Volume 329, number 1,2, 9–12 FEBS 12847 August 1993

Use of bimanyl actin derivative (TMB-actin) for studying complexation of β-thymosins

Inhibition of actin polymerization by thymosin β9

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Received 1 June 1993; revised version received 30 June 1993

By reacting trimethylammoniobromobimane bromide (TMB bromide) with rabbit muscle actin, a fluorescent reporter group was linked to cysteine at position 374. Fluorescence of TMB-actin decreased significantly on addition of thymosin β4 (Tβ4), a peptide of 43 amino acid residues reported to bind to monomeric actin and to prevent filament formation. Based on this effect, we determined the K_D value of the thymosin β4 complex as 0.8 μM, a value that is in agreement with previous determinations. In addition to the main compound thymosin β4, bovine tissue contains a related peptide, thymosin β9 (Tβ9), which has 41 amino acid residues and ca. 75% sequence homology. In the present study we show for the first time that Tβ9, similar to Tβ4, forms a 1:1 complex with monomeric actin, and hereby inhibits actin polymerization. With a K_D value of 1.1 μM the affinity of Tβ9 is in the same range as that of Tβ4, suggesting that Tβ9, like Tβ4, contributes to maintaining the pool of monomeric actin in bovine non-muscle cells. Further proof of the interaction of Tβ9 with actin was provided by native PAGE, where the complex showed the reported higher mobility, as well as by crosslinking experiments. Using different crosslinking reagents, like water-soluble carbodiimide (EDC), *m*-maleimidobenzoyl-*N*-hydroxysuccinimidate (MBS), and disuccinimidylsuberate (DSS), we were able to produce conjugates of 47 kDa. In one of these (from MBS) both actin and Tβ9 could be identified by immunoblotting. When, in the MBS crosslinking experiments, native actin was replaced with (374-NEM)-actin, the 47 kDa band was not seen, indicating that Cys-374 takes part in the thiol-specific crosslinking reaction. This suggests that part of the binding site of Tβ9 must be located close to the carboxy-terminus.

Muscle actin; Polymerization inhibition; β-Thymosin; Binding constant; Bimanylfluorescence; Thiol spcificity

1. INTRODUCTION

In non-muscle cells the microfilament system is dynamic in nature. Rapid appearance of filaments in response to various stimuli to the cell is made possible by the existence of a pool of actin monomers which may account for up to 50–60% of total actin in the cell. A high concentration of monomeric actin is not expected to exist in the cytoplasm, since the ionic conditions favor polymerization; it is therefore believed that in non-muscle cells the pool of unpolymerized actin is maintained through complexation of monomers with small actin-sequestering proteins, such as profilin or actobindin [1].

Very recently Safer et al. [2,3] reported that thymosin β4 (Tβ4) has actin-sequestering properties. Tβ4 is the main component of a family of peptides (4–5 kDa) which originally were isolated from calf thymus [4] and became known for their T-cell-modulating activities. Besides Tβ4, a second peptide, Tβ9, was found associated with Tβ4 in bovine tissue and has ca. 75% sequence homology [5,6]. Other species, such as man and rat, contain Tβ10 instead of Tβ9 [7]. While in plasma the concentration of Tβ4 is low (ca. 2.5 nM [8]), a much higher concentration was found inside cells (ca. 600 μM in the cytoplasm of platelets [7,9]). This led to the suggestion that the main physiological role of β-thymosins may be other than in modulating immune activities. One such possibility is that β-thymosins play a role in the regulation of the microfilament system.

Here we report that the action of Tβ9 resembles that known for Tβ4 [2,3] and recently reported for Tβ10 [10]. For studying the complexes of β-thymosins with monomeric actin we established an assay system based on a change in fluorescence that is induced in a TMB derivative of actin on the addition of β-thymosins.

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Abbreviations: TMB bromide, trimethylammoniobromobimane bromide = 3,7-methyl-7-trimethylammoniomethyl-1,5-diazabicyclo [3,3,0] octadiene-2,8-dione [22].

2. MATERIALS AND METHODS

Actin was prepared as described by Spudich and Watt [11] and further purified by a gel-filtration step on a Fractogel TSK HW 55 (MERCK; Darmstadt) column (3 × 120 cm) in buffer G (2 mM Tris, 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.02% NaN_3, pH 7.8).

Tβ9 was isolated together with Tβ4 from bovine lung, following a procedure described for the isolation of these peptides from bovine tissue [15]. Separation of Tβ9 from Tβ4 was achieved using preparative isoelectric focussing based on different pI values of the two peptides, which are 4.6 and 5.1, respectively. After semipreparative HPLC on RP18, Tβ9 was obtained pure as assessed by analytical HPLC.

2.1. *(Cys-374)TMB-actin*

To a solution of G-actin (ca. 3 mg protein per ml) in buffer G an 8-fold excess of TMB bromide (Thiolyte MQ; Calbiochem, Bad Soden, Germany) in methanol was added and reacted at 4°C for 2 h. The mixture was applied to a Sephadex G-25 column (1 × 25 cm) equilibrated with buffer G in order to remove excess reagent.

2.2. *Fluorescence measurements*

The fluorescence of TMB-actin in buffer G (2 μM in fluorescence cuvettes) was measured in a Spex fluorolog (Spex Industries Inc., New York) between 385 and 600 nm (excitation 370 nm). Then β-thymosins (0–12 μM) were added, and after incubation for 15 min at room temperature the changed fluorescence spectra were recorded and the K_D values were calculated from the integrated spectra according to Pesce et al. [12].

2.3. *Viscosimetric measurements*

Polymerization was monitored in a Cannon Capillary viscosimeter, using a 9.5 μM concentration of G-actin in buffer G, to which $MgCl_2$ was added to a final concentration of 1 mM. For studying the polymerization in the presence of Tβ9, the peptide was added to the actin solution to final concentrations of 2.4, 4.8, 7.1 and 9.5 μM, and allowed to stand for 15 min at room temperature (RT) before polymerization was started.

2.4. *Native PAGE*

This was performed as described by Safer et al. [2,13] using a buffer containing 25 mM Tris, 0.194 M glycine, 0.3 mM ATP, 0.1 mM $CaCl_2$. Actin and Tβ9 in equimolar amounts were incubated for 30 min at 4°C before the start. In experiments without Ca^{2+}, 1 mM EGTA was added.

2.5. *Crosslinking experiments with MBS*

To a mixture of equimolar amounts of G-actin and Tβ9 in buffer G, preincubated for 15 min at RT, a 50-fold excess of *m*-maleimidobenzoyl-*N*-hydroxysuccinimidate dissolved in dimethylformamide/water (50/50 vol%) was added. After 3 h the reaction was stopped with a 200-fold excess of 2-mercaptoethanol. The mixture was analysed by SDS-PAGE (12.5%). In a control experiment (Cys-374)*N*-ethylmaleimide actin was used instead of native actin.

2.6. *Crosslinking experiments with EDC/N-hydroxysuccinimide*

G-actin in 2 mM MOPS pH 6.0 containing 0.2 mM ATP and 0.1 mM $CaCl_2$ was reacted with 2 mM EDC/5 mM *N*-hydroxysuccinimide for 30 min at 4°C. After the pH had been adjusted to 7.8 by adding 2 mM MOPS buffer pH 9.5, an equimolar amount of Tβ9 was added. Reaction was stopped after 30 min, and the mixture analyzed as above.

2.7. *Crosslinking experiments with DSS*

A 1:1 mixture of G-actin in buffer G and Tβ9 was incubated at 4°C for 15 min with a 50-fold molar excess of disuccinimidylsuberate; the reaction was stopped by the addition of Tris and the mixture analysed by SDS-PAGE.

2.8. *SDS-PAGE and immunoblotting*

SDS-PAGE was conducted on 12.5% gels. Immunoblotting was done on Immobilon membranes (Millipore); non-specific binding was blocked by 3% skimmed milk in PBS. Mouse anti-actin antibodies (Amersham) and rabbit anti-Tβ4 antiserum were used as primary antibodies. The bound antibodies were detected by peroxidase-conjugated second antibodies (anti-mouse, and anti-rabbit IgG, respectively; Dianova, Hamburg).

3. RESULTS AND DISCUSSION

In G-actin in the presence of ATP, only the thiol group in position 374 is exposed [14]. Alkylation of this SH group with Thiolyte MQ yielded a conjugate containing 0.7 moles of label per mol of actin. As Fig. 1 shows, the fluorescence of this label was decreased on the addition of Tβ4 in a dose-dependent way. Evaluation of the fluorescence data yielded an apparent K_D value of 0.8 ± 0.10 μM. This value is in the same range as that reported by Weber et al. [9] on the basis of kinetic measurements with gelsolin-capped filaments (K_D = 2 μM). A similar set of fluorescence data as shown in Fig. 1 was obtained with Tβ9 (not shown). The apparent K_D value of Tβ9 was 1.1 ± 0.11 μM, indicating that this peptide interacts with monomeric actin in a way similar to Tβ4.

The biological activity of Tβ9 was assessed by subjecting equimolar amounts of the peptide and G-actin to polymerization conditions. On the addition of 1 mM $MgCl_2$ no increase in viscosity was observed in a capillary viscosimeter. This indicates that Tβ9, like Tβ4, is able to prevent actin polymerization. As shown in Fig. 2 the effect is dose-dependent and suggests the existence of a 1:1 complex, similar to Tβ4. The fact that relative viscosity falls to zero already at a ratio of 0.75:1 is explained by the presence of uncomplexed monomers (critical concentration) and oligomers that do not contribute to viscosity.

Evidence for the physical interaction of Tβ9 and actin was obtained from native gel electrophoresis, as described recently for Tβ4 by Safer and co-workers [2,13]. Fig. 3 shows that the complex of Tβ9 and actin can easily be identified on the basis of its mobility, which exceeds that of pure actin. Gels run in the presence or in the absence of 0.1 mM $CaCl_2$ showed no difference, suggesting that formation of the complex between Tβ9 and actin is independent of Ca^{2+} ions. This is in agreement with data published by Weber et al. [9] who found

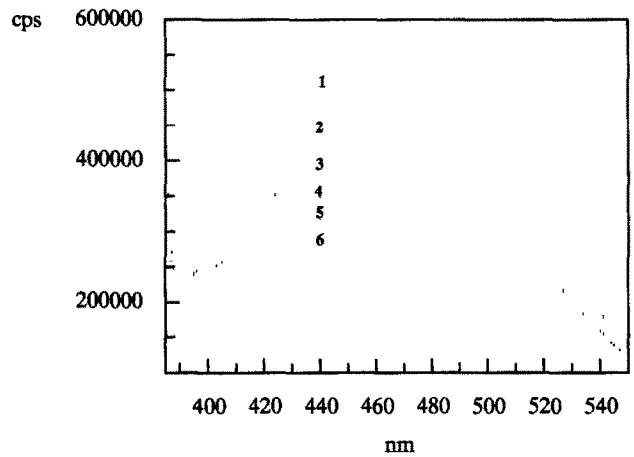

Fig. 1. Change in the fluorescence spectrum of TMB-actin (trace 1) with increasing amounts of Tβ4 added (traces 2–6).

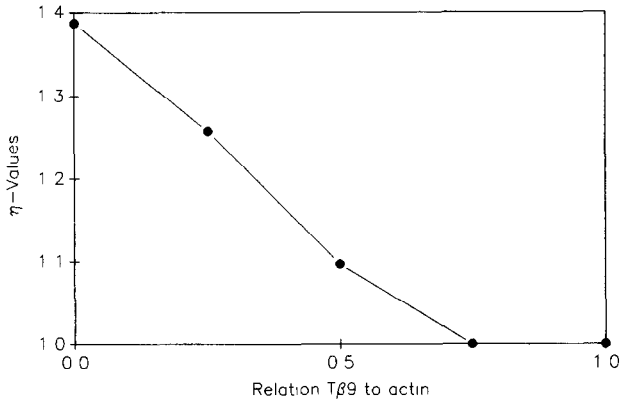

Fig. 2. Plot of the relative viscosity values η (plateau values) against the ratio of Tβ9 and actin.

that the K_D of Tβ4 was identical in the presence of 0.2 mM Ca^{2+} or 5 mM EGTA.

Additional evidence for the existence of a complex of Tβ9 and actin was obtained from crosslinking experiments. We examined three different crosslinking reagents: the zero-length crosslinker, EDC, leading to the formation of an isopeptide bond; the amino-specific 8-atom crosslinker, DSS; and the heterobifunctional 7-atom crosslinker, MBS, which allows crosslinking between a thiol and an amino group. All three crosslinking experiments were positive, as shown by the appearance of a 47 kDa band in SDS-PAGE (Fig. 4). The conjugate obtained by crosslinking with MBS was further characterized by Western blotting. Using antiserum against Tβ4 exhibiting 35% cross-reactivity against Tβ9 [15] in an ELISA, we were able to detect Tβ9 in the covalently

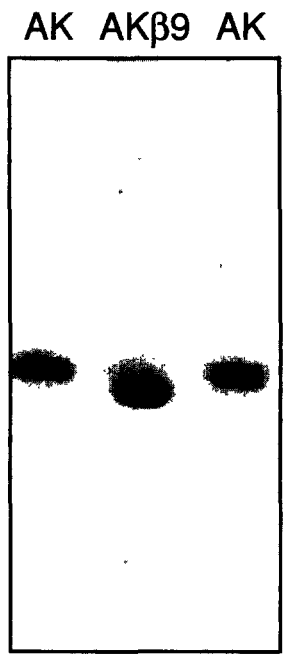

Fig. 3. Native PAGE of actin and the actin–Tβ9 complex. Lanes 1, 3, actin; lane 2, complex of actin and Tβ9. Stained with Coomassie blue.

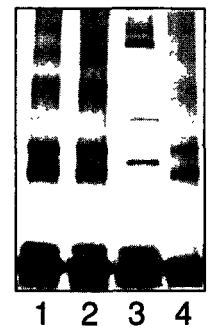

Fig. 4. 12.5% SDS-PAGE on the crosslinking experiments of actin with Tβ9. Lane 1, actin, Tβ9 (1:1) with EDC/NHS, lane 2, actin, Tβ9 (1:1) with DSS; lane 3, actin, Tβ9 (1:1) with MBS; lane 4, actin.

linked state (data not shown). Based on this possibility, and using the same antiserum in combination with antibodies against actin, we could show that the 47 kDa band seen in SDS-PAGE contains both actin and Tβ9 (Fig. 5).

Crosslinking was likewise obtained with Tβ9 and ADP–G-actin. This reaction occurred even when an ADP–G-actin was used in which the reported conformational transition had uncovered Cys-10 [16] (data not shown). Crosslinking experiments clearly give no information on the stability of the complexes formed from Tβ9 and the various actin species, like an ATP–actin and the two ADP–actins; this question is interesting in the light of the suggestion that a retarded exchange of the nucleotide in the actin–thymosin complex (in combination with accelerated exchange in the profilin–actin complex) may be of importance for the regulation of the actin equilibrium in non-muscle cells [17].

From the reaction product with one of the crosslinkers, MBS, preliminary information could be obtained on the binding site of β-thymosins on the actin surface. Since Tβ9 contains no cysteine residues, and actin under the given conditions exposes only one thiol group, the successful crosslinking reaction with MBS necessarily

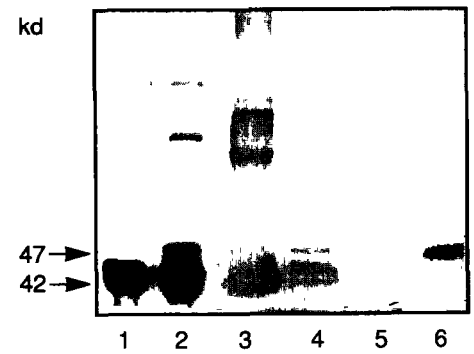

Fig. 5. SDS-PAGE and immunoblot of actin (lanes 1,3,5) and actin crosslinked with Tβ9 (MBS) (lanes 2,4,6). Lanes 1,2, SDS-PAGE (stained with Coomassie blue); lanes 3,4, immunoblot using mouse anti-actin antibody; lanes 5,6, immunoblot using rabbit anti-Tβ4 antiserum.

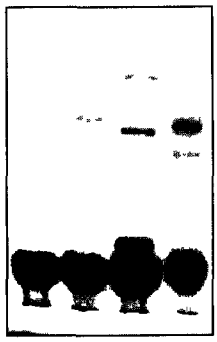

Fig. 6. 12.5% SDS-PAGE of the MBS crosslinking experiment. Lanes: (1) actin; (2) ^{374}NEM–actin; (3) actin, Tβ9 (1:1) with MBS added; (4) ^{374}NEM–actin, Tβ9 (1:1) with MBS added.

involves the thiol group of Cys-374. This was shown by replacing native actin by (374-NEM)–actin in the crosslinking reaction. As expected, the 47 kDa band of the complex was not seen in the SDS-PAGE (Fig. 6), suggesting that crosslinking failed because the thiol group 374 was not available. In a control experiment it was shown that Tβ9 inhibits the polymerization of (Cys-374) NEM-actin as efficiently as that of the native actin, excluding the possibility that the negative result of the crosslinking experiment was due to a loss of thymosin-binding capacity in the (374-NEM)–actin. From of these results we thus conclude that at least a part of the β-thymosin molecule must be located in close vicinity to the carboxy-terminus of actin.

More information on the binding site of Tβ9 on the actin molecule was obtained from studying the DNase I inhibition capacity of actin in the presence of the peptide. Using the DNase I assay of Blikstadt et al. [18] we were able to show that G-actin in the presence of Tβ9 is unable to inhibit the enzymatic activity. This suggests that actin cannot bind Tβ9 and DNase I at the same time. This is difficult to understand because DNase I has been reported to bind to subdomains 2 and 4 of the actin monomer [19], distant from the carboxy-terminus. Possible explanations for this inconsistency are that either the binding sites of DNase I and Tβ9 overlap, despite the large distance, which would be in line with the stretched conformation of the peptide suggested on the basis of NMR studies [20]. Alternatively binding of Tβ9 may induce a conformational change in the actin monomer that excludes simultaneous binding of DNase I.

In conclusion, it has been shown that not only the major component of the β-thymosins, Tβ4, but also a minor component such as Tβ9, which is associated with Tβ4, has an actin-sequestering activity. This result is in line with the recent finding that Tβ10 also has the same biological activity [10,21]. By using a novel fluorescence assay system we have shown that the complexes of actin with Tβ9 and Tβ4 are of comparable stability. The question as to whether or not the cellular concentration of β-thymosins is high enough to match the concentration of actin monomers now has to consider the contribution of other components of the β-thymosin family as well. The fact that various β-thymosins possessing different lengths and up to 30% variation in primary structure can have similar or identical biological activities strengthens the idea that sequestration of actin is the major physiological role of these peptides.

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