The Actin Filament-severing Domain of Plasma Gelsolin

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Abstract. Gelsolin, a multifunctional actin-modulating protein, has two actin-binding sites which may interact cooperatively. Native gelsolin requires micromolar Ca^{2+} for optimal binding of actin to both sites, and for expression of its actin filament-severing function. Recent work has shown that an NH_2-terminal chymotryptic 17-kD fragment of human plasma gelsolin contains one of the actin-binding sites, and that this fragment binds to and severs actin filaments weakly irrespective of whether Ca^{2+} is present. The other binding site is Ca^{2+} sensitive, and is found in a chymotryptic peptide derived from the COOH-terminal two-thirds of plasma gelsolin; this fragment does not sever F-actin or accelerate the polymerization of actin. This paper documents that larger thermolysin-derived fragments encompassing the NH_2-terminal half of gelsolin sever actin filaments as effectively as native plasma gelsolin, although in a Ca^{2+}-insensitive manner. This result indicates that the NH_2-terminal half of gelsolin is the actin-severing domain. The stringent Ca^{2+} requirement for actin severing found in intact gelsolin is not due to a direct effect of Ca^{2+} on the severing domain, but indirectly through an effect on domains in the COOH-terminal half of the molecule to allow exposure of both actin-binding sites.

GELSOLIN is a Ca^{2+}- and actin-binding protein first purified from the cytoplasm of rabbit lung macrophages (Yin and Stossel, 1979). A similar protein called plasma actin depolymerizing factor was also identified in plasma (Chaponnier et al., 1979; Norberg et al., 1979). It is homologous to cytoplasmic gelsolin (Yin et al., 1984), and has been called plasma gelsolin (Harris and Weeds, 1984; Harris, 1985; Coue and Korn, 1985) or brevin (Harris and Schwartz, 1981; Doi and Frieden, 1984; Lees et al., 1984; Bryan and Hwo, 1985; Kilhoffer and Gerard, 1985). Both cytoplasmic and plasma gelsolins fragment actin filaments, nucleate actin filament assembly, and block their fast growing (+) ends. Of gelsolin's actin-modulating functions, severing of filaments has the most stringent Ca^{2+} requirement, being completely abolished in its absence (Yin et al., 1980; Harris and Weeds, 1983; Bryan and Coluccio, 1985; Janmey et al., 1985). Functional studies indicate that gelsolin has two actin-binding sites (Yin and Stossel, 1980; Bryan and Kurth, 1984) and indirect evidence suggests that they may bind actin in a cooperative manner (Coue and Korn, 1985; Harris, 1985; Janmey et al., 1986*b*). After a 2:1 actin/gelsolin complex is formed in the presence of Ca^{2+} at a limiting actin concentration, removal of free Ca^{2+} with EGTA releases actin from one site but not from the other, as reflected by the persistence of a 1:1 actin/gelsolin complex in EGTA (Bryan and Kurth, 1984; Kurth and Bryan, 1984). In macrophages, an as yet undefined mechanism dissociates this EGTA-resistant actin/gelsolin complex (Chaponnier et al., 1985).

Structural analyses support the existence of two distinct actin-binding domains in human plasma gelsolin (Kwiatkowski et al., 1985). One actin-binding site is found in a 17-kD peptide derived from the extreme NH_2-terminus of plasma gelsolin by chymotrypsin cleavage. This peptide binds to actin immobilized on Sepharose beads equally well in the presence of 0.2 mM $CaCl_2$ or 1 mM EGTA. It binds to actin monomers in solution to form a 1:1 complex, and it can sever actin filaments. However, compared with native gelsolin, its severing ability is decreased ~100-fold and is no longer regulated by Ca^{2+}. The other actin-binding domain is located away from the NH_2-terminus in a 47-kD fragment generated by chymotrypsin digestion. It binds an actin monomer only in the presence of micromolar Ca^{2+}, and releases the actin when the Ca^{2+} concentration is decreased. It does not sever actin filaments. Since intact gelsolin requires Ca^{2+} to sever actin filaments, these observations raise the questions of why severing by the small NH_2-terminal fragment no longer requires Ca^{2+} and why its ability to sever is decreased. One possibility is that CT17 is part of a larger, more effective actin-severing domain which is Ca^{2+} sensitive. Alternatively, the severing domain may be inherently Ca^{2+} insensitive, and Ca^{2+} regulation is imposed on it by a separate part of the protein.

To examine these possibilities, larger NH_2-terminal actin-binding peptides were identified and their ability to sever actin filaments and to nucleate actin assembly in the presence and absence of Ca^{2+} were determined. The NH_2-terminal peptide has an M_r of 45,000 and is gener-

ated by cleavage at a site close to the middle of plasma gelsolin. It severs actin filaments as effectively as intact gelsolin but is no longer regulated by Ca^{2+}. The NH_2-terminal peptide/actin complexes formed in the presence of Ca^{2+} retain Ca^{2+} ions which cannot be chelated by EGTA. We conclude that the NH_2-terminal half of plasma gelsolin is responsible for the efficient actin-fragmenting activity of gelsolin and for the formation of an EGTA-irreversible complex with actin. Since the NH_2-terminal half peptide severs actin filaments even in the absence of Ca^{2+}, the stringent Ca^{2+} requirement for actin severing found in intact gelsolin must be imposed on the NH_2-terminal half by the COOH-terminal half of gelsolin, a region which contains a highly Ca^{2+}-sensitive actin-binding site with no severing ability. A model for the interaction of the two halves of plasma gelsolin is also proposed by Bryan and Hwo (1985), except that their data suggest that both actin-binding sites are located at the NH_2-terminal half of the molecule.

Materials and Methods

Purification of Plasma Gelsolin

Human plasma gelsolin was prepared as described previously by conventional column chromatography (Yin and Stossel, 1980) or by an immunoaffinity chromatographic method using the monoclonal anti-gelsolin antibody designated 2C4. IgG was purified from ascites fluid (prepared as described below) by DEAE Sepharose CL-6B chromatography, and coupled to cyanogen bromide-activated Sepharose CL4B beads (Pharmacia Fine Chemicals, Piscataway, NJ) at a ratio of 3 mg protein/ml packed beads. To affinity-purify plasma gelsolin, 300 ml human plasma was centrifuged at 10,000 *g* for 30 min and passed over a column packed with 30 ml of 2C4 antibody-Sepharose and equilibrated with 0.15 M NaCl, 20 mM Tris-HCl, pH 7.4 (TBS) containing 1 mM EGTA at 4°C. The column was washed sequentially with 10 vol of TBS-EGTA, and 3 vol of TBS-EGTA containing 1 M $MgCl_2$. Plasma gelsolin was eluted with TBS-EGTA containing 3 M $MgCl_2$, pH 7.4. Routinely, ~20-30 mg of 90-95% pure gelsolin was recovered by this procedure (see Fig. 3). The plasma gelsolin was dialyzed against TBS-EGTA and stored at −20°C in 40% glycerol. Plasma gelsolin prepared by this immunoaffinity procedure was functionally identical to that purified by conventional chromatography techniques, as judged by their ability to sever actin filaments and nucleate actin assembly (Doi and Frieden, 1984; Janmey and Stossel, 1986; Janmey et al., 1986*a*). Plasma gelsolin concentration was estimated using an extinction coefficient of A_{280} = 1.538 cm^{-1} for a 1-mg/ml solution (Kilhoffer and Gerard, 1985). In some cases, plasma gelsolin concentration was determined by the method of Bradford (1976), using IgG as a standard. Parallel determinations with both methods show that they gave similar protein concentrations (within 10%). The extinction coefficient for plasma gelsolin was used to estimate the protein content of purified NH_2-terminal half and COOH-terminal half gelsolin peptides.

Production of Monoclonal Antibodies

8-wk-old BALB/c mice were injected intraperitoneally with 20 μg of human plasma gelsolin in complete Freund's adjuvant. They were boosted 3 wk later with 20 μg plasma gelsolin in complete Freund's adjuvant, and subsequently every 3 wk with antigen in incomplete Freund's adjuvant over a 3-mo period. 3 d before fusion, the immunized mouse was boosted intravenously with 10 μg of gelsolin in 0.15 M NaCl solution. The mouse was killed by cervical dislocation and its spleen cells were fused with NS1 mouse myeloma cells (Kohler and Milstein, 1975; Oi and Herzenberg, 1980). Hybridomas producing antibody against gelsolin were screened by the solid-phase enzyme-linked immunosorbent assay (ELISA) and positive clones were subcloned twice. Ascites fluid was produced by injecting cells into pristane-primed mice. The specificity of the monoclonal antibodies against gelsolin was assessed by immunoblotting with human plasma and human platelet extracts, after separation of proteins on SDS polyacrylamide gels. In each case, a single band co-migrating with either plasma or cytoplasmic gelsolin was detected (data not shown).

Production of Antibody against Synthetic Peptide

The NH_2-terminal amino acid sequence of human plasma gelsolin has been published (Yin et al., 1984). Based on this data, a synthetic peptide with a sequence consisting of the NH_2-terminal 15 residues (Ala-Thr-Ala-Ser-Arg-Gly-Ala-Ser-Gln-Ala-Gly-Ala-Pro-Gln-Gly) was generated by solid-phase technique, by the Peptide Synthesis Facility, Children's Hospital Medical Center, Boston, MA. The peptide was desalted on a P-2 (Bio-Rad Laboratories, Richmond, CA) column equilibrated with 5% acetic acid. 10 mg of the peptide was coupled to 40 mg of either keyhole limpet hemocyanin (Sigma Chemical Co., St. Louis, MO) or bovine serum albumin (BSA, radioimmune assay grade; Sigma Chemical Co.) in a vol of 16 ml by dropwise addition of glutaraldehyde (2%) to a final concentration of 0.1%. The solution was stirred for 1 h at room temperature, and dialyzed exhaustively against saline. Rabbits were immunized by subcutaneous injection of 500 μg peptide/keyhole limpet hemocyanin complex in complete Freund's adjuvent diluted with an equal volume of saline. After 1 mo, they were boosted three times at biweekly intervals with 200 μg protein in incomplete Freund's adjuvent. The presence of antibody against plasma gelsolin was determined by ELISA, using microtiter plates coated with 10 μg/ml human plasma gelsolin or the 15-mer synthetic peptide-BSA complex. To assess the specificity of the antibody against the NH_2-terminal peptide of human plasma gelsolin, the immune serum was tested against plates coated with the 15-mer peptide/BSA complex or human plasma gelsolin in the presence of 0-0.1 mg/ml of the peptide (without carrier).

Proteolytic Cleavage of Plasma Gelsolin

Human plasma gelsolin (0.6 mg/ml) was incubated with α-chymotrypsin (Worthington Biochemical Corp., Freehold, NJ) at a weight ratio of 230:1 in TBS containing 2 mM $MgCl_2$ and 0.2 mM $CaCl_2$. After 15 min at room temperature, freshly prepared phenylmethylsulfonyl fluoride (300 mM) in ethanol was added to a final concentration of 1 mM. Gelsolin was digested with thermolysin at wt/wt ratios ranging from 230:1 to 145,000:1, in TBS containing 2 mM $MgCl_2$ and 0.2 mM $CaCl_2$ for 15 min at room temperature. The digests were analyzed on 5-15% or 5-20% discontinuous pH gradient slab gels by the method of Laemmli (1970). The apparent molecular weights of the peptides were estimated by comparison with the mobility of molecular weight standards (Bio-Rad Laboratories).

Immunoblotting of Proteolytically Cleaved Peptides

Gelsolin peptides resolved by PAGE in SDS were transferred to nitrocellulose sheets (BA 85; Schleicher & Schuell, Inc., Keene, NH) according to the method of Towbin et al. (1979). The blots were washed with TBS and blocked for 1 h with 3% BSA in TBS. They were then incubated with hybridoma supernatants at 1:10 dilution in TBS containing 0.1% Triton X-100 (TX)[1] and 3% BSA or polyclonal anti-N serum at a 100-fold dilution with TBS-TX-BSA for 2 h at room temperature or overnight at 4°C. The blots were washed with TBS-TX and incubated with the appropriate secondary antibody conjugated with horseradish peroxidase (either goat anti-mouse IgG or goat anti-rabbit IgG, Cappel Laboratories, Cochranville, PA) at a 1:100-1:200 dilution in TBS-TX for 1 h at room temperature. The blots were then washed with three changes of TBS-TX, incubated with 0.5% TX in TBS for 30 min, and washed sequentially with three changes of TBS followed by 10 mM Tris-HCl, pH 7.4. Horseradish peroxidase bound to the peptides was reacted with O-dianisidine and hydrogen peroxide (250 μg/ml and 0.075%, respectively) for 1-2 min, and the reaction was stopped by washing in TBS for 5 min.

Binding of Gelsolin Peptides to Actin-Sepharose

Actin was coupled to cyanogen bromide-activated Sepharose CL4B as described in Kwiatkowski et al., 1985. 10-15 μg gelsolin or its cleaved products were incubated with 30 μl of actin-Sepharose in 200 μl of TBS-2 mM $MgCl_2$ containing either 0.2 mM $CaCl_2$ or 1 mM EGTA. The samples were mixed constantly with an end-over-end mixer for 30 min at 10°C and washed with buffers containing either $CaCl_2$ or EGTA, respectively. To determine if the peptides were bound irreversibly to the actin beads, peptide/actin bead complexes formed in the presence of 0.2 mM $CaCl_2$ were washed extensively with TBS-EGTA. Aliquots of proteins bound to the beads were analyzed on SDS polyacrylamide gels and, in some cases, electrophoretically transferred to nitrocellulose paper for staining with antibodies.

1. *Abbreviations used in this paper*: DBP, vitamin D-binding protein; TX, Triton X-100.

Presence of EGTA-resistant Ca^{2+} in Gelsolin–Actin Complexes

9 μg of gelsolin or gelsolin digests were incubated with 150 μl of actin–Sepharose at 4°C in 458 μl TBS, 1.18×10^{-5} M total $CaCl_2$, and ^{45}Ca at a specific activity of 8.69×10^2 μCi/μmol with constant mixing. At the end of a 30-min incubation, the beads were sedimented by centrifugation in an Eppendorf microfuge and washed twice with TBS–EGTA. The washed beads were incubated overnight at 4°C in TBS–EGTA to maximize elution of bound ^{45}Ca, and collected on GFA filters set on a Multiphore filtration apparatus (Millipore Corp., Bedford, MA). The beads were covered with an additional filter, washed with 10 ml of TBS–EGTA, and counted in a Packard liquid scintillation counter operating at 60% efficiency. Actin beads incubated with ^{45}Ca but no gelsolin were included as a control.

Interaction of Peptides with Pyrene-labeled Actin

G-actin, prepared by the method of Spudich and Watt (1971), was stored in a buffer containing 2 mM Tris–HCl, 0.2 mM $CaCl_2$, 0.2 mM 2-mercaptoethanol, 0.2 mM ATP, pH 7.6. It was labeled with *N*-(1-pyrenenyl)iodoacetamide by the method of Kouyama and Mihashi (1981). The relative amounts of G- and F-actin and the rate of change of these quantities were determined as previously described (Janmey et al., 1985). In the presence of Ca^{2+}, the rate of actin polymerization in the presence of gelsolin has been shown to be proportional to the gelsolin concentration (Doi and Frieden, 1984; Janmey and Stossel, 1986), and when actin filaments are diluted to below the critical monomer concentration the depolymerization rate is proportional to the number of filament ends created by gelsolin (Bryan and Coluccio, 1985; Coue and Korn, 1985; Janmey and Stossel, 1986).

Results

Specificity of Anti-gelsolin Antibodies

In an ELISA, serum from a rabbit immunized with the plasma gelsolin NH_2-terminus 15-mer peptide/KLH complex (anti-N-peptide antiserum) reacted positively with the same peptide coupled to a different carrier, BSA, as well as with plasma gelsolin. Binding of the anti-N serum to peptide/BSA and plasma gelsolin was reduced 75 and 85%, respectively, in the presence of 20 μg/ml of the synthetic NH_2-terminal peptide (without carrier BSA) antigen, and 100% with 50 μg/ml of the antigen. BSA alone, which was present throughout the assay, did not block the binding of the antibody to the antigen or to plasma gelsolin. Specificity of the anti-N peptide antibody against the NH_2-terminal residues of human plasma gelsolin was further ascertained by immunoblotting against chymotryptic peptides known to be derived from the NH_2-terminus of plasma gelsolin (Fig. 1). CT17 starts at residue 1 of plasma gelsolin while CT15 is offset by 23 amino acids, beginning at residue 24 (Kwiatkowski et al., 1985). The anti-N serum reacted with CT17 but not CT15. Since these two peptides were identical except for the absence of the synthetic peptide sequence in CT15, the antibody is specific for the NH_2-terminal peptide sequence of plasma gelsolin.

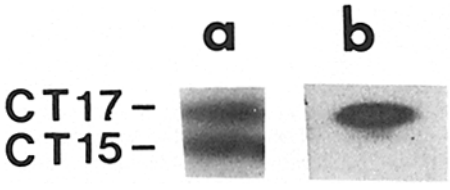

Figure 1. Specificity of the anti-N peptide antibody. EGTA-resistant CT17 and CT15 which were eluted from an actin–Sepharose column with acetic acid and sequenced as described previously (Kwiatkowski et al., 1985) were electrophoresed on an SDS polyacrylamide gel. One sample, containing 1 μg protein, was stained with Coomassie Blue (*a*), and the other, containing 0.33 μg protein, was blotted onto nitrocellulose paper and stained with the polyclonal rabbit anti-N peptide antibody.

Mapping of Proteolytic Fragments of Plasma Gelsolin

Digestion with Chymotrypsin. It was previously reported that chymotrypsin cleaves plasma gelsolin into two distinct actin-binding domains (Kwiatkowski et al., 1985). One domain is at the amino-terminal 17-kD peptide portion and the other resides in a 47-kD peptide derived at least one-third of the way from the NH_2-terminus. In this report, these chymotrypsin peptides were used to define the epitopes of anti-gelsolin antibodies which were in turn used to locate other hitherto uncharacterized peptides containing these domains. To maximize the yield of large fragments, gelsolin was digested for a shorter time than reported previously. When such a chymotrypsin digest of gelsolin (Fig. 2 *a*, lane *1*) was incubated with actin beads in the presence of 0.2 mM $CaCl_2$, a 47-, 45-, and 17-kD band bound to actin beads while a 31-kD band did not. The 45-kD band and the 17-kD band also bound to actin beads in the presence of EGTA. To sort out the relation between these peptides and distinguish between peptides with similar mobility on SDS gels but apparently different actin-binding characteristics, the bands were analyzed by immunoblotting. The anti-N peptide antibody reacted with the 45- and 17-kD peptides which bound to actin beads irrespective of the Ca^{2+} concentration (Fig. 2 *b*). The 17-kD peptide is therefore identical to CT17 which contains the NH_2-terminal amino acid sequence of plasma gelsolin (Kwiatkowski et al., 1985). The 45-kD actin-binding peptide which was also recognized by this antibody must contain the NH_2-terminus and the NH_2-terminal actin-

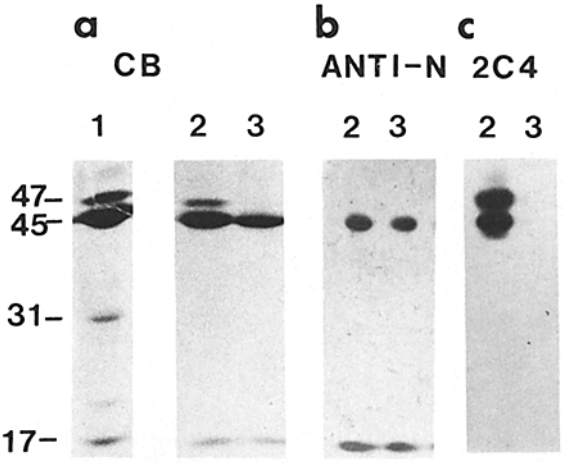

Figure 2. Effects of EGTA on the binding of chymotryptic peptides to actin–Sepharose. Aliquots of human plasma gelsolin digested with chymotrypsin in the presence of 0.2 mM $CaCl_2$ at a wt/wt ratio of 230:1 were incubated with actin–Sepharose in the presence of 0.2 mM $CaCl_2$ (lanes *2*) or 1 mM EGTA (lanes *3*) and washed with the appropriate buffers. Proteins bound to the beads were analyzed on an SDS polyacrylamide gel. (*a*) Coomassie Blue–stained gel. Lane *1*, 2 μg chymotrypsin digest. (*b* and *c*) Actin-binding peptides stained with anti-N peptide antiserum and 2C4 antibody, respectively. One third as much protein was loaded for immunoblotting as for Coomassie Blue staining. The M_r ($\times 10^{-3}$) of the chymotrypsin peptides are indicated.

binding domain as well. For the sake of clarity, peptides containing the NH_2-terminal peptide of plasma gelsolin are labeled with the suffix n.

The anti-N peptide antibody did not recognize the 47-kD peptide, which on the basis of its Ca^{2+}-dependent reversible binding to actin beads is equivalent to CT47 (Kwiatkowski et al., 1985). This peptide, and a 45-kD peptide with similar actin-binding properties reacted with the monoclonal antibody designated 2C4 (Fig. 2 *c*). These two peptides are therefore related and contain the same actin-binding domain which is shown in Kwiatkowski et al. (1985) to be derived from the carboxyl-terminal two-thirds of plasma gelsolin. Using full-length sequence data for plasma gelsolin derived from cDNA cloning (Kwiatkowski et al., 1986), the origin of CT47 can be located precisely. The deduced amino acid sequence shows that plasma gelsolin has 755 amino acid residues and CT47 originates at residue 407, as indicated in Fig. 8. This places the site of chymotrypsin cleavage at the peptide bond between residues 406–407, which is approximately in the middle of plasma gelsolin. The 31-kD peptide which does not bind actin begins at residue 151, suggesting that it is derived from CT45n after separation from the actin-binding CT17n.

Digestion with Thermolysin. Although CT17n severs actin filaments, its activity is much weaker than that of intact gelsolin and is no longer regulated by Ca^{2+} (Kwiatkowski et al., 1985). To identify a domain with activity more comparable to that of intact gelsolin, the severing ability of the larger NH_2-terminal half peptide was examined. Although chymotrypsin can generate the two gelsolin halves, it readily cleaves the NH_2-terminal half further to form smaller fragments (such as CT17n and CT31). Therefore, other proteases were used to digest gelsolin. Thermolysin gave a pattern of cleavage similar to that with chymotrypsin, but it did not degrade the NH_2-terminal half as readily. Consequently, using thermolysin, it is possible to cleave plasma gelsolin into its two halves quantitatively.

Fig. 3 shows thermolysin first cleaved plasma gelsolin into two fragments with M_r's of 47,000 and 45,000, and subsequently into smaller polypeptides. The origin of some of these thermolysin peptides and their relation to the chymotrypsin peptides could be deduced based upon their reactivity towards the two antibodies described above and their Ca^{2+} requirement for binding to actin. TL47 and TL45 bound to actin beads in the presence of 0.2 mM $CaCl_2$ (Fig. 4 *a*). TL47 required Ca^{2+} to bind actin and was eluted by EGTA. TL45 was not detectibly affected by Ca^{2+}. Anti-N peptide antibody recognized TL45, suggesting that it contained the NH_2-terminal actin-binding site (Fig. 4 *b*) and was equivalent to CT45n. 2C4 antibody recognized a 47-kD and a minor 45-kD band which bound to actin beads only in the presence of $CaCl_2$, suggesting that they contained the actin-binding site located in the COOH-terminal half of plasma gelsolin and were equivalent to CT47c and CT45c, respectively. These results showed that thermolysin, like chymotrypsin, cleaved gelsolin into peptides of 45 kD containing the NH_2-terminal actin-binding site of gelsolin and peptides of 47 and 45 kD containing the COOH-terminal half actin-binding site.

Effect of Thermolysin-derived Gelsolin Fragments on F-actin

The effects of gelsolin fragments, generated by various degrees of thermolysin digestion, on the kinetics of F-actin depolymerization are shown in Fig. 5. In this depolymerization assay, the rate at which the actin filaments depolymerize when diluted to their critical concentration (with the resultant loss of pyrene fluorescence) is proportional to the number of filament ends. In the presence of proteins such as gelsolin that block monomer exchange with the (+) filament

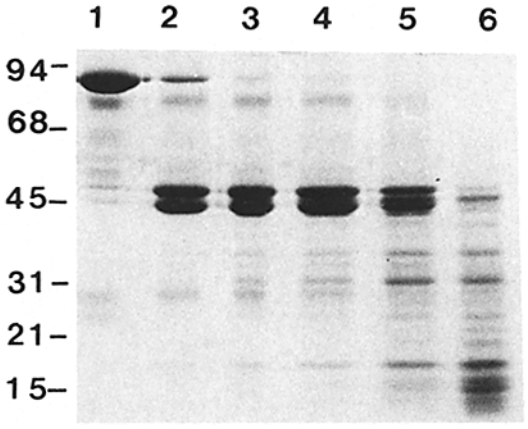

Figure 3. Thermolysin cleaved products of human plasma gelsolin. Plasma gelsolin (0.63 mg/ml) was digested with thermolysin at weight ratios of 145,000:1 (lane *2*), 29,000:1 (lane *3*), 5,800:1 (lane *4*), 1,200:1 (lane *5*), and 230:1 (lane *6*) in TBS-2 mM $MgCl_2$-0.2 mM $CaCl_2$ for 15 min at room temperature. 2 μg of intact gelsolin (lane *1*) or gelsolin digests were analyzed on an SDS polyacrylamide gel and stained with Coomassie Blue. M_r ($\times 10^{-3}$) of protein standards (phosphorylase b, *94*; bovine serum albumin, *68*; ovalbumin, *45*; carbonic anhydrase, *31*; soybean trypsin inhibitor, *21*; lysozyme, *15*) are indicated on the left margin.

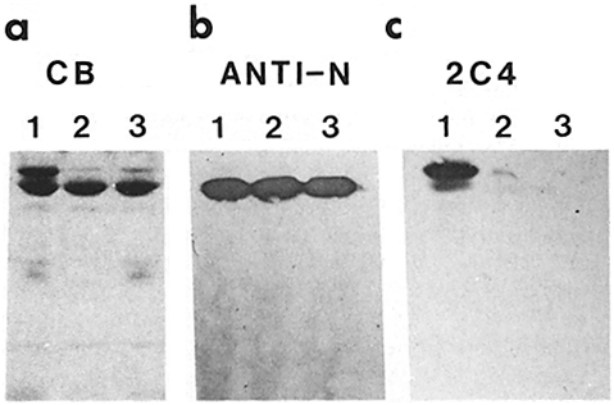

Figure 4. Effect of EGTA on the binding of thermolysin-cleaved gelsolin peptides to actin-Sepharose. Gelsolin digested with thermolysin at a weight ratio 5,800:1 was incubated with actin-Sepharose beads in the presence of 0.2 mM $CaCl_2$ (lanes *1* and *3*) or 1 mM EGTA (lane *2*). The beads were washed with buffer containing 0.2 mM $CaCl_2$ (lane *1*) or 1 mM EGTA (lanes *2* and *3*). Proteins bound to the beads were resolved on an SDS polyacrylamide gel in triplicate. One set was stained with Coomassie Blue (*a*). The other two sets, containing one third as much protein, were blotted onto nitrocellulose papers and stained separately with anti-N antiserum (*b*) or 2C4 antibody (*c*).

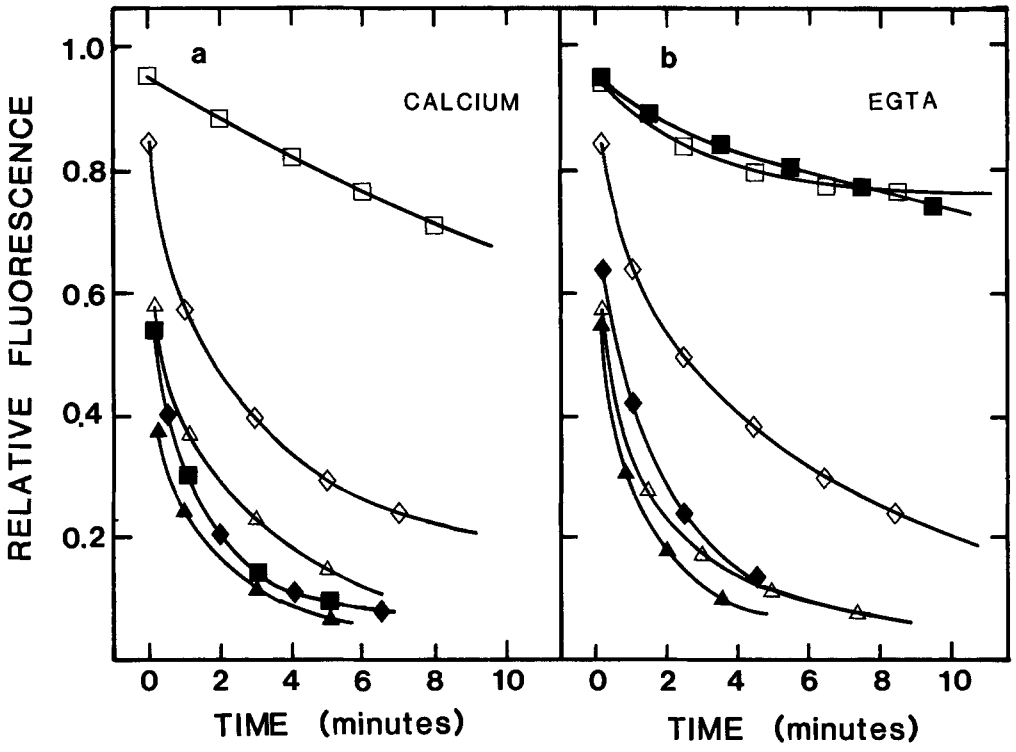

Figure 5. Effects of thermolysin-digested gelsolin on F-actin. Gelsolin samples digested with thermolysin (identical to those in Fig. 3) were diluted to a final concentration of 7.7 μg/ml (equivalent to 83 nM of plasma gelsolin) in polymerizing buffer (2 mM Tris-HCl, pH 7.6, 0.2 mM 2-mercaptoethanol, 0.2 mM ATP, 2 mM $MgCl_2$, and 150 mM KCl) containing either 0.2 mM $CaCl_2$ (*a*) or 1 mM EGTA (*b*) at 23°C. Pyrene-labeled actin was then added to a final concentration of 38.7 μg/ml (900 nM). The rate of decrease in pyrene-actin fluorescence, proportional to the fraction of polymerized actin, was then measured for each solution. Gelsolin digests generated with gelsolin/thermolysin weight ratios of 145,000:1, 29,000:1, and 5,900:1 (◆) had similar activity which was superimposable with that of undigested gelsolin (■). Gelsolin digested with thermolysin (wt/wt ratios of 230:1) was indicated by ◇ and that with wt ratio of 1,200:1 had slightly lower activity than intact gelsolin (similar to △), and was omitted from the figure for the sake of clarity. The depolymerization of F-actin (without gelsolin) in the presence of an amount of thermolysin equivalent to the highest concentration present in the gelsolin digests was indicated by □. The effects of thermolysin NH_2-terminal peptides purified as described in Fig. 6 after digestion with thermolysin at wt/wt ratios of 5,800:1 (5.0 μg/ml, 111 nM) and 1,200:1 (4.5 μg/ml, 100 nM) were indicated by ▲ and △, respectively.

ends, this critical concentration is ~0.7 μM, and the filaments depolymerize from their (−) ends (Bryan and Coluccio, 1985; Janmey et al., 1985; Cooper and Pollard, 1985; Mabuchi, 1983; Janmey and Stossel, 1986). Plasma gelsolin which had been cleaved by low amounts of thermolysin (weight ratios of gelsolin to thermolysin of 145,000:1–5,800:1) into peptides in the range of 47–45 kD (Fig. 4, lanes *2–4*) retained all of the apparent filament-severing activity of intact gelsolin (Fig. 5 *a*). More extensive proteolysis (gelsolin to thermolysin weight ratio of 230:1) resulting in almost complete loss of CT45n decreased but did not eliminate the ability to accelerate the depolymerization of F-actin. The severing activity of the thermolysin peptides was not abolished in the presence of EGTA, in contrast to the dependence of intact gelsolin on Ca^{2+} for actin filament severing (Fig. 5 *b*). Since there were equivalent amounts of gelsolin or gelsolin digests in each assay, these data suggest that the larger thermolysin-derived peptides had severing activity equivalent to that of intact gelsolin and the severing function was no longer regulated by Ca^{2+} (Fig. 5 *b*).

To identify the actin filament–severing peptide in the thermolysin digests, NH_2-terminal half peptide was separated from the COOH-terminal half peptide by affinity chromatography. Thermolysin digests were passed over a 2C4–Sepharose column to adsorb specifically peptides containing the 2C4 epitope. As shown in Fig. 6 *a*, the adherent fraction contains a major 47-kD and a minor 45-kD band, which by virtue of their ability to bind 2C4 but not anti–N peptide antibody (Fig. 6 *b*) could be identified as TL47c and TL45c, respectively. The nonadherent fraction contained mostly a 45-kD band and in the more digested sample, a minor 31-kD and a 17-kD band (data not shown). The 45-kD band and the 17-kD band (not shown) reacted with anti–N peptide antibody (Fig. 6 *b*) and were therefore TL45n and TL17n,

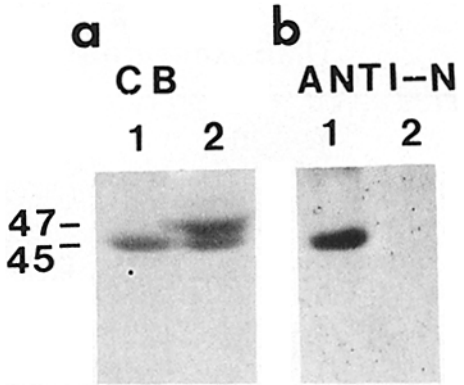

Figure 6. Separation of NH_2- and COOH-terminal plasma gelsolin peptides by affinity chromatography. Plasma gelsolin (0.63 mg in 1 ml) was digested with thermolysin (wt/wt ratio of 5,800:1) for 15 min and passed over a column containing 1.5 ml of 2C4 antibody–Sepharose beads equilibrated with TBS–EGTA. The nonadherent peptides were collected. The column was washed with TBS–EGTA and adsorbed proteins were eluted with 3 M $MgCl_2$ in TBS–EGTA. Aliquots from the nonadherent flow-through fractions (lanes *1*) and adherent fractions (lanes *2*) were analyzed on SDS polyacrylamide gels. Peptides were stained with Coomassie Blue dye (*a*) or immunoblotted with anti–N peptide antibody. M_r ($\times 10^{-3}$) of peptides are indicated.

respectively. The protein contents of the NH_2-terminal half and COOH-terminal half fractions were estimated using the extinction coefficient reported for plasma gelsolin (Kilhoffer and Gerard, 1985). This is justified because each half peptide contains similar mole percent of aromatic acids as intact plasma gelsolin (mole percents of tyrosine and tryptophan for plasma gelsolin are 2.0 and 2.9, respectively; for CT45n are 1.5 and 2.7, and for CT47c are 2.6 and 3.2) (Kwiatkowski et al., 1986).

In Fig. 5, the effect of two different affinity-purified TL45n preparations on actin filaments is examined. In the presence of Ca^{2+}, TL45n fractions derived from digestion with thermolysin (wt/wt ratios of 5,800:1 and 1,200:1) had severing activity comparable to that of their respective unfractionated digests (Fig. 5 *a*). Based on these data, it can be inferred that the actin-severing function of gelsolin resides entirely in the NH_2-terminal half of the molecule. Fig. 5 *b* shows that TL45n severed actin filaments in EGTA almost as effectively as it did in Ca^{2+}, confirming that the NH_2-terminal actin-severing domain of gelsolin is Ca^{2+} independent.

Gelsolin peptides from the COOH-terminal half (TL47c) had no effect on the rate of depolymerization of F-actin when added at a similar weight ratio (data not shown). An effect on the final extent of polymerization only became apparent at close to equimolar amounts of the COOH-terminal peptides to actin (see below).

Effects of Thermolysin-derived Gelsolin Fragments on Actin Polymerization

The effects of NH_2-terminal and COOH-terminal gelsolin fragments on the kinetics of actin polymerization are shown in Fig. 7. In the presence of Ca^{2+}, 6.2 μg/ml thermolysin NH_2-terminal peptides accelerated actin polymerization to

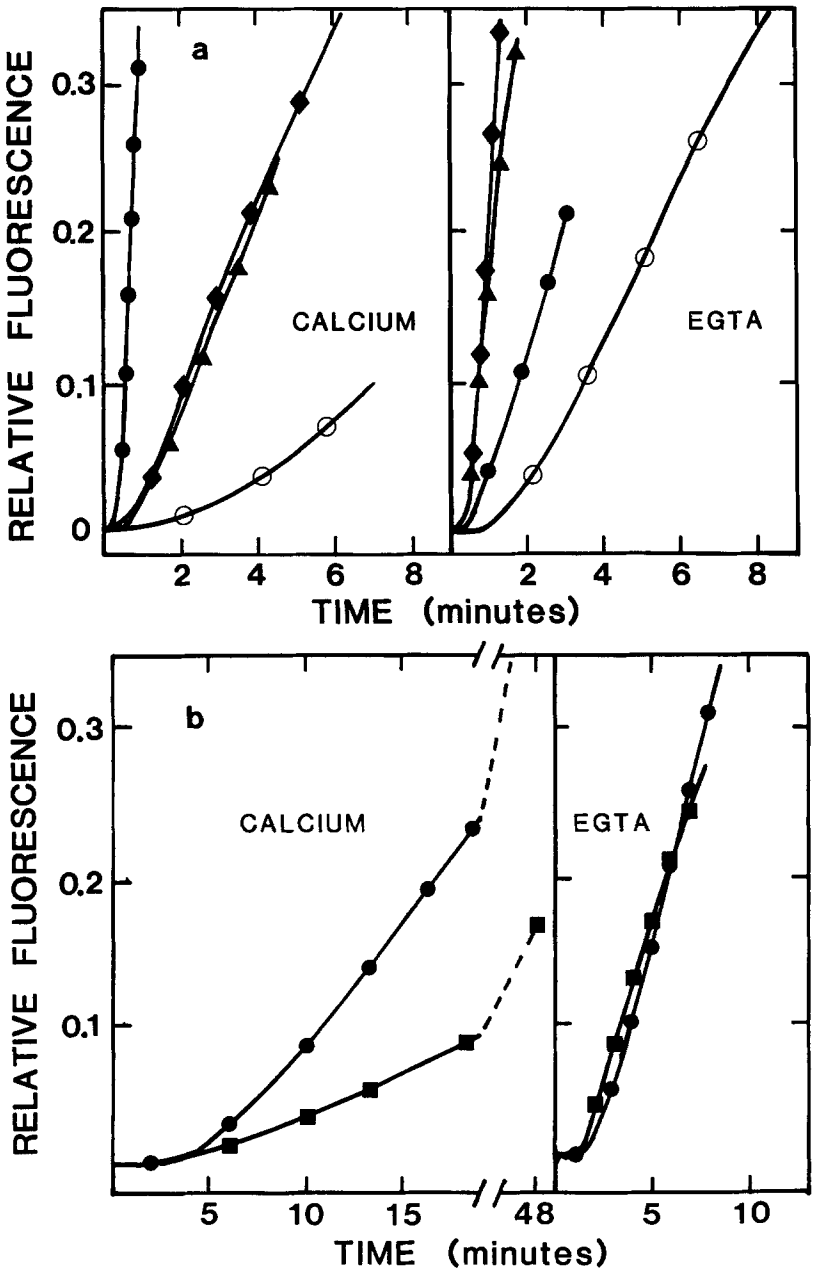

Figure 7. Effects of thermolysin NH_2- and COOH-terminal half peptides on actin polymerization. Gelsolin digested with thermolysin at a wt/wt ratio of 1,900:1 for 15 min was fractionated through a 2C4–Sepharose affinity column as described in Fig. 6. (*a*) The NH_2-terminal peptides of gelsolin (6.2 μg/ml, 137 nM, ▲), intact gelsolin (12.4 μg/ml, 133 nM, ●), unfractionated gelsolin digest (12.4 μg/ml, ♦), or thermolysin (an amount equivalent to that in the digests, ○) were added to polymerizing buffer containing 1.2 mM $CaCl_2$ (*left*) or 0.2 mM $CaCl_2$/0.8 mM EGTA (*right*). 3.3 μM (142 μg/ml) pyrene-labeled G-actin was added and the subsequent actin polymerization, proportional to the fluorescence, is shown as a function of time. Fluorescence intensity is reported relative to a value of 1.0 for fully polymerized actin in the absence of gelsolin. (*B*) The COOH-terminal half peptides of gelsolin, TL47-45c (100 μg/ml in polymerizing buffer in the presence of pyrene-actin as above, ■). Actin control (142 μg/ml, 3.3 μM, ●).

the same extent as 12.4 μg/ml of the total digest, but fivefold less than 12.4 μg/ml of intact gelsolin (Fig. 7 *a*). In the presence of EGTA, thermolysin NH_2-peptides, like the total digest, accelerated actin polymerization nearly eightfold more than intact gelsolin.

In contrast, TL47c retarded the polymerization of actin and its effect was highly Ca^{2+} dependent. In the presence of Ca^{2+}, the rate was diminished and the final extent of polymerization was lowered to an extent consistent with the formation of 1:1 complexes between monomeric actin and TL47c. The effect of TL47c, like that of CT47c (Kwiatkowski et al., 1985), on actin polymerization was not detected until close to an equimolar amount of peptides was added to the actin. No effect on either the kinetics or extent of actin polymerization was observed in the presence of EGTA, suggesting that the COOH-terminal actin-binding domain is regulated by Ca^{2+}.

Identification of an EGTA-resistant Ca^{2+} within the Actin–NH_2-Terminal Peptide Complex

The EGTA-stable 1:1 actin/gelsolin complex contains a sequestered Ca^{2+} (Bryan and Kurth, 1984). To determine if the EGTA-stable actin/NH_2-terminal peptides also contain nonexchangeable Ca^{2+}, complexes between affinity-purified NH_2-terminal peptides and actin beads were formed in the presence of 1.2×10^{-5} M $^{45}CaCl_2$, and the beads were washed extensively in EGTA. The actin bead assay detected Ca^{2+} sequestered by plasma gelsolin/actin complexes (Table I, first line). Under the conditions of the experiment, one mol of EGTA-resistant Ca^{2+} was bound to 2 mol of gelsolin/actin complexed on the beads. The explanation for the less than equimolar binding has not been determined. ^{45}Ca was bound specifically to the gelsolin/actin bead complexes, because very little ^{45}Ca was trapped in actin beads in the absence of gelsolin (fourth line).

A chymotryptic digest of plasma gelsolin with peptides no larger than 47 kD retained 80% of the EGTA-resistant ^{45}Ca radioactivity relative to that found in intact gelsolin/actin complexes (second line). The NH_2-terminal actin-binding peptides in the flow-through fraction of the 2C4-Sepharose column also retained ^{45}Ca when they were complexed with the actin beads. Since this fraction contained two peptides capable of binding actin (CT45n and CT17n), and it is not known whether both species bound Ca^{2+} irreversibly when complexed with actin, it was not possible to calculate the number of Ca^{2+} atoms bound per mol of NH_2-terminal actin-binding peptides. However, assuming that all of the actin-binding peptides in the sample were 45 kD, 1 mol of EGTA-resistant ^{45}Ca was estimated to be associated with 2.5 mol of actin/CT45n complex. A similar amount of ^{45}Ca was retained when the flow-through fraction of thermolysin-digested gelsolin was tested in this assay (Table I, third line). These data establish that the NH_2-terminal gelsolin peptide/actin complexes retain Ca^{2+}, even in the presence of EGTA. This technique could not be used to determine if the COOH-terminal peptide/actin complex might likewise retain ^{45}Ca, because it was eluted from the actin beads by EGTA. Consequently, ^{45}Ca would not be expected to be retained on the beads, as was documented (Table I, second and third lines).

Table I. Presence of EGTA-resistant ^{45}Ca in Actin/Plasma Gelsolin Complexes

	Unfractionated*	Nonadherent‡	Adherent§
	cpm‖	*cpm*	*cpm*
Intact gelsolin	54,560 + 50	–	–
Chemotrypsin digest	43,484 + 94	34,787 + 34	387 + 7
Thermolysin digest	ND	34,174 + 44	423 + 4
None	300	–	–

* 9 μg human plasma gelsolin or gelsolin digested with chymotrypsin (at wt/wt ratio of 380:1) or thermolysin (wt/wt ratio of 1900:1) as described in Fig. 5 were incubated with actin–Sepharose beads in the presence of ^{45}Ca, and washed subsequently in EGTA.

‡§ Gelsolin digests were passed over a 2C4 affinity column as described in Fig. 6. 4.5 μg each of the nonadherent and adherent peptides were used per assay.

‖ The ^{45}Ca bound to the beads were expressed in cpm, as mean + SEM of duplicate samples.

Discussion

The data presented here support other evidence that the two actin-binding domains of plasma gelsolin are functionally distinct, both with respect to Ca^{2+} sensitivity and with respect to the ability to shorten actin filaments and nucleate actin assembly. In addition, they show that the actin-severing domain of gelsolin is located entirely at the NH_2-terminal half of gelsolin. In the presence of Ca^{2+}, plasma gelsolin was readily cleaved in half by chymotrypsin or thermolysin to generate peptides containing the Ca^{2+}-insensitive, NH_2-terminal actin-severing domain and the Ca^{2+}-sensitive, COOH-terminal actin-binding domain with no severing function (Fig. 8). The NH_2-terminal half is cleaved further by these enzymes to generate a 17-kD and a 15-kD peptide containing the NH_2-terminal actin-severing site and a 31-kD peptide with no actin-binding activity. The COOH-terminal half of plasma gelsolin is more resistant to further proteolysis (Kwiatkowski et al., 1985). Plasma gelsolin is not as readily cleaved in half in EGTA, suggesting that the cleavage site is less accessible in the absence of Ca^{2+} (Bryan and Hwo, 1986).

The protease cleavage sites on the plasma gelsolin polypeptide have been defined because the primary structure of the protein is now known, based on nucleotide sequence analyses of plasma gelsolin cDNA clones (Kwiatkowski et al., 1986). These data also reveal that the two halves of the molecule have highly homologous amino acid sequences, particularly at the NH_2-terminal portions of each half of the molecule. Since the actin-binding domain of the NH_2-terminal half is located close to its NH_2-terminus (Kwiatkowski et al., 1985), by inference, the homologous region in the NH_2-terminal portion of the COOH-half may contain the other actin-binding domain. If the two halves of the molecule contain a duplicated actin-binding domain and yet have different actin-modulating properties, they are likely to have arisen from a gene duplication event, and diverged subsequently to adopt their respective unique functions.

The NH_2-terminal half peptide of gelsolin severs actin filaments as effectively as gelsolin, but it is no longer regulated by Ca^{2+}. These findings clearly establish that (*a*) the NH_2-terminal actin-binding domain is responsible for the severing function of gelsolin; (*b*) the NH_2-terminal 17-kD fragment contains the actin-binding site, but the contiguous

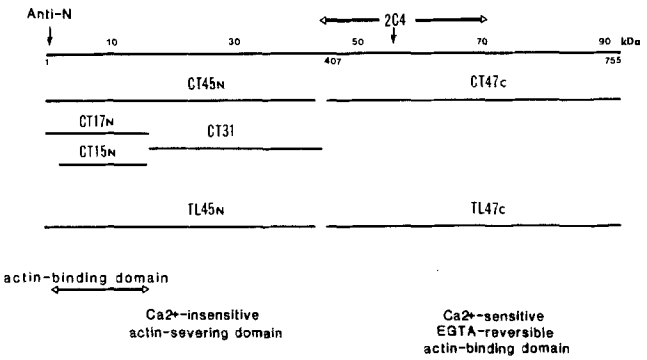

Figure 8. A model of the primary structure of human plasma gelsolin. The major peptides generated by chymotrypsin (*CT*) and thermolysin (*TL*) cleavage in the presence of Ca^{2+} are indicated. In the absence of Ca^{2+}, the cleavage site in the middle of the molecule is less accessible to the enzymes. The regions containing the epitopes for anti-N and 2C4 antibodies are indicated by vertical arrows. The epitope of 2C4 was narrowed down to a 25-kD peptide at the NH_2-terminus of CT47c (Kwiatkowski, D. J., unpublished data). The numbers on top of the plasma gelsolin indicate apparent molecular mass (in kD), estimated by comparison with the electrophoretic mobility of the peptides with molecular mass standards in SDS polyacrylamide gels. The amino acid residues, deduced from the primary sequence of plasma gelsolin, are indicated at the bottom. cDNA sequence analyses show that plasma gelsolin has a total of 755 amino acid residues, with CT45n and CT47c accounting for 406 and 349 residues, respectively. The molecular mass calculated from the deduced amino acid compositions of these peptides is 83 kD for plasma gelsolin, 45 kD for CT45n, and 38 kD for CT47c. The reason for the anomalous mobility of plasma gelsolin and CT47c on SDS polyacrylamide gels is not known, but for the sake of consistency, we have continued to refer to the M_r of the peptides according to their apparent mobility on SDS gels.

31 kD of the molecule, which by itself has no actin-binding ability, is required to promote optimal severing; (*c*) Ca^{2+} is not required for actin binding or severing by this domain. Since of all the interactions of gelsolin with actin, filament severing has the most stringent requirement for Ca^{2+} (Janmey et al., 1985), the lack of a Ca^{2+} requirement by the isolated actin-severing domain suggests that in the intact molecule, the NH_2-terminal-severing domain is influenced by the Ca^{2+}-regulated COOH-terminal half of the molecule. This effect could presumably be exerted through a conformational change of the COOH-half of the molecule to expose the NH_2-terminal actin-binding site. A Ca^{2+}-induced conformation change in plasma gelsolin is indicated by the fact that Ca^{2+} promotes the cleavage of gelsolin in the middle of the polypeptide (Bryan and Hwo, 1986), increases the reactivity of gelsolin sulfhydryl groups towards 5,5′ dithiobis(2-nitrobenzoic acid) (Kilhoffer and Gerard, 1985), and decreases its sedimentation coefficient (Soua et al., 1985). The conformation of CT47c changes in the presence of Ca^{2+}, based on circular dichroism studies (Kwiatkowski et al., 1985). The overall change in the conformation of plasma gelsolin however must be sufficiently subtle to account for the findings that Ca^{2+} produced no changes in the circular dichroism (Kwiatkowski et al., 1985) or tryptophan fluorescence spectrum (Kilhoffer and Gerard, 1985) of the protein.

Besides severing actin filaments, the NH_2-terminal half peptide also accelerated the rate of polymerization of actin, but to a smaller extent than intact plasma gelsolin in the presence of Ca^{2+}. Actin assembly may be promoted by one of two mechanisms: (*a*) the actin/peptide complex may not itself constitute a nucleus, but can trap an additional actin to form a stable nucleus, as has been suggested for an *Acanthamoeba* protein (Cooper and Pollard, 1985); (*b*) the peptides provide nucleating sites secondarily through severing of actin filaments grown from spontaneously formed nuclei, as has been suggested for other actin-severing proteins isolated from starfish oocytes (Mabuchi, 1983), brain (Maekawa et al., 1984), and *Acanthamoeba* (Cooper et al., 1986).

The finding that the binding of the NH_2-terminal domain to actin was not modulated by Ca^{2+} provides an explanation for why the 1:1 actin/gelsolin complex is not dissociated by EGTA. The NH_2-terminal peptide/actin complex retains a Ca^{2+} resistant to chelation by EGTA, as has been shown for the EGTA-resistant 1:1 actin/gelsolin complex. What is not clear at present is whether the Ca^{2+} is associated with actin or the NH_2-terminal peptide. Actin binds Ca^{2+} with high affinity and it may be induced to sequester Ca^{2+} when complexed with gelsolin. Gelsolin has two high-affinity Ca^{2+}-binding sites (Yin and Stossel, 1979; Bryan and Kurth, 1984). Since its COOH-terminal half is highly Ca^{2+} sensitive, at least one of these sites should be present at that half of the molecule. Whether the NH_2-terminal half also binds Ca^{2+} remains to be determined. A search of the plasma gelsolin sequence for Ca^{2+}-binding sites conforming to the EF-hand paradigm of calmodulin-like proteins (Kretsinger, 1980) reveals a potential site in the NH_2-terminal half but not the COOH-terminal half of plasma gelsolin (Kwiatkowski et al., 1986).

The COOH-terminal half peptide of gelsolin binds actin reversibly. When present at close to equimolar ratio with G-actin, it retards polymerization, apparently by sequestering actin monomers. Therefore, the binary complex of COOH-terminal peptide and actin is not an effective actin nucleus. Furthermore, it does not sever actin filaments. Its major contribution to gelsolin function apparently lies in its ability to be regulated by Ca^{2+} to influence the NH_2-terminal domain of the molecule.

In Fig. 8, we have integrated our structure and function data into a model for how gelsolin interacts with two actins in the presence of Ca^{2+}. Recent data suggest that binding of actin to the two sites may be cooperative, and binding is initiated at the Ca^{2+}-sensitive actin-binding domain. In the presence of Ca^{2+} and an equimolar or greater amount of gelsolin to actin, the predominant species formed is the 2:1 actin/gelsolin complex, as indicated by enhancement of actin fluorescence (Bryan and Kurth, 1984), equilibrium sedimentation (Coue and Korn, 1985), and chemical cross-linking studies (Harris, 1985). A similar conclusion is also reached by Janmey et al. (1986*b*) using the plasma vitamin D-binding protein (DBP) to compete with gelsolin for actin. This actin monomer-sequestering protein prevents the formation of either 1:1 or 2:1 actin/gelsolin complexes, and extracts one monomer from preformed 2:1 actin/gelsolin complexes even in the presence of Ca^{2+}. The resultant DBP-resistant 1:1 actin/gelsolin complex cannot be dissociated by EGTA, suggesting that the same actin-binding site resists elution by both agents. The EGTA-irreversible NH_2-terminal site therefore must have a higher affinity for actin than DBP while the EGTA-reversible COOH-terminal site must have a lower affinity for actin than DBP. Since neither site binds actin in

the presence of competitive amounts of DBP, occupation of the higher affinity NH_2-terminal site must depend on the prior occupation of the lower affinity Ca^{2+}-sensitive COOH-terminal site. The possibly cooperative nature of the binding, and the fact that the Ca^{2+}-sensitive COOH-terminal site is the first site to be occupied, explains why Ca^{2+} is required for expression of the severing activity of gelsolin.

Our model for plasma gelsolin structure is different in several respects from that proposed by Bryan and Kurth (1984) and Bryan and Hwo (1986). They suggested that actin first binds to a high-affinity site which is relatively Ca^{2+} insensitive, and then a lower affinity site which is Ca^{2+} sensitive and EGTA reversible. Occupation of the first actin site promotes trapping of a Ca^{2+} which cannot be eluted by EGTA, accounting for the persistence of a 1:1 actin/gelsolin complex in the presence of EGTA (Bryan and Kurth, 1984). Both actin-binding sites are located in a 40-kD peptide derived from the NH_2-terminus of plasma gelsolin, while a 45-kD peptide, presumably derived from the COOH-terminal half, has no actin-binding activity but contains two Ca^{2+}-binding sites (Bryan and Hwo, 1986). The reasons for the discrepancies with our results are not clear. One possibility is that due to the slightly different conditions used for proteolysis, the site of cleavage may not identical. This possibility can be investigated by determining the NH_2-terminal amino acid sequence of the 40-kD peptide, as we have done for CT47c. Another possibility is that different actin-binding assays for gelsolin were used, and the less dramatic effect of substoichiometric amounts of CT47c on actin may have escaped detection by the methods used by Bryan's group. Despite these differences in detail, both models describe a high degree of coordination between the two halves of gelsolin to account for the Ca^{2+}-regulated actin-binding properties of the intact molecule.

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