End-Label Fingerprintings Show That the N- and C-Termini of Actin Are in the Contact Site with Gelsolin†

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ABSTRACT: Gelsolin was cleaved by chymotrypsin or thermolysin into an N-terminal M_r 45 000 fragment (45N) and a C-terminal M_r 38 000 fragment (38C). The N-terminal half was further cleaved into two fragments with M_r 17 000 (17N) and M_r 28 000 (28N). These fragments were complexed with actin and cross-linked with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) to introduce covalent bonds into their contact sites. The location of these bonds was mapped along the actin sequence by end-label fingerprinting with highly sensitive probes for the N- and C-termini of actin. The mapping studies revealed that two gelsolin N-terminal fragments (17N and 28N) were cross-linked with the actin N-terminal segment, while the gelsolin C-terminal fragment (38C) was cross-linked with the actin C-terminal segment. The result indicates that the actin N- and C-terminal segments are in the binding site of gelsolin.

Gelsolin is a Ca^{2+}- and poly(phosphoinositide)-modulated actin binding protein found in the cytoplasm of a large variety of cells and in a variant form in blood plasma (plasma gelsolin or brevin). It severs actin filaments, nucleates actin assembly, and caps the "barbed" end of actin filaments. The multiple actions of gelsolin appear to involve different actin binding domains, and three such domains have been identified thus far by use of proteolytic cleavage [see Matsudaira and Janmey (1988) as a review for the actin-severing proteins].

In the presence of Ca^{2+}, gelsolin is rapidly cleaved by a variety of enzymes into halves (Kwiatkowski et al., 1985; Chaponnier et al., 1986; Bryan & Hwo, 1986). For example, chymotrypsin cleaves human plasma gelsolin between residues 406 and 407 to generate an N-terminal 45-kDa fragment (CT45N) and an C-terminal 38-kDa fragment (CT38C).[1] CT45N can be further cleaved between residues 149 and 150 to generate CT17N and CT28N. Thermolysin digestion also generates similar fragments, and they have been referred to as TL45N, TL17N, TL28N, and TL38C, respectively. The N-half of gelsolin (e.g., CT45N) severs actin filaments as effectively as intact gelsolin, although neither of its subfragments (CT17N or CT28N) does so in isolation. Therefore, severing appears to require a cooperative interaction between the two N-terminal actin binding domains. On the basis of the finding that CT28N can bind stoichiometrically to actin protomers within a filament and its binding is inhibited by poly(phosphoinositides) with a dose–response similar to that observed for severing, it is proposed that severing requires binding of gelsolin through a site within CT28N to the side of actin filaments initially and subsequently binding of another site within CT17N (Yin et al., 1988). A third actin binding domain is located in the C-terminal half of gelsolin (CT38C or TL38C). This site requires Ca^{2+} to bind actin and does not appear to participate in severing per se (Kwiatkowski et al., 1985; Chaponnier et al., 1986). However, CT38C may contribute to Ca^{2+} regulation of intact gelsolin, because the severing activity of the N-terminal-half peptide is not Ca^{2+} regulated while that of gelsolin is.

Therefore, proteolytic cleavage studies have established that there are at least three distinct actin binding domains in gelsolin which may have unique functional and regulatory properties. In the presence of micromolar Ca^{2+}, gelsolin associates with two, not three, actins. The isolated N-half of gelsolin also associates with two actins, so it is not clear which of the three actin binding sites are occupied at any moment in intact gelsolin. These observations raise the question of whether the three actin binding domains bind similar or distinct sites on actin.

In this paper, we have identified the actin segments which bind to each of the three actin binding domains of gelsolin, by using chemical cross-linking and end-label fingerprinting, which have been employed extensively to identify interactive sites between actin and a number of actin binding proteins (Sutoh, 1982, 1984; Sutoh & Mabuchi, 1984, 1986, 1989; Sutoh & Hatano, 1986; Doi et al., 1987; Mimura & Asano, 1987; Grabarek & Gergely, 1987; T. Pollard, D. Kaiser, C. Ampe, and J. Vandekerckhove, personal communication). Proteolytic fragments of gelsolin were cross-linked to actin by 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), and cross-linked sites were identified along the actin sequence by end-label fingerprinting with probes for both the N- and C-termini of actin. We showed that the N-terminal actin binding domains of gelsolin, located within gelsolin 17N and 28N peptides, are cross-linked to the actin N-terminal residues 1–18, while the C-terminal actin binding domain of gelsolin,

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[1] Abbreviations: LEP, lysyl endopeptidase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CNBr, cyanogen bromide; $NaDodSO_4$, sodium dodecyl sulfate; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide; CT28N, chymotryptic gelsolin N-terminal fragment with M_r 28 000; CT38C, chymotryptic gelsolin C-terminal fragment with M_r 38 000; TL17N, thermolysin-digested gelsolin N-terminal fragment with M_r 17 000; TL45N, thermolysin-digested gelsolin N-terminal fragment with M_r 45 000.

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located within gelsolin 38C, is cross-linked to the actin C-terminal residues 356–375.

Materials and Methods

Proteins and Reagents. Human plasma gelsolin was prepared as described (Chaponnier et al., 1986; Yin et al., 1988). Two proteolytic fragments of gelsolin (CT28N and CT38C) were prepared and purified by actin affinity chromatography as described (Kwiatkowski et al., 1985; Yin et al., 1988). A mixture of N-terminal gelsolin fragments was obtained by digesting gelsolin with thermolysin at a weight ratio of 1200:1 for 15 min at room temperature and passing the resultant digest over a 2C4 monoclonal antibody–Sepharose column that is specific for an epitope in the C-terminal half of gelsolin (Chaponnier et al., 1986). The nonadherent fraction contains a mixture of 45-, 28-, 17-, and 14-kDa peptides, which have been shown by amino acid sequencing and immunoblotting to be derived from the N-terminal half of gelsolin (Yin et al., 1988). These peptides are referred to as TL45N, TL28N, TL17N, and TL14N, respectively. Rabbit skeletal muscle actin was prepared according to the method of Spudich and Watt (1971).

Polyclonal antibodies against human plasma gelsolin and against residues 1–15 of human plasma gelsolin were raised in rabbits (Chaponnier et al., 1986). They were used in immunoblotting at a 1/1000 and a 1/100 dilution, respectively. Antibody against the N-terminus of actin was prepared as described (Sutoh & Mabuchi, 1986) and used in immunoblotting at a 1/2000 dilution. Lysyl endopeptidase (LEP) was purchased from Wako Chemical Co. (Osaka, Japan). Streptavidin–alkaline phosphatase conjugate (BlueGENE) was from BRL (Gaithersburg, MD) and used at a 1/1000 dilution. Anti-rabbit IgG–alkaline phosphatase conjugate was from KPL (Gaithersburg, MD) and used at a 1/1000 dilution.

The iodoacetamide derivative of biotin, *N*-(iodoacetyl)-*N'*-biotinylhexylenediamine (IAA-biotin), was synthesized as described (Sutoh et al., 1984). EDC was purchased from Wako Chemical Co. Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were from KPL.

Biotinylation of Cys-374 of Actin. F-Actin (1 mg/mL) in 50 mM NaCl, 20 mM Tris-HCl, and 3 mM $MgCl_2$ (pH 8.0) was mixed with IAA-biotin dissolved in dimethylformamide (10 mM) to a final concentration of 0.1 mM. The biotinylation reaction was allowed to proceed for 16 h at 0 °C and was quenched by addition of 2-mercaptoethanol to a final concentration of 10 mM.

Cross-Linking of Complexes of Actin and Gelsolin Fragments. Sixty microliters of the biotinylated actin (1 mg/mL) in 50 mM NaCl, 20 mM Tris-HCl, 3 mM $MgCl_2$, and 10 mM 2-mercaptoethanol (pH 8.0) was mixed with 600 μL of CT28N fragment (70 μg/mL) in 0.15 M NaCl, 1 mM Tris-HCl, and 2 mM $CaCl_2$ (pH 8.0). The mixture was dialyzed against 50 mM MOPS, 0.2 mM $CaCl_2$, and 1.5 mM $MgCl_2$ (pH 7.5). The resulting solution was mixed with 1/10 volume of freshly prepared 20 mM EDC in water. EDC cross-linking was allowed to proceed for 1 h at 25 °C and was quenched by boiling for 5 min immediately after addition of 2-mercaptoethanol (final concentration 140 mM) and $NaDodSO_4$ (2%).

Sixty microliters of the biotinylated F-actin (1 mg/mL) was mixed with 500 μL of CT38C fragment (70 μg/mL) in 0.15 M NaCl, 20 mM Tris-HCl, 1 mM EGTA, and 1 mM $CaCl_2$ (pH 8.0), dialyzed against the MOPS buffer, and cross-linked for 30 min at 25 °C with 2 mM EDC.

Three hundred microliters of the biotinylated F-actin (1 mg/mL) was mixed with 1.6 mL of thermolysin-digested gelsolin N-terminal peptide mixture (0.1 mg/mL) and dialyzed against the MOPS buffer. The resulting solution was centrifuged for 1 h at 100 000 rpm in a TL-100 ultracentrifuge (Beckman) to sediment F-actin. The proteins in the supernatant were cross-linked with 5 mM EDC for 30 min at 25 °C.

LEP Mappings of Cross-Linking Sites. Cross-linked products were separated on a $NaDodSO_4$–acrylamide slab gel [12.5% acrylamide–0.4% bis(acrylamide)] (Laemmli, 1970) in duplicate lanes. Prestained M_r standards (Bio-Rad) were used to estimate apparent M_r's of cross-linked products and their fragments. Duplicate gel strips were cut out and loaded onto two separate slab gels, which contained 0.2 μg/mL lysyl endopeptidase (LEP) in the stacking gel. Electric current was adjusted so that peptides electrophoresed through the stacking gel in 30 min, to ensure partial digestion by LEP prior to separation in the second-dimension gel [15% acrylamide–0.5% bis(acrylamide)]. After electrophoresis, peptides were transferred onto Durapore membranes (type GV, Millipore, Bedford, MA) (Towbin et al., 1979). The duplicate membranes were washed with 0.15 M NaCl and 20 mM Tris-HCl (pH 8.0) containing 0.05% Tween 20 (ELISA grade, Bio-Rad) (TTBS) for 15 min twice. One of the duplicate membranes was incubated with streptavidin–alkaline phosphatase conjugate (BRL) at a 1/1000 dilution for 1 h, washed with three changes of TTBS for 5 min, rinsed in water briefly, and stained with NBT and BCIP (KPL) as chromogens. The other membrane was incubated sequentially with rabbit antiserum against the N-terminus of actin (Sutoh & Mabuchi, 1986) and alkaline phosphatase conjugated anti-rabbit IgG (KPL). The blots were washed after each incubation in TTBS. Finally, they were washed with water briefly and stained as above.

CNBr Mappings of Cross-Linking Sites. Cross-linked products were separated on a $NaDodSO_4$ slab gel in duplicate. Duplicate gel strips were washed with 50% methanol for 30 min twice and 100% methanol for 30 min and dried in vacuo. These dried gels were soaked in 20 mM CNBr in 70% formic acid at 25 °C for 1 h to partially digest peptides in situ. After digestion, the gel strips were washed sequentially with 50% methanol containing 5% 2-mercaptoethanol for 1 h, 50% methanol for 30 min, and 100% methanol for 30 min. The gel strips were dried in vacuo, soaked in 20 mM Tris-HCl, 2% $NaDodSO_4$, 10% glycerol, and 1% 2-mercaptoethanol (pH 8.0) at 37 °C for 1 h, and loaded onto slab gels [15% acrylamide–0.5% bis(acrylamide)] for separation in the second dimension.

Results

Cross-Linking of Gelsolin Fragments and Actin with EDC. Figure 1, lane A, shows the $NaDodSO_4$–acrylamide gel of a solution containing an excess of actin and the gelsolin N-terminal peptide mixture before the EDC cross-linking. The gelsolin N-terminal mixture was obtained from total thermolysin digest by passing it through a monoclonal antibody column specific to the gelsolin C-terminal segment (CT38C). Thus the mixture contains various thermolysin fragments except the C-terminal fragment CT38C. They include predominantly TL45N, which migrates very closely to actin, TL28N (apparent M_r 30 000), and TL17N (barely visible on the Coomassie-stained gel) as well as other minor peptides whose identification is unknown. Lane B shows that the EDC cross-linking generated products with M_r 59 000 and 87 000. Each of these peptides contains actin, because they reacted positively with an anti-actin (N-terminus) antiserum (Figure 2, lanes C and D). Moreover, the 59- and 87-kDa peptides cross-reacted with antibody directed against the N-terminus

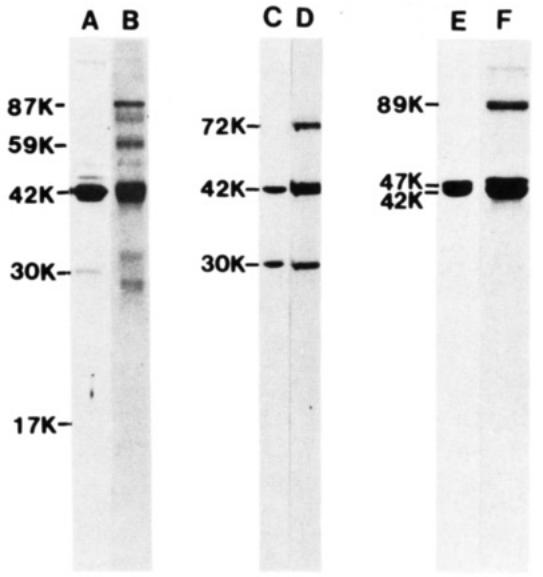

FIGURE 1: EDC cross-linking of gelsolin fragments to actin. Proteins were resolved on $NaDodSO_4$–acrylamide gels [12.5% acrylamide–0.4% bis(acrylamide)] and stained with Coomassie blue. Lanes A and B: actin and gelsolin N-terminal peptide mixture from thermolysin digestion before and after EDC cross-linking, respectively. Lanes C and D: actin and CT28N before and after cross-linking, respectively. Lanes E and F: actin and CT38C before and after cross-linking, respectively. Apparent molecular masses are indicated in daltons (K = 10^3).

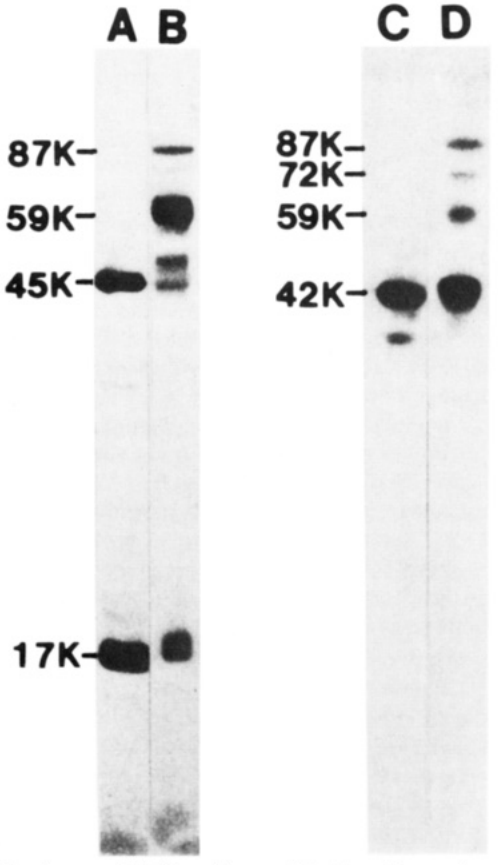

FIGURE 2: Immunoblotting of cross-linked products of actin and thermolysin-digested gelsolin N-terminal peptide mixture. Lanes A and C: actin and N-terminal peptide mixture before cross-linking (corresponds to Figure 1, lane A) stained with anti-gelsolin (N-terminus) antiserum and anti-actin (N-terminus) antiserum, respectively. Lanes B and D: actin and N-terminal peptide mixture after EDC cross-linking (corresponds to Figure 1, lane B) stained with anti-gelsolin (N-terminus) antiserum and anti-actin (N-terminus) antiserum, respectively.

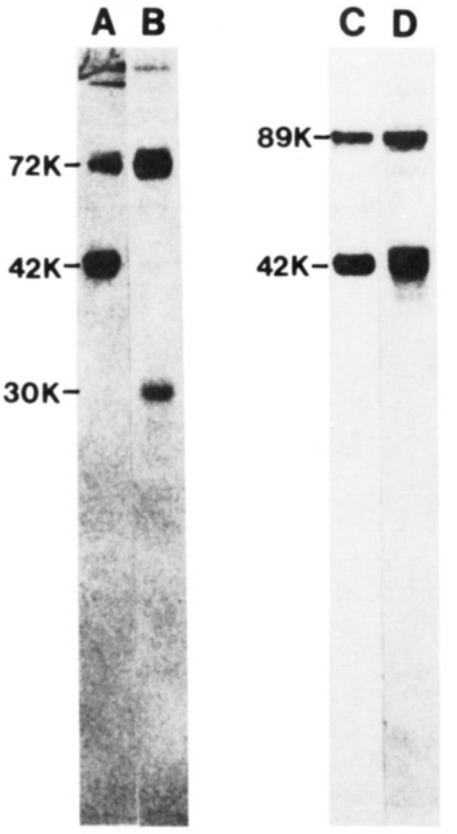

FIGURE 3: Immunoblotting of cross-linked actin–CT28N and actin–CT38C. Lanes A and B: actin–CT28N after EDC cross-linking (corresponds to Figure 1, lane D). They were stained with anti-actin (N-terminus) antiserum and anti-gelsolin antiserum, respectively. Lanes C and D: actin–CT38C after EDC cross-linking (corresponds to Figure 1, lane F). They were stained with anti-actin (N-terminus) antiserum and anti-gelsolin antiserum, respectively.

of human plasma gelsolin (Figure 2, lanes A and B). Therefore, on the basis of the estimated M_r's of the bands generated by EDC (59 000 and 87 000), they are most likely derived from cross-linking of TL17N and TL45N with actin. We cannot at present identify the origin of the 47-kDa cross-linked peptide which cross-reacts with anti-gelsolin (N-terminus) antiserum (lane B) but not with anti-actin (N-terminus) antiserum (lane D) but suggest that it is most likely derived from intermolecular or intramolecular cross-linking of gelsolin fragments.

The 72-kDa cross-linked peptide which was not stained with anti-gelsolin (N-terminus) antiserum (Figure 2, lane B) but cross-reacted with anti-actin (N-terminus) antiserum (lane D) is most likely derived from cross-linking of TL28N, which is missing the N-terminal epitope of gelsolin, with actin. This was directly established by cross-linking a similar peptide, CT28N, with actin. In Figure 1, lanes C and D, purified CT28N (apparent M_r 30 000) was incubated with approximately equal moles of actin in the absence or presence of EDC, respectively. Consistent with the results from cross-linking of the N-terminal peptide mixture, a 72-kDa cross-linked product was generated. This peptide cross-reacted with anti-actin (N-terminus) antiserum and a polyclonal antibody against the entire gelsolin molecule (Figure 3, lanes A and B), confirming that it is the covalently cross-linked product of actin and CT28N. Neither actin nor CT28N by itself was cross-linked with EDC (data not shown). In conclusion, our results demonstrate that each of the gelsolin N-terminal actin binding domains is cross-linked separately by EDC to actin.

Lanes E and F of Figure 1 compare the Coomassie staining pattern on $NaDodSO_4$ gels of a mixture of purified CT38C,

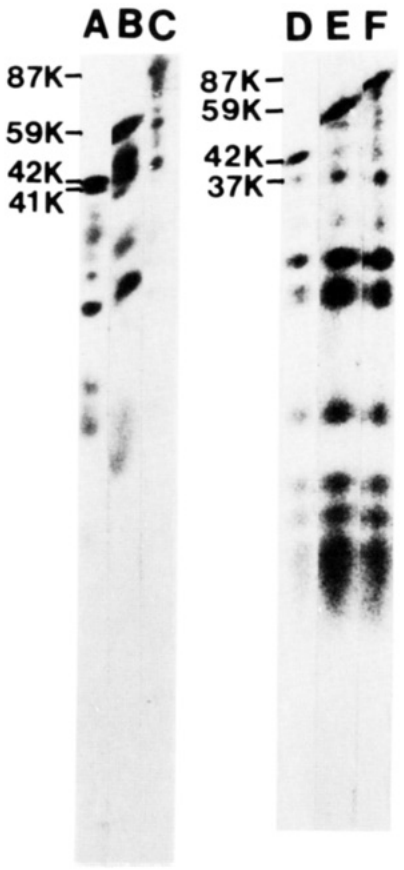

FIGURE 4: CNBr mapping of cross-linking sites of TL17N and TL45N along the actin sequence. Lanes A and D: CNBr fragments generated from non-cross-linked actin. Lanes B and E: CNBr fragments generated from the 59-kDa actin–TL17N conjugate. Lanes C and F: CNBr fragments generated from the 87-kDa actin–TL45N conjugates. Blots shown in lanes A–C were stained with the actin N-terminal probe, while blots shown in lanes D–F were stained with the actin C-terminal probe.

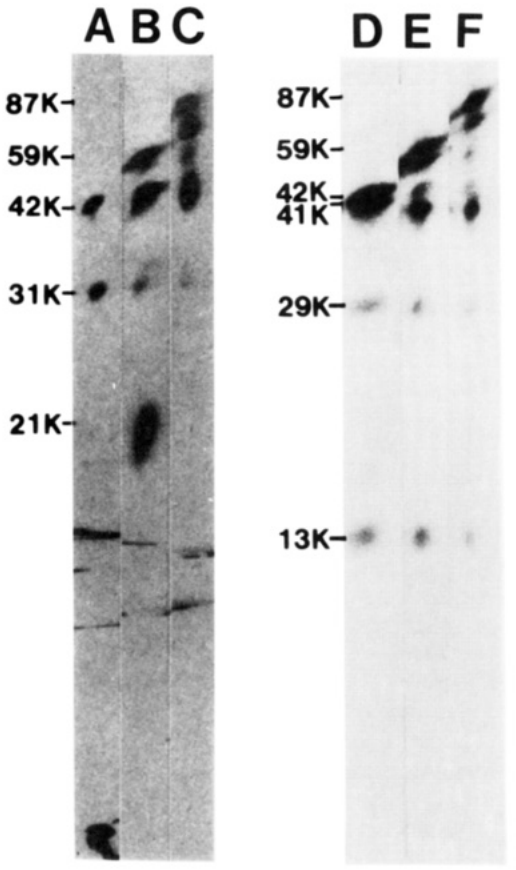

FIGURE 5: LEP mapping of cross-linking sites of TL17N and TL45N along the actin sequence. Lanes A and D: LEP fragments generated from non-cross-linked actin. Lanes B and E: LEP fragments generated from the 59-kDa actin–TL17N conjugate. Lanes C and F: LEP fragments generated from the 87-kDa actin–TL45N conjugate. Blots on the left-hand panel were stained with the actin N-terminal probe, while those on the right-hand panel were stained with the actin C-terminal probe.

a fragment derived from the C-terminal half of gelsolin, and actin in the absence and presence of EDC. CT38C has M_r 38 000 on the basis of its amino acid content and migrates anomalously on $NaDodSO_4$–acrylamide gels as a 47-kDa band (Chaponnier et al., 1986). In the presence of 0.2 mM $CaCl_2$, EDC generated an 89-kDa complex which cross-reacted with anti-actin (N-terminus) and anti-gelsolin antisera (Figure 3, lanes C and D). This complex was not formed in the presence of EGTA (data not shown), confirming that CT38C requires Ca^{2+} to bind actin.

Mapping of Cross-Linking Sites of Gelsolin N-Terminal Fragments along the Actin Sequence. (A) TL17N and TL45N, CNBr Mappings. Duplicate gel strips containing non-cross-linked actin, the 59-kDa actin-TL17N conjugate, and the 87-kDa actin–TL45N conjugate, similar to those shown in Figure 1, lane B, were treated with CNBr. The resultant CNBr fragments were resolved by electrophoresis and transferred onto membranes in duplicate for staining with streptavidin–alkaline phosphatase conjugate to visualize the biotin moiety on Cys-374 of actin (the C-terminal probe) or with the antibody against the actin N-terminus (the N-terminal probe). Staining of one of the duplicate membranes with the C-terminal probe shows that ladder-like bands are aligned with each other up to the 37-kDa fragment (Figure 4, lanes D–F), which corresponds to actin fragment of residues 45–375 and/or 48–375 (Sutoh, 1982, 1984). Therefore, the cross-linking sites in the actin–TL17N and actin–TL45N conjugates are outside the C-terminal 37-kDa fragment of actin, i.e., within the N-terminal fragment of residues 1–44 (Figure 10).

When the other blot was stained with the N-terminus probe, no alignment of ladder-like bands was possible even at the low M_r region, indicating that the cross-linking sites are close to the extreme N-terminus of actin.

(B) TL17N and TL45N, LEP Mappings. To obtain finer mapping, actin and actin–TL17N and actin–TL45N conjugates were partially digested with lysyl endopeptidase (LEP), electrophoresed, and then transferred onto membranes in duplicate. The blot stained with the C-terminal probe shows that the fragment pattern generated from non-cross-linked actin (Figure 5, lane D) is aligned, up to the 41-kDa fragment which emcompasses actin residues 19–375 (Sutoh & Mabuchi, 1986), with that generated from the 59-kDa (lane E) or the 87-kDa conjugate (lane F). The results indicate that their cross-linking sites are outside the 41-kDa C-terminal fragment of actin, i.e., within actin N-terminal residues 1–18 (Figure 10).

The other blot stained with the N-terminus probe shows that fragment patterns of actin and the 59- and 87-kDa conjugates are different from each other (Figure 5, lanes A–C). Digestion of the 59-kDa conjugate released a unique 21-kDa band (lane B). Since this is smaller than the smallest fragment detected in digestion products of actin (lane A), it is likely due to cross-linking between gelsolin fragment and a small actin N-terminal peptide, which, in its free form, is too small to be retained on the membrane. Therefore, it is clear that a cross-linking site in the actin–TL17N conjugate is located, at

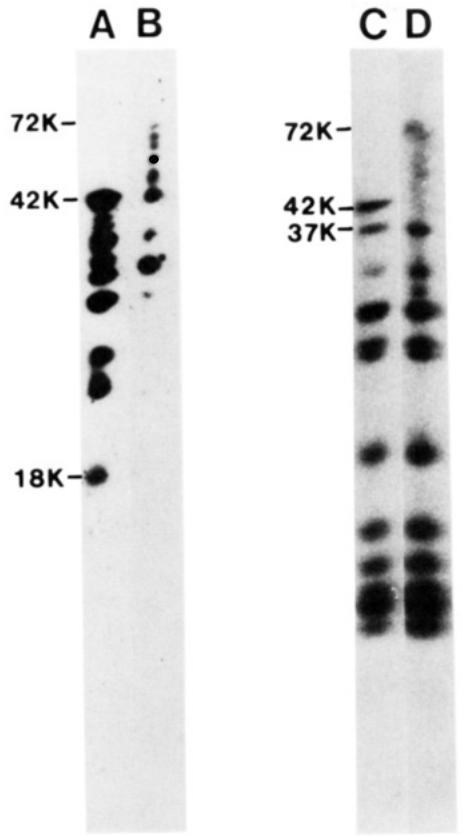

FIGURE 6: CNBr mapping of cross-linking site of CT28N along the actin sequence. Lanes A and C: CNBr fragments generated from non-cross-linked actin. Lanes B and D: CNBr fragments from the 72-kDa actin–CT28N conjugate. Blots on the left-hand panel were stained with the actin N-terminal probe, while those on the right-hand panel were stained with the actin C-terminal probe.

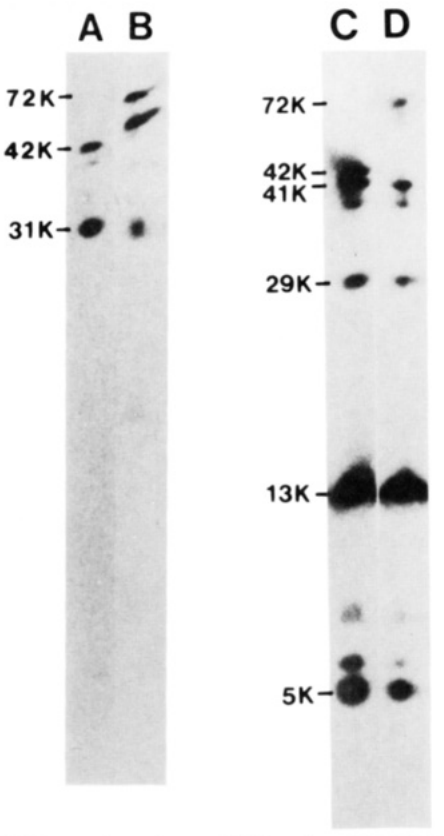

FIGURE 7: LEP mapping of cross-linking site of CT28N along the actin sequence. Lanes A and C: LEP fragments generated from non-cross-linked actin. Lanes B and D: LEP fragments from the 72-kDa actin–CT28N conjugate. Panels on the left were stained with the actin N-terminal probe, while those on the right were stained with the actin C-terminal probe.

most, within the N-terminal 21-kDa segment of actin (Figure 10). Lane C shows that LEP digestion of the 87-kDa actin–TL45N conjugate did not release the 31-kDa actin fragment, indicating that the cross-linking site is within the N-terminal 31-kDa segment of actin (Figure 10).

(*C*) *CT28N, CNBr Mappings.* A cross-linking site between actin and CT28N was mapped after CNBr cleavage. Lanes C and D of Figure 6 show that, after the actin C-terminal probing, CNBr fragments generated from non-cross-linked actin and the actin–CT28N conjugate are aligned with each other up to the 37-kDa fragment, indicating that a cross-linking site of CT28N is located within an actin segment of residues 1–44 (Figure 10).

Staining of the other duplicate blot with the N-terminal probe confirmed that cross-linking of CT28N is at the N-terminus of actin. Lanes A and B of Figure 6 show that the smallest CNBr fragment generated from non-cross-linked actin (18 kDa, lane A) does not have its counterpart in fragments from the 72-kDa actin–CT28N conjugate (lane B). Therefore, we deduce that the cross-linking site is, at most, within the N-terminal 18-kDa fragment of actin (Figure 10).

(*D*) *CT28N, LEP Mappings.* LEP digestion located the cross-linking site within the first 18 residues of actin. Lanes C and D of Figure 7 show fragments from non-cross-linked actin and the 72-kDa actin–CT28N conjugate after the C-terminal probing. These LEP fragments are perfectly aligned up to the 41-kDa fragment. Therefore, a cross-linking site is located within residues 1–18 of actin, similar to that of the other gelsolin N-terminal fragment, TL17N. The N-terminal probing shows that the 72-kDa conjugate released only a small fraction of the 31-kDa fragment (Figure 7, lane B) compared to that released from non-cross-linked actin (lane A), confirming that a major cross-linking site is located within the N-terminal 31-kDa fragment of actin.

Mappings of Cross-Linking Site of the C-Terminal Fragment of Gelsolin along the Actin Sequence. (*A*) *CT38C, CNBr Mappings.* The N-terminal probing shows that CNBr fragments derived from non-cross-linked actin and the 89-kDa actin–CT38C conjugate are perfectly aligned up to the 41-kDa fragment (Figure 8, lanes A and B), which encompasses actin residues 1–355. The results indicate that a cross-linking site is located within actin residues 356–375 (Figure 10).

The C-terminal probing shows that the CNBr fragments derived from non-cross-linked actin and the 89-kDa actin–CT38C conjugate are perfectly aligned up to the 42-kDa band, which corresponds to intact actin (Figure 8, lanes C and D). Cross-linking however did occur, because higher molecular weight products were generated. Note that the intensity ratio of the 42-kDa band to the smaller fragments is similar in lanes C and D. These results are expected only when the CNBr digestion rapidly generated a small gelsolin fragment carrying a cross-linking site.

(*B*) *CT38C, LEP Mappings.* Further mappings were carried out by partial LEP digestion. The N-terminal probing (Figure 9, lanes A and B) shows that upon LEP digestion non-cross-linked actin and the 89-kDa actin–CT38C conjugate both released the 31-kDa fragment, suggesting that a cross-linking site is outside the N-terminal 31-kDa fragment of actin (Figure 10). Probing the C-terminus (Figure 9, lanes C and D) shows that a distinct fragment pattern was generated from non-cross-linked actin compared with that from the actin–CT38C conjugate. The 5-kDa band generated from non-

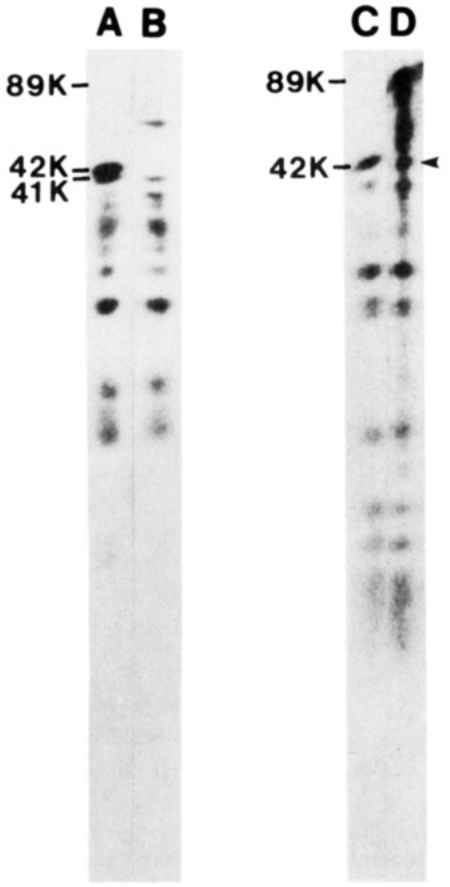

FIGURE 8: CNBr mapping of cross-linking site of CT38C along the actin sequence. Lanes A and C: CNBr fragments generated from non-cross-linked actin. Lanes B and D: CNBr fragments from the 89-kDa actin–CT38C conjugate. Panels on the left were stained with the actin N-terminal probe, while those on the right were stained with the actin C-terminal probe.

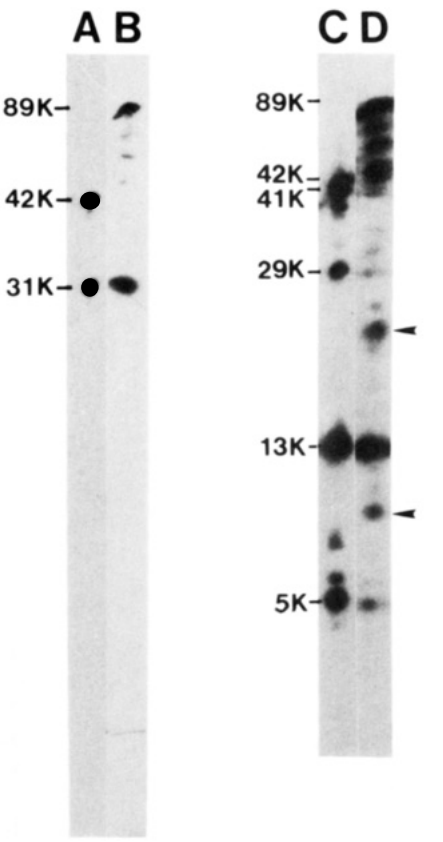

FIGURE 9: LEP mapping of cross-linking site of CT38C along the actin sequence. Lanes A and C: LEP fragments were generated from non-cross-linked actin. Lanes B and D: LEP fragments were from the 89-kDa actin–CT38C conjugate. Panels on the left were stained with the actin N-terminal probe, while those on the right were stained with the actin C-terminal probe.

cross-linked actin is almost absent in the LEP digest of the conjugate, indicating that a major cross-linking site is within the C-terminal 5-kDa fragment of actin (Figure 10). Consistent with the conclusion is the fact that small cross-linked fragments (indicated by arrowheads in lane D) were generated.

Discussion

The EDC cross-linking of four gelsolin fragments with actin has revealed that the two N-terminal actin binding domains (17N and 28N) of gelsolin are cross-linked with the N-terminal segment of actin (residues 1–18), while the C-terminal actin binding domain (38C) of gelsolin is cross-linked with the C-terminal segment of actin (residues 356–375). EDC is a zero-length cross-linking reagent which cross-links closely juxtaposed amino and carboxyl side chains. Examination of the actin sequence (Elzinga et al., 1973) reveals that Asp-1, Glu-2, Asp-3, Glu-4, and Asp-11 are potential candidates for cross-linking sites with each of the N-terminal gelsolin fragments, while Lys-359, Glu-361, Asp-363, Glu-364, Lys-373, and the C-terminal Phe-375 are candidates for cross-linking sites with the C-terminal gelsolin fragment. Considering the fact that both gelsolin and its N-terminal half associate with two actins, it seems that the two N-terminal actin binding domains (17N and 28N) bind to two different actins while the C-terminal actin binding domain (38C) shares one of these actins with the N-terminal one. Since N- and C-terminal segments of actin are in proximity with each other in an actin molecule as discussed below, it is likely that the N- and C-terminal halves of gelsolin are also in proximity with each other at least at the interface with actin.

EDC cross-links two proteins when they form a tight complex. As shown in this paper, actin and a gelsolin fragment (CT38C) are cross-linked only when they form a complex in the presence of Ca^{2+}. Another example is that actin and myosin are cross-linked only when they form a "rigor" complex (Mornet et al., 1981). Therefore, it is most likely that the cross-linked residues in the N- and C-terminal segments of actin are in the contact site with gelsolin. This notion is supported by results by Boyer et al. (1987), who have reported that an antibody directed against a C-terminal peptide of actin (residues 285–375) inhibits binding of gelsolin to actin and dissociates preformed actin–gelsolin complexes. Conversely, gelsolin interferes with the binding of anti-actin C-terminal fragment antibody to actin. It must be mentioned here that ionic interactions between amino and carboxyl side chains involved in the EDC cross-linking may not be the sole factor stabilizing the actin–gelsolin complex. For example, hydrophobic interactions between nonpolar side chains, which cannot be detected by the EDC cross-linking, must be taken into account to fully understand the actin–gelsolin interaction.

Chemical cross-linking studies have shown that almost all of the actin binding proteins examined to date are cross-linked to either or both of the actin terminal segments. These include myosin (Sutoh, 1982), depactin (Sutoh & Mabuchi, 1984, 1986), fragmin (Sutoh & Hatano, 1986), α-actinin and actinogelin (Mimura & Asano, 1987), troponin I (Grabarek & Gergely, 1987), and profilin (T. Pollard, D. Kaiser, C. Ampe, and J. Vandekerckhove, personal communication) as well as gelsolin. It is tempting to speculate that the two actin termini are juxtaposed to present a common site in the interactive

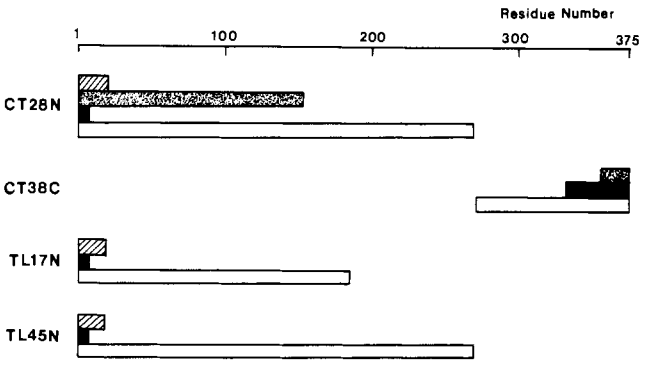

FIGURE 10: Maps of cross-linking sites of gelsolin fragments along the actin sequence. Bars indicate actin segments identified as participating in binding gelsolin fragments (CT28N, CT38C, TL17N, and TL45N). Cross-hatched bars: segments identified by the actin C-terminal probe after CNBr cleavage. Bars shaded with dots: segments identified by the actin N-terminal probe after CNBr cleavage. Closed bars: segments identified by the C-terminal probe after LEP digestion. Open bars: segments identified by the N-terminal probe after LEP digestion.

domain with actin binding proteins and that various actin binding proteins associating with this site have similar secondary structures to recognize the site. The putative site is likely to be located at the outer surface of F-actin, because Toyoshima and Wakabayashi (1985a,b) have shown that the actin–myosin interface is located there and Sutoh (1982) has found that the myosin head is cross-linked to both the N- and C-terminal actin segments. Various actin binding proteins might use the site composed of the N- and C-terminal segments for anchoring on F-actin before exerting their effects such as bundling or severing.

The idea that some actin binding proteins have similar actin binding domains is plausible at least for a class of actin binding proteins. It is now clear that gelsolin is a member of a large family of severing proteins which have similar multiple repeats within their primary sequences. These include villin, a 95-kDa protein found in brush border microvilli (Bretscher & Weber, 1979; Matsudaira & Burgess, 1979), and fragmin and severin, 40–42-kDa proteins isolated from *Physarum polycephalum* (Hasegawa et al., 1980) and *Dictyostelium discoideum* (Brown et al., 1982), respectively. The N- and C-terminal halves of gelsolin have 47% sequence homology to each other, and each half shows a 3-fold internal tandem repeat (Kwiatkowski et al., 1986). Similar repeat structures are found in villin (6-fold; Bazari et al., 1988), fragmin (3-fold; Andre et al., 1988), and severin (3-fold; Ampe & Vandekerckhove, 1987). The presence of 3- and 6-fold repeats suggests that the invertebrate and vertebrate proteins were evolved from a common ancestral actin binding protein of 14–17 kDa, initially to a trimeric unit like fragmin and severin and by further duplication to yield proteins like gelsolin and villin. The presence of common sequences for actin binding in some actin binding proteins is also supported by the findings that *Acanthameba* profilins, which sequester actin monomers and, like the proteins in the gelsolin family, cap the "barbed" end of actin filaments, have a 16-residue stretch which is homologous to those of the gelsolin family (Ampe & Vanderkerckhove, 1987) and, moreover, a Lys residue unique to profilin located within this homologous region (profilin residue 115) is cross-linked to the C-terminal segment of actin with EDC (Pollard et al., 1988). In this context, it is interesting to determine if the gelsolin sequences homologous to the profilin sequence mentioned above are also involved in the interaction with the N- and/or C-terminal segment of actin.

It has been recently shown that the N-terminal segment of depactin is cross-linked with the N- and C-terminal segment of actin (Sutoh & Mabuchi, 1989). The amino acid sequence of the N-terminal segment of depactin (Takagi et al., 1987), however, shows no homology to the profilin sequence and to the gelsolin (and its family member) sequence. Thus, although actin binding proteins in the gelsolin family may have a common actin binding sequence as suggested above, it may not be the case for other actin binding proteins.

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