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Polymerization of actin from the thymosin β4 complex initiated by the addition of actin nuclei, nuclei stabilizing agents or myosin S1

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Abstract

Thymosin β4 forms a 1:1 complex with actin and thereby prevents polymerization. Rapid formation of filaments from this complex was observed, however, when actin trimers were added. Polymerization can likewise be initiated by the addition of one equivalent of phalloidin or, less effectively, cytochalasin B. Since both toxins, which reportedly support nucleation, have similar effects as the covalently linked actin trimers, it appears that the formation of filaments from the actin–thymosin β4 complex depends on the availability of stable actin nuclei. Remarkably, rapid polymerization was also observed if small amounts of myosin S1 were added, suggesting that also myosin, a protein functionally connected with polymeric actin, can serve as a nucleation center. Considering the existence of thymosin β4 and related peptides in numerous mammalian tissues, our data suggest that spontaneous formation of microfilaments in non-muscle cells may be regulated at the level of nucleation. Uncontrolled polymerization induced by the formation of phalloidin-stabilized nuclei may explain the acute toxic effects of phalloidin in hepatocytes.

Key words: Actin polymerization; Thymosin β4; Actin nucleus; Myosin S1; Phalloidin

1. Introduction

In non-muscle cells a considerable part of actin exists in the monomeric state [1]. Under the ionic conditions of the cell, high concentrations of monomeric actin require sequestration by proteins like profilin [2] or actobindin [3]. Since the detection of Safer and coworkers [4,5] that non-muscle cells contain a 5 kDa peptide, thymosin β4, that is likewise able to prevent actin polymerization, evidence has been growing that at least in some tissues sequestration by thymosin β4 may be of physiological importance [6–10].

It has been reported that in some kinds of cells hormones [11] or chemotactic peptides [6] can lead to spontaneous formation of microfilaments. So far, neither the regulation of this process nor the nature of the complex from which the filaments arise, is known. From experiments with model systems it has been speculated that differences in the rate of nucleotide exchange in complexes of actin with profilin, or thymosin β4, may provide the basis for regulated polymerization [12]. Other arguments suggest that regulation may depend on the different affinities of the actin–thymosin β4 complex for ATP and ADP [13], or on the oxidation of the methionine residue in position 6 of thymosin β4 which strongly decreases the polymerization-inhibiting capacity of thymosin β4 (unpublished results). Our present data suggest that spontaneous polymerization from the thymosin β4 complex may simply depend on the availability of stable actin nuclei.

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2. Materials and methods

2.1. Protein purification

Actin was prepared as described by Spudich and Watt [14] 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).

Chemically crosslinked actin trimers (nuclei) were obtained from F-actin by the addition of a 2-fold excess of *N,N'*-p-phenylene-bis-maleimide at pH 9.2 for 15 min and purified according to Lal et al. [15]. Myosin S1 was prepared according to Weeds and Taylor [16].

Thymosin β4 was isolated from bovine lungs following a procedure described for the isolation of the peptides from bovine tissue [17]. Purification was achieved using preparative iso-electric focussing and semipreparative HPLC on RP18 (Parcosil Pro RP 300–5, C_{18}, Biotek).

2.2. Fluorescence measurements

Polymerization was monitored by pyrenyl fluorescence [18], using a 10 mM solution of actin, containing 5% pyrenyl actin (excitation 366 nm; emmission 406 nm). Equimolar amounts of thymosin β4 were added and incubated for 30 min before the polymerization was started by adding 0.1 M KCl. Phalloidin, myosin S1 or nuclei were added simultaneously with the salt.

2.3. Viscosimetric measurements

Polymerization was monitored in a Cannon capillary viscosimeter, using a 10 μM concentration of G-actin in buffer G to which KCl was added to a final concentration of 100 mM. For investigating the nucleation effect in actin–thymosin β4 solutions, thymosin β4 was added to the actin to a final concentration of 10 μM (1 : 1) and allowed to stand for 30 min at RT before polymerization was started. After 30 min incubation under polymerization conditions, phalloidin, actin nuclei, myosin S1 or cytochalasin B were added. In control assays with uncomplexed actin the nucleation factors were added together with KCl.

3. Results and discussion

It has been reported from several laboratories that when complexed with thymosin β4 muscle actin is unable to polymerize [6–10]. Nonetheless, in a 10 μM solution

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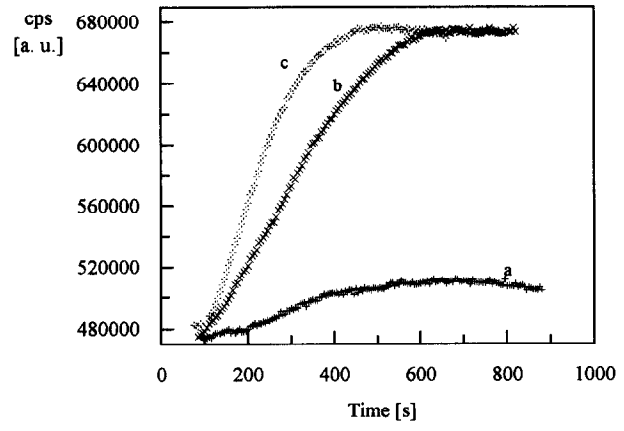

Fig. 1. Polymerization of actin (10 μM) with 100 mM KCl followed by fluorescence (5% pyrenyl actin) in the presence of thymosin β4 (1 equiv.) (a); thymosin β4 (1 equiv.) plus phalloidin (1 equiv.) (b); phalloidin (1 equiv.) (c).

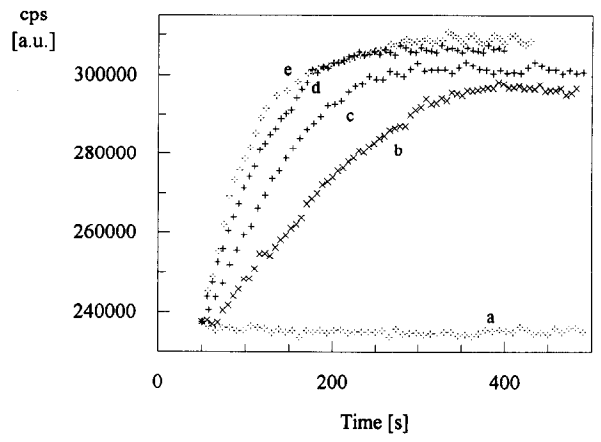

Fig. 3. Polymerization of actin–thymosin β4 upon the addition of various concentrations of actin nuclei followed by fluorescence. Actin and thymosin β4 (1 : 1) were incubated for 30 min before different amounts of nuclei were added together with 100 mM KCl. No nuclei added (a); 2.5%, 5%, 7.5% and 10% nuclei added, respectively (b–e).

of muscle actin and thymosin β4 in a ratio 1 : 1, polymeric actin is formed at a very low rate upon the addition of 0.1 M KCl, as shown by fluorescence measurement (5% pyrenyl actin) (Fig. 1). This is most simply explained as reflecting the polymerization of that amount of actin that according to the K_d (ca. 1 μM) is present in the uncomplexed state [19,20]. If to this solution one equivalent of phalloidin is added, the rate of polymerization is strongly enhanced, and the extent of polymerization reaches that of normal actin, as shown in Fig. 1. The effect of phalloidin could be confirmed by viscometry measurements (Fig. 2). Here, the actin–thymosin β4 complex was pretreated with 0.1 M KCl for 30 min before the phalloidin was added. Also in this system the

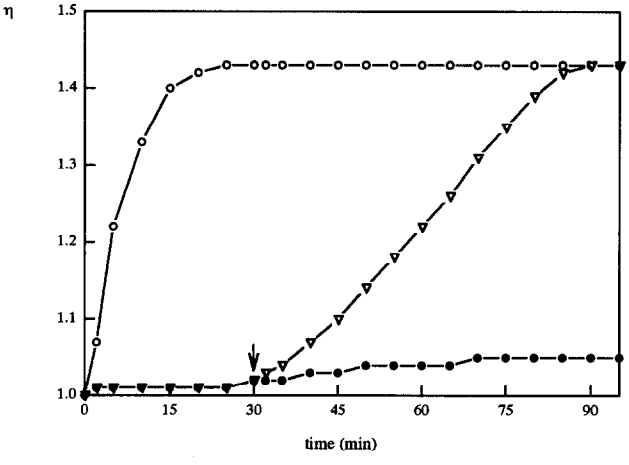

Fig. 2. Polymerization kinetics of actin–thymosin β4 (1 : 1) measured by capillary viscometry after the addition of 100 mM KCl at time zero, without phalloidin (●) and with 1 equiv. of phalloidin (▽). Phalloidin was added (↑) 30 min after the addition of KCl showing that in the absence of the toxin polymerization did not occur within this time. For comparison: pure actin with 1 equiv. of phalloidin (○), added together with the salt at time zero.

toxin caused a steep increase in both the rate and the absolute amount of polymerization.

It is generally agreed that the effect of phalloidin is to stabilize actin filaments [21]. From polymerization experiments in which phalloidin caused a distinct acceleration of the rate of polymerization [22] it was concluded that the stabilization effect observed for filaments would apply for actin nuclei as well. In accordance with these findings, the most likely explanation for the instant onset of polymerization in solutions containing the actin–thymosin β4 complex is the increase in the concentration of actin nuclei, due to their stabilization by phalloidin.

In order to substantiate our suggestion that nuclei might be involved in the phalloidin-accelerated polymerization we prepared actin trimers by reacting F-actin with *N-N'*-p-phenylene-bis-maleimide according to Lal et al. [15]. As proved by SDS-PAGE, our preparation consisted of ca. 50% of actin trimers, ca. 45% monomers and traces of dimers (data not shown). Addition of these nuclei (5% of total actin) to the actin–thymosin β4 complex accelerated polymerization at a rate even higher than that observed by the addition of phalloidin (Fig. 3). The acceleration depended on the amount of nuclei added, but not in a linear way. The increase in the polymerization rate upon the addition of actin nuclei was also confirmed by viscometry measurements (Fig. 4).

It has been reported that one effect of cytochalasin B on actin is to accelerate polymerization by nucleation [23,24]. We confirmed this effect (Fig. 5) by showing that in the presence of cytochalasin B the typical lag phase in the polymerization kinetics of actin was absent. When cytochalasin B was added to a solution of actin–thymosin β4, the toxin accelerated the polymerization also from the complex, although to a much smaller extent than when phalloidin or nuclei were added. Although quantitatively small in size, the effect of cytochalasin B

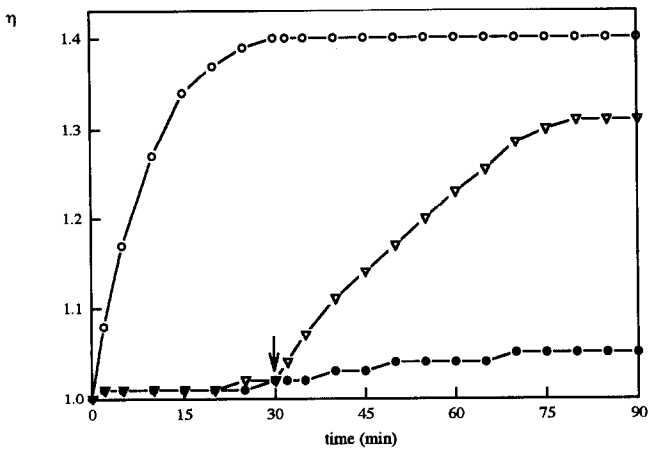

Fig. 4. Polymerization of actin and actin–thymosin β4 (1:1) upon the addition of actin nuclei followed by viscometry. Nuclei (5%) were added to actin (○) together with 100 mM KCl at time zero, or to actin–thymosin β4 (1 : 1) (▽) (↑) 30 min after the addition of 100 mM KCl showing that in the absence of nuclei polymerization did not occur within this time. For comparison: actin–thymosin β4 without added nuclei (●).

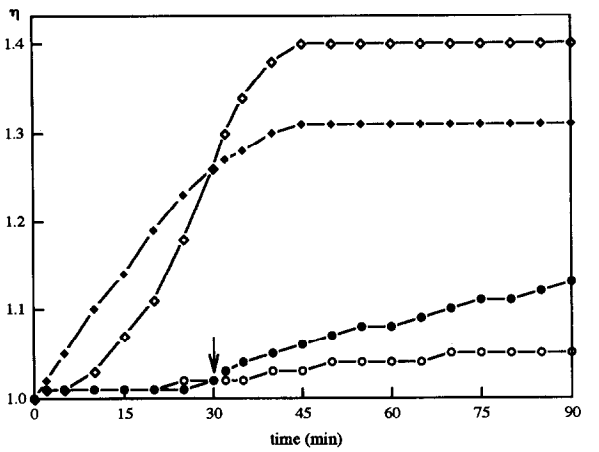

Fig. 5. Polymerization kinetics of actin (◇,◆) and actin–thymosin β4 (○,●) upon the addition of cytochalasin B (1 equiv.) followed by viscometry. Actin with (◆) or without (◇) cytochalasin B, added together with 100 mM KCl at time zero. Actin–thymosin β4 with (●) or without (○) cytochalasin B. Cytochalasin B was added (↑) 30 min after the addition of 100 mM KCl showing that in the absence of the toxin polymerization did not occur within this time.

supports the idea that formation of nuclei is the crucial event for initiating spontaneous polymerization from the actin–thymosin β4 complex.

It is known that myosin S1 is able to induce actin polymerization even under non-polymerizing conditions [25]. We therefore tested the effect of myosin S1 on a thymosin β4 inhibited actin solution by fluorescence and viscometry. Myosin S1 was added either simultaneously with 0.1 M KCl to the fluorescence assay (Fig. 6), or, after 30 min pretreatment with the salt, to the viscosimeter (Fig. 7). In both systems the addition of 10% myosin S1 caused the steepest increase in the rates of polymerization that we observed. Also here, the effect depended on the concentration. These experiments show that not only actin nuclei can serve as nucleation centers, but that this function can likewise be exhibited by a protein that is functionally connected with the polymeric form of actin.

While the experiments with phalloidin, cytochalasin B or covalently linked actin trimers represent non-physiological conditions, the experiments using myosin S1 are considered to be closest to the physiological situation. Anticipating that myosin itself has the same activity as the fragment and that thymosin β4 complexation is the predominant mechanism for inhibiting uncontrolled filament formation in non-muscle cells, our data open the possibility that cells can organize the formation of filaments from their pool of complexed monomeric actin simply by activating myosin at locations where the contractile machinery is required. Other nucleation centers of different structure and function may exist, and it cannot be excluded that cells may contain a component representing a physiological equivalent of phalloidin engaged in regulating filament formation. More arguments for the involvement of nuclei as organization centers of microfilaments are based on the identification of the ActA-protein of *Listeria monocytogenes* that induces the formation of a tail of polymerized actin on which the bacterium is propelled through the infected cell [26], as well as from the proposal that capped ends may serve as nucleation centers [27].

Cytotoxic effects of phalloidin have mainly been studied in hepatocytes since these cells possess a phallotoxin-transporting activity in their plasma membrane [28–30]. In freshly isolated rat hepatocytes the addition of phalloidin causes the formation of surface blebs within ca. 10 min [31]. Considering the reported filament stabilization of phalloidin that leads to a decrease in critical concentration of actin in vitro [22,32], the morphological changes in the hepatocytes were interpreted in terms of

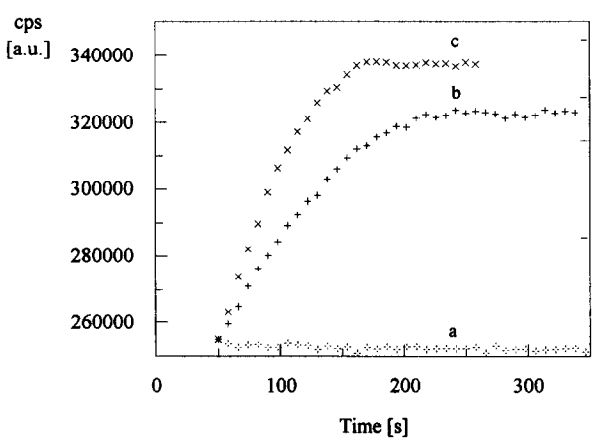

Fig. 6. Polymerization of actin–thymosin β4 with 100 mM KCl in the presence of various amounts of myosin S1 followed by fluorescence. Actin and thymosin β4 (1 : 1) were incubated for 30 min before myosin S1 was added together with the salt. Without myosin S1 (a); with 5% myosin S1 (b); with 10% myosin S1 (c).

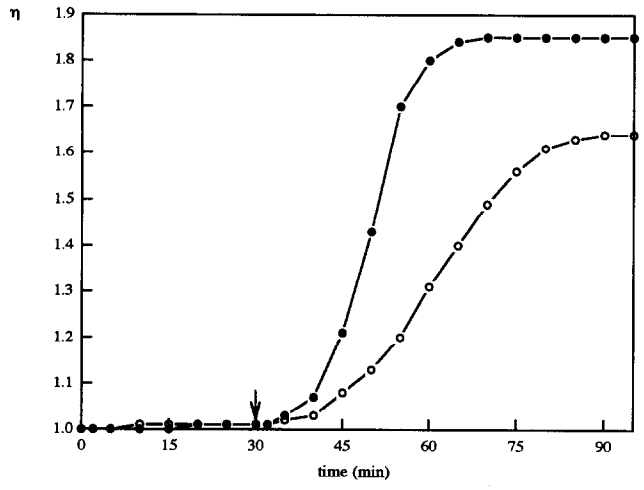

Fig. 7. Polymerization of actin–thymosin $\beta 4$ (1 : 1) upon the addition of various amounts of myosin S1, followed by viscometry. Myosin S1 (5% (○); 10% (●)) was added after 30 min incubation of actin–thymosin $\beta 4$ in the presence of 100 mM KCl.

delayed filament turnover. However, lack of actin monomers as a consequence of inhibited filament depolymerization might be expected to lead to long-term effects, but not to the formation of these blebs within a few minutes. On the other hand, the kinetics of polymerization of thymosin $\beta 4$ stabilized muscle actin by phalloidin are well consistent with this time scale. We therefore believe that formation of phalloidin-stabilized nuclei is the most likely mechanism to explain the phalloidin effect in isolated hepatocytes. This would imply, of course, that also in hepatocytes the pool of monomeric actin is stabilized by thymosin $\beta 4$, a possibility for which evidence has been provided recently [33]. A similar effect of phalloidin on the actin-profilin complex has so far not been reported.

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