Mapping the binding site of thymosin β_4 on actin by competition with G-actin binding proteins indicates negative co-operativity between binding sites located on opposite subdomains of actin

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The β -thymosins are small monomeric (G-)actin-binding proteins of 5 kDa that are supposed to act intracellularly as actin-sequestering factors stabilizing the cytoplasmic monomeric pool of actin. The binding region of thymosin β_4 was determined by analysing the binding of thymosin β_4 to actin complexed with DNase I, gelsolin or gelsolin segment 1. Binding was analysed by determining the increase in the critical concentration of actin polymerization by native gel electrophoresis or chemical cross-linking. The formation of a ternary complex including thymosin β_4 should indicate that the actin-binding proteins attach to different sites on actin. Competition would be indicative of binding to identical or overlapping sites on actin or of a negative co-operative linkage between the two binding sites. Competition of thymosin β_4 for actin binding was observed in the presence of

intact gelsolin or the N-terminal gelsolin fragment, segment 1, indicating that thymosin β_4 binds to a site close to or identical with the gelsolin segment 1-binding site. The ternary complex of actin–DNase I–thymosin β_4 was obtained only when using the chemically cross-linked actin–thymosin β_4 complex, indicating that thymosin β_4 is dissociated by the binding of DNase I to actin. It is suggested that the dissociation of thymosin β_4 by DNase I binding to actin is caused by negative co-operativity between their spatially separated binding sites on actin. A similar negative co-operativity was observed between DNase I and gelsolin segment 1 binding to actin. The results therefore indicate that the respective binding sites for DNase I and segment 1 on subdomains 1 and 2 of actin are linked in a negative co-operative manner.

INTRODUCTION

Thymosin β_4 ($T\beta_4$) and related peptides (the β -thymosins) are small G-actin-binding proteins (AbPs) of molecular mass 5 kDa. They are supposed to be the main actin-sequestering factors in many eukaryotic non-muscle cells, where they are present in sufficiently high concentration to stabilize approx. 50% of the total actin in monomeric form. They bind monomeric actin with intermediate affinity (K_d in the region of 0.2–2 μ M), forming a stoichiometric 1:1 complex. On cell activation, actin can be mobilized from the β -thymosin complex and enter the polymeric pool.

The exact mechanism by which the actin– $T\beta_4$ complex is separated *in vivo* is still not clear, although considerable evidence from experiments *in vitro* indicates that profilin [1] and a number of other F-actin-stabilizing proteins [2] can stimulate actin repolymerization from the actin– $T\beta_4$ complex. It has been shown that profilin is able to lower the critical concentration of actin polymerization (C_c) [1] and might thus shift the equilibrium between free G-actin and actin– β -thymosin, although the exact mechanism of its effect is still unclear. Besides profilin a number of other G-AbPs can influence the stability of the actin– $T\beta_4$ complex by direct competition. Indeed, it was recently demonstrated that F-actin-stabilizing factors such as myosin subfragment 1 or phalloidin induce actin repolymerization from the actin– $T\beta_4$ complex [2]. In addition, a sudden increase in the number of free actin filament ends also leads to repolymerization of actin from its complex with $T\beta_4$ [2]. Similar observations were reported when using extracts obtained from activated neutrophils containing an elevated concentration of free F-actin ends [3]. The repolymerizing effect of free F-actin ends could also be due to a

shift of the G/F equilibrium, because the affinity of monomeric actin for filament ends seems to be higher than that for $T\beta_4$. Another mechanism could be the alteration of the stability of the actin– $T\beta_4$ complex by direct interaction of AbPs with the complexed actin. Furthermore the exact knowledge of the $T\beta_4$ binding site on actin will also allow conclusions to be drawn about the mechanism of β -thymosin function. We have therefore analysed the interaction of the actin– $T\beta_4$ complex with other G-AbPs by measuring the formation of ternary complexes with a number of different techniques. This analysis allowed the determination of the binding region of $T\beta_4$ on actin and in addition indicated the existence of negative co-operativity between binding sites located on different subdomains of actin.

MATERIALS AND METHODS

Materials

The chemical cross-linkers FFD (1,5-difluoro-2,4-dinitrobenzene) and EDL {1-ethyl-3[3-(dimethylamino)propyl]carbodiimide} were obtained from Serva (Heidelberg, Germany) and Pierce (Rockford, IL, U.S.A.) respectively. Pyrenyliodoacetamide was purchased from Paesel & Lorei (Frankfurt, Germany).

Protein preparations

Skeletal muscle actin was purified from acetone powder prepared from rabbit psoas muscle as described in [4]. Pyrene-labelled actin (pyrenyl-actin) was prepared exactly as detailed by Kojima and Mihashi [5].

Abbreviations used: AbP, actin-binding protein; C_c , critical concentration of actin polymerization; DbP, vitamin D-binding protein; FFD, 1,5-difluoro-2,4-dinitrobenzene; $T\beta_4$, thymosin β_4 .

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Bovine pancreatic DNase I (EC 3.1.21.1) was a commercial product (Paesel & Lorei) and further purified by ion-exchange chromatography with hydroxylapatite as detailed previously [6].

Human gelsolin and the N-terminal domain of human plasminic gelsolin (segment 1) were expressed in *Escherichia coli* and purified as described by Way et al. [7].

$T\beta_4$ was isolated from bovine spleen in accordance with the method described for thymosin β_{9met} from pig spleen [8]. Briefly, the tissue was homogenized in 5 vol. of ice-cold 0.5 M $HClO_4$ and kept on ice for 30 min. The precipitate was removed by centrifugation at 4 °C and the supernatant was adjusted to pH 4 with 10 M KOH. After 30 min on ice the insoluble $KClO_4$ was removed by centrifugation. The peptides were extracted from the supernatant solution by solid-phase extraction on LiChroprep RP-18, which was primed with methanol followed by water. To remove the salts the RP-18 material was washed several times with water and then the peptides were eluted with 30 % (v/v) n-propanol. The eluate was concentrated under reduced pressure and then applied to preparative isoelectric focusing (power 8 W; temperature 8 °C; running time overnight) in a granulated gel (Sephadex G75). Thereafter the gel was sliced into 30 sections with a grid and the gel sections were transferred into small plastic columns containing 1 g of primed RP-18 between two frits. The columns were washed with 10 vol. of water to elute peptides and ampholines and the pH of the eluate was determined. The peptides bound to the RP-18 material were eluted with 30 % (v/v) n-propanol. In a last purification step the peptides were separated by preparative reverse-phase HPLC on a Pharmacia Super-Pac PrepS column (pore size 5 μ m; 4.0 mm $\times$ 250 mm) at a flow rate of 0.75 ml/min by applying a linear gradient from 0.1 % (w/v) trifluoroacetic acid in water to 0.1 % (w/v) trifluoroacetic acid in 40 % (v/v) acetonitrile within 2 h. Detection was performed by measuring the absorbance at 215 nm.

Radiolabelled $T\beta_4$ was prepared after biolabelling the human lymphoblast cell line IM9 (A.T.C.C. CCL 159) with [35 S]methionine. To this end the cells were initially kept in methionine-free RPMI 1640 medium supplemented with 10 % (v/v) heat-inactivated fetal calf serum for 3 h. After the addition of 0.7 mCi of [35 S]methionine they were cultured for a further 36 h. Then the cells were pelleted at 300 g and carefully washed with PBS by repeated centrifugation. The pelleted cells were resuspended in 0.4 M $HClO_4$. After 30 min the pH was adjusted to 3.5 by the addition of 10 M KOH and after a further incubation of 30 min the precipitated $KClO_4$ was removed by centrifugation. The clear supernatant was loaded on an RP-18 column (1 g) and after being washed with distilled water to remove salts, the bound material was eluted with 40 % (v/v) n-propanol. The eluate was concentrated by vacuum centrifugation, and labelled $T\beta_4$ was purified by reverse-phase HPLC with an acetonitrile gradient in 0.1 % (w/v) trifluoroacetic acid.

Vitamin D-binding protein (DbP) was purified from outdated human plasma as described previously [9] and generously provided by Dr. H. van Baelen (Liège, Belgium). Before use it was further purified with a DEAE-52 ion-exchange column (13 cm $\times$ 1.8 cm) equilibrated with 50 mM Tris/HCl (pH 8.0)/0.1 mM $CaCl_2$ /1 mM NaN_3 . The absorbed DbP was eluted at approx. 0.2 M NaCl with a linear gradient from zero to 0.5 M NaCl.

Quantitative protein determination

Protein concentrations were determined by following the procedure given by Bradford [10], with BSA as standard. This technique leads to an underestimation of the concentration of DNase I by a factor of approx. 2.3 compared with measuring the

absorbance at 280 nm [6]. However, we used the colorimetric procedure throughout this work to avoid effects of the nucleotide-containing buffers.

Enzymic analysis

DNase I activity was determined by measuring the increase in absorbance at 260 nm (hyperchromicity test) as introduced by Kunitz [11] and modified by Mannherz et al. [12], with a Shimadzu UV 300 spectrophotometer.

Electrophoretic techniques

SDS/PAGE was performed by the method of Laemmli [13]. Immunoblots were performed as described previously [14]. $T\beta_4$ was identified by using a monoclonal antibody prepared against thymosin β_4 kindly provided by Professor B. Jockusch (Braunschweig, Germany). Native gel electrophoresis [15] was performed as detailed previously [16]. Formation of native complexes was achieved by incubation of the proteins for 30 min at room temperature. A control lane containing sample buffer with Bromophenol Blue to indicate the electrophoretic progress was replenished with dye after its complete elution. The separation was stopped when the dye had passed through the gel twice (native actin- $T\beta_4$ complex) or 1.5 times (mixtures of native and cross-linked actin- $T\beta_4$ complex). The gels were stained with Coomassie Blue. When SDS/PAGE as second dimension was to follow, the native gels were stained briefly with Coomassie Blue. Bands to be analysed by SDS/PAGE as second dimension were carefully excised with a razor blade and placed into the slots of the stacking gel.

Fluorescence measurements of polymerized actin

C_e for actin alone or in the presence of a monomer-binding protein was determined by measuring the fluorescence increase of native actin supplemented with 4 % of pyrenyl-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. The amount of unpolymerized actin in equilibrium with F-actin was derived from pyrene fluorescence measurements of serially diluted solutions of F-actin containing a constant amount of DNase I, DbP, segment 1 or $T\beta_4$. For these experiments the proteins were taken up in a buffer containing 5 mM Hepes/NaOH, pH 7.4, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.2 mM ATP and 0.2 mM NaN_3 (buffer A). When employing segment 1 in the kinetic experiments, the plus ends of F-actin were capped by gelsolin at a ratio of 300:1.

Chemical cross-linking

Chemical cross-linking was performed in buffer A with FFD, which bridges a distance of 0.3 nm. For cross-linking, defined concentrations of actin and $T\beta_4$ (as described in the Figure legends) were preincubated for 30 min at room temperature. The pH of the samples was raised by adding triethanolamine, pH 8.5, to 0.1 M final concentration; FFD dissolved in dimethylformamide was then added to give a final concentration of 0.1 mg/ml and further incubated at room temperature for the times indicated in the Figures. The cross-linked actin- $T\beta_4$ complex used for native gel electrophoresis was generated by an overnight incubation with FFD at room temperature.

RESULTS

Ternary complex formation of actin, DNase I and DbP

The methods of detecting ternary complex formation were first tested with two AbPs that bind to actin with similar affinities and whose binding sites on actin are known to be spatially separated. For this reason DNase I and the DbP were used. First we used a kinetic approach to measure the increase of C_c in the absence or presence of these AbPs. Figure 1(A) gives the results of experiments in which F-actin containing co-polymerized 5% pyrene-labelled actin was incubated with constant concentrations of either DNase I, DbP or both proteins. The decrease in pyrene fluorescence was measured after equilibrium had been reached (normally after 16 h of incubation at room temperature). It can be seen that either AbP leads to an increase in C_c . When both

Figure 1 Ternary complex formation of actin, DNase I and DbP

(A) Effect of DNase I and DbP on C_c : determination of C_c for actin alone (line A), in the presence of 0.6 μM DNase I (line B), 2.04 μM DbP (line C) or both AbPs (line D). For details see the Materials and methods section. (B) Cross-linking of actin with DNase I and DbP. Lanes 1–4 show the components before cross-linking with FFD: 23.7 μM actin and 20.5 μM DNase I (lane 1), 23.7 μM actin and 23.7 μM DbP (lane 2), actin with both AbPs (lane 3), and both AbPs without actin (lane 4). The same incubation mixtures after cross-linking with FFD are shown in lanes 5–8. Note the absence of cross-linked adducts in lane 8 (DbP and DNase I). Protein concentrations were determined with the colorimetric method of Bradford [10] as detailed in the Materials and methods section. Lane M indicates the positions of prestained molecular mass marker proteins (Sigma, Munich, Germany), from top to bottom: α_2 -macroglobulin (180 kDa), β -galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactic dehydrogenase (36.5 kDa) and triose-phosphate isomerase (26.5 kDa; co-migrated with dye front). Abbreviation: A, actin.

Figure 2 Effect of DNase I and $T\beta_4$ on C_c

Determination of C_c on actin alone (line A), in the presence of 0.6 μM DNase I (line B), 3.15 μM $T\beta_4$ (line C) or both AbPs at identical concentrations (line D).

AbPs were added no further increase in C_c was obtained, indicating that both AbPs bind simultaneously to the same actin molecule. Ternary complex formation of DNase I and DbP with actin was further verified by gel filtration (results not shown) and chemical cross-linking with FFD as described in the Materials and methods section (Figure 1B).

No stable ternary complex of actin, $T\beta_4$ and DNase I is formed under native conditions

First we used the kinetic procedure to test for ternary complex formation between actin, $T\beta_4$ and DNase I. An additional increase in C_c was obtained in the simultaneous presence of both $T\beta_4$ and DNase I (Figure 2), indicating that both AbPs do not bind simultaneously to the same actin molecule. Indeed, the final increase in C_c is almost the sum of the increases in C_c produced by each AbP on its own. When radiolabelled $T\beta_4$ was used to analyse its binding to actin by gel filtration, it was found that in the absence of DNase I the radioactivity co-eluted with actin (Figure 3A). After the addition of DNase I, however, the radiolabelled $T\beta_4$ did not elute with the actin–DNase I complex; instead it was shifted to lower molecular mass, indicating its dissociation from actin (Figure 3B).

DNase I is inhibited by G-actin and actin– $T\beta_4$ complex

Next we tested the ability of actin on its own or actin– $T\beta_4$ complex to inhibit the DNase I activity. In agreement with previous reports [17,18] it was found that the inhibitory capacity of G-actin on DNase I is not impaired even when preincubated for 60 min in the presence of a 2-fold molar excess of $T\beta_4$. The addition of equimolar G-actin or actin– $T\beta_4$ resulted in an identical DNase I inhibition of approx. 90%. In contrast, when F-actin was preincubated with $T\beta_4$ for 60 min and then mixed rapidly with DNase I (within 3–5 s, to minimize F-actin depolymerization by DNase I during the test), a dependence of the DNase I inhibitory capacity on the $T\beta_4$ concentration was obtained. The observed values were approx. 40%, 62% and 85% ($n = 4$) at molar actin-to- $T\beta_4$ ratios of 13:1, 6:1 and 0.5:1 respectively. In contrast, the addition of F-actin on its own did not lead to a significant DNase I inhibition under these conditions. The inhibition of DNase I by F-actin in the presence of $T\beta_4$ can only be attributed to an increasing shift of the G/F equilibrium after the addition of $T\beta_4$ towards monomeric actin,

Figure 3 Dissociation of the actin- $T\beta_4$ complex after the addition of DNase I

(A) Actin (1 mg) and $T\beta_4$ (64 μ g) supplemented with 35 S- $T\beta_4$ were mixed in a final volume of 0.15 ml and incubated in buffer A for 2 h on ice. Then the reaction mixture was separated on an AcA-44 column (1 cm $\times$ 40 cm) at a flow rate of 10 ml/h and 30 fractions of 3 ml each were collected. The protein concentration (A_{290}) and the radioactivity (c.p.m.) were plotted against the fraction number. Portions (300 μ l) of the fractions indicated were analysed further by SDS/PAGE [15% (w/v) gel] as shown in the inset. (B) Identical experiment to that shown in (A) except that, after a preincubation period of 2 h, DNase I (23.8 nM) was added. The mixture was incubated for a further 30 min. Gel filtration on the AcA-44 column and the evaluation were performed as detailed above. The fraction size was 2 ml. The inset gives the SDS/PAGE analysis of the fractions indicated. The 180 kDa marker did not migrate into gel. Symbols: ■, A_{290} ; ×, c.p.m./0.5 ml of fractions collected.

because the formation of the actin- $T\beta_4$ complex removes free G-actin from its equilibrium with F-actin, which subsequently depolymerizes to re-establish the G/F equilibrium. If only G-actin formed inhibitory complexes with DNase I, the inhibition should not be altered by the addition of $T\beta_4$. However, increasing the $T\beta_4$ concentration resulted in a further increase in DNase I inhibition. This result can be interpreted in two ways: (1) both G-actin and the actin- $T\beta_4$ complex are able to inhibit DNase I, or (2) DNase I rapidly shifts the equilibrium between G-actin and the actin- $T\beta_4$ complex. To decide between these possibilities, we performed the following experiments.

Formation of a chemically cross-linked ternary actin-DNase I- $T\beta_4$ complex

Direct interaction of the actin- $T\beta_4$ complex with DNase I was

Figure 4 Demonstration of the formation of a ternary cross-linked complex consisting of actin, $T\beta_4$ and DNase I

Actin (A) (46.6 μ M) and $T\beta_4$ (93 μ M) were incubated for 30 min at room temperature. The cross-linking reaction was performed as described in the Materials and methods section. After 30 and 60 min, 3.2 μ l of the reaction mixtures were treated with SDS sample buffer and analysed by SDS/PAGE [10% (w/v) gel] (lanes 1 and 2). Lanes 3 and 4 show an immunoblot of an identical gel reacted with the monoclonal antibody against $T\beta_4$. After 2.5 h, DNase I was added to the cross-linking reaction. The protein concentrations were changed to 18.4 μ M actin, 36.6 μ M $T\beta_4$ and 21.9 μ M DNase I. For analysing the progress of the cross-linking reaction, 8.2 μ l samples were removed, mixed with SDS sample buffer and analysed by SDS/PAGE (lanes 5 and 6) and immunoblotting (lanes 7 and 8). The cross-linking pattern obtained 30 min after the addition of DNase I is shown in lanes 5 and 7 and after 60 min in lanes 6 and 8.

Figure 5 Interaction of cross-linked actin- $T\beta_4$ complex with DNase I

(A) Native gel electrophoresis [7.5% (w/v) polyacrylamide] of a mixture of native and cross-linked actin- $T\beta_4$ complex (12.3 μ M actin and 24.6 μ M $T\beta_4$) that reacted with 2.34 μ M DNase I (lane 2). Lane 1 shows DNase I on its own and lane 3 the mixture of native and cross-linked actin- $T\beta_4$ complexes. (B) The protein spots were stained, excised, incubated for 2 h with non-boiling SDS sample buffer and analysed by SDS/PAGE [10% (w/v) gel]. Protein spots in (A) and corresponding lanes in (B) were marked identically. Protein concentrations were determined by the method given by Bradford [10]. As detailed in the Materials and methods section this procedure leads to an underestimation of the DNase I concentration. Abbreviations: A, actin; cross-l., cross-linked.

demonstrated by chemical cross-linking. The results in Figure 4 (lanes 1, 2, 5 and 6) show that FFD is able to generate binary actin- $T\beta_4$ and actin-DNase I complexes. In the presence of both AbPs a new species of approx. 80 kDa was generated (Figure 4, lanes 5 and 6). Immunoblots with the monoclonal antibody against $T\beta_4$ clearly demonstrated that this 80 kDa band contains $T\beta_4$ (Figure 4, lanes 7 and 8).

Binding of DNase I to chemically cross-linked actin- $T\beta_4$ complex

Ternary complex formation was further analysed by native gel electrophoresis. The addition of DNase I to preformed native

Figure 6 Interaction of the actin- $T\beta_4$ (A: $T\beta_4$) complex with gelsolin

(A) Native gel electrophoresis [7.5% (w/v) polyacrylamide] of an incubation mixture containing native and cross-linked actin- $T\beta_4$ complex (12.3 μ M actin and 24.6 μ M $T\beta_4$) and 2.58 μ M gelsolin (lane 2). The position of free gelsolin and actin- $T\beta_4$ complex are shown in lanes 1 and 3 respectively. (B) The marked spots in (A) were excised, treated as detailed above and analysed by SDS/PAGE [10% (w/v) gel]. (C) Immunoblot of an SDS/polyacrylamide gel parallel to (B) after reaction with the monoclonal $T\beta_4$ antibody. Abbreviation: cross-l., cross-linked.

Figure 7 Effect of gelsolin segment 1 and $T\beta_4$ on C_c

Determination of C_c on actin alone (line A), in the presence of 0.75 μ M gelsolin segment 1 (seg 1) (line B), 3.1 μ M $T\beta_4$ (line C) and both AbPs (line D).

actin- $T\beta_4$ generated a new spot; however, this exhibited the same mobility as the binary actin-DNase I complex (results not shown). To prove ternary complex formation, we used actin- $T\beta_4$ complex that had been treated with FFD and therefore contained

a mixture of cross-linked and native complex. After incubation of this material with DNase I, a shift in mobility on native gels was observed that was identical with that obtained after the addition of DNase I to untreated actin- $T\beta_4$ (Figure 5A). The spots separated by native gel electrophoresis were then excised and loaded on SDS/polyacrylamide gel as the second dimension. The results obtained are given in Figure 5(B). They demonstrate that the product spot 2a generated by the addition of DNase I contained cross-linked actin- $T\beta_4$ complex together with DNase I, indicating that a ternary actin- $T\beta_4$ -DNase I complex can be formed or rather that DNase I can bind to actin chemically cross-linked to $T\beta_4$.

Actin- $T\beta_4$ complex does not bind to gelsolin

Gelsolin contains three actin-binding sites. Ternary complex formation between actin, $T\beta_4$ and gelsolin (82 kDa) was analysed by native gel electrophoresis followed by SDS/PAGE. The experiments shown were performed in the presence of 0.1 mM CaCl_2 , which normally induces the formation of the gelsolin-actin complex [7,16]. It was found that the addition of gelsolin to a mixture of native and cross-linked actin- $T\beta_4$ did not induce the formation of a new species (Figure 6A). After excision, analysis of these bands by SDS/PAGE indicated that the complex formed in spot 2b does not contain cross-linked actin- $T\beta_4$ complex (Figure 6B). This was again verified by immunoblotting

Figure 8 Interaction of gelsolin segment 1 with the actin- $T\beta_4$ (A: $T\beta_4$) complex

Native gel electrophoresis [7.5% (w/v) polyacrylamide] of the mixture of native and cross-linked actin- $T\beta_4$ complexes (12.3 μ M actin and 24.6 μ M $T\beta_4$) is shown in lanes 1 and 2 after preincubation with 10.7 μ M gelsolin segment 1. Under these conditions free gelsolin segment 1 does not migrate into the gel (lane 3). The compositions of the protein spots (2a and 2b) of the native gel were analysed by SDS/PAGE [5–20% (w/v) gradient gel]; these are shown in lanes 2a and 2b respectively. Lane M indicates the positions of prestained molecular mass marker proteins as in Figure 1(B).

an identical gel and reacting with the monoclonal antibody against $T\beta_4$ (Figure 6C).

Actin- $T\beta_4$ complex does not bind to gelsolin segment 1

Actin binds to the N-terminal 14 kDa domain of gelsolin (segment 1) independently of Ca^{2+} ions with high affinity. Furthermore the three-dimensional structure of the complex of segment 1 and actin has been solved [19]. Therefore binding of actin- $T\beta_4$ to segment 1 was tested. With the kinetic approach no indication of ternary complex formation was obtained; instead we observed an additional increase in C_c in the presence of both AbPs (Figure 7). Analysis by native gel electrophoresis followed by SDS/PAGE as second dimension clearly demonstrated that cross-linked actin- $T\beta_4$ does not bind to segment 1 (Figure 8). In contrast, the cross-linked actin- $T\beta_4$ complex remained at its original position in the native gel. Furthermore, when gelsolin segment 1 was added to actin complexed with radiolabelled $T\beta_4$ and analysed by gel filtration, we observed, as with DNase I, a complete dissociation of the radiolabelled $T\beta_4$ (results not shown).

Ternary complex formation of actin, DNase I and gelsolin segment 1

The experiments conducted to determine ternary complex formation of actin with DNase I and $T\beta_4$ indicated a negative allosteric coupling of the DNase I- and $T\beta_4$ -binding sites. Because $T\beta_4$ binding to actin is completely suppressed by segment 1, we assume that segment 1 and $T\beta_4$ bind to the same or closely spaced sites on actin. Therefore we analysed whether the binding of segment 1 to actin, like that of $T\beta_4$, is also negatively influenced by DNase I. Figure 9 demonstrates an additional increase in C_c in the presence of both AbPs, although it is well known, from the structure analysis of actin-DNase I and actin-segment 1 complexes [19,20], that their binding sites are spatially separated. This increase is, however, not completely additive like that of the pairing of $T\beta_4$ and gelsolin segment 1 (Figure 7). Instead, the formation of a ternary actin-DNase

Figure 9 Effect of gelsolin segment 1 and DNase I on C_c

Determination of C_c on actin alone (line A), in the presence of 1.3 μ M gelsolin segment 1 (line B), of 0.26 μ M DNase I (line C) and of both AbPs (line D).

I-segment 1 complex can be demonstrated by gel filtration, indicating that the affinity of these proteins is still sufficiently high for ternary complex formation (results not shown). The kinetic results, however, suggest that the binding sites for both proteins are also linked in a negative co-operative manner.

DISCUSSION

The initial aim of this investigation was to map the binding site for $T\beta_4$ on actin. Because there is no direct structural information available about the binding site for $T\beta_4$ on actin, its identification relied mainly on chemical cross-linking studies. From such experiments a number of studies [21–23] concluded that $T\beta_4$ interacts with the C-terminus, whereas Safer and Nachmias [24] presented data indicating cross-linking to the N-terminus of actin. These conflicting results might be explained by the fact that the N- and C-termini of actin are both located on subdomain 1 and are highly mobile, or they might be due to the use of different chemical cross-linkers. Here we report a different approach: measuring ternary complex formation or competition of $T\beta_4$ with DNase I or gelsolin segment 1, whose actin-binding sites are known by structure determination [19,20]. The results obtained demonstrate the complete abolition of binding of actin- $T\beta_4$ to segment 1. Therefore we deduce that the actin-binding site of $T\beta_4$ is within or close to the contact region of segment 1 on actin, namely the cleft between subdomains 1 and 3 [19]. Recent results obtained by chemical cross-linking support the assumption that the $T\beta_4$ -binding site on actin is close to that of segment 1. Safer and Elzinga [25] identified Asp-154, Asp-155 and Glu-167, located in subdomain 3 of actin, as residues forming part of the $T\beta_4$ -binding site. Glu-167 directly contacts the actin-binding helix of segment 1 via the shared Ca^{2+} atom [19]. A definitive answer, however, about the actin- $T\beta_4$ interface will be possible only by direct structure determination.

Previous reports have indicated that $T\beta_4$ binding to actin is inhibited by DNase I [26]. Because we demonstrate that DNase I binds to the cross-linked actin- $T\beta_4$ complex, the binding site for $T\beta_4$ must be different from the DNase I-binding site. Both sites seem to be allosterically linked and to control each other negatively. We assume that the binding of DNase I decreases the affinity of $T\beta_4$ for actin, most probably by increasing the rate of $T\beta_4$ dissociation. Indeed, Huff et al. [27] have recently shown by equilibrium ultracentrifugation that the K_d of $T\beta_4$ to actin is increased approx. 57-fold when complexed with DNase I. Our

gel-filtration experiments with radiolabelled $T\beta_4$ also demonstrate that binding of DNase I to actin- $T\beta_4$ complex leads to the dissociation of $T\beta_4$.

Our results are in contrast with recently published results indicating the independent interaction of DNase I and $T\beta_4$ with actin [18]. In these experiments cross-linked actin-DNase I complex was employed, to which a modified $T\beta_4$ was chemically cross-linked. As shown in Figure 4, we were also able to generate a cross-linked actin-DNase I- $T\beta_4$ complex. However, chemical cross-linking perturbs the true equilibrium and might produce complexes that normally are only short-lived and/or of low affinity.

Negative co-operativity between different binding sites of actin was also indicated by the kinetic procedure when analysing ternary complex formation between DNase I and segment 1, which bind separately to actin with high affinity (K_d approx. 1 nM). The negative co-operativity does not seem to decrease the affinity of the second binding protein so much as to prevent the formation of a stable ternary actin-DNase I-segment 1 complex. In contrast, the affinity of $T\beta_4$ for actin (K_d approx. 1 μ M) is decreased by DNase I binding to such an extent that it apparently excludes the formation of a stable ternary complex.

DNase I and segment 1 bind to two oppositely spaced subdomains of actin bridging an interdomain cleft (reviewed in [28]). Their binding sites are part of the actin/actin interfaces along the long-pitch F-actin helix [29]. DNase I has been shown to bind to the pointed end of F-actin [30]. In addition, it has been demonstrated that DNase I also increases the rate of monomer dissociation from the pointed end of F-actin [31]. In view of our results, this effect might also be due to negative co-operativity between the DNase I-binding site and the opposite actin/actin interface. The underlying mechanism of the observed negative co-operativity is unclear. The structure analysis of both complexes had not indicated drastic differences in the three-dimensional structure of actin when complexed with either protein [19,20]. However, it was not possible to trace the binding loop for DNase I on subdomain 2 in the segment 1 complex, most probably owing to high mobility in this region [19]. Indeed, this loop has been shown to adopt a different orientation in F-actin than in the DNase I complex [32]. In the actin-segment 1 complex only Tyr-169 in subdomain 3, a residue involved in segment 1 binding, adopted a rotamer orientation different from the actin-DNase I complex [19]. Otherwise no conformational alterations of loop regions or side chains in subdomain 1 were noted between the two binary complexes.

DNase I, segment 1 and $T\beta_4$ are actin-sequestering proteins and their binding sites overlap with the actin/actin interfaces along the long-pitch F-actin helix [29]. Each sequestering protein can bind to only one side of actin. For effective sequestration it will be necessary to inactivate the opposite actin-binding site so as not to act as a capping protein. This might be achieved by negative allosteric coupling of the oppositely spaced interface as described here. It will be interesting to analyse other actin-sequestering or actin-capping proteins for an allosteric coupling of their binding sites to DNase I, segment 1 or $T\beta_4$.

The function of $T\beta_4$ *in vivo* is the intracellular sequestration of monomeric actin. The intermediate affinity of $T\beta_4$ to actin (K_d

approx. 1 μ M) allows the rapid dissociation of this complex and the repolymerization of actin. Its dissociation could also be accelerated by proteins that bind to spatially separated sites on actin and decrease the affinity of $T\beta_4$ for actin by negative co-operativity.

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