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The amino-terminal fragment of gelsolin is cross-linked to Cys-374 of actin in the EGTA-resistant actin–gelsolin complex

Yukio Doi[a,c], Yumiko Kanatani[a] and Fimi Kim[b]

[a]*Department of Food Science, Kyoto Women's University, Higashiyama-ku, Kyoto 605, Japan,* [b]*Protein Engineering Research Institute, 6-2-3 Furuedai, Suita-shi, Osaka 565, Japan* and [c]*Laboratoire d'Enzymologie, CNRS, 91198 Gif-sur-Yvette, France*

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It has been shown that the EGTA-resistant actin, one of the two actin molecules associated to gelsolin, can be predominantly cross-linked to gelsolin by benzophenone-4-maleimide (BPM), a photoaffinity-labeling reagent, which was conjugated to Cys-374 of actin prior to cross-linking (Doi, Y., Banba, M. and Vertut-Doï, A. (1991) Biochemistry 30, 5769–5777). When a chymotryptic digest of gelsolin containing the amino-terminal 15-kDa fragment was mixed with BPM-actin (42 kDa) and irradiated for cross-linking, a band of 58 kDa appeared on SDS-PAGE which was shown to contain actin molecule by using fluorescently labeled actin. The amino-terminal sequence of the 58-kDa complex was identical to that of gelsolin, confirming that the amino-terminal segment (residues 1–133) of pig plasma gelsolin lies closely to Cys-374 of actin in the EGTA-resistant complex.

Gelsolin; Actin; Actin binding protein

1. INTRODUCTION

Gelsolin belongs to a group of the actin-binding proteins that can sever F-actin and cap the barbed end of actin filaments [1]. It is encoded by a single gene and expressed as a cytoplasmic form and a secreted form [2]. Both isoforms have identical functional properties although the secreted form has additional residues at the amino-terminal end [3,4]. Gelsolin consists of six repeated homologous domains, and limited proteolysis dissects gelsolin in between these domains [5–8]. For instance, in the presence of Ca^{2+}, chymotrypsin treatment of human plasma gelsolin yields three actin binding fragments: the amino-terminal CT 17N fragment (the nomenclature according to Yin et al. [7]) forms a 1:1 complex with G-actin in a Ca-independent manner, the CT 28N fragment binds to F-actin, and the CT 38C fragment binds actin only in the presence of Ca^{2+}. In spite of three actin binding sites manifested by the fragments of gelsolin, intact gelsolin interacts with only two actin molecules in the presence of Ca^{2+} forming an actin–gelsolin 2:1 complex [9–11]. Removal of Ca^{2+} by adding EGTA dissociates one actin molecule resulting in the formation of an EGTA-resistant actin–gelsolin 1:1 complex [12–14]. To understand the molecular mechanism of gelsolin function, it is necessary to elucidate how these three binding sites interact with two actins. So far it has been found that CT 17N and CT 28N were close to the amino terminal segment of actin [15,16] and CT 38C to the carboxyl-terminal segment [16,17]. These observations, however, did not distinguish two actin molecules interacting with gelsolin.

Recently we showed that among the two actin molecules associated to gelsolin only the EGTA-resistant actin was effectively cross-linked to gelsolin when benzophenone-4-maleimide-actin (BPM-actin) was used [18]. This finding provides a means to discriminate the two actin molecules in the actin–gelsolin 2:1 complex and enables to study a spatial relationship of the actin molecules relative to gelsolin by a chemical cross-linking method. In the present communication we identified that Cys-374 of the EGTA-actin is in proximity to the amino-terminal CT 17N of gelsolin.

Abbreviations: BPM, benzophenone-4-maleimide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EGTA, [ethylenebis(oxyethelenenitrilo)]tetraacetic acid; F-actin, filamentous actin; G-actin, globular actin; NBD-Cl, 7-chloro-4-nitrobenzeno-2-oxa-1,3-diazole chloride; NBD, 7-nitrobenz-2-oxa-1,3-diazole-4-yl; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

Correspondence address: Y. Doi, Department of Food Science, Kyoto Women's University, Higashiyama-ku, Kyoto 605, Japan. Fax: (81) (75) 531-7216.

2. MATERIALS AND METHODS

2.1. *Proteins*

Gelsolin was purified from pig plasma as described elsewhere [19]. The concentration of gelsolin was determined by using $\varepsilon_{280} = 1.16 \times 10^5\ M^{-1} \cdot cm^{-1}$ [19]. The activity of gelsolin was determined as described previously [20]. Actin from rabbit skeletal muscle was prepared by the method of Spudich and Watt [21] and purified by Sephadex G-200 chromatography in G-buffer (2 mM Tris-HCl pH 8.0, 0.2 mM ATP, 0.2 mM $CaCl_2$, 1 mM NaN_3). The concentration of actin was estimated by using $\varepsilon_{290} = 2.66 \times 10^4\ M^{-1} \cdot cm^{-1}$ [22]. BPM-actin was prepared according to Tao et al. [23] with a slight modification as described previously [18].

2.2. *Limited chymotryptic cleavage of gelsolin*

As described later, the susceptibility of gelsolin to chymotrypsin was greatly affected by Ca^{2+}; therefore digestion was carried out in the absence and in the presence of Ca^{2+}. To obtain a chymotryptic digest to be used for cross-linking experiments, the digestion was done without Ca^{2+}; gelsolin was treated with chymotrypsin in G-buffer containing 1 mM EGTA. Chymotrypsin treatment in the presence of Ca^{2+} was performed in 10 mM Tris-HCl pH 7.8, 0.2 M NaCl, and 2 mM $CaCl_2$. The mass ratio of gelsolin to protease was 100:1 in both cases and the incubation was carried out at 25°C. The reaction was terminated by the addition of 1 mM phenylmethylsulfonyl fluoride.

2.3. *Fluorescent labeling of BPM-actin*

BPM-F-Actin was labeled by NBD-Cl essentially according to Detmers et al. [24]. By this procedure most NBD should be incorporated into Lys-372 since Cys-374 is already blocked by BPM. The degree of labeling was typically 0.6.

2.4. *Photo-cross-linking between BPM-actin and the chymotryptic digest of gelsolin*

To gelsolin and its chymotryptic digest in G-buffer containing 1 mM EGTA and 0.4 mM $MgCl_2$, $CaCl_2$ was added to a final concentration of 1.4 mM to ensure the interaction between them. Then BPM-actin was added to an actin/gelsolin ratio of 2:1 and incubated for 30 min at room temperature. To dissociate the Ca-sensitive actin from the actin–gelsolin complex, EGTA was then added to a final concentration of 2 mM. After incubating for 30 min at room temperature, samples were illuminated for 1 h at 0°C by a long wavelength ultraviolet lamp for cross-linking as described elsewhere [18]. Cross-linking of NBD-BPM-actin was performed in the same manner except that the duration of exposure to the lamp was limited to 30 min in order to prevent excess photo-bleaching of a fluorescence dye.

2.5. *SDS-PAGE and Western blotting*

To identify the cross-linked products, samples were prepared and separated on SDS-PAGE using a gradient gel essentially as described by Laemmli [25]. The gel to be subjected to transblotting for amino acid sequence analysis was stained for 5 min with Coomassie blue, destained and equilibrated by incubation in 0.1% SDS, 25 mM Tris-borate buffer pH 9.5, prior to electroblotting. Electrophoretic transfer to polyvinylidene difluoride (PVDF) membranes was carried out essentially as described [26] using the transfer buffer of 25 mM Tris-borate pH 9.5, containing 20% methanol.

2.6. *Amino-acid sequence determination*

The portion of PVDF membrane containing the immobilized polypeptide, which could be identified by the co-adsorbed dye originally bound to the polypeptide in the gel, was cut out and mounted in the reaction chamber of the gas-phase sequenator (Applied Biosystem) equipped with an on-line high-performance liquid chromatography column for analyzing phenylthiohydantoin derivatives of amino acids.

3 RESULTS

3.1 *Chymotryptic digestion of gelsolin*

Chymotrypsin treatment of human plasma gelsolin yields three major fragments, CT 17N, CT 28N and CT 38C, in this order, to form the amino terminus [5–8]. When pig plasma gelsolin is digested with chymotrypsin in the presence of Ca^{2+} (Fig. 1, lane b), three main bands of 47 kDa, 31 kDa and 15 kDa are observed, which are corresponding to CT 38C, CT 28N and CT 17N. (CT 38C migrates with much slower mobility on SDS-PAGE than expected from its 47 kDa mass [7].) However, in the absence of Ca^{2+}, gelsolin is cleaved exclusively into two fragments, 15 kDa and 75 kDa (Fig. 1, lanes e–g). The amino-acid sequence analysis of the 15-kDa fragment showed a sequence that matches the first four amino-terminal residues of pig plasma gelsolin (Table I) [4]. The first five residues of the 75-kDa fragment was found to correspond to the residues 134–139 of gelsolin (Table I). Therefore, in the absence of Ca^{2+} chymotrypsin selectively cleaves gelsolin at the peptide bond between residues 133 and 134, yielding the amino-terminal 15-kDa fragment and the carboxyl-terminal 75-kDa fragment.

3.2. *Cross-linking between BPM-actin and chymotryptic fragments of gelsolin*

Previously we showed that one of the two actin molecules associated to gelsolin was predominantly cross-linked by BPM, forming a cross-linked actin–gelsolin 1:1 complex with apparent molecular mass of 130 kDa (Fig. 1, lane h). Moreover, since the cross-linking occurred even after removal of the Ca-dependent actin from the actin–gelsolin 2:1 complex, it was concluded that the EGTA resistant actin is the one which is cross-linked. When BPM-actin was incubated with the chymotryptic digest composed of the 15-kDa and 75-kDa fragments (Fig. 1, lanes e–g) and irradiated for cross-linking, a band with a molecular mass of 58 kDa appeared, its intensity being increased as the digestion proceeded (Fig. 1, lanes i–k). The intensity of the 15-kDa band in the presence of actin seemed to be less than those without actin, indicating that the 15-kDa fragment was consumed for the crosslink. The 58-kDa band did not appear without illumination (data not shown). From its apparent molecular mass the 58-kDa band seems to represent a cross-linked complex between BPM-actin and the 15-kDa fragment. If the speculation is correct the sequence analysis should reveal the same sequence as that of the 15-kDa fragment since the amino terminus of actin is acetylated and is not available for Edman degradation. The sequence of the 58-kDa band was found to match to that of gelsolin (Table I), confirming that the 58-kDa band is in fact a cross-linked product between BPM-actin and the amino-terminal fragment of gelsolin.

3.3 *Cross-linking between NBD-BPM-actin and the 15-kDa fragment*

To ascertain that the 58-kDa cross-linked product contains the actin molecule, actin was fluorescently labeled by NBD and the experiment was repeated (data not shown). The protein-staining pattern of the cross-linked products with NBD-BPM-actin was essentially identical to that obtained with BPM-actin (Fig. 1, lanes i–k) although the amount of the 58-kDa cross-linked product is considerably less, probably because the introduction of NBD at Lys-373, next to Cys-374 to which BPM is attached, somewhat impairs photocross-linking reaction. Observation of the same gel under a ultraviolet lamp shows that the 58-kDa band is indeed fluorescent,

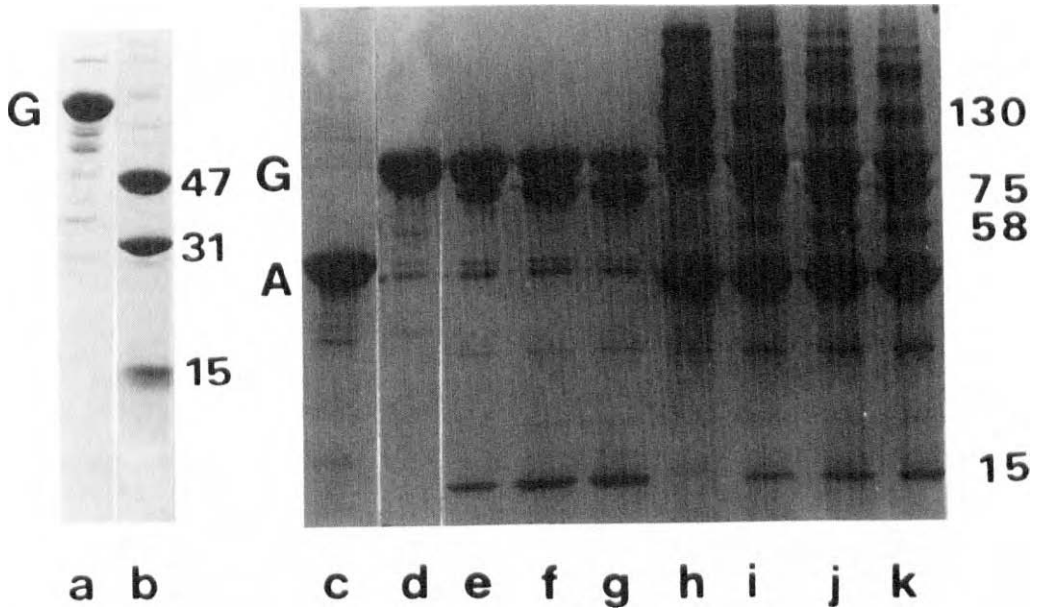

Fig. 1. SDS-PAGE patterns of chymotryptic fragments of gelsolin and their cross-linked complexes with BPM-actin. Lane a, gelsolin before treatment with chymotrypsin; lane b, chymotryptic fragments of gelsolin digested in the presence of Ca^{2+}; lane c, BPM-actin used for cross-linking; lane d, gelsolin; lanes e to g, chymotryptic fragments of gelsolin digested in the presence of 1 mM EGTA for 5 (e), 15 (f) and 30 min (g) at 25°C; lane h, cross-linked complex between gelsolin and BPM-actin; lanes i to k, cross-linked products between the gelsolin digests (same as those in lanes e to g) and BPM-actin. A 10–20% gradient gel was used for samples in lanes a and b, and a 6–18% gel for those in lanes c to k. G and A indicate the bands corresponding to gelsolin and actin, respectively. The numbers on the right of the panels show the migration positions of bands with apparent molecular masses indicated in thousands.

confirming that it is a cross-linked complex between actin and the amino-terminal 15-kDa fragment of gelsolin.

4. DISCUSSION

The apparent irreversibility of one of the two actin molecules associating to gelsolin has been well documented [12–14], and previously we showed that this actin molecule (the EGTA-resistant actin) can be distinguished from the other by using BPM, a photo-sensitive cross-linking reagent [18]. The present study shows that BPM-actin is conjugated to the amino-terminal fragment (residues 1–133) of gelsolin, indicating that Cys-374 of the EGTA-resistant actin resides within a distance of 9–10 Å from the amino-terminal fragment of gelsolin. The conclusion is consistent with the observations that CT 17N showed high-affinity Ca-insensitive binding to G-actin [8] and that CT 17N retains Ca-resistance to chelation by EGTA upon binding to actin [27]. Other barbed end-capping proteins such as severin [28], *Acanthamoeba* profilin [29] and fragmin [30] are also shown to bind at the carboxyl-terminal area. Regarding the sequence similarities at actin binding regions among the capping proteins, including probably adseverin [31], it is tempting to generalize that the capping proteins bind to the carboxyl-terminal segment of the actin molecule topologically equivalent to the EGTA-resistant actin between the two actin molecules found at the barbed end of an actin filament. It is worth noting that the BPM-actin dimer composed of BPM-actin and unlabeled actin which are covalently conjugated by phenylenedimaleimide can be cross-linked to gelsolin (unpublished results). Therefore, the topologically equivalent actin molecule can be defined as the subunit which has Cys-374 freely available on an actin filament since Cys- 374 of all the other subunits face closely to Lys-191 of the successive subunit [32].

Sutoh and Yin [16] showed that the isolated carboxyl-terminal fragment (CT 38C) of gelsolin was cross-linked with the actin carboxyl-terminal segment (residues 356–375) by a zero-length cross-linking reagent, EDC. Since the cross-linked product was observed only in the presence of Ca^{2+}, it is likely that CT 38C lies closely to the carboxyl-terminal segment of the EGTA-sensitive actin. Furthermore, we have shown that the amino-terminal 12 residues of both actin molecules in the actin–gelsolin

Table I

Amino-terminal sequence analysis of chymotryptic fragments of gelsolin and the cross-linked complex with BPM-actin

Cycle	15 kDa fragment	75 kDa fragment	58 kDa complex
1	Val (935)	Lys (446)	Val (316)
2	Ser (256)	His (89)	Ser (109)
3	Pro (198)	Val (963)	Pro (129)
4	Met (180)	Val (534)	Met (74)
5		Pro (384)	Arg (85)

The yields in pmol for each amino acid are given in parentheses.

2:1 complex are in close contact with gelsolin [15], and later it was found that in fact these residues were in proximity to the amino-terminal fragments of gelsolin (CT 17N and CT 28N) [16]. Summing up all these observations together with the one obtained in the present study, the spatial arrangements of the segments participating at the contact region of the actin–gelsolin 2:1 complex are as follows: in the EGTA-resistant actin both termini are in proximity to the amino-terminal fragment (CT 17N) of gelsolin whereas in the EGTA-sensitive actin the amino-terminal segment (residues 1–44) is close to the amino-terminal fragment (CT 17N or CT 28N) and the carboxyl-terminal segment (residues 356–375) to the carboxyl-terminal fragment (CT 38C). The spatial model of the actin–gelsolin complex proposed by Pope et al. [33] fits to this view.

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