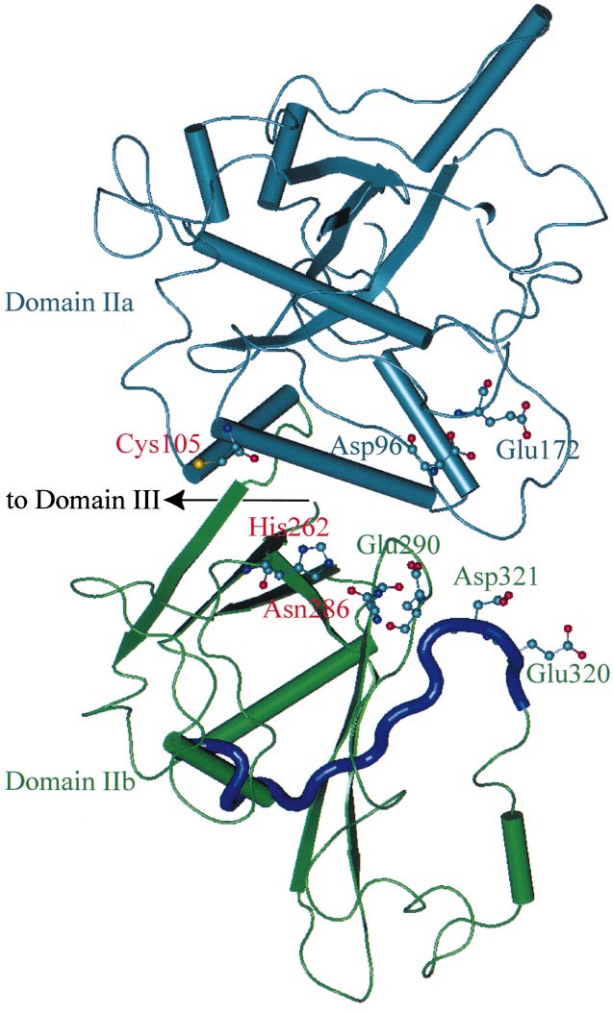

Fig. 4. Amplified view of an 'open' structure of domain II in the absence of Ca^{2+}. The 3D structure of domain II in the absence of Ca^{2+} is divided into subdomains IIa (cyan, upper side) and IIb (green, lower side) Catalytic residues (Cys105, His262, and Asn286), together with acidic residues (Asp96, Glu172, Glu290, Glu320, and Asp321) possibly involved in ionic repulsion between these subdomains are shown. Yellow, red, blue, and gray balls represent sulfur, oxygen, nitrogen, and carbon, respectively. A blue tube in domain IIb indicates the position (residues 319–331) where an EF-hand motif was once predicted. This figure was prepared using VMD Software, version 1.6.1 [21].

(lane 6). Moreover, calpastatin was partially proteolysed by domain II.

As shown in Fig. 3b, a clear singlet shift in mobility was observed only when domain II was incubated in the presence of Ca^{2+} and E-64c (lane 4), indicating that E-64c was covalently bound to the active site of domain II only in the presence of Ca^{2+}. Thus, it was shown that domain II forms an active site in a Ca^{2+}-dependent manner, and that 100% of the recombinant domain II was modified by E-64c. It was possible, however, that Ca^{2+} was not specific for activation of domain II, i.e. ionic strength resulted in the activation of domain II. To eliminate this possibility, we tested the effects of other ions, including K^+, Na^+, Mg^{2+}, Mn^{2+}, and Ba^{2+} (5 mM each), but these ions did not activate domain II nor cause a mobility shift (data not shown). Thus, the activation of the domain II was specific for Ca^{2+}.

4. Discussion

In this study, we have purified and characterized a recombinant domain II of m-calpain, and observed that domain II showed Ca^{2+}-dependent proteolytic activity. Although the specific activity of domain II was very low, a band shift by E-64c binding (see Fig. 3b) clearly indicated that all the recombinant domain II was correctly folded and active in the presence of Ca^{2+}, and that the low specific activity was not due to partial denaturation of the protein. Thus, we concluded that the default state of the domain II is 'open', and that it becomes 'closed' upon Ca^{2+} binding.

This raises the question: where is the Ca^{2+}-binding site in the domain II? Andresen et al. showed that only domain II and its flanking regions of schistosome calpain could bind Ca^{2+} [17], leading to their speculation that a 'sixth' EF-hand motif in the C-terminal region of domain IIb is a candidate for the Ca^{2+}-binding site. In the 3D structure of m-calpain, neither an EF-hand motif nor other Ca^{2+}-binding motifs were found in domain II (Fig. 4). The region (residues 319–331) is highly conserved in various calpain homologs, and Glu320 and Asp321 in this region and Glu290 in the adjacent loop are close to Asp96 and Glu172 in domain IIa, indicating ionic repulsion between domains IIa and IIb (Fig. 4). Therefore, it is possible that charge neutralization by Ca^{2+} binding to some of these acidic residues permits closure of domain II.

The low specific activity of domain II suggests that other domains assist formation of the 'closed' active site upon Ca^{2+} binding. Without other domains, domain II may not be able to form the 'closed' structure efficiently. Alternatively, other domains, especially domain III, may play a role identical to Ca^{2+} in activating domain II. In other words, domain II may be able to form the 'closed' structure without Ca^{2+} binding to domain II when the other domains exist. These points may be clarified by using m-calpain molecules that have mutations in possible Ca^{2+}-binding residues in the domain II. This is now being examined in our laboratory.

It has been assumed that several 'atypical' calpain homologs that lack domains III, IV, and T, such as stomach-specific nCL-2′ [19] and *Drosophila* SOL [20], might possess Ca^{2+}-independent activity. Our results however, indicate that these atypical calpains should have Ca^{2+}-dependent protease activity because these acidic residues, comprising a putative Ca^{2+}-binding site, are conserved among these calpain species. On the other hand, it should be noted that the domain I+II protein had no proteolytic activity even in the presence of Ca^{2+} (data not shown). Although the possibility that the domain I+II protein could not fold correctly in *E. coli* cells cannot be eliminated, it is possible that the unanchored domain I prevented domain II from forming the 'closed' structure.

It was surprising that domain II remained stable and active for more than 10 h without autolysis (see Fig. 1), compared with the very rapid inactivation of intact m-calpain (half-life is less than 1 h). This suggests that inactivation of the intact calpain is mainly caused by intra/inter-molecular autolytic degradation dependent upon domains other than the protease domain. In other words, the domains are important for intra/inter-molecular autolysis. These points will be clarified by resolving the 3D structure of calpain in the presence of Ca^{2+}.

Acknowledgements: We would like to thank Dr. Wolfram Bode and Dr. Hiroyuki Kawahara for valuable discussions. This work has been supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (Intracellular Proteolysis) from the Ministry of Education, Science, Sports and Culture, a Grant-in-Aid for Scientific Research from JSPS (Japan Society for the Promotion of Science), a Research Grant (11B-1) for Nervous and Mental Disorders from the Ministry of Health and Welfare, CREST of JST (Japan Science and Technology), a grant from the Kato Memorial Bioscience Foundation, and from the Human Frontier Science Programme.

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