Plant profilin induces actin polymerization from actin : β-thymosin complexes and competes directly with β-thymosins and with negative co-operativity with DNase I for binding to actin

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Abstract Recombinant plant (birch) profilin was analyzed for its ability to promote actin polymerization from the actin:thymosin β_4 and β_9 complex. Depending on the nature of the divalent cation, recombinant plant (birch) profilin exhibited two different modes of interaction with actin, like mammalian profilin. In the presence of magnesium ions birch profilin promoted the polymerization of actin from A:Tβ_4. In contrast, in the presence of calcium but absence of magnesium ions birch profilin was unable to initiate the polymerization of actin from the complex with Tβ_4. However, under these conditions profilin formed a stable stoichiometric complex with skeletal muscle α-actin, as verified by its ability to increase the critical concentration of actin polymerization. Chemical cross-linking indicated that birch profilin competes with Tβ_4 for actin binding. Ternary complex formation of birch profilin with actin:DNase I complex was suggested by chemical cross-linking. However, the determination of the critical concentrations of actin polymerization in the simultaneous presence of birch profilin and DNase I indicated that profilin and DNase I did not form a ternary complex. These data indicated a negative co-operativity between the profilin and DNase I binding sites on actin.

Key words: Actin; Plant profilin; DNase I; β-Thymosin

1. Introduction

The intracellular pool of monomeric (G-)actin is supposed to be maintained by a number of G-actin binding proteins. The first actin monomer stabilizing protein identified was profilin [1]. However, it was subsequently realized that the intracellular concentration of profilin is not high enough to sequester all the actin normally found in unpolymerized form (for review see [2]). Recently, a family of 5 kDa proteins was identified, the β-thymosins, that are able to stabilize G-actin by forming 1:1 stoichiometric complexes [3,4]. Their most prominent variant is thymosin β_4. In many vertebrate cell types the β-thymosins occur in sufficiently high concentration to explain the high concentration of G-actin. Furthermore, it was realized that profilin is not a mere actin sequestering protein, but instead is able to promote actin polymerization from the actin:thymosin complex [5]. Immunohistochemistry showed that profilin is particularly enriched in regions of rapid actin polymerization and depolymerization like in the cortical web of actively migrating cells [6].

In a previous publication [7], we showed that birch pollen profilin is able to interact with skeletal muscle α-actin. It was also shown that birch profilin is able to inhibit chemical cross-linking of Tβ_4 to actin [7]. These results were taken as evidence that this plant profilin exhibits binding characteristics to actin similar to mammalian profilin, although both profilins differ considerably in their primary structures. Recently, the tertiary structures of the complex of bovine spleen profilin and cytoplasmic β-actin [8] and of human [9], *Acanthamoeba* [10], birch pollen [11] and *Arabidopsis* profilin [12] have been solved by X-ray crystallography or NMR techniques. These results have indeed demonstrated that the tertiary structures of these profilins are almost identical in spite of their sequence differences.

Here, we analyzed the interaction of birch profilin with actin, actin:thymosin and actin:DNase I complexes quantitatively. We demonstrate that birch profilin is also able to promote actin polymerization from the actin:thymosin complex. This effect was, however, found to be restricted to certain ionic conditions, i.e. it was only observed in the presence of Mg^{2+}, but not in the presence of Ca^{2+} ions.

2. Materials and methods

2.1. Protein preparations

Rabbit skeletal muscle actin was purified as described previously [13] and taken up in 5 mM HEPES-OH, pH 7.4, 0.1 mM $CaCl_2$, 0.5 mM NaN_3, and 0.2 mM ATP (G-buffer). Birch pollen profilin was expressed in *Escherichia coli* and isolated as detailed previously [7]. Human plasma gelsolin was expressed in *E. coli* and purified as described in [14]. Bovine pancreatic deoxyribonuclease I (DNase I; EC 3.1.21.1) was a commercial product (Paesel and Lorei, Frankfurt, Germany) and further purified by ion-exchange chromatography on hydroxylapatite as described [13]. Tβ_4 and Tβ_{9met} were isolated from bovine and pig spleen, respectively, as given in [15]. Pyrene-labeled actin (PI-actin) was prepared by following exactly the procedure described in [16].

2.2. Gel electrophoretic procedures

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) was performed as given by [17].

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Abbreviations: A:P, actin:profilin complex; A:Tβ_4, actin:thymosin β_4 complex; A:D, actin:DNase I complex; A:D:P, actin:DNase I:profilin complex; C_c, critical concentration of actin polymerization; DNase I, deoxyribonuclease I (EC 3.1.21.1); FFD, 1,5-difluoro-2,4 dinitrobenzene; HEPES, 4-(hydroxyethyl)-1-piperazineethanesulfonic acid; PI-actin, pyrenyl-actin; Tβ_4 or Tβ_9, thymosin β_4 or β_9

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2.3. Chemical cross-linking

Chemical cross-linking with FFD (1,5-difluoro-2,4-dinitrobenzene) was performed exactly as detailed previously [18].

2.4. Determination of the critical concentration of actin polymerization (C_c)

C_c for actin alone or in the presence of constant amounts of monomer binding protein was determined by measuring the fluorescence decrease of serially diluted samples of F-actin supplemented with 4% of PI-actin after a preincubation period of 16 h at room temperature. The excitation and emission wavelengths were set at 365 nm and 385.5 nm, respectively. Relative fluorescence readings were done on a Shimadzu RF-5001-PC spectrofluorometer as detailed in [18].

The time course of actin polymerization from A:Tβ_4 induced by birch profilin was determined after depolymerization of F-actin containing 4% PI-actin by Tβ_4 to produce free plus ends of F-actin in equilibrium with A:Tβ_4. The depolymerization process was allowed to continue until reaching the equilibrium (after about 16 h) at room temperature. Then profilin was added and the relative fluorescence increase was followed with time.

2.5. Materials

The chemical cross-linker FFD was obtained from Serva (Heidelberg, Germany). Pyrenyliodoacetamide was purchased from Paesel and Lorei (Frankfurt, Germany). All other reagents were of analytical grade.

3. Results

3.1. Plant profilin binds to mammalian α-actin

The affinity of birch profilin to rabbit skeletal muscle actin was determined under a number of different ionic conditions by determining its effect on C_c. When gelsolin capped F-actin was used, profilin increased the critical concentration of actin polymerization in the presence of 2 mM $CaCl_2$ (Fig. 1a). This effect was attributed to the formation of a stable actin:profilin complex. It can also be seen from Fig. 1a that profilin apparently increased the extent of actin polymerization above C_c (steeper slope of the C_c plot). This effect has been observed previously for mammalian profilin and was attributed to its preferential binding to unmodified actin thus increasing the concentration of free PI-actin available for the polymerization reaction [19]. Here we demonstrate the identical behavior for birch profilin. From the shift of the C_c the dissociation constant K_D of birch profilin to α-actin was calculated to be 1.83 μM. A similar result was obtained by using uncapped actin filaments (Fig. 1b), although a significantly higher affinity for profilin binding to actin was expected. Under these conditions and in the presence of 2 mM $CaCl_2$ or 50 mM KCl, the K_D of birch profilin to α-actin were determined to be 3.25 μM and 6.53 μM, respectively, indicating a lower affinity in the absence of divalent and presence of high concentrations of monovalent cations. The determined affinities for birch profilin are in good agreement with our own previous results [7] and recently determined values for *Arabidopsis* [20] and maize pollen profilin [21], but lower than the affinities of mammalian (bovine spleen) profilin to actin (about 0.45 μM [5]).

3.2. Profilin induces actin repolymerization from the actin:thymosin β_9 complex

The β-thymosins inhibit the salt induced polymerization of actin [2–4,22]. When added to F-actin, β-thymosins were shown to induce its slow depolymerization [23]. Addition of plant profilin to preformed A:Tβ_4 under polymerizing conditions, i.e. in the presence of free filament ends and 2 mM $MgCl_2$, led to a partial repolymerization of actin that was followed fluorometrically with time (Fig. 2a). This polymerization effect was not observed in the presence of 2 mM $CaCl_2$ (Fig. 2a), as previously reported for mammalian profilin [20].

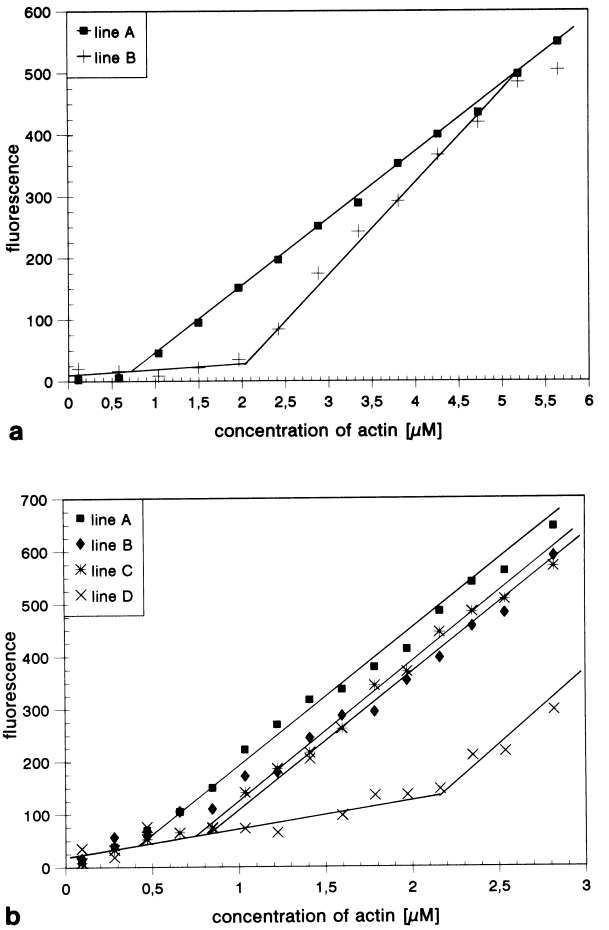

Fig. 1. a: Determination of K_D of birch profilin to α-actin C_c was determined as detailed in Section 2. Line A, actin alone (C_c: 0.7 μM) and line B, actin in the presence of 4.86 μM birch profilin (C_c: 2.04 μM). The reaction was carried out in a buffer containing 10 mM imidazole-HCl, pH 7.0, 1 mM ATP and 2 mM $CaCl_2$. The plus ends of F-actin were capped by gelsolin (molar ratio actin:gelsolin = 300:1) to prevent binding to A:P. b: Comparison of K_D values of birch profilin to α-actin under different ionic conditions. The reaction was carried out in a buffer containing 10 mM imidazole-HCl, pH 7.0, 1 mM ATP and 50 mM KCl (line A/B) or 2 mM $CaCl_2$ (lines C/D). Lines A/C, actin alone (C_c: 0.41 μM/0.76 μM) and lines B/D, actin in the presence of 7.275 μM birch profilin (C_c: 0.84 μM/2.14 μM)

The polymerizing effect of birch pollen profilin was also observed when F-actin had been depolymerized by Tβ_9 (Fig. 2b), which is known to bind to G-actin with a twofold higher affinity than Tβ_4 (see Fig. 2c and [24]). The mode of interaction of birch profilin with actin was further analyzed kinetically by determining C_c in the simultaneous presence of Tβ_9 and 2 mM $MgCl_2$ (Fig. 2c). It can be seen that profilin decreased C_c in the presence of Tβ_9. Under these experimental conditions the estimated C_c of actin alone was 0.235 μM and 0.8675 μM in the presence of 2.54 μM Tβ_9 [Tβ_{9o}]. The K_D of Tβ_9 to actin was calculated to be 0.708 μM, in good agreement with [24]. The critical concentration of actin polymerization in the presence of 4.33 μM birch profilin [A_p] equals $[K_T]\times[AT\beta_9]/([T\beta_{9o}]-[AT\beta_9])$ and was calculated to be

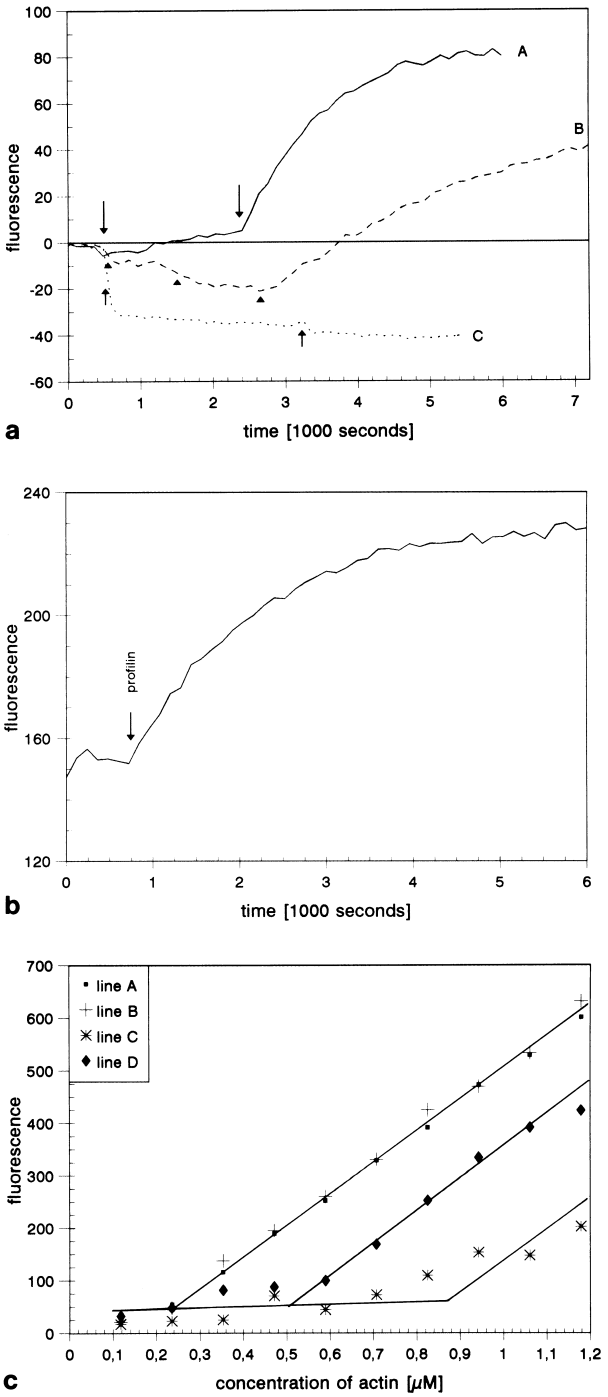

Fig. 2. a: Time course of actin polymerization from A:Tβ4 induced by birch profilin under different ionic conditions. 1.0 μM F-actin supplemented with 4% PI-actin was preincubated with 13.16 μM Tβ4 in G-buffer with 50 mM KCl (A and B). The same actin concentration was preincubated with 12 μM Tβ4 in G-buffer with 50 mM KCl and 2 mM $CaCl_2$ (C). After reaching the equilibrium the initial fluorescence was set to zero and the time course of the relative fluorescence was measured. Curve A, successive addition of 1 mM $MgCl_2$ and 5.1 μM profilin (long arrows); curve B, successive addition of 2.56 μM profilin, 5.1 μM profilin (final concentration), and finally 1 mM $MgCl_2$ (arrowheads); curve C, successive addition of 4.7 μM profilin and 7.05 μM profilin (short arrows). b: Time course of actin polymerization from A:Tβ9 induced by birch profilin. 1.08 μM F-actin (4% PI-actin) and 2.55 μM Tβ9 were preincubated in G-buffer supplemented with 50 mM KCl and 2 mM $MgCl_2$. After 16 h the repolymerization was started with 5.1 μM birch profilin. c: Effect of profilin and Tβ9 on C_c. The effect of profilin on C_c was determined as detailed in Section 2. The reaction was performed in G-buffer supplemented with 50 mM KCl and 2 mM $MgCl_2$. Line A, actin alone (C_c: 0.235 μM); line B, actin in the presence of 4.33 μM profilin (C_c: 0.235 μM); line C, actin in the presence of 2.54 μM Tβ9 (C_c: 0.8675 μM); line D, actin in the presence of 4.33 μM profilin and 2.54 μM Tβ9 (C_c: 0.505 μM). ←

0.0842 μM. Thus our data indicated a 2.8-fold decrease of C_c by birch profilin in the presence of Tβ9. Furthermore, the results given in Fig. 2a–c indicate that profilin and Tβ4 or Tβ9 did not bind simultaneously and independently to the same actin molecule, but competed in a mutually exclusive manner.

3.3. Chemical cross-linking indicates competition between profilin and thymosin β_4 for actin binding

Competition of profilin and Tβ4 for actin binding was further analyzed by chemical cross-linking. To this end, actin was first cross-linked to Tβ4 and in a second reaction to birch profilin. The results obtained are shown in Fig. 3a,b and demonstrate that binary complexes of actin with either Tβ4 or birch profilin were obtained. After addition of the second actin binding protein, it was not possible to generate the A:P:Tβ4 ternary complex which was also not obtained

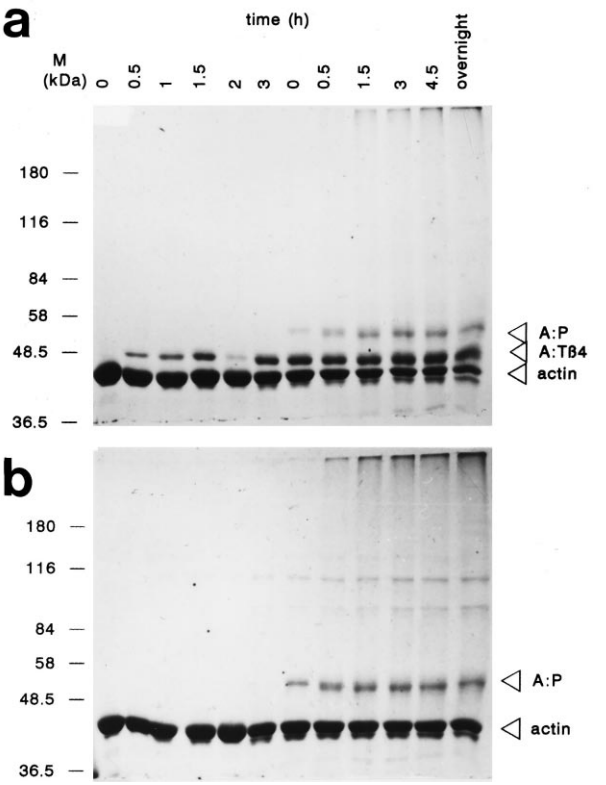

Fig. 3. Interaction of cross-linked A:Tβ4-complex with profilin. a: Time course of cross-linking A:Tβ4 (4.66×10^{-5} M actin, 9.3×10^{-5} M Tβ4, lanes 1–6). After 3 h the resulting mixture of native and cross-linked A:Tβ4 was treated with profilin (lanes 7–12). The protein concentrations changed to 2.79×10^{-5} M actin, 5.59×10^{-5} M Tβ4 and 5.6×10^{-5} M profilin. At the times indicated in the figure 3.2 μl (lanes 1–6) or 5.3 μl (lanes 7–12) were removed and analyzed by SDS-PAGE (7.5% acrylamide). b: Identical experiment to that shown in a, except that Tβ4 was omitted.

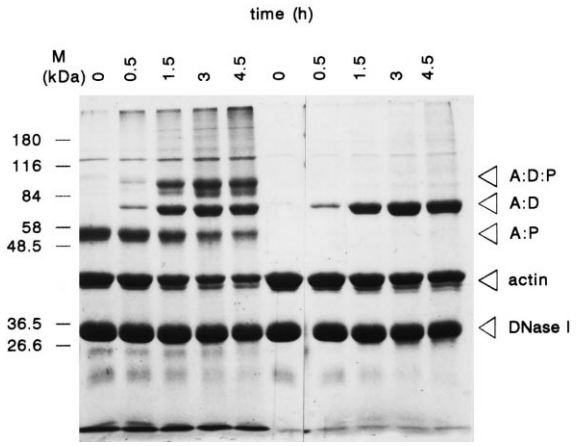

Fig. 4. Interaction of cross-linked actin:profilin-complex with DNase I. A mixture of cross-linked and native actin:profilin complex (2.5×10^{-5} M actin, 5.4×10^{-5} M profilin) was treated with DNase I (2.5×10^{-5} M, lanes 1–5). At the times indicated in the figure 6 μl of the samples were removed and analyzed by SDS-PAGE (10% acrylamide). Lanes 6–10 give the same experiment except that profilin was omitted.

when the cross-linking reaction was carried out in the simultaneous presence of $T\beta_4$ and profilin [7].

3.4. Chemical cross-linking indicates ternary complex formation of profilin with A:D

Recent 3D structure analyses had demonstrated that DNase I and bovine spleen profilin bind to opposite sites on actin [8,25]. It appeared therefore not surprising to generate a ternary complex between actin, profilin and DNase I by chemical cross-linking (Fig. 4).

3.5. Negative co-operativity between the profilin and DNase I binding sites on actin

However, when the kinetic procedure (determination of C_c) was used to analyze ternary complex formation between actin and birch profilin and DNase I, a separate slope for the C_c in the presence of both DNase I and profilin was obtained (Fig. 5). Since the shift in C_c was the sum of the C_cs of profilin and DNase I on their own, these data suggest that binding of birch profilin and DNase I was mutually exclusive. Negative co-operativity between birch profilin and DNase I binding had to be assumed, since the binding sites of profilin and DNase I on actin are spatially separated [8,25]. This assumption was supported by gel filtration experiments using mixtures of actin, plant profilin and DNase I after preincubation for 2 h in G-buffer. The results obtained indicated complex formation between actin and DNase I (not shown) or profilin (Fig. 5b), whereas profilin elution was shifted to later fractions after addition of DNase I (Fig. 5c), although cross-linking indicated ternary complex formation. However, it has recently been shown that chemical cross-linking may also stabilize short-lived and/or low affinity complexes [18].

4. Discussion

Here we demonstrate that birch profilin interacted with rabbit skeletal muscle α-actin in a manner identical to the behavior reported for mammalian profilin. Birch profilin was able to promote the polymerization of actin from β-thymosin complexes. As reported previously for mammalian profilin [20], the repolymerization ability depended on the nature of the divalent cation. Indeed, the recent structure determination of birch profilin by NMR techniques [11] has shown a protein fold identical to mammalian spleen [8] or even *Acanthamoeba* profilins [10], although they differ considerably in their primary sequences. The estimated dissociation constant of birch profilin to α-actin is considerably lower than for mammalian profilin, but in good agreement with a previous determination for recombinant birch pollen [7] and *Arabidopsis* profilin [19], which in its primary sequence is highly homologous (90%) to birch profilin [20].

Like mammalian spleen profilin, birch profilin also promoted the polymerization of actin from the β-thymosin com-

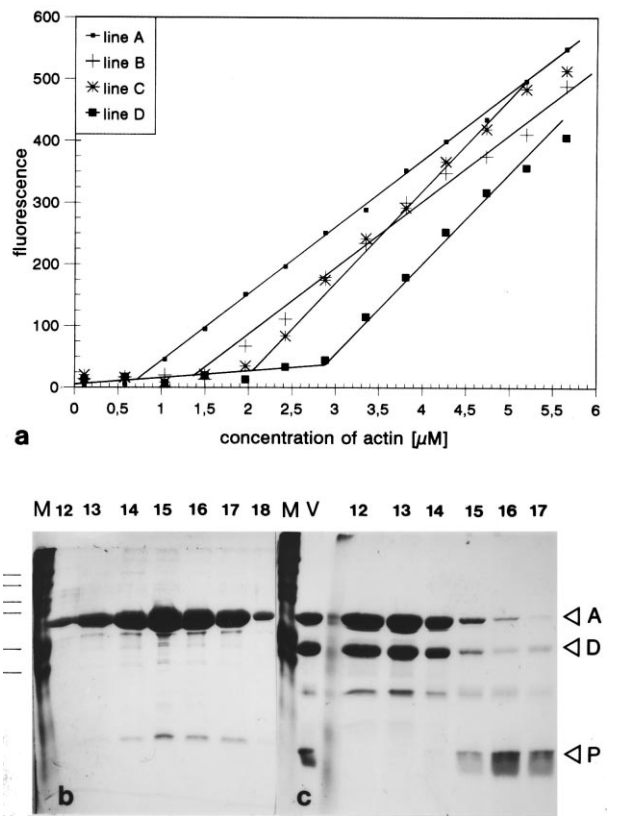

Fig. 5. a: Effect of DNase I and profilin on C_c. The reaction was carried out in a buffer consisting of 10 mM imidazole-HCl, pH 7.0, 1 mM ATP and 2 mM $CaCl_2$. All samples contained gelsolin (molar ratio actin:gelsolin=300:1) to prevent binding of A:P to plus ends of F-actin. Line A, actin alone (C_c: 0.7 μM); line B, actin in the presence of 0.6 μM DNase I (C_c: 1.34 μM); line C, actin in the presence of 4.86 μM profilin (C_c: 2.04 μM); line D, actin in the presence of both actin binding proteins at identical concentrations (C_c: 2.86 μM). b and c: Gel filtration experiments. Actin (7.9 mg) was mixed with birch profilin (2.46 mg), preincubated for 2 h on ice and passed over a Sephacryl S 300 (Pharmacia, Freiburg, Germany) column (1.5 cm, 45 cm) equilibrated and eluted with G-buffer. c: Actin (3.3 mg) was mixed with birch profilin (1.1 mg), incubated overnight and then supplemented with DNase I (2.4 mg) and preincubated for 2 h on ice. The mixture (V) was then subjected to gel filtration under identical conditions as in b. Fractions of 3.4 ml were collected. Aliquots of 20 μl were analyzed by SDS-PAGE (15% acrylamide). Fraction numbers are indicated at the top of the gels. M gives prestained molecular mass marker proteins (Sigma, Munich, Germany), top to bottom: 116, 84, 58, 48.5, 36.5, and 26.6 kDa.

plex. It might therefore be interesting to search for β-thymosin-like proteins in plants. The polymerization promoting ability was only observed in the presence of magnesium ions in agreement with data from [5,20]. The dependence of the ability of birch profilin to promote actin polymerization from A:Tβ_4 on the nature of the divalent cation is difficult to explain, since profilin itself has not been reported to contain cation binding sites. However, it has repeatedly been shown that Ca^{2+}-actin (containing a calcium ion at its high affinity site) differed structurally and in many functional respects from Mg^{2+}-actin (for review see [26]). Our data therefore suggest that the cation induced different conformational states of actin also influence its mode of interaction with profilin. Another explanation of the Mg^{2+}-dependent induction of actin polymerization by profilin has been given by [20]. These authors assume that the plus ends have to contain Mg-ADP-actin for the profilin:actin complex to associate to F-actin. In the presence of Mg^{2+} ions, the ATP of filament associated actin:profilin complex is rapidly hydrolyzed which then leads to the rapid dissociation of profilin. In contrast, Ca^{2+} ions uncouple ATP hydrolysis and actin polymerization leading to a predominance of Ca-ATP-actin containing filament ends to which the profilin:actin complex is unable to bind [20].

Chemical cross-linking experiments indicated that profilin does not form a ternary complex with A:Tβ_4. Indeed, a recent study has given evidence that the binding sites of Tβ_4 and profilin overlap [7] in agreement with our data indicating direct competition of profilin and Tβ_4 for binding to actin. The decrease in the critical concentration of actin polymerization effected by profilin in the presence of $MgCl_2$ can only be due to a shift in the G-F equilibrium induced by increased binding of actin monomers or A:P to filament ends.

Structural analyses have shown that the binding sites for gelsolin segment 1 and profilin (bovine spleen) on actin are not identical, but overlap considerably around the cleft between subdomains 1 and 3 of actin [8,27]. The data presented demonstrate that like gelsolin segment 1 [18] plant profilin also influences the DNase I binding site on subdomain 2 in a negative co-operative manner. Therefore, it can be inferred that profilin binding to actin induces conformational changes in subdomain 2 of actin. From fiber diffraction and modelling data of F-actin this area is supposed to be directly involved in the actin:actin interaction and to alter its conformation during polymerization [28]. It is therefore possible that the decrease in the critical concentration of Mg-actin polymerization in the presence of profilin is mediated by an allosterically induced structural change of this region of actin promoted by profilin binding.

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