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Calpain proteolysis of free and bound forms of calponin, a troponin T-like protein in smooth muscle

Shoji Tsunekawa*+, Katsuhito Takahashi°, Masahiro Abe°, Kunio Hiwada°, Kazue Ozawa+ and Takashi Murachi*

**Department of Clinical Science and Laboratory Medicine and +Second Department of Surgery, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan* and *°Second Department of Internal Medicine, Ehime University School of Medicine, Onsen-gun, Ehime 791-02, Japan*

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Calponin, a novel homologue of troponin T, purified from chicken gizzard was found to be one of the most susceptible proteins among smooth muscle contraction-associated proteins to hydrolysis by calpain I purified from human red blood cells. The high susceptibility of calponin was comparable to that reported for troponin T. The rate of degradation of calponin, unlike caldesmon and myosin light chain kinase, was accelerated when bound to calmodulin. When calponin existed as a bound form in both reconstituted actin filament and native thin filament, the rate of proteolysis was markedly retarded, indicating close association of calponin with actin filament. These observations are compatible with the view that calponin is an integral part of the actin-linked contractile machinery in smooth muscle.

Calpain; Proteolysis; Smooth muscle; Calmodulin-binding protein; Troponin T; Thin filament

1. INTRODUCTION

Recent discovery of a troponin T-like protein in smooth muscle, referred to as calponin [1,2], elicited novel approaches to the study of smooth muscle contraction. Calponin isolated from chicken gizzard smooth muscle was found to bind calmodulin, F-actin, and tropomyosin, suggesting its actual involvement in smooth muscle contraction in a way equivalent to that for troponin T in skeletal muscle [3,4].

Calpain (EC 3.4.22.17) is a Ca^{2+}-dependent cysteine endopeptidase, which is known to be very widely distributed in animal tissues (for review see [5]), including smooth muscles of bovine blood vessels [6] and chicken gizzard [7]. Increased degradation of myofibrillar proteins at elevated intracellular Ca^{2+} concentrations have been frequently reported [8,9]. Thus, the purpose of the present study was to determine if calponin could be proteolyzed by calpain and, if so, how such proteolysis would be affected by calponin binding to other contraction-associated proteins.

Correspondence address: T. Murachi, Department of Clinical Science and Laboratory Medicine, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan

2. MATERIALS AND METHODS

2.1. *Smooth muscle proteins*

Actin [10], myosin [11], myosin light chain kinase [12], calponin [3], calmodulin (Takahashi, K. et al., unpublished), tropomyosin and caldesmon [13] were purified from chicken gizzard. They were practically devoid of contamination (fig.1). The thin filaments were prepared in the 'native' state from chicken gizzard as described [14]. F-actin-bound calponin was prepared at a molar ratio of actin monomer to calponin of 7 [15].

2.2. *Proteolysis by calpain*

Proteolysis was carried out at 25°C in 1.0 ml incubation mixture containing 0.4 mg of the substrate protein, 0.49 unit (or 3.3 μg) of calpain I purified from human red blood cells [16], 20 mM Tris-HCl buffer (pH 7.5), 100 mM NaCl, 0.5 mM DTT, 5 mM cysteine and 0.5 mM $CaCl_2$. Calmodulin was added to the reaction mixture to saturate the calmodulin-binding sites of the substrate protein: molar ratios to calmodulin were 0.25 for calponin, and 0.5 for caldesmon monomer and myosin

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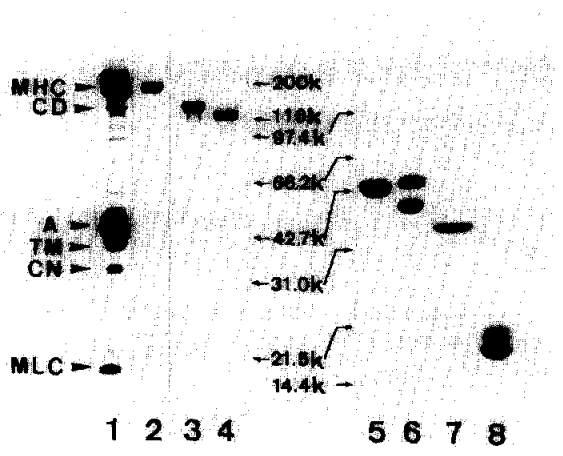

Fig.1. SDS-PAGE of crude actomyosin and purified proteins from chicken gizzard smooth muscle. Lane 1, crude actomyosin (40 μg protein); lane 2, myosin (7 μg); lane 3, caldesmon (7 μg); lane 4, myosin light chain kinase (6 μg); lane 5, actin (10 μg); lane 6, tropomyosin (10 μg); lane 7, calponin (8 μg); lane 8, calmodulin (9 μg). The acrylamide concentration used for lanes 1–4 was 10% and for lanes 5–8, 12.5%. MHC, myosin heavy chain; CD, caldesmon; A, actin; TM, β subunit of tropomyosin; CN, calponin; MLC, myosin light chain; k, kDa.

light chain kinase. For F-actin-bound calponin, the incubation mixture contained 4.3 mg of substrate protein, corresponding to 0.4 mg of calponin as estimated by densitometric analysis on SDS-PAGE. For the proteolysis of native thin filament, the final protein concentration was 3.5 mg/ml, corresponding to approximately 0.15 mg of calponin and 0.15 mg of caldesmon. The reaction was stopped at time intervals by transferring an aliquot of the reaction mixture into SDS-PAGE sample buffer.

2.3. *Other materials and methods*

The pattern of proteolysis was analyzed by SDS-PAGE in the buffer system of Laemmli [17]. Densitometric scanning was performed with a Toyo digital densitometer, Densitol, model DMU-33C (Tokyo, Japan) at 620 nm. Protein concentration was determined by the method of Bradford [18] using bovine immunoglobulin as the standard. Immunoblot analysis was carried out with anti-calponin and anti-caldesmon antibody as described [19].

3. RESULTS

3.1. *Susceptibility to calpain*

Among the proteins shown in fig.1, calponin and caldesmon were the most susceptible to hydrolysis by calpain I, followed by myosin light chain kinase (fig.2). The percentage of densitometric intensity on SDS-PAGE of a remaining band, which retained the same mobility as the starting material, was plotted as a function of the incubation time. Semi-logarithmic plots gave an almost linear decrease in the early phase of the reaction, indicating that the proteolytic reaction followed apparent first-order kinetics. This led us to calculate, in common logarithms, apparent first-order rate constant, k_{app}, for each reaction. The k_{app} values for calponin, caldesmon and myosin light chain kinase were 8.1×10^{-3} s^{-1}, 9.6×10^{-3} s^{-1} and 3.1×10^{-3} s^{-1}, respectively.

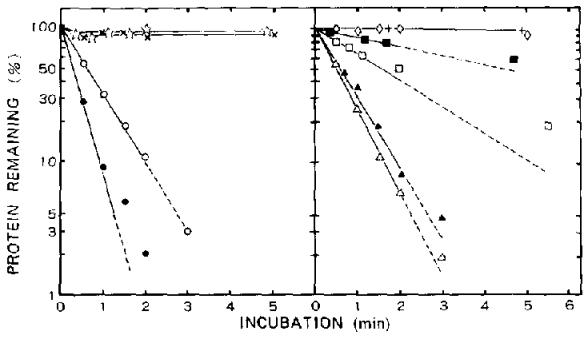

Fig.2. Susceptibility of individual proteins purified from smooth muscle to calpain I. The logarithmic ordinate shows the percentage of densitometric intensity of a remaining protein band having the same mobility on SDS-PAGE as the starting material after various times of incubation. (☆) Actin; (×) myosin; (◇) tropomyosin; (+) calmodulin; (○,●) calponin; (□, ■) myosin light chain kinase; (△, ▲) caldesmon. For the last three substrate proteins: open symbols indicate an incubation in the absence of calmodulin; closed symbols indicate an incubation in the presence of calmodulin.

Calponin (34 kDa) was degraded into 30, 27, and 19.5 kDa fragments before further extensive digestion (fig.3A). Although tropomyosin remained intact within 90 s, a smaller portion of its γ subunit with an apparent molecular mass of 50 kDa was gradually degraded thereafter, producing a major fragment at 43 kDa and several minor fragments at 30–20 kDa. F-actin, myosin, and calmodulin were resistant to calpain under the same conditions (fig.2).

3.2. *Effect of calmodulin*

Calponin was degraded more rapidly when incubated with calpain in the presence of 4 times molar excess of calmodulin. The k_{app} value calculated was 1.8×10^{-2} s^{-1}, which was 2.2 times higher than that found in the absence of calmodulin (fig.2). However, the degradation pat-

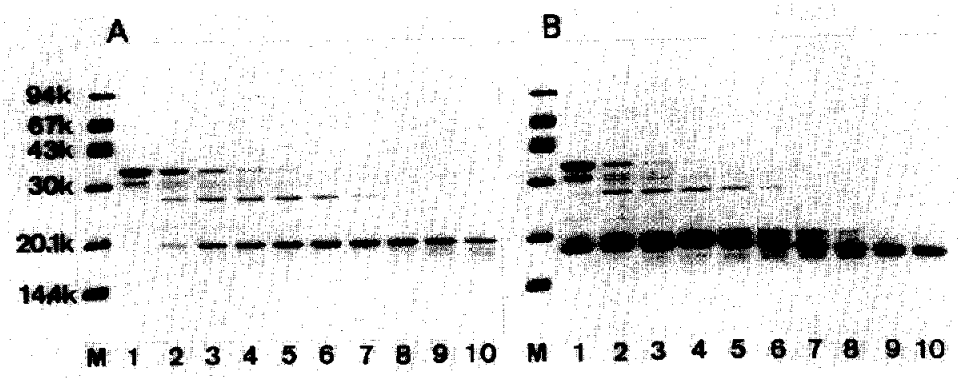

Fig.3. Degradation pattern of calponin by calpain I in the absence (A) and in the presence (B) of calmodulin (12.5% SDS-PAGE gels). The incubation periods were: lane 1, 0 min; lane 2, 30 s; lane 3, 60 s; lane 4, 90 s; lane 5, 2 min; lane 6, 3 min; lane 7, 5 min; lane 8, 15 min; lane 9, 30 min; lane 10, 60 min. Lane M, molecular mass markers (k, kDa). The weakly stained proteins of 31, 30 and 22 kDa found even at 0 min represent the contamination of degradation products of calponin (34 kDa) during the purification procedures.

terns of calponin on SDS-PAGE were similar in the presence and absence of calmodulin (fig.3).

Calmodulin did not markedly affect the degradation of caldesmon ($k_{app} = 8.3 \times 10^{-3}\ s^{-1}$), while it decreased the rate of degradation of myosin light chain kinase ($k_{app} = 1.1 \times 10^{-3}\ s^{-1}$). These observations were in agreement with the previous report of Kosaki et al. [20].

3.3. *Degradation of F-actin-bound calponin*

The degradation rate of F-actin-bound calponin was remarkably retarded. The degradation of calponin became visible on SDS-PAGE only after 10 min of incubation (graphic data not shown), and calculated k_{app} value was $5.8 \times 10^{-5}\ s^{-1}$, which was 1/140th of that found without F-actin, i.e. free form of calponin.

3.4. *Degradation of calponin located on native thin filament*

Since F-actin-bound calponin was highly resistant to calpain digestion, 9.8 times more calpain I was added to the incubation mixture. Calponin was degraded completely after 3 h-incubation ($k_{app} = 1.6 \times 10^{-4}\ s^{-1}$), while caldesmon was degraded almost completely within 3 min ($k_{app} = 7.3 \times 10^{-3}\ s^{-1}$). Actin and tropomyosin were well preserved throughout the incubation period (fig.4). By immunoblot analysis, several fragments of 17–14 kDa seen after 20-s incubation were identified as the degradation products of actin. The origin of 31 kDa fragment (lanes 2–5, fig.4) could not be identified, because none of anti-actin (Amersham), anti-caldesmon, or anti-calponin antibodies corss-reacted with 31 kDa band. It could have originated from intermediate filaments possibly contaminating the native thin filament preparation used, since intermediate filaments are also known to be good substrates of calpain [21].

4. DISCUSSION

The present study demonstrated that calponin was a good substrate for calpain I among the

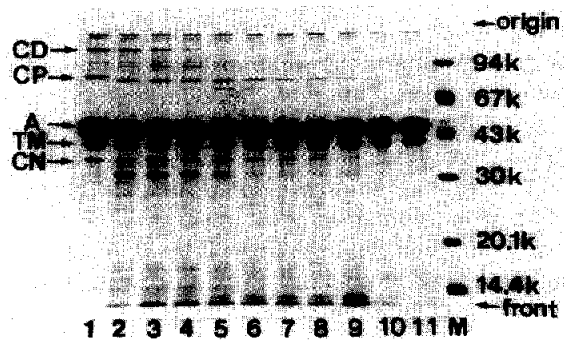

Fig.4. Degradation of native thin filament by calpain I (12.5% SDS-PAGE gel). The incubation periods were: lane 1, 0 min; lane 2, 20 s; lane 3, 50 s; lane 4, 80 s; lane 5, 3 min; lane 6, 20 min; lane 7, 40 min; lane 8, 60 min; lane 9, 2 h; lane 10, 3 h; lane 11, 4 h. Lane M, molecular mass markers (k, kDa). CD, caldesmon; CP, heavy subunit of calpain I; A, actin; TM, β subunit of tropomyosin; CN, calponin.

smooth muscle contraction-associated proteins. Note that calponin is similar in its characteristics to troponin T of skeletal and cardiac muscle [1], and troponin T is also reported to be quite susceptible to calpain [8,22].

In the presence of calmodulin, calponin was degraded by calpain about twice more rapidly than in the absence of calmodulin when k_{app} values were compared. Calmodulin may have caused a conformational change of the calponin molecule resulting in higher susceptibility to calpain. This is compatible with the loss of the tropomyosin-binding ability of calponin in the presence of Ca^{2+} and calmodulin [4].

When calponin was bound to F-actin, it became markedly resistant to calpain digestion. It was degraded about 140 times more slowly in terms of k_{app} values. The k_{app} value for calponin in the native thin filaments was 1.6×10^{-4} s^{-1}, which may correspond to 1.6×10^{-5} s^{-1} under the assay conditions used for free calponin, since 9.8 times more enzyme was added in the experiment with native thin filament (fig.4) than in the experiment with free calponin (figs 2 and 3). We concluded that the thin filament-bound form of calponin was degraded 500 times more slowly than the free form of calponin. Such a marked decrease in susceptibility may have been caused not only by actin filament but also by the other components such as tropomyosin and caldesmon.

Caldesmon bound to the thin filaments was degraded 13 times more slowly than the free form of caldesmon. Calponin and caldesmon are F-actin-binding proteins [3,13,23] and susceptible to calpain to almost the same extent when tested in each free form. However, calponin was degraded about 50 times less rapidly than caldesmon when tested as the thin filament-bound forms. This may be related to the structural difference between caldesmon and calponin in constituting thin filament. For example, caldesmon is an elongated and highly flexible protein [24–26] which would extend over the length of 70–150 nm along the axis of the actin filaments, while calponin may be a globular protein [1,3] which is more deeply buried in actin filaments than caldesmon. Such close association of calponin with actin filament could further support the view that calponin is an integral part of smooth muscle thin filament, which is vital to the actin-linked mechanism of contraction [3,15].

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