Identification of a Polyphosphoinositide-modulated Domain in Gelsolin Which Binds to the Sides of Actin Filaments

H. L. Yin, K. Iida, and P. A. Janmey
Hematology-Oncology Unit, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114

Abstract. Gelsolin is a Ca²⁺- and polyphosphoinositide-modulated actin-binding protein which severs actin filaments, nucleates actin assembly, and caps the “barbed” end of actin filaments. Proteolytic cleavage analysis of human plasma gelsolin has shown that the NH₂-terminal half of the molecule severs actin filaments almost as effectively as native gelsolin in a Ca²⁺-insensitive but polyphosphoinositide-inhibited manner. Further proteolysis of the NH₂-terminal half generates two unique fragments (CT14N and CT28N), which have minimal severing activity. Under physiological salt conditions, CT14N binds monomeric actin coupled to Sepharose but CT28N does not. In this paper, we show that CT28N binds stoichiometrically and with high affinity to actin subunits in filaments, suggesting that it preferentially recognizes the conformation of polymerized actin. Analysis of the binding data shows that actin filaments have one class of CT28N binding sites with \( K_d = 2.0 \times 10^{-7} \) M, which saturates at a CT28N/actin subunit ratio of 0.8. Binding of CT28N to actin filaments is inhibited by phosphatidylinositol 4,5-bisphosphate micelles. In contrast, neither CT14N nor another actin-binding domain located in the COOH-terminal half of gelsolin form stable stoichiometric complexes with actin along the filaments, and their binding to actin monomers is not inhibited by PIP2. Based on these observations, we propose that CT28N is the polyphosphoinositide-regulated actin-binding domain which allows gelsolin to bind to actin subunits within a filament before severing.

Gelsolin is a multifunctional act binding protein of mammalian cytoplasm and extracellular fluids. It can interact with actin in at least three ways (reviewed in Stossel et al., 1985). First, it binds to actin monomers and oligomers to promote actin nucleation. Second, it blocks the “barbed” end (with reference to the orientation of heavy meromyosin arrowheads) of actin filaments, preventing monomer exchange from that end. Third, it severs actin filaments by nonproteolytically breaking actin–actin bonds between adjacent actin molecules. Each gelsolin can cause a single break within a filament, suggesting that there is a stoichiometric interaction between gelsolin and actin molecules within a filament before severing (Yin et al., 1980; Janmey et al., 1986a, b). Once the filament is severed, gelsolin remains bound to the barbed end of the filament, preventing reannealing. Severing is activated by micromolar Ca²⁺ (Yin and Stossel, 1979; Bryan and Kurth, 1984; Janmey et al., 1985; Bryan and Coluccio, 1985) and inhibited by phosphatidylinositol 4,5 bisphosphate (PIP2) and phosphatidylinositol monophosphate (Janmey and Stossel, 1987, Janmey et al., 1987). Although the interaction of gelsolin with actin is strongly Ca²⁺ dependent, complete reversal of binding was not observed after chelation of Ca²⁺ with EGTA. Only one of the two actins bound to gelsolin dissociates, resulting in a 1:1 EGTA-resistant gelsolin–actin complex (Kurth and Bryan, 1984; Janmey et al., 1985) which does not sever actin filaments. The complex can be dissociated with PIP2 micelles, and severing activity is restored after disruption of micelles with nonionic detergents. These data suggest that gelsolin may be regulated by a two signal mechanism involving changes in Ca²⁺ and polyphosphoinositide concentrations, and may have a critical role in mediating the reorganization of the cytoskeleton after agonist stimulation of cells (Chaponnier et al., 1987; Lind et al., 1987).

We and others have sought to understand the structural basis for the many functions of gelsolin and its complex regulation. One approach has been to examine the actin binding and functional properties of proteolytically derived fragments of human plasma gelsolin, and their regulation by Ca²⁺ or polyphosphoinositides. The primary structure of human plasma gelsolin (previously called actin-destabilizing factor or brevin [Chaponnier et al., 1979; Norberg et al., 1979; Harris et al., 1980; Harris and Schwartz, 1981]) is identical to that of cytoplasmic gelsolin, with the exception of a 25-amino acid extension at the NH₂ terminus (Yin et al., 1984; Kwiatkowski et al., 1988), so we shall refer to it as gelsolin. The actin filament (F-actin)–severing domain of gelsolin has been localized to the NH₂-terminal half of the molecule (Kwiatkowski et al., 1985; Bryan and Hwo, 1986; Chaponnier et al., 1986; Rouyrec et al., 1986). A 45-kD NH₂-terminal peptide derived by digesting human plasma...
gelsolin with chymotrypsin or thermolysin severs actin filaments almost as effectively as intact gelsolin (Bryan and Hwo, 1986; Chaponnier et al., 1986). Severing by the NH₂-terminal half fragment is inhibited by PI (2) (Janmey and Stossel, 1987; Janmey et al., 1987), but Ca²⁺ is no longer required. Ca²⁺ regulation of the intact gelsolin appears to reside in the COOH-half of the molecule that exhibits a required Ca²⁺-dependent conformational change (Hwo and Bryan, 1985; Kwiatkowski et al., 1985) and contains a Ca²⁺-sensitive actin-binding domain (Kwiatkowski et al., 1985). These observations led to several models for the interaction of the two halves of gelsolin to account for the stringent Ca²⁺ requirement of gelsolin's severing function, and the persistence of monovalent actin–gelsolin complexes when EGTA is added subsequent to Ca²⁺ activation of gelsolin–actin binding (Kwiatkowski et al., 1985; Bryan and Hwo, 1986; Chaponnier et al., 1986, Janmey et al., 1986).

Further proteolytic digestion of the NH₂-terminal half yields two major species, a 17–14 kDa doublet from the NH₂ terminus (amino acid residues 1–150/24–150 of plasma gelsolin, deduced from amino acid sequence analysis and the cDNA sequence shown in Kwiatkowski et al., 1986) and a 28-kDa fragment encompassing residues 151–406 (Table I). CT4–17N binds actin coupled to Sepharose, inhibits actin polymerization and accelerates the depolymerization of F-actin with kinetics consistent with severing (Kwiatkowski et al., 1985). Its severing activity however is weak, being ~1% as effective as CT45N on a molar basis. Therefore, an additional domain in the NH₂ terminus may be necessary to account for the potent severing activity of CT45N. In this paper, we report that CT28N (referring to the 28-kDa chymotryptic fragment derived from the NH₂ terminus; formerly called CT30N because it has an apparent mobility of 30 kDa on an SDS–polyacrylamide gel) binds stoichiometrically and with high affinity to actin molecules within a filament (see Table I). Binding of CT28N to actin is more sensitive to inhibition by PI than CT45N, CT45N, and CT38C. CT28N itself does not sever actin filaments. Because the severing function of gelsolin is likewise more sensitive to inhibition by PI than other gelsolin functions examined, we propose that CT28N is the actin binding domain that allows gelsolin or CT45N to bind to the side of actin filaments before severing. A similar fragment has been identified by Bryan (1988).

### Materials and Methods

#### Gelsolin Preparation

Human plasma gelsolin was purified in two steps: affinity chromatography over a monoclonal antibody (2C4) column (Chaponnier et al., 1986) and further purification by DEAE–Sepharose ion exchange chromatography. The gelsolin concentration was estimated using an extinction coefficient, ε⁰⁰⁰ of 117.580 M⁻¹ cm⁻¹, calculated from the number of tyrosine and tryptophan residues present in the human plasma gelsolin (Kwiatkowski et al., 1985). The ε⁰⁰⁰ for tyrosine and tryptophan are 1,390 and 5,800 M⁻¹ cm⁻¹, respectively, taking an average between that for exposed and buried residues (Magne et al., 1977). The gelsolin was >99% pure, as judged by Coomassie Blue staining of SDS–polyacrylamide gels. 108 μg of gelsolin was iodinated with 0.5 mCi of I² by the Bolten Hunter procedure (Amerham kit; Amerham Corp., Arlington Heights, IL), as described by Harris (1985). Iodinated gelsolin was separated from unincorporated NaI by passage through a Sepharose G-25 column equilibrated with 10 mM Tris–HCl, pH 8.0, 0.1 M NaCl, 5 mM MgCl₂, 1 mg/ml gelatin (Bio–Rad Laboratories, Richmond, CA) and dialysis against TBS–EGTA. The specific activity was 0.9 μCi/μg, or 1 mol I² per 24 mol gelsolin. Autoradiography of the labeled gelsolin confirmed that >99% of the radioactivity was associated with the 93-kD gelsolin band, and a small amount of label was associated with a 40-kD band that did not bind actin.

To prepare I²-labeled gelsolin peptides, 5.4 μg of I²-labeled gelsolin was mixed with 542 μg unlabeled gelsolin (0.55 mg/ml) and digested with 4 μg of a-chymotrypsin (CooperBiomedical, Inc., Malvern, PA) (gelsolin/chymotrypsin weight ratio of 136:1) in 10 mM Tris–HCl, pH 7.4, 1 mM EGTA, 1.5 mM CaCl₂, and 2 mM MgCl₂. Digestion was allowed to proceed for 30 min at room temperature, and the reaction was stopped by addition of phenylmethyl sulfonyl fluoride to a final concentration of 2 mM. The mixture of peptides was fractionated in either of two ways.

(a) To separate NH₂- and COOH-terminal half derived peptides, the digest was passed over a 2-ml monoclonal anti-gelsolin (2C4) column that is specific for an epitope in the COOH-terminal half of gelsolin (Chaponnier et al., 1986). The adherent and nonadherent peptides were analyzed by SDS-polyacrylamide gel electrophoresis and radiolabeled bands were detected by autoradiography (see Table I). Binding of CT28N to actin is more sensitive to inhibition by PI than CT45N, CT45N, and CT38C. CT28N itself does not sever actin filaments. Because the severing function of gelsolin is likewise more sensitive to inhibition by PI than other gelsolin functions examined, we propose that CT28N is the actin binding domain that allows gelsolin or CT45N to bind to the side of actin filaments before severing. A similar fragment has been identified by Bryan (1988).

### Table I. Properties of Gelsolin Peptides

| Parameters       | Gelsolin | CT45N | CT14N | CT28N | CT38C |
|------------------|----------|-------|-------|-------|-------|
| Residues*        | 1–756    | 1–496 | 24–150| 151–406| 407–756|
| Molecular mass   | (x 10⁻⁵) | 83.0  | 44.8  | 14.3  | 28.2  | 38.2  |
| Ca²⁺ activation  | +        | +     | +     | +     | +     |
| PI activation    | +        | +     | +     | +     | +     |
| Monomer binding  | +        | +     | +     | +     | +     |
| Filament binding | +        | +     | +     | +     | +     |
| Nucleation       | +        | +     | +     | +     | +     |
| Severing         | +        | +     | ? | ? | ? | ? |
| Capping          | +        | +     | +     | +     | +     |

In the presence of Ca²⁺, gelsolin is cleaved into four major groups of peptides by chymotrypsin (Kwiatkowski et al., 1985; Bryan and Hwo, 1986; Chaponnier et al., 1986). These peptides are designated CT, followed by molar mass (determined from deduced amino acid sequence), and either N or C, to denote their origin from the NH₂- or COOH-terminal half of the molecule.

* Amino acid residues, numbered from the NH₂ terminus. The origin of the peptide was determined by amino acid sequencing, and their location in the protein deduced from cDNA data (Kwiatkowski et al., 1985, 1986). Two Gelsolin and CT38C migrated with slower mobility on SDS–polyacrylamide gels than expected from their mass (as 93- and 47-kDa bands). The reason for the anomalous mobility is not known.

| Only 1% of the severing activity of CT45N or intact gelsolin. See discussion.|

Kwiatkowski et al. (1985) showed that there is no change in critical monomer concentration in the presence of these peptides. However, only high affinity capping proteins are expected to cause detectable changes in monomer concentration, so that weak capping activity would not be detected by measuring changes in critical monomer concentration at steady state.
same as CT30N (amino acids 151-406) described previously (Kwiatkowski et al., 1985), because it was isolated by the same procedure, and recognized by the same panel of monoclonal antibodies. These preparations contain a small amount (<5%) of a 45-kD band, part of which is due to CT45N (based on antibody cross-reactivity).

The concentrations of CT45N and CT28N were determined by calculating the theoretical extinction coefficient as described above. $E_{280}$ for CT45N and CT28N are 50,090 and 28,760 M$^{-1}$ cm$^{-1}$, respectively. The concentration of the NH$_2$-terminal peptide mix was determined using the $E_{280}$ for CT45N, and expressed as mg/ml.

**Actin-binding Assays**

**Cosedimention with F-Actin.** This assay has been described for $^{125}$I-pig plasma gelsolin by Harris (1985). Actin for these studies was gel-filtered through a Sephacry G50 column, and its concentration determined using $E_{280}$ = 26,655 M$^{-1}$ cm$^{-1}$. Actin (1.67 x 10$^{-6}$ M) was polymerized by addition of KCl and MgCl$_2$ to 0.15 M and 2 mM, respectively. The actin was added to $^{125}$I-labeled gelsolin or gelsolin fragments in 100 $\mu$L of 0.15 M KCl, 2 mM Tris-HCl, pH 7.6, 0.5 mM ATP, 0.1% gelatin, and either 0.2 mM CaCl$_2$ or 1 mM EGTA for 30 min at 37°C. The reaction mixtures were centrifuged at 20 psi in an airfuge for 30-60 min, and radioactivity in the supernatant fluid and pellet was determined in a gamma counter. The supernatant and pellet were boiled in equal volumes of gel sample buffer, and the radioactivity amount of each was subjected to electrophoresis on SDS-polyacrylamide slab gels. Radioactive bands were detected by autoradiography.

**Binding to Actin–Sepharose.** NH$_2$-terminal half peptide mix (at a concentration equivalent to 0.15 $\mu$M CT45N; 9,000 cpm) was incubated with actin–Sepharose beads in 10 mM Tris-HCl, pH 7.5, 0.15 M KCl, 0.2 mM CaCl$_2$ (TBS–Ca$^{2+}$) containing 0.1% Triton X-100. After a 45-min incubation at 4°C, the beads were sedimented by centrifugation for 1 min, and washed with 0.1% Triton X-100 solution. The beads were resuspended in the same buffer, sedimented once more, and counted in the gamma counter.

**Preparation of Phospholipids**

PIP2, purchased from Sigma Chemical Co., St. Louis, MO, was mixed with water to a final concentration of 1 mg/ml and sonicated for 30 sec at maximum power in a sonicator (model W185; Heat Systems-Ultrasonics, Inc., Farmingdale, NY). The suspension was frozen in 0.2 ml aliquots at -70°C. The lipid solution was quickly thawed in warm water and sonicated in a water bath sonicator for 30 min immediately before use. In some cases, the PIP2 suspension was examined by quasielastic light scattering.

**Interaction of Peptides with Pyrene-labeled Actin**

G-actin was labeled with N-(1-pyrenyl)iodoacetamide by the method of Kounaka and Mihashi (1981). The relative amounts of G- and F-actin and the rate of change were determined as described in Janmey et al. (1985). The buffer used contains 2 mM MgCl$_2$, 0.5 mM ATP, 0.2 mM CaCl$_2$, 150 mM KCl, and 20 mM Tris-HCl, pH 7.5 (buffer B). The rate of actin polymerization in the presence of gelsolin has been shown to be proportional to the gelsolin concentration (Doi and Frieden, 1984; Coue and Korn, 1985; Janney 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; Janney and Stossel, 1986).

**Results**

**Identification of F-Actin-binding Gelsolin Peptides**

Fig. 1 A shows that $^{125}$I-labeled human plasma gelsolin binds F-actin in the presence of Ca$^{2+}$ but only minimally in its absence. These results are consistent with previous data using unlabeled rabbit macrophage and human plasma gelsolin (Yin and Stossel, 1979; Janney et al., 1985), but different from that with pig plasma gelsolin (Harris, 1985) which is also less stringent in its Ca$^{2+}$ requirement functionally (Weeds et al., 1986). Autoradiography of the $^{125}$I-gelsolin shows that the major band is 93 kD, and there is a minor contaminant of ~40 kD which does not cosediment with F-actin (Fig. 1 A, lane O). In the presence of 0.2 mM CaCl$_2$, a large proportion of gelsolin cosedimented with F-actin (Fig. 1 A, lanes C). Gamma counting of the pellets showed that 82% of the input gelsolin radioactivity was recovered in the presence of Ca$^{2+}$, and only 10% pelleted in the presence of EGTA.

Similar amounts of actin were sedimented, as judged by the intensity of the Coomassie Blue–stained actin band in the pellet and supernatants. The small amount of gelsolin left in the supernatant in Ca$^{2+}$ was most likely bound to actin oligomers that were too short to be sedimented. Sedimentation without actin pelleted 5% of the radioactivity, part of
which may be attributed to residual supernatant and non-specific sticking of $^{125}$I-gelsolin to the side of the tube.

Fig. 1 B shows an autoradiogram of NH$_2$-terminal half peptides that have been centrifuged in the presence of F-actin. The bulk of CT45N and CT28N cosedimented with actin (Fig. 1 B, lane PC), while most of CT14-17N remained in the supernatant (lane SC). A similar pattern was observed in EGTA (Fig. 1 B, lanes SE and PE). These results demonstrate that CT45N and CT28N form stable complexes with F-actin in a Ca$^{2+}$-independent manner, while CT14-17N does not.

Cosedimentation of gelsolin and its fragments with F-actin may reflect binding along the length of the filament (side binding) as well as to the end of a filament. Since gelsolin and CT45N sever actin filaments stoichiometrically, the species that cosediment with F-actin are most likely located at the barbed end of the newly severed actin filaments that are sufficiently long to be sedimented (note low ratios of gelsolin–actin and CT45N–actin used). The quantitative cosedimentation of CT28N with F-actin, however, is more likely to result from binding to the side of actin filaments, because CT28N has minimal actin severing activity (Kwiatkowski et al. 1985; also see below). The lack of binding of CT14-I7N suggests either that they do not bind to the side of the filaments, or if they do, the complex is not stable. However, it does not rule out binding to the end of filaments, because CT14-I7N does not sever filaments well, and the number of unoccupied barbed ends is small so little binding is predicted. The question of whether CT14N caps actin filament ends is not settled. Our previous functional studies indicate that CT14N does not cap actin filaments with sufficiently high affinity to increase the critical monomer concentration (Kwiatkowski et al., 1985; however also see Table 1).

**Binding of CT28N to Actin Filaments**

To examine further the interaction of CT28N with actin filaments, increasing amounts of purified CT28N were added to F-actin, and binding was monitored by the cosedimentation
Effect of PIP2 on the binding of gelsolin and gelsolin peptides to F-actin. 125I-labeled gelsolin or fragments were incubated with 12.1 μM F-actin (a-c) or actin-Sepharose (d) in the presence of PIP2 as described in Materials and Methods. (a) 0.00026 μM 125I-labeled gelsolin was centrifuged with F-actin in the presence of 0, 14, 42, 70, and 98 μM PIP2 respectively in a buffer containing 0.15 mM CaCl2/0.1 mM EGTA. Equivalent aliquots of supernatant (S) and pellet (P) were analyzed by autoradiography (after solubilization with SDS-gel sample buffer and PAGE on a slab gel), and the intensity of the gelsolin band was determined by densitometer scanning. Similar results were obtained by determining radioactivity in a gamma counter. In the absence of PIP2, 80% of gelsolin was sedimented. PIP2 decreased the sedimentable gelsolin without changing the amount of sedimentable actin (data not shown). G, gelsolin. (b) The amount of sedimentable gelsolin or 125I-labeled CT28N in the presence of PIP2 was expressed as percent of that sedimented in its absence, and plotted as a function of PIP2 concentration. The data for gelsolin was derived from experiment shown in a by gamma counting. The data for CT28N was obtained by cosedimenting purified 125I-labeled CT28N (0.18 μM) with actin in the presence of 1.0 mM EGTA. Radioactivity was determined by gamma counting, and 100% of the input CT28N was sedimented in the absence of PIP2. (c) 0.16 μM of 125I-NH2-terminal half peptide mix was incubated with F-actin in the presence of 0, 4.4, 13.7, and 27 μM PIP2 (lanes 1-4, respectively). The ratios of sedimented CT28N to CT45N, determined by densitometry, changed from 1.1 to 0.71, 0.46, and 0.36 with increasing amounts of PIP2. (d) 125I-NH2-terminal peptides (equivalent to 0.22 μM CT45N) were incubated with 75 μl of packed actin-Sepharose beads in the presence of TBS-0.2 mM CaCl2. The beads were washed with TBS-EGTA, divided into five aliquots, and incubated with 0.5 ml of solutions containing increasing amounts of PIP2 in the presence of 1 mM EGTA. (Inset) NH2-terminal peptides remaining on actin-Sepharose after incubation with 0, 0.22, 0.44, 0.88, and 1.76 mM PIP2 (lanes 1-4, respectively). Radioactivity associated with CT45N (which was resolved into two bands in this experiment) and CT17-15N was determined by cutting the bands from the gel and counting in a gamma counter. In the absence of PIP2, 540 and 180 cpm were associated with CT45N and CT17-15N, respectively. The amount of each peptide sedimented in the presence of PIP2 was expressed as a percent of control.

Fig. 2 A shows a Coomassie Blue-stained gel of supernatant and pellet fractions. CT28N cosedimented with F-actin and saturation of binding was observed when 2.9 μM CT28N was added to 2.2 μM actin (Fig. 2 A, lanes 3). At saturation, the molar ratios of CT28N to actin, determined by densitometric scanning of lanes 3 and 4, were 0.80 and 0.84, respectively. A similar ratio (0.81) was obtained with less sample was loaded on the gel (Fig. 2 A, lane 4'), confirming the linearity of the assay. The amount of actin sedimented remained constant (82 ± 5%) as judged by its intensity in the supernatant and pellet fractions. A 1.7-fold molar excess of CT28N to actin did not decrease the amount of sedimentable actin significantly, suggesting that CT28N does not sever actin filaments nor depolymerize them by binding to actin monomers. Direct measurement of actin severing, using a fluorimetric assay, showed that CT28N (not iodinated) contained <10% of the severing activity of an equivalent amount of intact gelsolin or CT45N, which can
be attributed to contamination by CT45N. The close to 1:1 binding of CT28N to actin monomers within a filament suggests that CT28N binds stoichiometrically to actin along the length of actin filaments.

Additional binding experiments using a wider concentration range of $^{125}$I-CT28N and two different concentrations of actin were performed. Fig. 2B shows that binding of $^{125}$I-CT28N to F-actin is saturable. The Fig. 2B, inset shows an autoradiogram of CT28N in the pelleted fraction, to demonstrate the purity of the CT28N preparation and confirm saturable binding of CT28N. Analysis of the binding data shown in Fig. 2B by the method of Scatchard (1949) suggests one class of CT28N binding sites on actin, which became saturated when 0.85 mol of CT28N was bound to each mole of actin subunit in the filaments (Fig. 2C). Since the Scatchard method is not strictly applicable to the sedimentation assay presented here (the concentration of actin, the "receptor", is similar to that of CT28N, the ligand, so that the free ligand does not approximate total ligand), the dissociation constant was calculated directly from individual data point from the relation $K_d = \frac{[\text{free CT28N}] [\text{free actin}]}{[\text{complex}]}$ where $[\text{free CT28N}] = [\text{total CT28N}] - [\text{complex}]; [\text{free actin}] = [\text{total actin}] - [\text{complex}];$ and $[\text{complex}]$ was taken as the amount of CT28N cosedimenting with actin divided by the initial volume. The result, $K_d = 2.0 \times 10^{-7} \text{ M} \pm 0.8 \times 10^{-7}$ $\text{ M} \ (n = 10)$. Three data points shown in Fig. 2B were omitted because they gave $K_d$ values considerably different from that of the rest of the data points, reflecting large errors in the calculation of $[\text{free CT28N}]$ at the plateau. If these points were included, $K_d = 2.8 \times 10^{-7} \text{ M} \pm 1.9 \times 10^{-7} \text{ M} \ (n = 13)$.

Effect of PIP2 on Gelsolin and Gelsolin Peptide Binding to Actin

Fig. 3a shows that PIP2 decreases the amount of gelsolin that cosedimented with F-actin, with a corresponding increase in nonsedimentable gelsolin. The effect of PIP2 on the percent of $^{125}$I-gelsolin bound to F-actin from this experiment is plotted on Fig. 3b. The amount of PIP2 required to inhibit gelsolin binding is much higher than that required to inhibit binding of CT28N, determined by cosedimentation of purified $^{125}$I-CT28N with F-actin (Fig. 3b). Binding of CT28N to F-actin is also more sensitive to inhibition by PIP2 than CT45N, from which it is derived. This was demonstrated by studying the sedimentation of the NH2-terminal half peptide mix (Fig. 3c). At the concentrations of PIP2 used, the intensity of the CT45N band remained relatively constant while that of CT28N decreased. This difference is quantitated by taking the ratios CT28N/CT45N intensity (determined by densitometer scanning), which decrease from 1.1 to 0.4.

Fig. 3d examines the ability of PIP2 to inhibit binding of CT14-17N to actin. Actin-Sepharose is used instead of actin filament to bind the gelsolin fragments because CT14-17N does not bind detectably to actin filaments (see Fig. 1). As shown previously, in the presence of 0.15 M KCl, CT45N and CT14-17N bind actin-Sepharose but CT28N does not (Kwiatkowski et al., 1985). PIP2 added to these complexes eluted more CT45N than CT14-17N from the beads, suggesting that the latter is less sensitive to PIP2. The elution curve for CT45N is similar to that for EGTA-resistant gelsolin-actin-Sepharose (data not shown), supporting the notion that PIP2 dissociates EGTA-resistant gelsolin-actin complexes through its effect on an NH2-terminal actin-binding site (Janmey et al., 1987). The high concentration of PIP2 required to cause dissociation of gelsolin or CT45N is presumably because protein-coated Sepharose beads bind PIP2 nonspecifically (Janmey and Stossel, 1987; Iida, K., unpublished observation), decreasing the actual amount available to gelsolin.

Janmey and Stossel (1987) have shown that the severing function of gelsolin is most sensitive to inhibition by PIP2 whereas nucleation and capping are less so. Thus, both functional and binding data suggest that different actin binding domains with differential sensitivity to PIP2 mediate the separate functions of gelsolin. The observations that PIP2 inhibits most effectively gelsolin's severing function as well as the stoichiometric binding of CT28N to actin within a filament suggest that PIP2 may inhibit gelsolin or CT45N severing by preventing binding of the CT28N domain to the side of actin filaments. The smaller effect of PIP2 on binding of CT45N to filaments per se indicates that although CT45N can no longer bind to the side of the filaments (through CT28N), it can still bind to the ends of actin filaments.

Binding of CT28N to Actin Monomers

Fig. 4 shows that CT28N does not cosediment with monomeric actin complexed with DNase-Sepharose in the presence of a solution containing 0.15 M NaCl. In contrast, a large proportion of CT45N and CT14-17N cosedimented with actin bound to DNase-Sepharose beads. The failure of CT28N to cosediment with actin complexed to DNase-Sepharose indicates that either CT28N does not bind stably to actin monomers at physiological salt concentrations, or that the CT28N-actin complex cannot bind DNase-Sepharose because of steric hindrance or competition for the same binding site on actin. The latter alternative is unlikely because the amount of actin bound to DNase I beads is not decreased in the presence of nonsedimentable CT28N (data not shown). The lack of association of CT28N to actin-DNase I and actin-Sepharose strongly suggests that CT28N does not bind to monomeric actin under physiological salt conditions, but preferentially binds F-actin. A lack of binding to monomeric actin is supported by our previous finding that CT28N does not bind actin-Sepharose (Kwiatkowski et al., 1985).

Effect of PIP2 on NH2-terminal Half Peptide Functions

Fig. 5 shows that PIP2 inhibits the severing and the weak nucleating activity of CT45N equally. Half maximal inhibition was observed at 3 mol of PIP2 micelle per mole of

S T P

-45 -30 -15

Figure 4. Binding of NH2-terminal peptides to actin complexed to DNase-Sepharose. NH2-terminal peptides (0.05 μM) were incubated with actin (0.12 μM) and then 15 μl DNase I-beads in buffer A containing 0.15 M KC1. The pellet (P) and supernatant (S) fractions were analyzed on SDS-PAGE, and radiolabeled polypeptides detected by autoradiography. T, NH2-terminal peptides input. Actin was quantitatively recovered in the DNase-Sepharose fraction (data not shown).
The data presented here show that human plasma gelsolin has at least three distinct actin binding sites (Table I), and identify a 28-kD peptide from the NH2-terminal half of gelsolin as the domain that binds to the sides of actin filaments, a step which may be required to account for the rapid stoichiometric severing of actin filaments by gelsolin (Yin et al., 1980; Janmey et al., 1986a). Previous proteolytic digestion studies have shown that the 45-kD NH2-terminal half of gelsolin severs actin filaments almost as effectively as intact gelsolin (Bryan and Hwo, 1986; Chaponnier et al., 1986), and that one of its actin binding domains is localized within CT14N (Kwiatkowski et al., 1985). It should be pointed out that although we have originally suggested that CT14N severs actin filaments (Kwiatkowski et al., 1985), its severing activity is only 1% (on a molar basis) of that of intact gelsolin or CT45N. Since we cannot rule out a small (3% by weight) contamination of our CT14N preparation with CT45N, we are not certain whether CT14N can sever. It is, however, clear that efficient severing requires both CT14N and another NH2-terminal half domain. Our present data show that CT28N has a role in promoting efficient severing by gelsolin: (a) CT28N binds stoichiometrically and with high affinity to actin molecules within a filament; (b) CT28N does not sever actin filaments, and neither does a 70-kD gelsolin fragment missing CT14N (Bryan, J., personal communications); (c) CT45N, which is required for severing, does not bind well (or stably) to the side of actin filaments and is relativelyPIP2 insensitive; (d) binding of CT28N to F-actin and severing by gelsolin are inhibited by PIP2 with comparable dose response. Since PIP2 inhibits CT28N binding to F-actin and severing as well, the simplest explanation is that PIP2 inhibits severing by preventing CT28N binding to F-actin.

The F-actin binding affinity of CT28N, determined here by direct binding assays, is at least two orders of magnitude lower than that estimated for gelsolin (and by inference, CT45N) by severing function assays (5 × 10^8 M^-1 and >10^9 M^-1, respectively; Coue and Korn, 1985; Janmey et al., 1985). This difference suggests that there may be a high degree of interaction between CT28N and CT45N to increase the affinity of the NH2-terminal half domain for F-actin and maximally express the severing function. It is not clear whether CT28N and CT45N bind to the same or different actin subunit. Since CT28N binds preferentially to filamentous actin, it presumably recognizes an epitope or conformation found on F-actin only. It follows that CT45N, which binds actin to the NH2-terminal half fragment, a ratio similar to that reported for inhibition of severing by intact gelsolin (0.5:1), but much lower than that for inhibition of nucleation by intact gelsolin (>3,200:1; Janmey and Stossel, 1987). The similarity in dose response for inhibition of severing by gelsolin and CT45N confirms that PIP2 acts on the NH2-terminal domain to inhibit severing. On the other hand, the difference in the ability of CT45N and gelsolin to nucleate actin assembly (Chaponnier et al., 1986), as well as their different sensitivity to PIP2, show that the nucleating ability of gelsolin is modified by removal of the COOH-half domain. Occupation of the COOH terminal site however is not sufficient by itself, because CT38C does not nucleate actin assembly (Kwiatkowski et al., 1985).

Although PIP2 inhibits severing and nucleation by the NH2-terminal half, a third function, blocking of the barbed end of actin filaments, is less affected. Fig. 6 shows that CT45N, which is inhibited from severing actin filaments by PIP2, decreases the initial rate of depolymerization of F-actin after dilution (left) and reduces the initial rate of actin polymerization to below that observed with actin alone (right). Furthermore, the final level of fluorescence attained at steady state is only slightly higher than that observed with CT45N in the absence of PIP2 (data not shown). These data suggest that the barbed ends of the filaments are blocked by CT45N in spite of inhibition of severing and nucleation by PIP2.

In contrast to the results obtained with the NH2-terminal half peptide, PIP2 has little effect on CT38C. We have shown previously that CT38C (formerly called CT47C) decreased the initial rate and final level of polymerization to an extent consistent with the formation of stable 1:1 actin-CT38C complexes that cannot polymerize (Kwiatkowski et al., 1985). The presence of 100 µM PIP2 (a nearly equimolar ratio of micelles to CT38C) did not change either parameter (data not shown), demonstrating that PIP2 does not inhibit the binding of CT38C to actin monomers.

Discussion

The data presented here show that human plasma gelsolin has at least three distinct actin binding sites (Table I), and identify a 28-kD peptide from the NH2-terminal half of gelsolin as the domain that binds to the sides of actin filaments, a step which may be required to account for the rapid stoichiometric severing of actin filaments by gelsolin (Yin et al., 1980; Janmey et al., 1986a). Previous proteolytic digestion studies have shown that the 45-kD NH2-terminal half of gelsolin severs actin filaments almost as effectively as intact gelsolin (Bryan and Hwo, 1986; Chaponnier et al., 1986), and that one of its actin binding domains is localized within CT14N (Kwiatkowski et al., 1985). It should be pointed out that although we have originally suggested that CT14N severs actin filaments (Kwiatkowski et al., 1985), its severing activity is only 1% (on a molar basis) of that of intact gelsolin or CT45N. Since we cannot rule out a small (3% by weight) contamination of our CT14N preparation with CT45N, we are not certain whether CT14N can sever. It is, however, clear that efficient severing requires both CT14N and another NH2-terminal half domain. Our present data show that CT28N has a role in promoting efficient severing by gelsolin: (a) CT28N binds stoichiometrically and with high affinity to actin molecules within a filament; (b) CT28N does not sever actin filaments, and neither does a 70-kD gelsolin fragment missing CT14N (Bryan, J., personal communications); (c) CT45N, which is required for severing, does not bind well (or stably) to the side of actin filaments and is relatively PIP2 insensitive; (d) binding of CT28N to F-actin and severing by gelsolin are inhibited by PIP2 with comparable dose response. Since PIP2 inhibits CT28N binding to F-actin and severing as well, the simplest explanation is that PIP2 inhibits severing by preventing CT28N binding to F-actin.

The F-actin binding affinity of CT28N, determined here by direct binding assays, is at least two orders of magnitude lower than that estimated for gelsolin (and by inference, CT45N) by severing function assays (5 × 10^8 M^-1 and >10^9 M^-1, respectively; Coue and Korn, 1985; Janmey et al., 1985). This difference suggests that there may be a high degree of interaction between CT28N and CT45N to increase the affinity of the NH2-terminal half domain for F-actin and maximally express the severing function. It is not clear whether CT28N and CT45N bind to the same or different actin subunit. Since CT28N binds preferentially to filamentous actin, it presumably recognizes an epitope or conformation found on F-actin only. It follows that CT45N, which binds actin

![Figure 5](https://example.com/figure5.png)

**Figure 5.** PIP2 inhibits severing and nucleation by the NH2-terminal half peptide. CT45N was diluted to 0.11 µM (for severing) or 0.15 µM (for nucleating) in buffer B containing 1 mM EGTA. PIP2 was added from a stock solution of 0.9 mM in water to the final concentrations shown. After a 15-s incubation, either F-actin (0.21 µM) or G-actin (4.1 µM) was added and the maximal rate of depolymerization (solid circles) or polymerization (open circles) was measured. The rates are relative to 100% for polymerization or depolymerization in the presence of the NH2-terminal half peptide and no PIP2. The rates for actin alone were 56% for polymerization and 10% for depolymerization.

![Figure 6](https://example.com/figure6.png)

**Figure 6.** CT45N blocks monomer exchange from the fast growing filament end in the presence of PIP2. (Left) The initial time course of actin depolymerization is shown for actin diluted in the presence of CT45N in the absence (solid circles) or presence of 22 µM PIP2 (solid triangles). The rate for actin alone in the presence of PIP2 is shown (open circles), and is indistinguishable from that in the absence of PIP2. (Right) The initial rate of polymerization of actin in the presence of CT45N in the presence (solid triangle) and absence (solid circles) of 22 µM PIP2, compared with that of actin alone (open circles). Reaction conditions are as described for Fig. 5.
tin monomer but not (stably) to the sides of actin filaments, but not to the sides of actin filaments, which seem to contribute to severing of gelsolin, except conferring amino acids at the NH₂ terminus of actin, but there is no information on which actin binding domains are cross-linked to actin under those circumstances.

Although the COOH-terminal half of gelsolin does not appear to contribute to severing of gelsolin, except conferring Ca²⁺ sensitivity (Kwiatkowski et al., 1985; Hwo and Bryan, 1986; Chaponnier et al., 1986), our current data indicate that it contributes significantly to the nucleating activity of intact gelsolin. This conclusion is based on the following reasoning: (a) CT45N has only 10% of the nucleating activity of intact gelsolin (Chaponnier et al., 1986); (b) the two nucleating activities are different in that Ca²⁺ promotes nucleation by intact gelsolin but not by CT45N, andPIP2 inhibits the latter more potently (Janmey and Stossel, 1987); (c) Ca²⁺ has no effect on CT45N binding to actin but PIP2 does; (d) Ca²⁺ is required for CT38C binding to actin, but PIP2 has no effect. Therefore, it is reasonable to hypothesize that occupation of the COOH-terminal actin binding domain is required for optimal nucleation. Additional NH₂-terminal domain must also be involved however, since CT38C does not itself promote nucleation (Kwiatkowski et al., 1985).

The question of which of the three currently identified actin binding sites are involved in capping of the barbed end of the filament is also not resolved. The actin filament end blocking function of gelsolin is most resistant to inhibition by itself promote nucleation (Kwiatkowski et al., 1985).

We thank T. P. Stossel for helpful discussions. We appreciate the excellent assistance of M. Nguyen.

This research was funded by grants HL29113 and AR38910 from the United States Public Health Services, and the Whitaker Health Science Fund. K. Iida was partially supported by the Yamamoto Foundation and Tokyo Metropolitan Institute of Medical Science. H. L. Yin is an Established Investigator of the American Heart Association.

Received for publication 4 August 1987, and in revised form 9 November 1987.

References

Bryan, J. 1988. Gelsolin has three actin binding sites. J. Cell Biol. In press.
Bryan, J., and L. M. Coluccio. 1985. Kinetic analysis of F-actin depolymerization in the presence of platelet gelsolin and gelsolin-actin complexes. J. Cell Biol. 101:1236-1244.
Bryan, J., and S. Hwo. 1986. Definition of an NH₂-terminal actin-binding domain and a COOH-terminal Ca²⁺ regulatory domain in human gelsolin. J. Cell Biol. 102:1439-1446.
Bryan, J., and M. Kurth. 1984. Actin-gelsolin interactions: evidence for two actin-binding sites. J. Biol. Chem. 259:7480-7487.
Chaponnier, C., R. Borgia, E. Ruggner-Brandle, R. Weil, and G. Gabbanini. 1979. An actin-destabilizing factor is present in human plasma. Experientia (Basel). 35:1039-1040.
Chaponnier, C., P. A. Janmey, and H. L. Yin. 1986. The actin filament-severing domain of plasma gelsolin. J. Cell Biol. 103:1473-1481.
Chaponnier, C., H. L. Yin, and T. P. Stossel. 1987. Reversibility of gelsolin/actin interaction in macrophages. J. Exp. Med. 165:97-106.
Coue, M., and E. D. Korn. 1985. Interaction of plasma gelsolin with G-actin and F-actin in the presence and absence of calcium ions. J. Biol. Chem. 260:15033-15041.
Doi, Y., and S. Frieden. 1984. Actin polymerization: the effect of brevin on filament size and rate of polymerization. J. Biol. Chem. 259:11868-11875.
Doi, Y., M. Higashida, and S. Kido. 1987. Plasma-gelsolin-binding sites on the actin sequence. Eur. J. Biochem. 164:89-94.
Harris, H. E. 1985. CovaJnc complexes formed between plasma gelsolin and actin with a zero-length cross-linking compound. Biochemistry. 24:6613-6618.
Harris, D. A., and J. H. Schwartz. 1981. Characterization of brevin, a serum protein that shortens actin filaments. Proc. Natl. Acad. Sci. USA. 78:6799-6802.
Harris, H. E., J. R. Bamberg, and A. G. Weeds. 1980. Actin filament disassembly in blood plasma. FEBS (Fed. Eur. BioL Soc.) Lett. 121:175-177.
Hwo, S., and J. Bryan. 1986. Immunochemical evidence for cross-linking studies using G-actin-gelsolin complexes by Doi et al. (1987) suggest that gelsolin binds to the first 12 amino acids at the NH₂ terminus of actin, but there is no information on which actin binding domains are cross-linked to actin under those circumstances.

Although the COOH-terminal half of gelsolin does not appear to contribute to severing of gelsolin, except conferring Ca²⁺ sensitivity (Kwiatkowski et al., 1985; Hwo and Bryan, 1986; Chaponnier et al., 1986), our current data indicate that it contributes significantly to the nucleating activity of intact gelsolin. This conclusion is based on the following reasoning: (a) CT45N has only 10% of the nucleating activity of intact gelsolin (Chaponnier et al., 1986); (b) the two nucleating activities are different in that Ca²⁺ promotes nucleation by intact gelsolin but not by CT45N, and PIP2 inhibits the latter more potently (Janmey and Stossel, 1987); (c) Ca²⁺ has no effect on CT45N binding to actin but PIP2 does; (d) Ca²⁺ is required for CT38C binding to actin, but PIP2 has no effect. Therefore, it is reasonable to hypothesize that occupation of the COOH-terminal actin binding domain is required for optimal nucleation. Additional NH₂-terminal domain must also be involved however, since CT38C does not itself promote nucleation (Kwiatkowski et al., 1985).

The question of which of the three currently identified actin binding sites are involved in capping of the barbed end of the filament is also not resolved. The actin filament end blocking function of gelsolin is most resistant to inhibition by PIP2, and also has the least stringent requirement for Ca²⁺ (Janmey et al., 1985) when compared with severing and nucleation. In this paper, we have shown that binding of isolated CT45N and CT38C to actin are relatively more resistant to inhibition by PIP2 than CT28N, so actin binding sites located in those peptides may contribute to PIP2-resistant capping observed at steady state. PIP2 however does appear to have some effect on the capping process, because it can temporarily allow elongation of actin filaments off the barbed ends of gelsolin-capped filaments (Janmy et al., 1987).

We thank T. P. Stossel for helpful discussions. We appreciate the excellent assistance of M. Nguyen.

This research was funded by grants HL29113 and AR38910 from the United States Public Health Services, and the Whitaker Health Science Fund. K. Iida was partially supported by the Yamamoto Foundation and Tokyo Metropolitan Institute of Medical Science. H. L. Yin is an Established Investigator of the American Heart Association.

Received for publication 4 August 1987, and in revised form 9 November 1987.

References

Bryan, J. 1988. Gelsolin has three actin binding sites. J. Cell Biol. In press.
Bryan, J., and L. M. Coluccio. 1985. Kinetic analysis of F-actin depolymerization in the presence of platelet gelsolin and gelsolin-actin complexes. J. Cell Biol. 101:1236-1244.
Bryan, J., and S. Hwo. 1986. Definition of an NH₂-terminal actin-binding domain and a COOH-terminal Ca²⁺ regulatory domain in human gelsolin. J. Cell Biol. 102:1439-1446.
Bryan, J., and M. Kurth. 1984. Actin-gelsolin interactions: evidence for two actin-binding sites. J. Biol. Chem. 259:7480-7487.
Chaponnier, C., R. Borgia, E. Ruggner-Brandle, R. Weil, and G. Gabbanini. 1979. An actin-destabilizing factor is present in human plasma. Experientia (Basel). 35:1039-1040.