Volume 259, number 1, 185–188 FEB 07952 December 1989

Loss of calcium sensitivity of plasma gelsolin is associated with the presence of calcium ions during preparation

B. Pope, J. Gooch, H. Hinssen* and A.G. Weeds

MRC Laboratory of Molecular Biology, Hills Road, Cambridge, England CB2 2QH and **Developmental Biology Unit, University of Bielefeld, D4800 Bielefeld, FRG*

Received 28 August 1989; revised version received 26 October 1989

Gelsolin is a calcium-dependent actin severing and capping protein. Calcium 'opens' the molecule to make actin binding sites accessible, but removal of calcium from the medium does not necessarily fully reverse this process. The calcium sensitivity of actin monomer binding and actin filament severing is here shown to vary considerably with the source of gelsolin and conditions of preparation. Plasma gelsolin undergoes irreversible loss of calcium sensitivity when prepared in the presence of calcium ions. This is not due solely to effects of bound calcium, because purified human plasma gelsolin expressed in *E. coli* and stored in calcium shows no comparable loss of calcium sensitivity when prepared or stored in calcium. These results suggest the presence of factors in plasma which, in the presence of calcium, promote an irreversible structural change in gelsolin resulting in permanent loss of calcium sensitivity.

Gelsolin; Actin binding protein; Actin filament severing; Calcium sensitivity

1. INTRODUCTION

Gelsolin interacts with both polymeric and monomeric forms of actin. It severs filaments and caps their barbed ends, thereby increasing the critical monomer concentration and preventing re-annealing. It binds 2 actin monomoners in the presence of calcium ions, forming ternary complexes which, under polymerizing conditions, form nuclei and thereby accelerate filament formation [1–4]. The nucleating and severing activities are calcium dependent, but removal of calcium does not reverse all actin binding [3–5]. For example, only one monomer dissociates from the ternary complex, leaving a binary complex containing trapped calcium, which cannot be dissociated without denaturation [3]. Similar properties have been reported for gelsolins from a variety of sources, including rabbit macrophage [6], human plasma [7] and pig stomach smooth muscle [8]. In contrast, pig plasma gelsolin has been shown to interact with actin both in the presence and absence of calcium [9–11]. Indeed calcium-dependent and calcium-independent forms of this gelsolin have been separated on actin–Sepharose [11].

Recent analysis of cDNA sequences has shown that the derived amino acid sequences of human and pig plasma gelsolins are 98% homologous [12,13]. Furthermore, there is no evidence for two different genotypes of pig gelsolin which might account for calcium-dependent and calcium-independent forms. Thus changes in calcium sensitivity are likely to arise from the conditions of purification and/or storage.

Purification of cytoplasmic gelsolins has conventionally been carried out in the presence of EGTA (to prevent interaction with cellular actin) [6], while plasma gelsolin has been purified by affinity chromatography on actin-Sepharose in the presence of calcium [14] (blood plasma contains about 2 mM calcium). Cytoplasmic gelsolin-actin complexes have also been purified in the presence of calcium by affinity chromatography on DNase I-agarose [15], but subsequent separation of the complexes in 6 M urea was done in 1 mM EGTA [3]. Hydroxyapatite has been used by a number of workers [e.g. 9,16], but extreme care is needed to avoid heavy losses and proteolysis [9].

Here we compare the calcium sensitivity of F-actin severing by a number of different gelsolins using a variety of preparative methods. Measurements are also made of monomer binding in EGTA. Results show that irreversible loss of calcium sensitivity of plasma gelsolin is associated with the presence of calcium during preparation and storage. However, prolonged exposure of purified plasma gelsolin to calcium does not reproduce this effect. The results suggest the presence of fac-

Correspondence address: A.G. Weeds, MRC Laboratory of Molecular Biology, Hills Road, Cambridge, England, CB2 2QH

Abbreviations: Fxgelsolin, human plasma gelsolin expressed in *E. coli* using the pLcIIFX expression vector [19], which directs synthesis of a hybrid protein containing the first 31-amino terminal residues of lambda cII protein, a Factor Xa recognition sequence (FX) as well as gelsolin. PI-actin, actin reacted on Cys 374 with N-(1-pyrenyl)iodoacetamide; NBD-actin, actin reacted with N-ethylmaleimide on Cys 374 then on Lys 373 with 7-chloro-4-nitrobenzeno-2-oxa-1,3-diazole

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

tors in plasma which, in the presence of calcium, promote the irreversible loss of calcium sensitivity of gelsolin.

2. MATERIALS AND METHODS

Pig plasma gelsolin (Ca) was prepared from fresh or frozen plasma using actin-Sepharose in the presence of calcium as described previously [14] (the parentheses are used to indicate whether the preparation was made throughout in calcium or otherwise). Pig plasma gelsolin (Ca/EGTA) was prepared by the method of Bryan [17], using DEAE-Sephacel columns first in 0.5 mM $CaCl_2$, (conditions in which gelsolin is not retarded), then in 0.1 mM EGTA, with the bound gelsolin eluted by a salt gradient. The gelsolin was further purified on CM-cellulose as described [14], except that 0.2 mM EGTA was included in all the buffers. These gelsolins were stored at −20°C.

Human plasma gelsolin (Ca) was also prepared as in [14]. Human plasma gelsolin (EGTA) was purified in the presence of 1 mM EGTA on an anti-gelsolin affinity column [7].

Pig stomach muscle gelsolin (EGTA) was purified by modification of earlier methods [16]. Briefly, stomach muscle was extracted with 5 vols of 40 mM imidazole-HCl pH 7.2, 20 mM KCl, 25 mM $MgCl_2$, 8 mM EGTA and 1 mM phenyl methane sulphonyl fluoride. (All buffers also contained 0.5 mM dithioerythritol.) The high [$MgCl_2$] minimises the extraction of myofibrillar proteins. After centrifugation at 10000 × *g* for 1 h, the supernatant was fractionated by ammonium sulphate precipitation. Protein precipitating between 40 and 55% was dissolved in 20 mM Imidazole-HCl pH 7.6, 50 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA (Buffer A) and, after dialysis, fractionated on a 30 × 3.5 cm column of DEAE Sepharose CL6B using a KCl gradient. After concentrating fractions with severing activity by ammonium sulphate precipitation, the gelsolin was further purified on a 90 × 2.5 cm column of Sephacryl S300 in Buffer A containing 100 mM KCl. It was then dialysed against 10 mM potassium phosphate pH 7.0, 1 mM EGTA. 1.5 mM $CaCl_2$ was added immediately prior to application to a 30 × 3.2 cm hydroxyapatite column, which was eluted with a gradient of 10–300 mM potassium phosphate, 0.5 mM $CaCl_2$. 1 mM EGTA was added to the gelsolin, which was finally chromatographed on a 1 ml Mono Q column (Pharmacia FPLC) in 20 mM Imidazole HCl pH 7.0, 50 mM KCl, 2 mM $MgCl_2$ and 1 mM EGTA, eluting with a 20 ml gradient to 400 mM KCl. The protein was stored as an ammonium sulphate precipitate at −70°C.

Fxgelsolin (human plasma gelsolin expressed as a fusion product in *E. coli*) was isolated as in [18] but purified without actin-Sepharose affinity chromatography either in the presence of calcium (fxgelsolin (Ca)) or in EGTA (fxgelsolin (EGTA)) using the following modifications: (i) fxgelsolin (Ca) was purified initially on a 30 × 1.5 cm column of DEAE-Sephacel in 10 mM imidazole-HCl pH 7.0, 0.1 mM $CaCl_2$ with a gradient from 0–150 mM NaCl (gelsolin elutes at ~ 55 mM NaCl), then, after dialysis into 10 mM sodium succinate pH 6.0, 35 mM NaCl, 0.1 mM $CaCl_2$, it was applied to a 30 × 1.5 cm column of Whatman CM-52 and eluted with a gradient to 200 mM NaCl (elution at ~80 mM NaCl); (ii) fxgelsion (EGTA) was purified on similar columns using the same buffers but with 0.2 mM EGTA in place of $CaCl_2$. The gelsolin elutes from DEAE-Sephacel ~ 150 mM CaCl and from CM-cellulose in ~ 80 mM NaCl. Proteins were concentrated by Centricon filtration and stored at −20°C. All gelsolins were analysed by SDS polyacrylamide gel electrophoresis and the DNase I inhibition assay was carried out as described previously [14].

The calcium dependence of the actin-severing activities of the gelsolins was assayed by viscometry using 22 μM F-actin in F-buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM ATP, 0.1 mM dithiothreitol, 1 mM sodium azide and either 0.1 mM $CaCl_2$ or 0.5 mM EGTA) as described earlier [11]. Flow times for individual assays were reproducible to <1%, giving an error rate of <4% in specific viscosities. Calcium sensitivity is defined as: (1- (% reduction of viscosity in EGTA/% reduction of viscosity in calcium). Values given were standardly calculated at a molar ratio of gelsolin to actin subunits of 1:70.

The effects of long term storage in calcium of fxgelsolin (EGTA) were assessed as follows: 2 mM $CaCl_2$ was added to fxgelsolin (EGTA) in 10 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM EGTA 3 mM sodium azide and 0.5 μg/ml leupeptin and the protein was stored for up to 3 weeks at 4°C. Samples taken after various times for viscometry were preincubated for 10 min in F-buffer containing 1 mM EGTA (> 10-fold excess over calcium present) and assayed either in EGTA or calcium as described [11].

The binding of gelsolin to monomeric actin was measured by fluorescence enhancement as described previously [10], using 200 nM NBD-actin in 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, 0.8 mM EGTA, 0.2 mM ATP and 0.2 mM dithiothreitol.

3. RESULTS

All gelsolins prepared by the different methods used here gave a single band on gel electrophoresis (see [14,18], also Huckriede et al., in preparation). Fxgelsolin has identical actin severing, binding and nucleating properties in calcium as pig plasma and human plasma gelsolins [18].

Fig.1A shows the effects of gelsolins from pig plasma and pig stomach muscle on the viscosity of 22 μM F-actin in calcium and EGTA. All three preparations gave

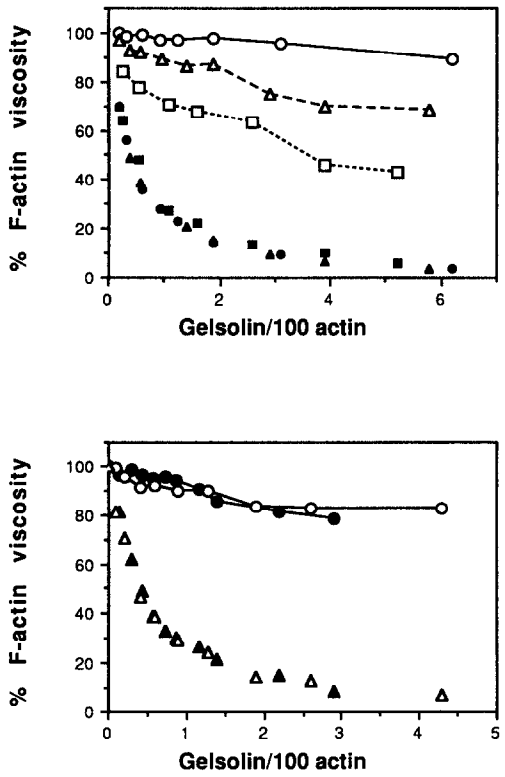

Fig.1. Effects of different preparations of gelsolin on the viscosity of F-actin. (A) Gelsolins were mixed with F-actin in either 0.1 mM $CaCl_2$ (solid symbols) or 0.5 mM EGTA (open symbols) at the molar ratios shown and measurements completed within 2.5 min: pig stomach gelsolin (EGTA) (● ○); pig plasma gelsolin (Ca/EGTA) (▲ △); pig plasma gelsolin (Ca) (■ □) (B) Similar experiments using fxgelsolin (Ca) (solid symbols) and fxgelsolin (EGTA) (open symbols). Measurements were made in calcium (▲ △) or EGTA (● (○) as above.

virtually identical effects in calcium, showing that their severing activities were similar. However, the extent of severing in EGTA differed considerably. Pig stomach gelsolin (EGTA) showed the highest level of calcium sensitivity and pig plasma gelsolin (Ca) the lowest. For example, at a molar ratio of gelsolin to actin subunits of 1:70, conditions under which the viscosity in calcium was reduced to about 20% of the F-actin control, the calcium sensitivities were as follows: pig stomach gelsolin (EGTA)>95%, pig plasma gelsolin (Ca/EGTA) = 83% and pig plasma gelsolin (Ca) = 58%. This value for pig plasma gelsolin is similar to that reported earlier [11]. These results suggest that loss of calcium sensitivity is associated with exposure to calcium ions during gelsolin preparation.

To test whether the presence of calcium during purification is the cause of this effect, viscometric assays were carried out on fxgelsolin prepared by ion exchange chromatography either in calcium or EGTA (fig.1B). The calcium sensitivities of each preparation was ~ 90% at a gelsolin:actin ratio of 1/70.

Prolonged exposure of purified fxgelsolin (EGTA) to calcium was assessed over a 3 week period at 4°C. The calcium sensitivity after 10 days in a calcium-containing solution was 95% (similar to that measured initially for this preparation). After 16 and 21 days this value had dropped to 91% and 86% respectively (compared to control values after storage in EGTA of 96% and 94%). Thus exposure of fxgelsolin to calcium either during preparation or prolonged storage is not sufficient to induce a significant shift in calcium sensitivity.

Calcium-insensitive gelsolin has been shown to bind monomeric actin in the absence of calcium with a K_d ~ 30–60 nM [10]. Fig.2 shows fluorescence enhancement of NBD-actin when mixed with a number of gelsolin preparations in EGTA. Human plasma gelsolin (Ca) gave a maximum fluorescence increase of over 130%, similar to that observed for at least 4 different preparations of pig plasma gelsolin (Ca). By contrast, human plasma gelsolin (EGTA) gave maximally little over 20% fluorescence enhancement and pig stomach gelsolin (EGTA) even less. All these gelsolins gave a maximum enhancement of 130–140% in 0.1 mM $CaCl_2$. From these experiments it is evident that the decreased calcium sensitivity affects not only the severing activity of gelsolin but also its monomer binding.

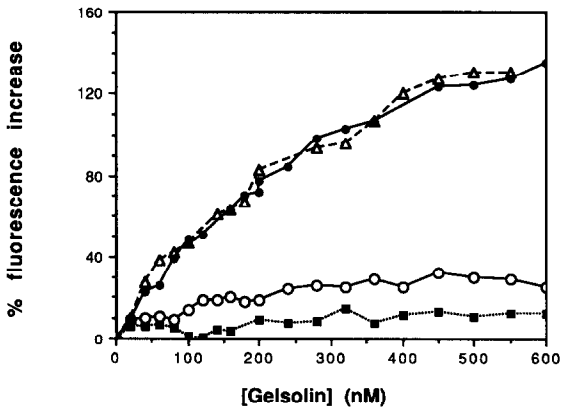

Fig.2. Fluorescence titrations of different gelsolins with 200 nM NBD-actin in EGTA. Human plasma gelsolin (Ca) (—●—); human plasma gelsolin (EGTA) (—○—); pig stomach gelsolin (EGTA) (... ■ ...); pig plasma gelsolin (Ca) (—△—).

4. DISCUSSION

These results show that the calcium sensitivity of both the F-actin severing and G-actin binding properties of gelsolins depends critically on the method of preparation. Pig stomach muscle gelsolin (EGTA) showed the highest level of calcium sensitivity for interaction with both F- and G-actins. Human plasma gelsolin (EGTA) prepared on antibody affinity columns or cloned in *E. coli* also showed high calcium sensitivity (see also [18]). By contrast, both porcine gelsolin (Ca) and human gelsolin (Ca) prepared from plasma in the presence of calcium showed considerable loss of calcium sensitivity in binding and severing assays. Pig plasma gelsolin (Ca/EGTA) isolated initially in calcium but with final purification stages in EGTA gave an intermediate level (fig.1A).

Loss of calcium sensitivity does not depend solely on prolonged exposure to calcium ions. This is evident both from the identical properties of fxgelsolin prepared either in calcium or EGTA (fig.1B) and from experiments in which fxgelsolin was stored in the presence of calcium. Although there was some deterioration in the level of calcium sensitivity after 3 weeks in calcium, this was not as marked as that observed with gelsolin prepared from plasma in calcium. Loss of calcium sensitivity must therefore be associated in addition with other factors in the plasma preparation. One possibility is that loss of calcium sensitivity is associated with structural changes when bound gelsolin is eluted from actin-Sepharose in 4 M $MgCl_2$. However, fxgelsolin purified in a similar manner is fully calcium-sensitive [18], which shows that this treatment per se has no deleterious effect on gelsolin structure. The intermediate level of calcium sensitivity of plasma gelsolin (Ca/EGTA) suggests the involvement of as yet unidentified components from plasma. This is consistent with the observation that the level of calcium sensitivity of pig plasma gelsolin (Ca) varied between different preparations [9,11,14] (usually in the range 50–70%). It is not yet clear whether these components are activated only in the presence of calcium ions or whether the conformational changes that occur when gelsolin binds calcium renders the molecule more sensitive to irreversible structural modifications.

Although blood plasma contains about 2 mM calcium and there is no evidence for any deleterious effects of the ionic milieu in blood on the calcium sensitivity of gelsolin in situ, these results emphasize the

importance of preparing gelsolin in the absence of calcium.

Acknowledgements: We thank Dr Christine Chaponnier for providing human plasma gelsolin prepared on anti-gelsolin columns and Dr R. Cross for critically reading the manuscript.

REFERENCES

[1] Yin, H.L. (1988) Bioessays 7, 176–179.
[2] Way, M., Gooch, J., Pope, B. and Weeds, A.G. (1988) In: Structure and Functions of the Cytoskeleton, Biological and Physiopathological Aspects, (Rousset, B., ed.) 171, 401–413.
[3] Bryan, J. and Kurth, M.C. (1984) J. Biol. Chem. 259, 7480–7487.
[4] Doi, Y. and Frieden, C. (1984) J. Biol. Chem. 259, 11868–11875.
[5] Coué, M. and Korn, E.D. (1985) J. Biol. Chem. 260, 15033–15041.
[6] Janmey, P.A., Chaponnier, C., Lind, S.E., Zaner, K.S., Stossel, T.P. and Yin, H.L. (1985) Biochemistry 24, 3714–3723.
[7] Chaponnier, C., Janmey, P.A. and Yin. H.L. (1986) J. Cell Biol. 103, 1473–1481.
[8] Hinssen, H. (1987) Fortschritte der Zoologie, Band 34, 53–63. Wolhlfarth-Bottermann (Hsrg): Nature and Function of Cytoskeletal Protein in Motility and Transport. Fischer, Stuttgart.
[9] Harris, H.E. and Weeds, A.G. (1983) Biochemistry 22, 2728–2741.
[10] Weeds, A.G., Harris, H., Gratzer, W.B. and Gooch, J. (1986) Eur. J. Biochem. 161, 77–84.
[11] Pope, B. and Weeds, A.G. (1986) Eur. J. Biochem. 161, 85–93.
[12] Kwiatkowski, D.J., Stossel, T.P., Orkin, S.H., Mole, J.E., Colten, H.R. and Yin, H.L. (1986) Nature 323, 455–458.
[13] Way, M. and Weeds, A.G. (1988) J. Mol. Biol. 203, 1127–1133.
[14] Weeds, A.G., Gooch, J., Pope, B. and Harris, H.E. (1986) Eur. J. Biochem. 161, 69–76.
[15] Kurth, M.C., Wang, L.-L., Dingus, J. and Bryan, J. (1983) J. Biol. Chem. 258, 10895–10903.
[16] Hinssen, H., Small, J.V. and Sobieszek, A. (1984) FEBS Lett. 166, 90–95.
[17] Bryan, J. (1988) J. Cell Biol. 106, 1553–1562.
[18] Way, M., Gooch, J., Pope, B., and Weeds, A.G. (1989) J. Cell Biol. 109, 593–605.
[19] Nagai, K. and Thøgersen, H.C. (1984) Nature 309, 810–812.