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Limited digestion of α-actinin in the presence of F-actin

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N-terminal actin-binding domain of α-actinin is connected to central rod domain through flexible neck region that is susceptible to proteolysis. It is suggested that the neck region assumes variable orientations by actin binding. In order to examine the effect of actin binding to α-actinin, we carried out limited digestion of α-actinin by chymotrypsin in the presence and absence of F-actin. Although the cleavage process was retarded when bound to F-actin, digestion to 32 kDa-head and 55 kDa-rod domains occurred through the same intermediate products as the digestion in the absence of F-actin. N-terminal sequencing of 55 kDa-fragment showed the neck region was cleaved at 276-Leu. The cleavage site was not affected by binding to F-actin nor ionic strength of the solvent. It was also indicated that α-actinin was cleaved at 15-Tyr by chymotrypsin. Quantitation of the cleavage products by densitometry of the SDS-gels suggested the conformational change of α-actinin at domain-connecting regions by F-actin binding.

Key words: actin binding domain, chymotrypsin digestion

α-Actinin is an actin binding protein that plays a critical role for the integration of actin cytoskeleton. α-Actinin crosslinks actin filaments and causes bundling and gelation of actin filaments[1,2]. In muscle cells α-actinin localizes at actin bundling structure as Z-disks of striated muscles[3,4] and dense bodies in smooth muscle[5,6]. Non-muscle isoforms of α-actinin distribute periodically along actin filament bundles like stress fibers and then participate in anchoring structure of these actin bundles to membrane, adhesion plaques or focal contacts[7,8]. Moreover, recent works have shown that α-actinins offer binding sites to multiple structural and regulatory proteins as multi-talent platform[9–12].

α-Actinin is a rod shaped homodimer of polypeptides of ~100 kD each that are oriented anti-parallel[1,2]. Based on its domain structure, α-actinin is classified as a member of spectrin superfamily. Each subunit consists of three functional domains; N-terminal containing actin binding domain (ABD) with tandem calponin homology motifs, rod domain with four spectrin-repeats, and regulatory tail domain with EF-hand like motifs (Fig. 1). The binding activity of non-muscle α-actinins was shown to be Ca^{2+}-sensitive[13,14] while that of muscle isoforms was regulated by phosphoinositides[15,16].

Structural analysis on α-actinin molecules indicates that the central rod domain of the dimer registered[18–20], thus suggesting the interaction of ABD and regulatory domain between the opposite subunits regulates the binding activity of α-actinin to actin filaments. The linker regions between the domains are assumed to be flexible and are susceptible to limited digestion by proteases. Taylor and his colleagues[21,22] have proposed that flexible joint connecting ABD and rod domain assume variable orientations by actin binding. Although crystallography has revealed detailed structure of isolated ABDs[23–25] and rod domains[26,27], the conformational change of this region by actin binding is not known well.

In this paper, we carried out the limited digestion of α-actinin that bound to F-actin. In the presence of F-actin, the cleavage process became retarded when compared with that in the absence. However, N-terminal sequence study showed that cleavage site between actin binding domain and rod-domain was not affected by F-actin binding.

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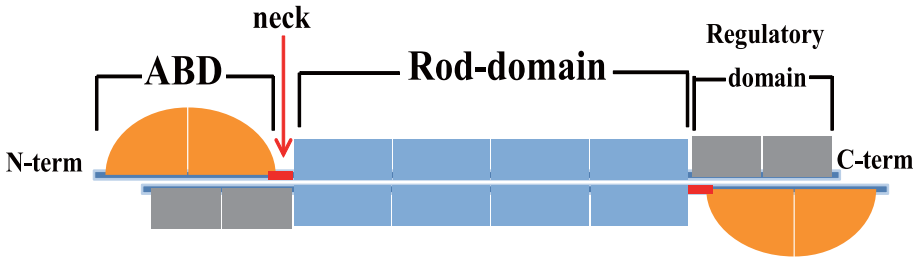

Figure 1 A schematic model of α-actinin molecule. N-terminal containing actin-binding domain (ABD) is connected to rod-domain through a short flexible segment (neck). Dimers are formed by lateral interaction between rod-domains.

Materials and Methods

Protein purification

Purification of α-actinin: α-Actinin was prepared from glycerinated chicken breast muscle as described previously[28]. Briefly, myofibrils were extracted with high salt solution containing 0.6 M KCl, 5 mM Mg-pyrophosphate, and 50 mM Tris-maleate pH 6.5, and resulting I-Z-I brush was extracted with 5 mM Tris-KCl (pH 8.0) and 0.1 mM phenylmethylsuflonyl fluoride (PMSF). The extract was fractionated with ammonium sulfate fractionation and precipitates obtained between 40–60% ammonium sulfate saturation were applied to DEAE-cellulose column equilibrated with 5 mM Tris-HCl (pH 8.0), 0.2 mM DTT and 0.5 mM PMSF. α-Actinin was eluted with linear KCl gradient and fractions eluted between 0.15–0.20 M KCl.

Purification of actin: Actin was extracted from an acetone-dried powder of chicken skeletal muscle by the method of Spudich and Watt[29] and purified by Sephadex G-150 column chromatography equilibrated with 0.2 mM ATP, 0.01% sodium azide and 5 mM Tris-HCl, pH 8.0.

SDS-polyacrylamide gel electrophoresis and densitometry

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out according to Laemmli[30] with a 10% separating gel. Protein samples were treated for three minutes over boiling water with one-third volumes of SDS-solution containing 5% SDS, 5% 2-mercaptoethanol, 0.1 mM PMSF, and 50% glycerol. Samples for protein sequencing studies were treated with the SDS-solution for 10 min at 37°C. After electrophoresis, the gels were fixed for 10 min with 50% ethanol and 12% acetic acid and stained with 0.2% Coomassie Brilliant Blue (CBB). Molecular weights of protein bands were determined using molecular weight standard purchased from Sigma (Broad Range).

CBB-stained gels were photographed with a digital camera (Canon Power Shot G-40) at the resolution of 300 dpi. Amount of proteins detected on a SDS-gel was quantitated from the digitized files in tif-format using Image J (ver. 1.4).

Protein Sequencing

Protein bands resolved on a SDS-gel were electrically transferred to apolyvinylidene fluoride (BioRad) membrane for 3 h at a constant current of 80 mA using 25 mM Tris-glycine (pH 8.3), 20% ethanol and 0.1 mM ethylene dithioglycol as an electrolyte[31]. Protein bands on transferred polyvinylidene fluoride (PVDF) membranes were visualized by brief staining with 0.1% CBB, and 32 kDa (head domain) and 55 kDa (rod domain) bands were excised from PVDF membranes. Five amino acid residues of N-terminal sequences of those bands were identified by Edman degradations using Shimazu PSQ-1 gas phase protein sequencer.

Other methods

Protein concentrations were determined by DC-Protein assay (BioRad) standardized with bovine serum albumin (Sigma). Concentration of purified α-actinin and actin were determined spectrophotometrically using extinction coefficient at 278 nm of 0.97 mg^{-1} ml cm^{-1} for α-actinin[32] and at 290 nm of 0.62 mg^{-1} ml cm^{-1} for actin[33] respectively.

Results and Discussion

Time course of α-actinin digestion in the presence of F-actin

Limited digestion with a protease is a useful method to access conformation of native proteins. α-Actinin is cleaved by chymotrypsin into protease resistant domains; namely N-terminal containing actin binding and rod-domain[1]. In a previous paper, we have shown that ionic strength of the solvents affects the conformation of skeletal muscle α-actinin molecule[28]. In the presence of 0.1 M KCl, the conformation of α-actinin is more compact than that in the absence of added salts. Reflecting the conformation, α-actinin becomes less susceptible to limited digestion by chymotrypsin although cleavage sites between domains were not affected (refer cleavage map shown in Fig. 4).

In this report, we examined the cleavage pattern of α-actinin that bound to F-actin. α-Actinin (0.82 mg/ml) was mixed with 0.93 mg/ml of F-actin and centrifuged for 1 hr with a Hitachi 55P-72 ultracentrigue with a 40P rotor at 300,000 rpm. Pelleted α-actinin-F-actin complex was dispersed in 0.1 M KCl and 20 mM Tris-HCl, pH 7.5. Densitometry of the SDS-gel of the mixture indicated that the molar ratio of α-actinin to actin was 0.13, showing saturated binding of α-actinin to F-actin[34,35]. As F-actin was fairly resistant

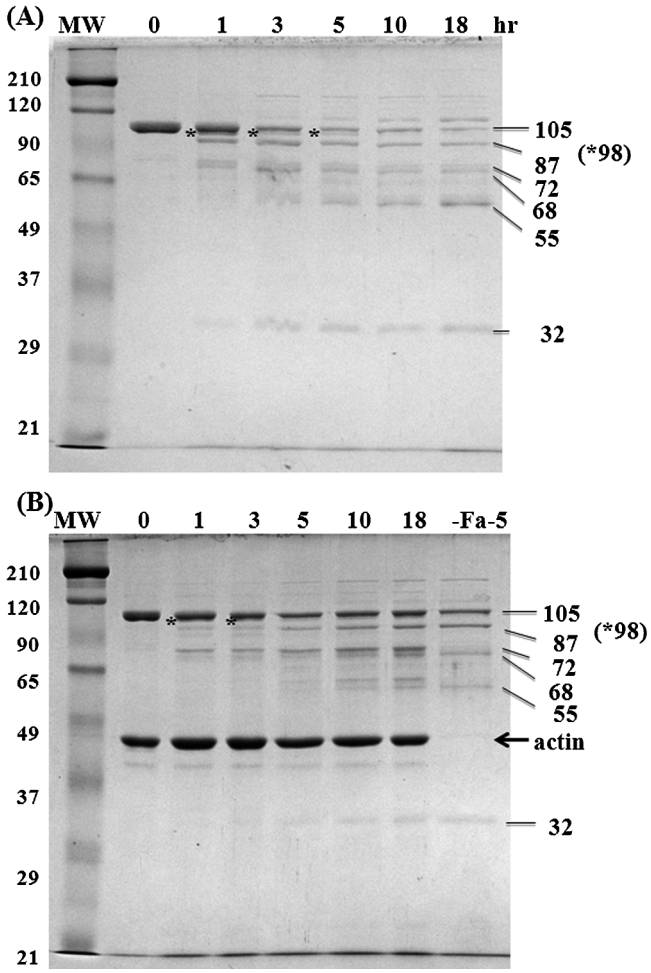

Figure 2 Time course of α-actinin digestion. α-Actinin was digested with 1/100 (w/w) chymotrypsin in 0.1 M KCl and 20 mM Tris-HCl (pH 7.5) at 25°C. (A) α-Actinin (0.42 mg/ml) was subjected to chymotrypitic digestion after clarification of α-actinin for 1 hr at 30,000 rpm, (B) α-Actinin-F-actin complex was pelleted by the ultra-centrifugation and dispersed in 0.1 M KCl and 20 mM Tris-HCl (pH 7.5) at a protein concentration of 0.80 mg/ml. α-Actinin in the complex was digested with chymotrypsin. Numbers on the top of gel images indicate incubation time in hour. Numbers on the side of the image show molecular weight of marker proteins (MW) and those of cleavage products shown in k ($\times 10^3$). The 98 kDa-fragment that appeared only early period of digestion in small amount was marked by asterisk. The last lane in Plate B (-Fa5) showed the 5h-cleavage product without actin just shown for comparison of products. Poly-acrylamide concentration, 10%. See the text for detail.

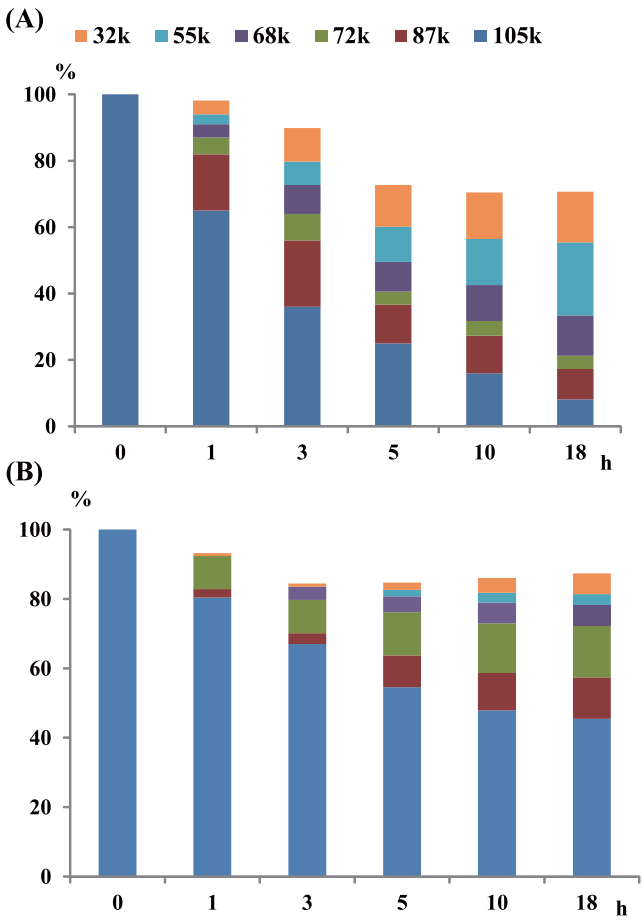

Figure 3 Change in the amount of cleavage products detected during digestion. The amount of cleavage products were measured from digitized images of the SDS-gel with NIH ImageJ in the presence (A) and the absence (B) of F-actin. *Ordinate*, the relative amount of each product to the amount of 105 k-band in zero-time was expressed in percentage.

to chymotryptic digestion (refer Fig. 1B), concentration of α-actinin in the complex was estimated from the molar ratio and 1/100 (w/w) chymotrypsin (Sigma, type I-S) was added to the amount of α-actinin. Aliquot was withdrawn from the reaction mixture at reaction time shown in Figure 2 and the digestion was quenched with the SDS-solution. In control experiments, α-actinin in 0.1 M KCl and 20 mM Tris-HCl (pH 7.5) was clarified by the centrifugation at 30,000 rpm and subjected to chymotrypsin cleavage. In the presence of 0.1 M KCl, the 105 kDa-subunit remained as a major component even after digestion over 10 hours with 1/100 (w/w) chymotrypsin (Fig. 1A). The 55 kDa-rod and 32 kDa-head domains were detected from one hour in digestion and their amounts increased gradually with time.

When bound to F-actin, the digestion of α-actinin was further retarded (Fig. 2B). In order to compare the amount of subfragments, we quantitated the amount of 105 k-, 87 k-, 72 k-, 68 k-, 55 k-, and 32 k-band from the recorded image of SDS-gels with NIH ImageJ. It was shown that 50% of the 105 kDa-subunit was digested after 10 hours of digestion in the presence of F-actin, while 85% was cleaved in the absence of actin (Fig. 3). Formation of 32 kDa-head and 55 kDa-rod domain were also retarded when α-actinin-F-actin complex was digested. Although F-actin affected the rate of the α-actinin digestion by chymotrypsin, the digestion to 55 kDa-rod and 32 kDa-head domains proceeded through common intermediate products with chain molecular weights of 98 k, 87 k, 72 k, and 68 k.

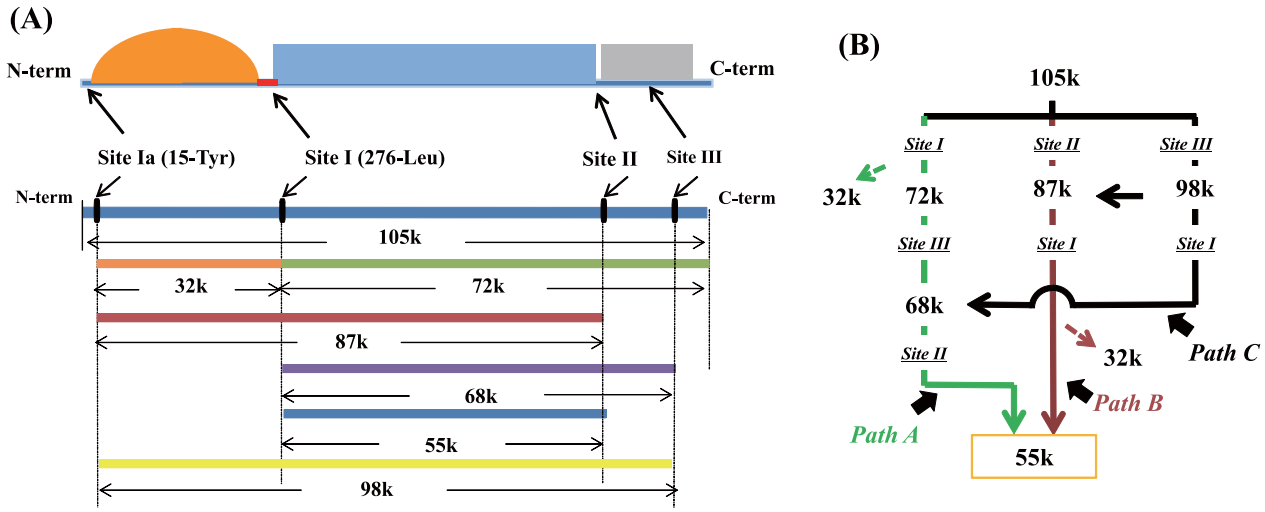

Figure 4 Cleavage map of skeletal muscle α-actinin. Cleavage sites of α-actinin by chymotrypsin were mapped on the primary sequence of skeletal muscle α-actinin (A). Flow chart for the degradation of 105 k-subunit into 55 k- and 32 k-domain (B). See the text for details.

Determination of N-terminal peptide sequences of digested products

The actin-binding domain and the rod domain of α-actinin is linked by about 30 residues protease-sensitive linker[1,2,11]. The neck region is flexible and actin-binding domain can assume variable conformations over rod domain[19,21,36]. Therefore it is suggested that binding of α-actinin to F-actin induced conformational changes on this flexible neck region. Corgan et al. showed that binding of phosphoinositides to smooth muscle α-actinin affected the cleavage site on the neck region[17]. Then, the question arises whether the binding of α-actinin to F-actin or ionic strength of the solvent influences the cleavage site on the neck region.

We examined N-terminal sequence of 32 kDa head and 55 kDa rod by Edman reaction and assigned the cleavage site on the reported primary sequence of chicken skeletal α-actinin[37,38]. As shown in Table 1, N-terminal sequence of 55 kDa-rod domains was Ala-Val-Asn-Gln-Glu-, indicating chymotrypsin hydrolyzed carbonyl side of 276-Leu. This cleavage site was not affected by actin binding nor by ionic strength of the solvent. It is interesting that this cleavage site was identical to the cleavage site reported to smooth muscle α-actinin[39]. In addition, it was shown that chymotrypsin also cleavaged at 15-Tyr. N-terminal sequence of the 32 kDa-actin-binding domain was Asn-Tyr-Glu–Glu-Asp-Glu- irrespective to the experimental conditions examined. Cleavage of N-terminal fragment of actin-binding domain with thermolysin at 24-Leu was reported for smooth muscle α-actinin[40,41]. Figure 4A illustrates the cleavage map of chicken skeletal α-actinin. Site Ia located at 15-Tyr and Site I at 276-Leu. Judged by the primary sequence of skeletal muscle α-actinin[37,38], Site II and Site III was assigned around 750th and 850th residue, respectively. Regulatory domain with EF-hand-like motifs exits between Site II and near C-terminal end

Table 1 Amino acid sequences of 32 k and 55 k fragments

	32 k fragment	55 k fragment
–KCl	Asn-Tyr-Glu–Glu-Asp	Ala-Val-Asn-Gln-Glu
0.1 M KCl	Asn-Tyr-Glu–Glu-Asp	Ala-Val-Asn-Gln-Glu
+FA	Asn-Tyr-Glu–Glu-Asp	Ala-Val-Asn-Gln-Glu

Limited digestion with chymotrypsin was carried out in the absence of added salt (–KCl), in the presence of 0.1 M KCl, and in the presence of F-actin.

32 k-ABD and C-terminal containing 72 kDa-fragments are formed by cleavage of native α-actinin at Site I. Cleavage of native α-actinin at Site II and Site III result in the formation of ABD-containing 87 kDa- and 98 kDa-fragment respectively. Formation of 98 kDa-fragment was limited only at early periods of the digestion and the amount of the 98 k was very small (refer Fig. 2). Flow chart of the degradation of α-actinin into actin-binding and rod-domain through sub-fragments are summarized in Figure 4B. There are three unique pathways of the degradation of native α-actinin. As the amount of 98 kDa-fragment was very small and limited, contribution of Path C for accumulation of other subfragments would be negligible for the present experiment.

As shown in Figure 3, the ratios of subfragments detected during digestion were affected by binding of α-actinin to F-actin. In the absence of F-actin, 87 kDa-fragment was a major product throughout the digestion process. 72 k- and 68 k-fragment were also detected continuously during the cleavage. Therefore, it is suggested that both Path A and B were available for the digestion of α-actinin into 32 k- and 55 k-domain in the absence of F-actin. On the other hand, in the presence of F-actin, 72 kDa-fragment was detected as a major product and its amount increased with time of digestion. These results suggest that binding to F-actin caused some conformational change at the neck region of α-actinin

and enhanced the cleavage at Site I. In addition, the formation of 87 kDa-fragment was limited at early period of digestion in the presence of F-actin. As shown in Figure 1, the central rod domain of the dimer registered[18–20]. This model indicates that Site I of one subunit locates close vicinity to Site II of another subunit. Therefore, it is quite probable that conformational change on neck region of one subunit affect the conformation of Site II of counterpart subunit, then reduce the formation of 87 kDa-fragment.

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