Artificial Phosphorylation Removes Gelsolin's Dependence on Calcium

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ABSTRACT. Gelsolin is one of the best known actin-binding proteins with several distinct activities regulated by calcium. Using a kinase fraction isolated from mitotic HeLa cells, we found that the plasma form of gelsolin can be phosphorylated at a site located within the NH₂-terminus region which does not exist in the cytoplasmic form. After this phosphorylation, gelsolin no longer requires Ca²⁺ for activity; it severs and subsequently caps actin filaments, and nucleates filament formation in Ca²⁺-free solution. These findings may clarify the mechanism of gelsolin regulation by Ca²⁺, and indicate that changes in electrical interactions between the NH₂- and COOH-terminal ends are important for this regulation. Moreover, since only a single site is phosphorylated, and since the phosphorylated region does not contribute to this protein's own activity, the results suggest that a single chemical charge modification at a site away from the protein's core structure, such as this phosphorylation site, is sufficient to alter the protein's function.

Key words: gelsolin/actin-severing activity/calcium regulation/phosphorylation/flow birefringence/protein engineering

Gelsolin is a 90 kDa Ca²⁺-regulated actin-binding protein found in the cytoplasm of eukaryotes as well as in blood plasma (Kwiatkowski *et al.*, 1988; Yin and Stossel, 1979; Yin *et al.*, 1984; Yin, 1987). Both the plasma and the cytoplasmic forms of gelsolin can be isolated from the same source, are encoded by a single gene and differ as the result of alternative splicing (Kwiatkowski *et al.*, 1986; Stella *et al.*, 1994). The cytoplasmic form of gelsolin has been implicated in the regulation of the actin cytoskeleton. It is thought to control the length and number of actin filaments (F-actin) (Lin *et al.*, 1997), and it is required for the rapid locomotion of moving cells (Cunningham *et al.*, 1991; Witke *et al.*, 1995). On the other hand, gelsolin secreted in the plasma plays a key role in scavenging F-actin from the circulation. It rapidly severs plasma F-actin released from dead cells and prevents the elevation of blood viscosity (Haddad *et al.*, 1990; Vasconcellos and Lind, 1993).

Only when micromolar concentrations of Ca²⁺ are present in solution can gelsolin sever F-actin and subsequently form

a cap on its newly exposed barbed end, which blocks the monomer association at that end. Because the two ends of F-actin have significantly different rates of assembly and disassembly, not only is the length of F-actin shortened, but the total amount of assembled actin is decreased, when gelsolin is active. This results in an increase in the critical concentration necessary for actin polymerization. In addition to these two activities, gelsolin has a third activity: stimulation of actin nucleation. Formation of the nucleation site, which consists of an actin trimer, is necessary for actin assembly before its elongation. Because the assembly of a nucleation site is relatively slower than actin elongation, a lag phase is seen in the early stages of polymerization. When Ca²⁺ is present, gelsolin can shorten this lag phase by its actin-nucleation activity, while the amount of F-actin at equilibrium is decreased by the other activities of gelsolin.

Gelsolin has six structurally related segments. An analysis of the properties of the proteolytically derived fragments of gelsolin (Bryan and Hwo, 1986; Chaponnier *et al.*, 1986; Kwiatkowski *et al.*, 1985) suggests that Ca²⁺ binds to the COOH-terminal half of gelsolin and induces a conformational change (Bryan and Hwo, 1986; Kilhoffer and Gerard, 1985; Kwiatkowski *et al.*, 1986; Lamb *et al.*, 1993) which disrupts connections (binding or interaction) between the NH₂-terminal and COOH-terminal halves of the protein, permitting the NH₂-terminal half to sever F-actin (Burtnick *et al.*, 1997; Lin *et al.*, 1997). Severing by this NH₂-terminal

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Abbreviations: PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; CBB, Coomassie brilliant blue.

region is Ca^{2+} independent, and requires the participation of two actin binding domains located within the NH_2 -terminal segment (S1) and segment 2 and 3 (S2-3). The S1 region binds to actin monomers and to the ends of filaments with high affinity, while S2-3 binds to the side of F-actin (Bryan, 1988; Yin, 1987).

We report here that plasma gelsolin isolated from the serum, but not from the cytoplasm, is phosphorylated with kinase activity isolated from mitotic HeLa cells. Phosphorylated gelsolin becomes active in the absence of calcium, but shows the same CD spectra as the unphosphorylated form. An analysis of proteolytically derived fragments of phosphorylated plasma gelsolin suggests that this phosphorylation occurs within an additional leader sequence at its NH_2 -terminus which exists only in the plasma form of gelsolin. These results suggest that this artificial phosphorylation at the NH_2 -terminus liberates gelsolin from its calcium dependence.

Materials and Methods

Protein

Actin was purified from rabbit skeletal muscle as previously described (Takiguchi, 1991). Plasma and cytoplasmic gelsolin were purified from calf or porcine serum and porcine heart, respectively (Ishikawa *et al.*, 1989; Rouayrenc *et al.*, 1984). Gelsolin was phosphorylated as described previously (Yamashiro *et al.*, 1990). Briefly, gelsolin (0.8 mg/ml) was incubated at 22°C for 2 hr in Buffer A (15 mM imidazole pH 7.0, 5 mM MgCl_2 , 0.2 mM EGTA, 75 mM KCl, 500 nM protein kinase A inhibitor peptide and 2 mM ATP) with the kinase fraction isolated from mitotic HeLa cells. The samples were then dialyzed against 25 mM Tris-HCl pH 7.6, 0.5 mM CaCl_2 and 0.2 mM PMSF at 4°C overnight and then used. The amount of phosphorylation was determined using $[\gamma\text{-}^{32}\text{P}]$ ATP.

Occasionally, to further purify phosphorylated calf serum gelsolin, the sample was applied to a DEAE-cellulose column equilibrated with the dialysis buffer noted above. Phosphorylated calf serum gelsolin was eluted with the same dialysis buffer containing 50 mM NaCl, and then dialysed against 20 mM imidazole pH 7.0, 100 mM KCl, and 0.2 mM PMSF. This purified fraction is termed DEAE-purified phosphorylated gelsolin. Using this procedure, a gelsolin sample in which 80–90 % of the protein was phosphorylated could be obtained. For control experiments, an unphosphorylated gelsolin sample was applied to the DEAE-cellulose column and then used in the same way. This sample was termed DEAE-purified unphosphorylated gelsolin. The actin-binding activity of gelsolin was assayed as described previously (Yamashiro-Matsumura and Matsumura, 1985).

Gelsolin was also phosphorylated with cdc2 beads using the same conditions as noted above using the kinase fraction isolated from mitotic HeLa cells. Briefly, gelsolin was incubated with cdc2 beads for 1 h at room temperature. Those cdc2 beads were prepared as described previously (Yamashiro *et al.*, 1990), and essentially were protein A beads bound to p34 cdc2 kinase antibodies.

The beads were subsequently incubated with the kinase fraction from mitotic HeLa cells, and after washing with phosphate-buffered saline, they were used as cdc2 beads. It is noted here that essentially the same results were obtained using either the kinase fraction or the cdc2 beads for gelsolin phosphorylation.

Proteolytic analysis of phosphorylated plasma gelsolin using α -chymotrypsin was performed according to Bryan and Hwo (1986).

Assays for gelsolin activities

Gelsolin activities were monitored by flow birefringence (Arisaka *et al.*, 1975; Ishikawa *et al.*, 1989). For severing activity, 12 μM F-actin and various concentrations of phosphorylated or unphosphorylated gelsolin were incubated in 20 mM imidazole pH 7.0, 100 mM KCl, 2 mM MgCl_2 , 0.2 mM DTT, 0.25 mM ATP and 0.2 mM CaCl_2 or 2 mM EGTA at 22°C for 30 min, and then each sample was assayed.

To follow the severing activity of plasma gelsolin during its phosphorylation, 0.25 mg/ml unphosphorylated gelsolin was incubated at 22°C in Buffer A with the kinase fraction. Aliquots (15 μl) of the mixture were withdrawn at various times and mixed with 135 μl of an F-actin-containing solution (final concentration of 20 mM imidazole pH 7.0, 100 mM KCl, 2 mM MgCl_2 , 0.2 mM DTT, 0.25 mM ATP, 2 mM EGTA and 12 μM F-actin). After incubation at 22°C for 40 min, the flow birefringence of the sample was measured. Conversely, to follow the severing activity of phosphorylated plasma gelsolin during its dephosphorylation, 0.2 mg/ml phosphorylated gelsolin was incubated at 22°C in 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES) pH 6.0 and 0.1 mM PMSF with 50 $\mu\text{g}/\text{ml}$ potato acidic phosphatase. Aliquots (25 μl) of the mixture were withdrawn at various times and mixed with 125 μl of the F-actin-containing solution (final concentration of 70 mM imidazole pH 7.0, 60 mM KCl, 2 mM MgCl_2 , 0.2 mM DTT, 0.2 mM ATP, 2 mM EGTA or 0.2 mM CaCl_2 and 12 μM F-actin). After incubation at 22°C for 5 min, the flow birefringence of each sample was measured.

For evaluating the capping activity, various concentrations of actin and 0.3 μM DEAE-purified phosphorylated or unphosphorylated gelsolin were incubated in 20 mM imidazole pH 7.0, 80 mM KCl, 5 mM MgCl_2 , 0.2 mM DTT, 1.2 mM ATP and 0.2 mM CaCl_2 or 2 mM EGTA at 4°C overnight and then at 20°C for 3 hr. The flow birefringence of each sample was then assayed and the critical concentration of actin polymerization was calculated. Because flow birefringence was difficult to measure at lower actin concentrations when only about 25 % of the gelsolin in the sample tested was phosphorylated, we used DEAE-purified phosphorylated gelsolin to evaluate capping activity, with or without calcium. Most of the gelsolin molecules in that sample were phosphorylated, as described above.

For nucleation activity, 12 μM G-actin and 0.15 μM phosphorylated or unphosphorylated gelsolin were incubated in 7.5 mM Tris-HCl pH 8.0, 2 mM MgCl_2 , 0.3 mM DTT, 1 mM ATP and 0.2 mM CaCl_2 or 2 mM EGTA at 22°C for 15 min. Actin polymerization was initiated by the addition of 2 mM MgCl_2 and 0.15 M KCl (final concentrations), and the increase of flow birefringence was

followed.

Results

Phosphorylation of plasma gelsolin

The kinase activity isolated from mitotic HeLa cells and cdc2 beads can phosphorylate plasma gelsolin isolated from porcine or calf serum (Fig. 1A), but can not phosphorylate cytoplasmic gelsolin isolated from porcine heart (Fig. 1E). Since gelsolin exists in two forms (plasma and cytoplasmic), that are encoded by a single gene and that differ as the result of alternative splicing, both forms contain the same core structure and differ only at the NH₂-terminus (Bryan and Hwo, 1986; Kwiatkowski *et al.*, 1985, 1986; Stella *et al.*, 1994). Only plasma gelsolin has an extended leader sequence at its NH₂-terminus, and therefore, the potential phosphorylation site was expected to reside in that sequence. To confirm the phosphorylation site, calf and por-

cine plasma gelsolins phosphorylated in the presence of [γ -³²P] ATP were treated with α -chymotrypsin (Bryan and Hwo, 1986), and the fragments were then analyzed by western blotting using a gelsolin antibody that recognizes the COOH-terminus, followed by autoradiography (Fig. 1, B and C). Since the amino acid sequence of the core structure of gelsolin is highly conserved, this 90 kDa protein generally produces five well-known major fragments after α -chymotryptic digestion (Fig. 1D) (Bryan and Hwo, 1986; Bryan 1988; Chaponnier *et al.*, 1986; Kwiatkowski *et al.*, 1985; Rouayrenc *et al.*, 1986; Way and Weeds, 1988). Gelsolin was initially cleaved into a 40 kDa NH₂-half and a 45 kDa COOH-terminal half. The 40 kDa NH₂-half was further proteolyzed into a 17 kDa fragment (called CT17N) and a 30 kDa fragment, while the 45 kDa COOH-terminal half was subsequently processed into a 40 kDa fragment. The CT17N fragment, the 30 kDa NH₂-half fragment, and the COOH-terminal half roughly correspond to the S-1, S2-3, and S4-6 segments of gelsolin, respectively (Yin, 1987). It

Fig. 1. Plasma gelsolin is phosphorylated by a kinase fraction isolated from mitotic HeLa cells. (A) Calf serum gelsolin phosphorylated by cdc2 beads. The left side panel shows CBB staining of SDS-PAGE of histone H1 (left lane) and calf serum gelsolin (right lane). The right panel shows an autoradiograph of the same gel (32P). Histone H1 (180 μ g/ml) and gelsolin (0.4 mg/ml) were incubated with cdc2 beads and [γ -³²P] ATP for 1 h at room temperature. Protein A beads binding a p34 cdc2 kinase antibody were incubated with a kinase fraction from mitotic HeLa cells. After washing with phosphate-buffered saline, the beads were used as cdc2 beads. The conditions are the same as phosphorylation using the kinase fraction. (B) Proteolytic analysis of phosphorylated calf serum gelsolin. The left side panel shows CBB staining of SDS-PAGE of phosphorylated calf serum gelsolin incubated with α -chymotrypsin (lanes, left to right, show incubation times of 0, 40, and 120 min, respectively). The center panel shows western blotting of the gel using an antibody which recognizes the COOH-terminal half of gelsolin (Anti-Ct). The right side panel shows an autoradiograph of the same gel (32P). (C) Proteolytic analysis of phosphorylated porcine serum gelsolin. The left panel shows CBB staining of SDS-PAGE of phosphorylated porcine serum gelsolin incubated with α -chymotrypsin (left and right lanes show incubation times of 0 and 120 min, respectively). The right panel shows an autoradiograph of the same gel (32P). Left side numbers indicate the positions of each molecular weight (kDa). Identified bands are, G, gelsolin; 45C, CT45C fragment; 40C, CT40C fragment; 40N, CT40N fragment; 30N, 30 kDa NH₂-half fragment; 17N, CT17N fragment (see Kwiatkowski *et al.*, 1985). (D) A schematic model shows the five major fragments generally produced after α -chymotryptic treatment of gelsolin (Bryan and Hwo, 1986; Kwiatkowski *et al.*, 1985; Rouayrenc *et al.*, 1986). (E) Porcine heart gelsolin is not phosphorylated by the same kinase fraction. Left panel shows CBB staining of SDS-PAGE of partially purified porcine heart gelsolin. Right panel shows an autoradiograph of the same gel (32P). Porcine heart gelsolin was incubated with the kinase fraction for 2 h at room temperature.

can be seen that only the CT17N fragment was phosphorylated (Fig. 1B). Similar results were obtained when porcine serum gelsolin rather than calf serum gelsolin was phosphorylated (Fig. 1, B and C). Since the kinase fraction isolated from mitotic HeLa cells contains mainly *cdc2* kinase activity (Yamashiro *et al.*, 1990, 1991), and since plasma gelsolin was also phosphorylated by *cdc2* beads (Fig. 1A), *cdc2* kinase is responsible for this gelsolin phosphorylation (Yamakita *et al.*, 1994; Yamashiro *et al.*, 1990, 1991). The consensus phosphorylation sequence of this kinase can be found in gelsolin at several sites (Way and Weeds, 1988; Weeds *et al.*, 1986). Our results, however, show that the phosphorylation site is located near the NH₂-terminus of plasma gelsolin, because the CT17N fragment was produced from the NH₂-terminal region (Fig. 1D). Phosphorylation at this site was confirmed by an experiment in which cytoplasmic gelsolin isolated from porcine heart was not phosphorylated by this kinase (Fig. 1E). Furthermore, the plasma and cytoplasmic forms contain the same core structure but only the plasma gelsolin form has an extended sequence at its NH₂-terminus (Bryan and Hwo, 1986; Kwiatkowski *et al.*, 1985, 1986). Therefore, these results indicate that the phosphorylation site is located within the excessive sequence which only plasma gelsolin has at its NH₂-terminus. It is noted here that, since the maximum efficiency of gelsolin phosphorylation did not exceed one mole/mole gelsolin (data not shown), only a single site might be phosphorylated.

Actin-severing activity of phosphorylated gelsolin

Only in the presence of Ca²⁺ can gelsolin sever F-actin and form a cap on its newly exposed barbed end, or stimulate nucleation for actin polymerization (Yin, 1987). Phosphorylated plasma gelsolin, however, also becomes active in the absence of Ca²⁺. In the presence of Ca²⁺, since gelsolin can sever F-actin regardless of whether it is phosphorylated or not, the phosphorylated and the unphosphorylated forms can decrease the amount of F-actin in a similar and concentration dependent manner. On the other hand, in the absence of Ca²⁺, although the unphosphorylated form completely lost its severing activity, the phosphorylated form could still sever F-actin (Fig. 2A). The severing activity of the phosphorylated sample in the absence of Ca²⁺ was about 25% that measured in the presence of Ca²⁺. This is however, consistent with the fact that about 25 % of the gelsolin in this sample was phosphorylated (data not shown).

This was further confirmed by following the severing activity during gelsolin phosphorylation and dephosphorylation (Fig. 2, B and C, respectively). In the absence of Ca²⁺ the severing activity increased or decreased with incubation time in the presence of kinase or phosphatase, respectively. The loss of severing activity after phosphatase treatment was due to the decrease in the phosphorylated form of gelsolin molecules as shown by the fact that the activity of

Fig. 2. The actin severing activity of phosphorylated plasma (calf serum) gelsolin. (A) The severing activities at various concentrations of gelsolin. The closed and open circles show the activities of unphosphorylated and phosphorylated gelsolin samples in the presence of 2 mM EGTA, respectively. The closed and open squares show the activities of unphosphorylated and phosphorylated gelsolin samples in the presence of 0.2 mM CaCl₂, respectively. Note here that similar results were obtained when porcine serum gelsolin was phosphorylated instead of gelsolin from calf serum. (B) The change of the actin severing activity of gelsolin during its phosphorylation by the kinase fraction in the presence of 2 mM EGTA (open circles). (C) The change of the actin severing activity of phosphorylated gelsolin during its dephosphorylation by potato acidic phosphatase in the presence of 2 mM EGTA (open circles). The closed circle indicated by the arrow shows the severing activity of the dephosphorylated plasma gelsolin in the presence of Ca²⁺. In (B) and (C) the broken line shows flow birefringence under the same conditions when no gelsolin was added.

the dephosphorylated sample remained in the presence of Ca^{2+} (Fig. 2C, closed circle). This restoration of the Ca^{2+} -dependence of gelsolin by phosphatase treatment indicates that the effect of phosphorylation on this protein is reversible.

The actin-severing speed of phosphorylated gelsolin in the presence of Ca^{2+} was indistinguishable from that of the unphosphorylated protein in the presence of Ca^{2+} , while the activity of the phosphorylated sample in the absence of Ca^{2+} was 25% that in the presence of Ca^{2+} (Fig. 3). This decreased activity of the phosphorylated sample is also consistent with the fact that about 25 % of the gelsolin in this sample was phosphorylated. The results shown in Figure 2A and 3 can be readily explained if the net concentration of gelsolin which can sever F-actin is equal to the phosphorylated gelsolin concentration. These results suggest that the actin-severing mechanism of phosphorylated gelsolin in the absence of calcium, and that of unphosphorylated gelsolin in the presence of calcium are probably the same.

Actin-capping activity of phosphorylated gelsolin

The two ends of actin filaments have significantly different rates of assembly and disassembly. The barbed ends of F-actin polymerize 10 times faster than the pointed ends. Because gelsolin can not only sever actin filaments but also can cap their barbed ends and block the monomer association at those ends, the critical concentration for actin polymerization is increased when gelsolin is active. Therefore,

Fig. 3. Time course of actin severing by calf serum gelsolin. The closed and open circles show the time course of the activities of unphosphorylated and phosphorylated gelsolin samples in the presence of 2 mM EGTA, respectively. The closed and open squares show the time course of the activities of unphosphorylated and phosphorylated gelsolin samples in the presence of 1.5 mM CaCl_2 , respectively. In either case, the concentration of calf serum gelsolin was 0.3 μM . Note here that similar results were obtained when porcine serum gelsolin was phosphorylated instead of gelsolin from calf serum.

under our experimental conditions, both phosphorylated and unphosphorylated gelsolin increased the critical concentration 10-fold in the presence of Ca^{2+} (Fig. 4A). On the other hand, when Ca^{2+} was absent, gelsolin which was not phosphorylated lost virtually all its activity (Fig. 4B). The phosphorylated gelsolin, however, still showed its capping activity and increased the actin critical concentration 8-fold in the absence of Ca^{2+} . It is noted here that the increasing rate of the actin critical concentration when the DEAE-purified

Fig. 4. Actin capping activity of phosphorylated and unphosphorylated plasma (calf serum) gelsolin. This activity was evaluated by increasing the critical concentration for actin polymerization when gelsolin was added. (A) Results obtained in the presence of 0.2 mM CaCl_2 . (B) Results obtained in the presence of 2 mM EGTA. To obtain each critical concentration, the amount of polymerized actin at five different actin concentrations (3, 6, 12, 24, or 36 μM) in the presence of unphosphorylated (closed circles) or phosphorylated (open circles) gelsolin, or in the absence of gelsolin (crosses), were determined by flow birefringence. These results were averages of three different sets of experiments. When no actin was present or polymerized, the flow birefringence is 4.3. The calculated critical concentrations (C_c) are shown in the insets. None, gelsolin was absent; -P and +P, unphosphorylated and phosphorylated gelsolin were added, respectively. In this case, to obtain clearer results, all gelsolin samples used were DEAE-purified phosphorylated or unphosphorylated calf serum gelsolin (see *Materials and Methods*).

phosphorylated gelsolin was used in the absence of Ca^{2+} was consistent with the fact that about 90 % of the gelsolin in that sample was phosphorylated (see *Material and Methods*).

Actin-nucleation activity of phosphorylated gelsolin

Gelsolin has yet another activity in addition to the above two activities. This third activity also stimulates actin nucleation in a Ca^{2+} -dependent manner. When Ca^{2+} was present, gelsolin shortened the lag phase which was seen in the early stages of actin polymerization (Fig. 5A). When Ca^{2+} was absent, unphosphorylated gelsolin had no effect on actin nucleation, and only phosphorylated gelsolin stimulated actin nucleation and assembly (Fig. 5B). It is noted here that the amount of assembled actin at equilibrium using phosphorylated gelsolin in the absence of Ca^{2+} was consistent with the fact that about one third of the gelsolin in that sample was

phosphorylated.

Discussion

Since the plasma and cytoplasmic forms of gelsolin are encoded by a single gene and differ as the result of alternative splicing, both forms of gelsolin contain the same highly conserved core structure and differ only at the extended leader sequence of the plasma form (Bryan and Hwo, 1986; Kwiatkowski *et al.*, 1985, 1986; Stella *et al.*, 1994; Way and Weeds, 1998). This additional NH_2 -terminus region consists of at least 9 additional amino acid residues and occasionally contains signal and propeptide sequences. The consensus phosphorylation sequence of cdc2 kinase (Ser/Thr-Pro) is found in this region of porcine plasma gelsolin (Way and Weeds, 1988; Weeds *et al.*, 1986; Yamakita *et al.*, 1994; Yamashiro *et al.*, 1990). This leader sequence region of plasma gelsolin is much less conserved and the amino acid sequence of calf serum gelsolin has not yet been solved. However, identical results were obtained in this study when calf plasma or porcine plasma gelsolin was phosphorylated. Additionally, it has recently been reported that cdc2 kinase can phosphorylate several sites besides the well-known consensus phosphorylation sequence of Ser/Thr-Pro (Yamakita *et al.*, 1994). Therefore, our results that only porcine plasma gelsolin but not porcine cytoplasmic gelsolin could be phosphorylated, indicate that the phosphorylation site is probably located within the extended leader sequence which exists only at the NH_2 -terminus of the plasma form of gelsolin. This was confirmed by the result that the CT17N fragments of calf and porcine plasma gelsolins were phosphorylated. Moreover, since the maximum efficiency of gelsolin phosphorylation did not exceed one mole/mole gelsolin, only a single site might be phosphorylated.

The activities of phosphorylated samples of gelsolin in the absence of Ca^{2+} were consistent with the percentage of phosphorylated protein in the total gelsolin sample (Figs. 2–5). These results suggest that the activities of phosphorylated gelsolin in the absence of calcium, and of unphosphorylated gelsolin in the presence of calcium, are essentially the same. Gelsolin has a 40 kDa NH_2 -terminal half and a 45 kDa COOH -terminal half. Severing and subsequent capping, and nucleation activities are located in the NH_2 -terminal domain (Bryan and Hwo, 1986; Kwiatkowski *et al.*, 1985, 1989; Yin *et al.*, 1988), while Ca^{2+} -binding sites are located in the COOH -terminal domain (Bryan and Hwo, 1986; Pope *et al.*, 1995; Way *et al.*, 1989; Weeds *et al.*, 1995). It has been demonstrated that the NH_2 -terminal fragment shows the same activities as intact gelsolin but in a Ca^{2+} -independent manner (Bryan and Hwo, 1986; Chaponnier *et al.*, 1986; Yin *et al.*, 1988). The NH_2 -terminal domain is thought to be involved in side-binding to F-actin, and probably penetrates into a filament to break a bond between two actin monomers, while continuously capping its barbed end. The COOH -terminal half of gelsolin inhibits

Fig. 5. The actin nucleating activity of phosphorylated and unphosphorylated plasma (calf serum) gelsolin. (A) The time course of actin polymerization in the presence of 0.2 mM CaCl_2 . (B) The time course of actin polymerization in the absence of Ca^{2+} (2 mM EGTA). The closed and open circles show the amount of F-actin polymerized in the presence of unphosphorylated and phosphorylated gelsolin, respectively. The crosses show the amount of F-actin polymerized when no gelsolin was added. Note here that similar results were obtained when porcine serum gelsolin was phosphorylated instead of gelsolin from calf serum.

these interactions between actin and the NH₂-terminal of gelsolin by masking the actin-binding sites of the NH₂-terminal domain (Kwiatkowski *et al.*, 1989). When micromolar concentrations of Ca²⁺ are added, a large conformational change, which permits the NH₂-terminal half of gelsolin to interact with F-actin by removing the masking, is induced in the COOH-terminal half by the binding of Ca²⁺ to this region (Fig. 6) (Bryan and How, 1986; Chaponnier *et al.*, 1986; Feinberg *et al.*, 1998; Hellweg *et al.*, 1993; Weber *et al.*, 1991). Phosphorylated and unphosphorylated gelsolin, however, showed the same CD spectra in either the presence or absence of Ca²⁺ (data not shown). These results suggest either that the phosphorylation at the NH₂-terminus of plasma gelsolin affects the Ca²⁺-dependence of this protein not by changing the structure of the COOH-terminal half, or that the conformational change induced may be restricted to a small part of the COOH-terminal region and/or is too small to be detected by CD spectra. For this reason, the affinity of phosphorylated gelsolin for F-actin might not be distinguishable from that of unphosphorylated gelsolin, even when Ca²⁺ is absent (data not shown).

The three-dimensional structure of gelsolin has been speculated to be "claw-like" in that its NH₂- and COOH-termini are positioned near each other (Fig. 6); this speculation was recently confirmed by crystallography (Burnick *et al.*, 1997). This closed claw-like structure can be opened by Ca²⁺-binding as described above and this opening may expose the gelsolin active sites to actin. Moreover, it has been reported that there is a pseudo-actin sequence with several

acidic amino residues similar to that found on the NH₂-terminus of actin, near the carboxyl-end of the gelsolin COOH-terminal, and that sequence seems to be important for the Ca²⁺-regulation of gelsolin (Kwiatkowski *et al.*, 1989). When the NH₂-terminus of gelsolin is phosphorylated, gelsolin shows its various activities even when Ca²⁺ is absent, and this may result from opening of the claw-like structure of gelsolin by electrical repulsive forces between both peptide ends of this protein (Fig. 6). Similarly, when the pH is lower than 6.0, gelsolin becomes active even when Ca²⁺ is absent (Lamb *et al.*, 1993), probably since the negative charges of the COOH-terminus pseudo-actin domain would be cancelled (Fig. 6) (Chaponnier *et al.*, 1986, Kwiatkowski *et al.*, 1989). It has recently been reported that human gelsolin could be phosphorylated by c-Src kinase at the Tyr438 residue located in the S4 segment, and the property of this phosphorylation has been revealed (De Corte *et al.*, 1997, 1999). This phosphorylation is stimulated by phosphatidylinositol 4,5-bisphosphate (PIP₂), a negative regulator of gelsolin activities (De Corte *et al.*, 1997). It is thought that phosphorylation at this tyrosine residue and actin-binding of gelsolin hinder each other sterically, because tyrosine phosphorylation of gelsolin in the gelsolin-actin₂ complex is almost completely inhibited (De Corte *et al.*, 1999). This opposing effect of tyrosine phosphorylation may be due to the location of its phosphorylation site. The S4 segment that contains the tyrosine phosphorylation site is located near the root of the claw-like structure of gelsolin, while the site of phosphorylation found in our study is located at the tip of the claw-like structure.

These findings may help us understand the mechanism of Ca²⁺-regulation of this well-known actin-binding protein found widely in cells and tissues, and also may help us understand general mechanisms of how protein phosphorylation can modify protein function, and may even provide a novel possible approach for the modification of enzyme activities. To investigate more detailed structural and mechanistic information concerning how proteins work, or to construct useful new proteins, several approaches have been developed by protein engineering using the genetic and chemical modification of proteins (Douglas, 1992; Fersht and Winter, 1992; Neideman, 1994; Shao and Arnold, 1996). Since the site of phosphorylation studied in this report appears to be a single one and since it is located within an extended sequence that only plasma gelsolins have at their NH₂-terminus, a region which does not contribute to the activity of gelsolin, our results suggests that single charge modifications, such as phosphorylation, at sites away from a protein's core structure can alter a protein's function.

For the same reason, it is not likely that the phosphorylation studied in this report is actually functioning in living cells, since the kinase fraction used contains primarily cdc2 kinase activity. However, regulation of the activities of gelsolin using this artificial phosphorylation system is very simple and reversible (Fig. 2, B and C). On the other hand,

Fig. 6. A schematic model shows the conformational change of plasma gelsolin induced by the addition of Ca²⁺, by decreasing the pH, or by phosphorylation.

the reported mechanisms of gelsolin regulation that depend on calcium and PIP2 are very complicated and still remain unclear (DiNubile 1998; Janmey and Stossel, 1987, Lin *et al.*, 1997; Yin, 1987). As described above, human gelsolin can also be phosphorylated by c-Src kinase, as reported recently (De Corte *et al.*, 1997, 1999). Therefore, there is a possibility that still undiscovered kinases phosphorylate and regulate cytoplasmic gelsolin in living cells, and that cells have evolved mechanisms for regulating these proteins.

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