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Distinct kinetics of subunit autolysis in mammalian m-calpain activation

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Abstract

Subunit autolysis of mammalian m-calpain upon activation was examined in kinetic terms using a set of antibodies recognizing different portions of the protease. Activation of m-calpain by calcium resulted in no apparent autolysis in the large catalytic subunit, whereas the small regulatory subunit underwent immediate autolysis followed by substrate proteolysis. This profile of subunit autolysis is distinct from that of the other ubiquitous isozyme, μ-calpain, in which autolysis of the large subunit and then of the small subunit precedes substrate proteolysis under the normal conditions. The activation state of m-calpain thus is not reflected by the large subunit autolysis. The mode and role of autolysis may vary among calpain isozymes.

Key words: m-Calpain; Autolysis; Proteolysis; Calcium

1. Introduction

Calpain [1–3], calcium-activated neutral endopeptidase, is a unique receptor for calcium signals in biological systems since its activation leads to irreversible modification of substrate proteins through limited proteolysis. Because calpain seems to be involved not only in physiological signal transduction processes [2,4] but also in pathological states, such as ischemia-induced delayed neuronal death [5], calpain research has attracted interest from a wide range of the life sciences. Its in vivo mode of action, however, remains unclear, due in part to the technical difficulty in resolving the activation processes. The elucidation of the mechanism by which this protease is activated, therefore, is an essential step towards an understanding of its roles in cells and tissues.

One of the notable features in calpain activation is that it undergoes subunit autolysis in the presence of calcium, as initially reported by Suzuki et al. using purified chicken calpain [6]. Autolysis results in reduction of the calcium concentration required for the proteolytic activity to arise and thus is considered an important process in the activation mechanism. This 'autolytic activation' applies to the mammalian calpain isozymes, μ- and m-calpain, as well [7–9]. Both the large catalytic (80 kDa) and small regulatory (30 kDa) subunits are autolyzed to release amino-terminal fragments. However, the fine details and specific roles of this process are not yet fully understood. For instance, controversy over which of the two subunits undergoing autolysis directly modifies the enzyme activity has not yet been settled [8,10,11]. In fact, we have not yet defined the relationship between the initiation of proteolytic activity (enzyme activation) and the autolysis-induced reduction of the calcium requirement. They are undoubtedly closely related but may not necessarily be equivalent to each other.

Because the large subunit autolysis of m-calpain can not be resolved by electrophoresis, it has been only poorly analyzed in kinetic terms [7,8,12]. In contrast, autolysis of μ-calpain is electrophoretically detectable and thus has been much better characterized [9,13]. Using antibodies that distinguish between the pre- and post-autolysis forms of μ-calpain, we have demonstrated that subunit autolysis precedes substrate proteolysis in test tubes [9], and that μ-calpain undergoes autolysis at membranes upon activation in platelets [13]. Similar approaches, if applicable, should be helpful in resolving the autolytic process of m-calpain.

In the present study, we employed antibodies that recognize different portions of the m-calpain molecule to kinetically analyze its autolytic process. A rather unexpected conclusion was drawn from our results.

2. Experimental

2.1. Reagents

Casein was purchased from Merck. The restriction enzymes, nucleases, and ligases were from Takara Shuzo. Both m-calpain (920 U/mg) and μ-calpain (680 U/mg) were purified from rabbit skeletal muscles as previously described [9]. An antipeptidic antibody against the amino-terminal 18-mer segment of pre-autolysis m-calpain (anti-pre-m80K$_{18}$) was developed using a synthetic peptide, AGIAAKLAKDREAAEG-

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LGC, conjugated to keyhole limpet hemocyanine and affinity-purified as previously described [9,14]. A portion of the antibody was further affinity-purified on an immobilized peptide, AGIAAKLAKC, to produce an antibody specific to the amino-terminal 9-mer segment of pre-autolysis m-calpain (anti-pre-m80K_9). Antibodies to the pre- and post-autolysis forms of μ-calpain and to the whole molecule of m-calpain were previously described [9].

2.2. Expression of recombinant m-calpain large subunit in E. coli

Bacterial expression vectors for the large subunit of m-calpain were constructed as follows. The cDNA encoding the large subunit of human m-calpain [15] was treated successively with a restriction enzyme, *Bbe*I, with S1 nuclease, and with another restriction enzyme, *Spe*I, to produce a fragment covering the entire coding region. This fragment was ligated with a vector, pTV119N (Takara Shuzo), which had been digested with restriction enzymes *Hinc*II and *Xba*I. To match the frames of the vector- derived initiation codon and the cDNA, the product was further subjected to digestion with *Nco*I, filled-in with Klenow fragment of DNA polymerase I, partially digested with *Eco*52I to conserve the in-frame *Eco*52I site, filled-in with Klenow fragment, and self-ligated. The final product, m80k/pTV119N, contains a segment derived from the non-coding 5′ region of the calpain cDNA (GGCCGCCGGGACCGCAGC) between the vector-derived initiation codon and the cDNA-derived one. The recombinant protein therefore possesses an extra amino acid sequence, MGRRDRS, at the amino-terminus. Another expression vector (δm80k/pTV119N) to produce a truncated m-calpain devoid of the amino-terminal 12-mer segment (GIAAKLKDREA) was produced as follows. The m80k/PTV119N was subjected to digestion with *Nco*I, filled-in with Klenow fragment, digested with *Eco*52I, digested with S1 nuclease, and self-ligated. These vectors were used to transfect *E. coli* to produce full-length and truncated large subunits of m-calpain as described [16].

2.3. Enzyme reactions and other procedures

Calpain-catalyzed proteolysis of casein was performed in a volume of 100 μl containing 3 mg/ml casein, 10 μg/ml μ- or m-calpain, 1 mg/ml bovine serum albumin (BSA), 10 mM β-mercaptoethanol, and 100 mM Tris-HCl (pH 7.5) under the conditions indicated in the figure legends [9]. SDS-PAGE and immunoblotting were performed as previously described [9,14].

3. Results

The specificity of the three anti-m-calpain antibodies employed (anti-whole m-calpain, anti-pre-m80K_{18}, anti-pre-m80K_9) was first examined using the recombinant m-calpain large subunits produced in *E. coli* (Fig. 1). While the anti-whole m-calpain antibody reacted with both the full-length and truncated forms of the large subunit, the anti-pre-m80K_{18} and anti-pre-m80K_9 antibodies recognized only the full-length one, not the truncated one devoid of the amino-terminal 12-mer segment. This certifies that the antipeptidic antibodies are solely specific to the amino-terminal portion of the m-calpain large subunit. Because the autolytic product of m-calpain exists in two forms, partially autolyzed and fully autolyzed, devoid of the terminal 8mer segment and of 18mer segment, respectively [8], the anti-pre-m80K_9 anti-

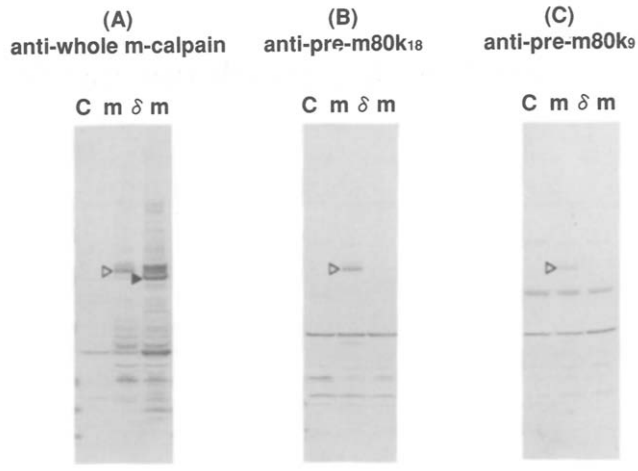

Fig. 1. Specificity of the anti-m-calpain antibodies. Lysates of *E. coli* transfected with PTV119N vector only (c: control), m80K/PTV119N (m: full-length m-calpain large subunit), or δm80k/PTV119N (δm: truncated m-calpain large subunit) were subjected to Western blot analysis using anti-whole m80k (A), anti-pre-m80k_{18} (B), and anti-pre-m80k_9 (C) antibodies. The open and closed arrowheads indicate the products of m80k/PTV119N and δm80k/PTV119N, respectively.

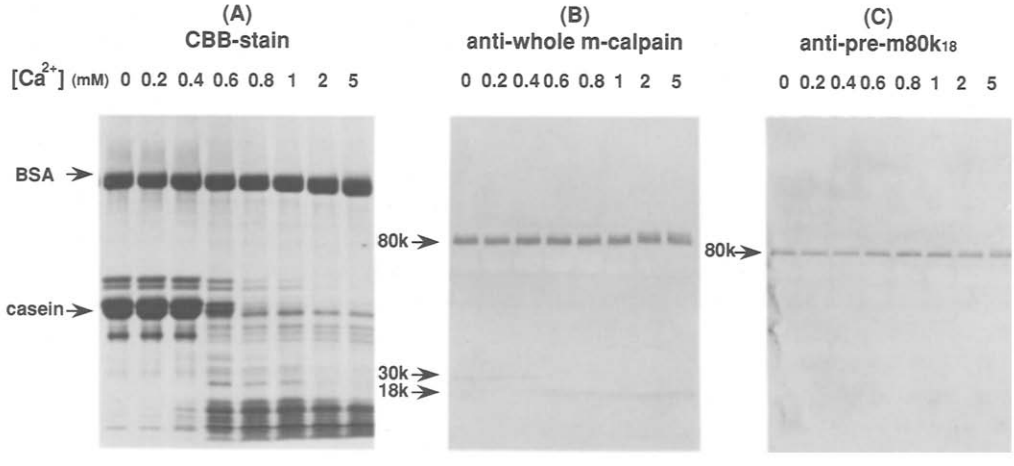

Fig. 2. Effect of calcium concentration on proteolytic activity and subunit autolysis of m-calpain. Casein, m-calpain, and BSA were incubated at 30°C for 5 min (see section 2) in the presence of various calcium concentrations and then subjected to SDS-PAGE on a 10–20% gradient gel (A) or 4–20% gels (B and C). The proteins were visualized by CBB (Coomasie brilliant blue) staining (A) or immunoblot analysis using anti-whole m-calpain antibody (B) and anti-pre-m80k_{18} (C). Note that the anti-whole m-calpain antibody reacts with both the large and small subunits [9]. BSA added to minimize calpain-catalyzed degradation of calpain. The arrows indicate the mobility of indicated proteins or relative molecular weights.

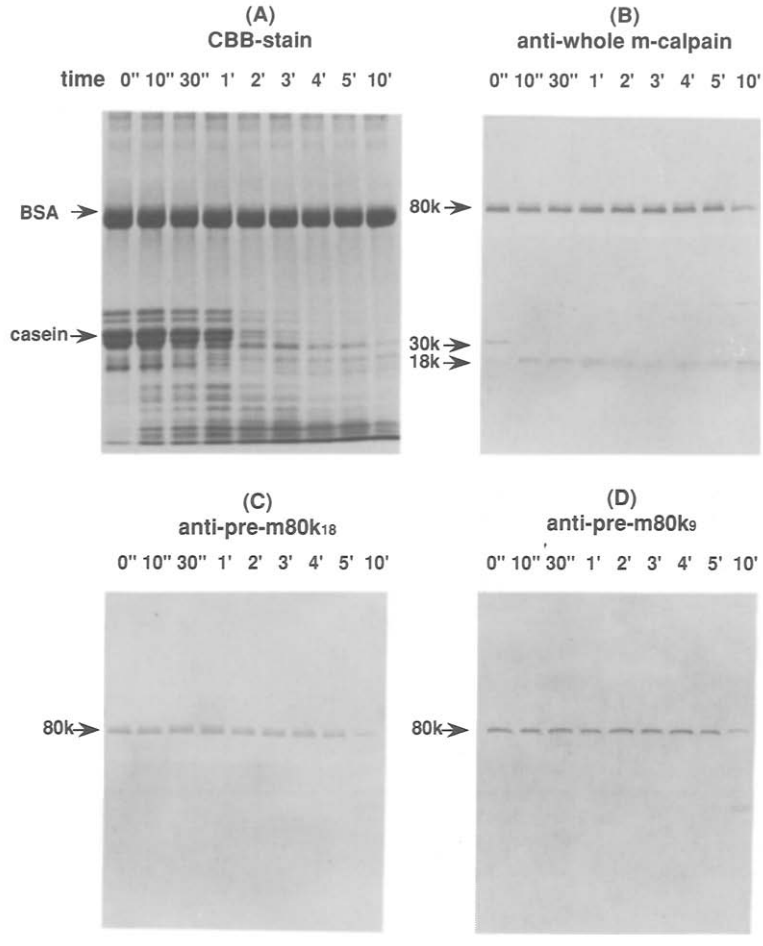

Fig. 3. Time–course of substrate proteolysis and subunit autolysis upon activation of m-calpain. The experiments were performed as described in the legend to Fig. 2 in the presence of 5 mM Ca^{2+} at 30°C. The reactions were terminated at indicated times. The results were visualized by CBB-staining (A) and by immunoblot analysis using the anti-whole m-calpain (B), anti-pre-m80k_{18} (C) and anti-pre-m80k_9 antibodies.

body recognizes neither of the autolyzed forms whereas the anti-pre-m80K_{18} antibody may recognize the partially autolyzed one. Removal of these amino-terminal segments in m-calpain by autolysis should result in reduced reactivity to the anti-pre-m80K_9 and anti-pre-m80K_{18} antibodies while the anti-whole m-calpain antibody binding should remain unchanged. These anti-m-calpain antibodies were employed to analyze autolysis of m-calpain subunits.

Fig. 2 shows the effect of calcium concentration on proteolysis of casein and autolysis of the m-calpain large and small subunits in test tubes. In accordance with casein proteolysis observed in the presence of 0.4 mM or greater Ca^{2+} (panel A), the small subunit of 30 kDa was converted to a 18 kDa form by autolysis (panel B). In contrast, the large subunit showed no sign of autolysis as analyzed by the anti-pre-m80K_{18} antibody. This relationship between the substrate proteolysis and the subunit autolysis was further examined in chronological terms (Fig. 3). In the presence of sufficient calcium, casein started to be fragmented within 10 s (panel A). By this time, the small subunit was fully autolyzed (panel B). In clear contrast again, the large subunit remained unautolyzed even at the time when casein was fully proteolyzed (panel C). At 10 min, the anti-pre-m80K_{18} antibody reactivity was significantly reduced. This seems to be mainly due to calpain-catalyzed degradation of calpain rather than intramolecular autolysis because the anti-whole m-calpain antibody reactivity was also diminished in a similar manner. Western blot analysis using the anti-pre-m80K_9 antibody gave similar results (panel D). The antibody binding remained unchanged, indicating that the majority of m-calpain did not even lose the amino-terminal 8-mer portion of the large subunit under these conditions. These results suggest that the large subunit of m-calpain does not undergo autolysis at the previously described cleavage sites within the time span during which substrate proteolysis proceeds.

Fig. 4 shows a comparison of the profiles of subunit autolysis and substrate proteolysis at 4.5°C in μ- and m-calpain. These conditions allow closer examination of the chronological relationship between autolysis and proteolysis [9]. Clearly, the kinetics of subunit autolysis is different between these isozymes whereas the casein

proteolysis arose similarly. A common feature shared by both the calpain isozymes is that the small subunit autolysis preceded the casein proteolysis. Notably, the small subunit autolysis was completed much earlier in m-calpain than in μ-calpain, indicating that the dynamic processes involved in the enzyme activation may vary substantially among calpain isozymes.

4. Discussion

We have demonstrated that the large subunit of mammalian m-calpain does not undergo autolysis within the time span during which substrate proteolysis proceeds, whereas the small subunit autolysis arises prior to the substrate proteolysis. The results agree with the observation by Hathaway and McClelland [7] showing that the amino-terminus of the large subunit remained blocked after incubation with Ca^{2+} under the conditions allowing full autolysis of the small subunit.

Our observation does not necessarily contradict the data presented by Brown and Crawford [8] and by Croall et al. [12] showing that autolysis of m-calpain large subunit proceeded very slowly in the time span 2–10 min. Because no exogenous substrates were added in the reaction mixture in which calpain was subjected to activation in their experiments, it is likely that the 'autolysis' of the large subunit was caused by intermolecular reactions between activated calpains. This again contrasts with the μ-calpain autolysis which is catalyzed by both intra- and intermolecular reactions [17]. At any rate, the large subunit autolysis is not an early pre-activation event in mammalian m-calpain. Therefore, the large subunit autolysis can not be used as a hallmark for m-calpain activation unlike the case with μ-calpain [9].

Brown and Crawford [8] demonstrated the correlation between the partial autolysis of the large subunit of m-calpain and the change in its Ca^{2+} requirement. Their results and ours taken together suggest that the enzyme activation defined as initiation of proteolytic activity is not equivalent to the reduction of the Ca^{2+} requirement in the case of m-calpain. It is as yet unclear whether this assumption applies to μ-calpain because the enzyme activation and the large subunit autolysis are inseparable in this case under the experimental conditions thus far examined [9].

The difference in the profile of the subunit autolysis between μ- and m-calpain brings up the following implications. One possibility is that the mode and role of autolysis may have diversified during the molecular evolution of the calpain family now consisting of at least six distinct isozymes [3]. A plausible interpretation based on this would be that the prototype calpain isozymes, including μ-calpain, require autolysis of both the subunits for activation while more evolved ones no longer rely on the large subunit autolysis, or vice versa. A contrasting interpretation is also possible; the autolytic reactions may not actually play any role in the activation mechanism but rather have emerged as a consequence of activation associated with conformational changes and, therefore, can vary among isozymes.

We do not yet have sufficient data to evaluate these possibilities. Analysis of autolysis in calpain isozymes from the wide range of taxonomic origins [3], including the recently identified tissue-specific ones [16,18], is necessary to address these questions. Mutational analysis

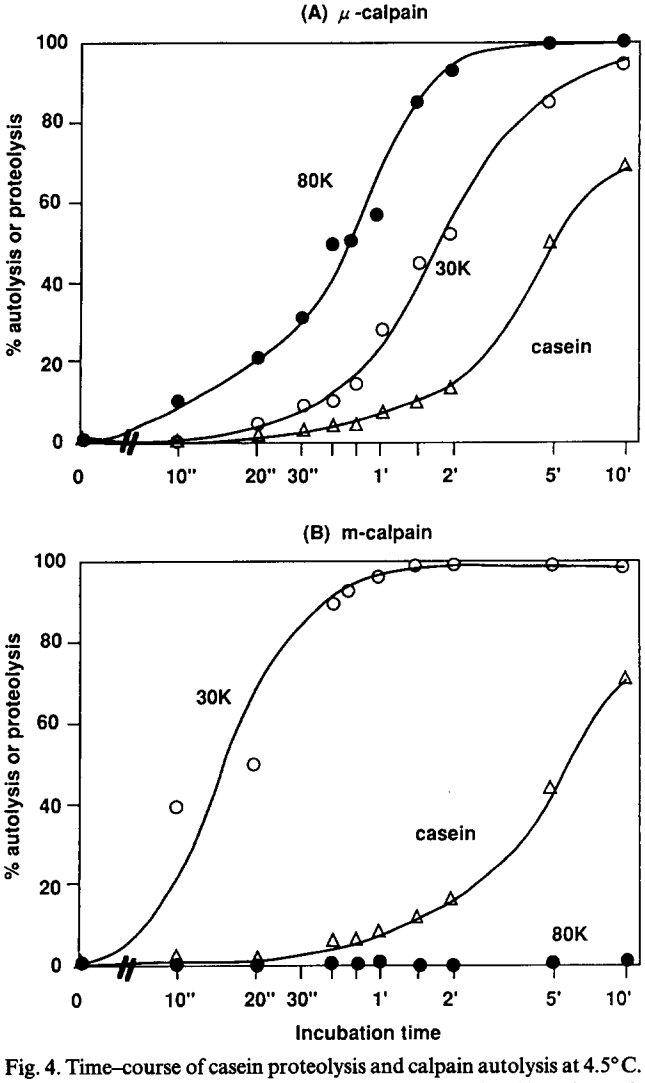

Fig. 4. Time–course of casein proteolysis and calpain autolysis at 4.5°C. Experiments were performed using purified μ-calpain (A) and m-calpain (B) in parallel similarly to those described in the legend to Fig. 3 except that the reaction mixtures were incubated at 4.5 °C. The anti-pre-m80k$_9$ antibody was used to analyze m-calpain autolysis. Results shown in panel A were described previously [9]. The data were densitometrically quantitated by a video densitometer (ACI Japan) and used to calculate % autolysis and proteolysis. % autolysis is given by B/(A+B) × 100, where A and B stand for the normalized density of the pre- and post-autolysis forms of the subunit, respectively. % proteolysis is given by D/(C+D) × 100, where C and D correspond to the density of intact casein bands and the increase in the proteolytic fragments, respectively.

using the cDNA expression system [16] should also be helpful.

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