Interaction of ADP-ribosylated actin with actin binding proteins

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Abstract Actin ADP-ribosylated at Arg177 was previously shown not to polymerise after increasing the ionic strength, but to cap the barbed ends of filaments. Here we confirm that the polymerisation of ADP-ribosylated actin is inhibited, however, under specific conditions the modified actin copolymerises with native actin, indicating that its ability to take part in normal subunit interactions within filaments is not fully eliminated. We also show that ADP-ribosylated actin forms antiparallel but not parallel dimers: the former are not able to form filaments. ADP-ribosylated actin interacts with deoxyribonuclease I, vitamin D binding protein, thymosin β_4, cofilin and gelsolin segment 1 like native actin. Interaction with myosin subfragment 1 revealed that the potential of the modified actin to aggregate into oligomers or short filaments is not fully eliminated. © **2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Actin binding protein; ADP-ribosylated actin

1. Introduction

Purified monomeric (G-)actin is only stable at low ionic strength, upon increasing the salt concentration it polymerises to filaments, F-actin, which in striated muscles form the core of the thin filaments. In non-muscle cells the organisation of actin is regulated by a large number of actin binding proteins (AbPs) which according to their specificity preferentially bind either to G- or F-actin. In non-muscle cells, a high percentage of the total cellular actin is in monomeric form, although the ionic strength would favour its complete polymerisation. The equilibrium between G- and F-actin is regulated by proteins which bind G-actin and sequester it from the G–F-equilibrium. There are a number of intra- and extracellular AbPs which are able to form 1:1 complexes with G-actin like thymosin β_4 ($T\beta_4$), vitamin D binding protein (DbP) or deoxyribonuclease I (DNase I). Other AbPs like cofilin or gelsolin interact with both G- and F-actin regulating its supramolecular organisation and filament turnover. Motor proteins like the myosins are assumed to solely interact with F-actin. Actin is a eukaryotic substrate of various bacterial toxins including *Clostridium botulinum* C2 toxin and *Clostridium perfringens* iota toxin [1]. These toxins ADP-ribosylate actin at Arg177 [2]. This actin has been reported to be unable to polymerise and to be transformed into a capping protein for the plus-ends of F-actin [3,4]. In cultured cells, these toxins induce the disappearance of intracellular microfilaments presumably by shifting the G–F-equilibrium to the monomeric state [5]. Subsequently the infected cells round up and lose contact to the substratum. It has been suggested that clostridial toxins mimic eukaryotic enzymes. Indeed, it has been shown that among the known eukaryotic ADP-ribosyltransferases an enzyme present in avian erythrocytes is able to ADP-ribosylate actin, although at different sites (Arg92 and 372) leading to a change in the actin turnover [6].

Here we analysed the ability of ADP-ribosylated skeletal muscle actin to polymerise and to interact with a number of AbPs. The results indicate that ADP-ribosylation of actin does not perturb its ability to bind to actin-sequestering proteins. Although polymerisation of ADP-rib-actin is reduced [3,4], we show that copolymerisation with native actin is still possible. ADP-rib-actin on its own forms the so-called lower dimers which were earlier identified in the initial phase of polymerisation but not during elongation [7]. Finally we show that the interaction with myosin subfragment 1 leads to its slow polymerisation.

2. Materials and methods

2.1. Materials

Nicotinamide adenindinucleotide (NAD), 1-ethyl-3[3(dimethylamino)propyl] carbodiimide (EDC), 1,5-difluoro-2,4-dinitrobenzene (FFD), and 1,4-phenylene-bismaleimide (1,4-PBM) were purchased from Sigma (Munich, Germany). All other reagents were of analytical grade.

2.2. Protein preparations

Rabbit skeletal muscle actin was prepared from fresh psoas muscle and dried acetone powder as detailed previously [8]. Subfragment 1 was obtained by digestion of purified rabbit skeletal muscle myosin with chymotrypsin. The subfragment 1 isoform containing the alkali light chain A1 (S1A1) was purified as described [9]. Iota toxin of *C. perfringens* was produced recombinantly as GST-fusion protein in *E. coli* and purified. Human cofilin, intact cytoplasmic gelsolin and gelsolin segment 1 (G 1) were all obtained recombinantly and purified from *E. coli* inclusion bodies as described [10]. Human Dbp was purified from outdated blood plasma [11] and a generous gift from Dr. Buch (Bochum, Germany). $T\beta_4$ was prepared from bovine spleen

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Abbreviations: DbP, vitamin D binding protein; DNase I, deoxyribonuclease I (EC 3.1.21.1.); EDC, 1-ethyl-3[3(dimethylamino)propyl] carbodiimide; FFD, 1,5-difluoro-2,4-dinitrobenzene; LD, lower dimer of actin; 1,4-PBM, 1,4-phenylene-bismaleimide; NAD, nicotinamide adenindinucleotide; $T\beta_4$, thymosin β_4

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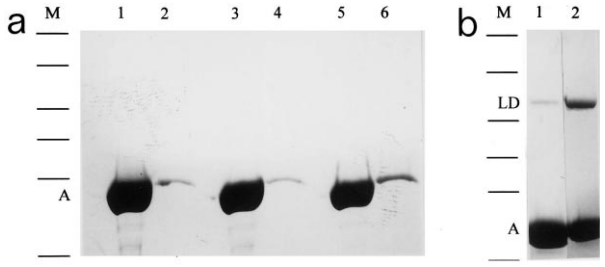

Fig. 1. a: SDS–PAGE of a sedimentation experiment of 11.7 μM ADP-rib-actin in the presence of no salt (1 and 2), 2 mM $MgCl_2$ (3 and 4) and 50 mM $MgCl_2$ (5 and 6). Lanes 1, 3 and 5 supernatants, lanes 2, 4 and 6 pellets. b: SDS–PAGE of 1,4-PBM cross-linked ADP-rib-actin (23, 7 μM) at 2 and 50 mM $MgCl_2$ (lanes 1 and 2, respectively). Bars give migration of prestained molecular mass markers (Sigma, Munich, Germany); from top to bottom: 230, 135, 97, 78, 57.5, and 38.5 kDa.

according [12] and kindly supplied by Dr. E. Hannappel (Erlangen, Germany). DNase I was obtained from Paesel (Frankfurt, Germany) and further purified [13]. Actin was fluorescently labelled at Cys374 by treatment with pyrenyl-iodoacetamide or dansylated at Gln41 as described in [14]. Chemical cross-linking by EDC, FFD or 1,4-PBM was performed as detailed [15].

2.3. ADP-ribosylation of G-actin

Actin at 5 mg/ml 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) was supplemented with 0.4 mM NAD. Then iota toxin (1 μg/5 mg actin) was added and further incubated at room temperature for 5 h. After this time the actin, was completely ADP-ribosylated as verified by its faster migration during native gel electrophoresis.

2.4. Analytical procedures

SDS–PAGE, native gel electrophoresis and immunoblotting were performed according to [16–18]. For co-sedimentation, the samples were centrifuged in a Beckman Optima TL ultracentrifuge for 1 h at $100\,000 \times g$. Changes in fluorescence intensity of pyrenyl- or dansyl-actin were measured in a Shimadzu RF 5001-PC spectrofluorometer at excitation and emission wavelengths of 365 or 383 nm and 340 or 500 nm, respectively. DNase I activity was determined by the hyperchromicity test [13] using a Beckman DU 640 spectrophotometer.

3. Results

3.1. ADP-ribosylated actin does not polymerise

In agreement with previous reports we observed no polymerisation of ADP-rib-actin after addition of 0.1 M KCl or 2 mM $MgCl_2$ as tested by sedimentation assays, Fig. 1a. At 50 mM $MgCl_2$ native F-actin precipitates as antiparallel paracrystals which can be cross-linked to the so-called lower dimer of actin (LD) [7,18]. By contrast, no increase of sedimentable ADP-rib-actin was found at 50 mM $MgCl_2$, Fig. 1a. Nevertheless, addition of the chemical cross-linker 1,4-PBM to ADP-rib-actin in 2 or 50 mM $MgCl_2$ led to the formation of the LD, a higher yield being obtained in 50 mM $MgCl_2$, Fig. 1b.

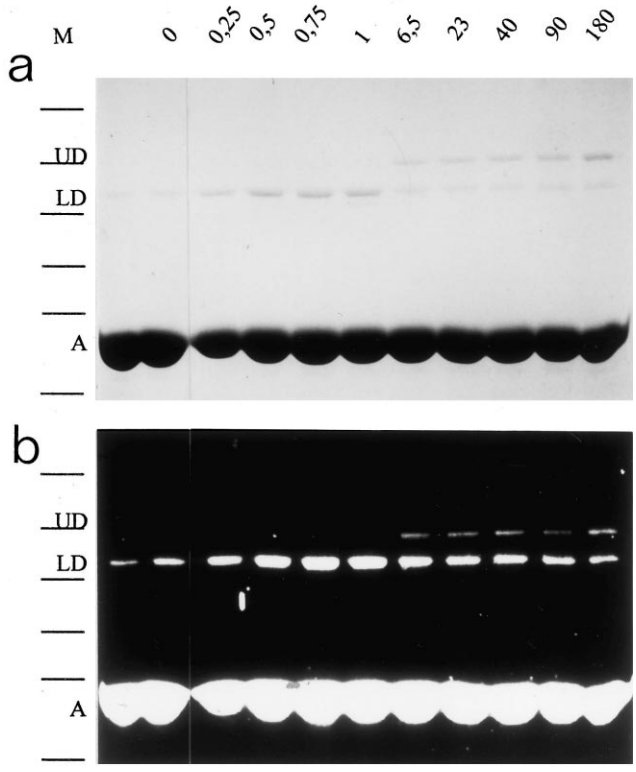

Fig. 3. Time dependent cross-linking of copolymerising dansyl-ADP-rib-actin and unlabelled native actin (11.6 μM each) analysed by SDS–PAGE. Gel stained with Coomassie blue (a) and (b) viewed on a fluorescent screen. Numbers on top indicate time of aliquot removal and mixing with boiling sample buffer. UD and LD denote upper and lower dimer, respectively. Bars indicate molecular mass markers as in Fig. 1.

3.2. Copolymerisation of ADP-ribosylated actin with native actin

Next we tested the ability of ADP-rib-actin to copolymerise with native actin. To test this we mixed 10 μM ADP-rib-actin with 10 μM native actin under polymerisation conditions and found that a considerable amount of actin remained in the

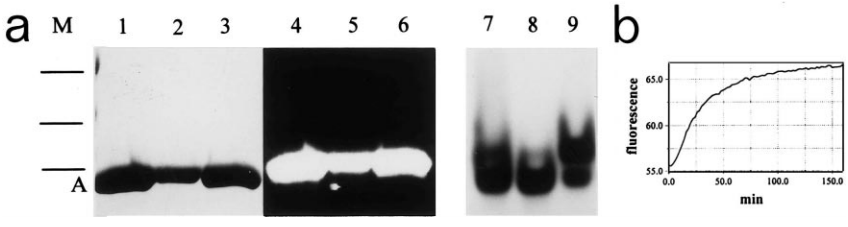

Fig. 2. a: SDS–PAGE of a co-sedimentation experiment of dansyl-ADP-rib- and native actin (11.6 μM each). Lanes 1, 4 and 7: sample before, lanes 2, 5 and 8: supernatant and lanes 3, 6 and 9: pellet after centrifugation. Lanes 1–3 gel stained with Coomassie blue, lanes 4 and 5 identical gel viewed on a fluorescent screen, and lanes 7–9 identical samples analysed by native gel electrophoresis. b: Measurement of the fluorescence increase of 10 μM dansyl-ADP-rib-actin copolymerising with 10 μM unlabelled native actin. Note the decrease in polymerisation speed: $t_{1/2}$ about 25 min. Independently determined $t_{1/2}$ for native dansyl-actin was about 1 min (not shown). Bars indicate 97, 78, and 57.5 kDa.

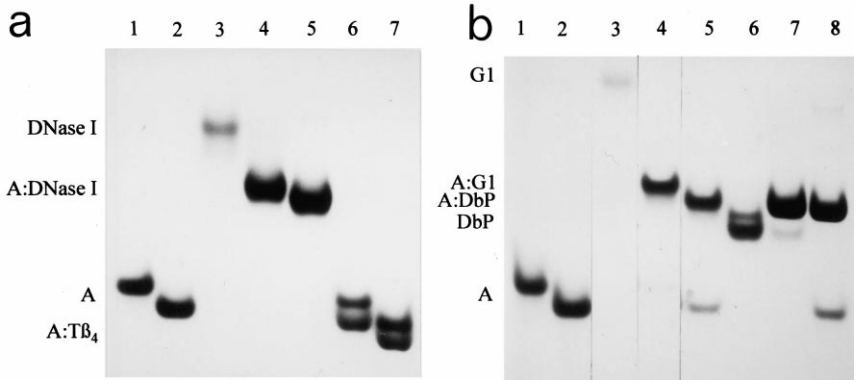

Fig. 4. Native gel analysis of the interaction of ADP-rib-actin with G-AbPs. a: Lanes: 1, native; 2, ADP-rib-actin; 3, DNase I; 4, DNase I complex with native; 5, ADP-rib-actin; 6, $T\beta_4$ complex with native; and 7, ADP-rib-actin. Actins, DNase I and $T\beta_4$ were at 6.8, 6.2 and 14 μM, respectively. b: Lanes: 1, native; 2, ADP-rib-actin; 3, G1; 4, complex of G1 with native; 5, with ADP-rib-actin; 6, DbP; 7, DbP complex with native; and 8, with ADP-rib-actin. Actins, DbP and G1 were at 6.8, 6.8 and 7.2 μM, respectively.

supernatant, Fig. 2a. Copolymerisation of native and dansyl-ADP-rib-actin in 2 mM $MgCl_2$ was verified by fluorescence of the pellets, Fig. 2a. Subjecting the pellets to native gel electrophoresis indicated a maximal ratio of 4:1 of native to ADP-rib-actin, Fig. 2a. Copolymerisation was also verified kinetically using dansyl-ADP-rib-actin whose fluorescence intensity increased during polymerisation like dansyl-actin, although with a considerably prolonged time course, Fig. 2b.

In the presence of 50 mM $MgCl_2$ ADP-rib-actin forms LD, Fig. 1b. In the case of native actin these dimers rearrange to upper dimers during filament elongation [7]. We therefore tested whether ADP-rib-actin was incorporated into the upper dimer moiety during polymerisation with native actin. The results indicated that after addition of 2 mM $MgCl_2$ dansyl-ADP-rib-actin was first cross-linked into the lower and then into the upper dimer, Fig. 3. In contrast to pure native actin [7], even 3 h after initiation of polymerisation a considerable amount of dansyl-ADP-rib-actin remained in the LD orientation.

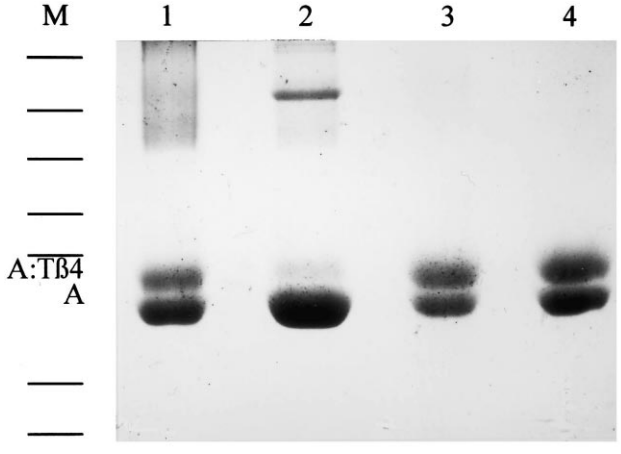

Fig. 5. SDS–PAGE of cross-linking of the $T\beta_4$ complex with native (lanes 1 and 2) and ADP-rib-actin (lanes 3 and 4) without (1 and 3) and in the presence of 0.1 M KCl (2 and 4). Actins and $T\beta_4$ were at 46.5 and 92 μM, respectively. Bars indicate 230, 135, 97, 78, 57.5, 38.5 and 33.5 kDa.

3.3. Interaction of ADP-ribosylated actin with monomer binding proteins

The interaction of ADP-rib-actin with monomer binding proteins was compared to native actin by native gel electrophoresis and chemical cross-linking followed by SDS–PAGE. In this analysis we included DNase I, $T\beta_4$, G1, and DbP, which all possess an almost exclusive binding preference for G-actin. During native gel electrophoresis ADP-rib-actin migrates faster than native actin. Fig. 4a,b gives a compilation of the data indicating that ADP-rib-actin interacts with these binding proteins equally well as with native actin. It was possible to cross-link all employed AbPs with identical efficiency to ADP-ribosylated and native actin (not shown).

DNase I, G 1 and DbP exhibit a high affinity for G-actin ($K_D \sim 10^{-10}$ to 10^{-11} M). In contrast, the affinity of $T\beta_4$ for monomeric actin is considerably lower ($K_D \sim 10^{-6}$ M) and sensitive to ionic strength. At high salt, the tendency of actin to polymerise leads to dissociation of this complex [8]. We therefore analysed the stability of the complexes of $T\beta_4$ with native and ADP-rib-actin by cross-linking assays. Fig. 5 demonstrates that addition of 0.1 M KCl reduced the amount of $T\beta_4$ cross-linked to native actin by about 80%, whereas no reduction was observed for the complex of $T\beta_4$ and ADP-rib-actin indicating a higher stability of this complex.

3.4. Interaction of ADP-rib-actin with F-AbPs

In this study, we used gelsolin, cofilin and S1A1 as F-AbPs. Gelsolin is a multifunctional AbP able to sever F-actin and to block barbed end growth by binding to this fast growing end. It also interacts co-operatively with two actin monomers in the presence of calcium, forming a nucleus that elongates in a manner similar to the slow growing pointed end of F-actin [19]. The binding of gelsolin to ADP-rib-actin was analysed by native gel electrophoresis. Fig. 6a demonstrates the formation of gelsolin:2actin complex. Cross-linking of the two actins of this complex by 1,4-PBM and subsequent immunoblotting with anti-actin (to differentiate LD from gelsolin) confirmed the formation of LD of ADP-rib-actin at both low and high salt (0.1 M KCl), Fig. 6c,d. When a mixture of monomeric and cross-linked LD of ADP-rib-actin was incubated with gelsolin, it preferentially bound LD, Fig. 6b.

Cofilin interacts with both G- and F-actin. It accelerates the dissociation of actin subunits from the pointed ends of fila-

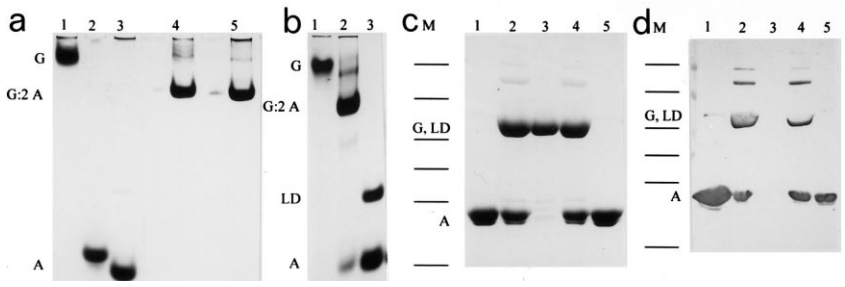

Fig. 6. Analysis of the interaction of ADP-rib-actin with gelsolin. a: Native gel: Lanes: 1, gelsolin; 2, 17.37 μM native; 3, ADP-rib-actin; 4, gelsolin complex with native; and 5, ADP-rib-actin. Actins and gesolin were at 17.37 and 8.7 μM, respectively. b: Native gel: Lanes: 1, 7 μM gelsolin; 2, gelsolin complex with LD of ADP-rib-actin; 3, 14 μM LD preparation of ADP-rib-actin. Note the complete disappearance of LD in lane 2. c: SDS–PAGE analysis of 1,4-PBM cross-linking ADP-rib-actin (lane 2) and native actin both at 21.3 μM (lane 4) to 10.7 μM gelsolin. Lanes: 1, ADP-rib-actin; 3, gelsolin; and 5, native actin on their own. d: Western blot of identical gel stained with anti-actin. Note the higher staining intensity of LD formed by ADP-rib-actin (2) in comparison to native actin (4). Bands of higher molecular mass than LD represent cross-linked products between gelsolin and actins which are not upper dimers. Bars in (c) and (d) indicate 205, 130, 90, 64, 53, and 37 kDa.

ments and promotes disassembly much more extensively at pH 8 than at pH 6.5 [20,21]. Native gel electrophoresis shows complex formation using native and ADP-rib-actin (Fig. 7). Cross-linking with EDC revealed higher molecular mass adducts for native but not for ADP-rib-actin (not shown). When the cofilin:ADP-rib-actin complex was shifted to pH 6.5, no polymerisation was observed within 30 min as determined by light scattering (not shown). Cofilin:actin complex can form a ternary complex with DNase I (not shown). Incubation of the cofilin:ADP-rib-actin complex with 2 mM $MgCl_2$ did not result in a significant loss of its DNase I inhibitory capacity.

Titration of pyrenyl-ADP-rib-actin with S1A1 resulted in an immediate fluorescence increase confirming an interaction with a calculated K_d of 50 nM, Fig. 8a. Electron microscopy showed a few short filaments indicating the formation of polymeric actin (not shown). When S1A1 was incubated with a 1:1 mixture of native actin and dansyl-ADP-rib-actin, co-sedimentation of both forms of actin with subfragment 1 was observed, Fig. 8b. The DNase I inhibition test showed a time dependent loss of the DNase I inhibitory capacity of ADP-rib-actin when incubated alone with S1A1 confirming formation of polymers over several hours, Fig. 8c.

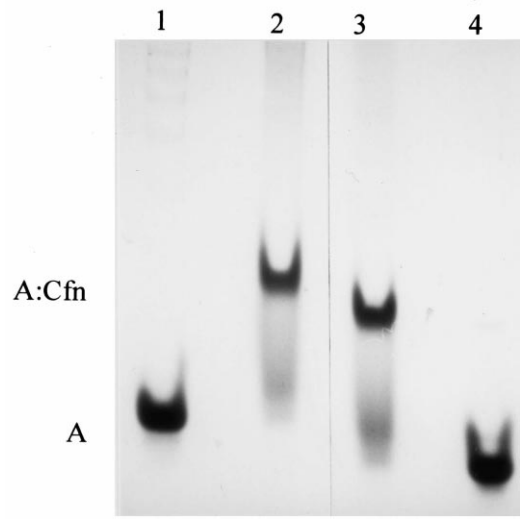

Fig. 7. Native gel analysis of the interaction of ADP-rib-actin with cofilin. Lanes: 1, native actin; 2, complex of cofilin with native; 3, with ADP-rib-actin; and 4, ADP-rib-actin on its own. Actins and cofilin were at 11.7 and 23.3 μM, respectively.

4. Discussion

In this report we analysed the polymerisability and interaction of ADP-rib-actin with a number of AbPs. The residue modified (Arg177) is located at the subunit interaction site along the one-start genetic helix [22] close to the region of the so-called hydrophobic finger. This finger extends from the junction between subdomains 3 and 4 contacting two adjacent actins of the opposing long pitch strands [23]. The introduction of a bulky group in this region by ADP-ribosylation is believed to interfere with the correct formation of the interface involving three actin subunits [24] and to inhibit the polymerisation of ADP-rib-actin [1,2]. In addition to these reports, we demonstrate that ADP-rib-actin is able to copolymerise with native actin. Thus ADP-ribosylation does not fully eliminate the polymerisability of actin.

ADP-rib-actin interacted with all the AbPs tested. The stability of ADP-rib-actin:$T\beta_4$ complex was increased at high salt suggesting that intracellularly ADP-ribosylation might reduce the level of assembled actin by both inhibiting polymerisation and stabilising sequestering complexes. Interaction with gelsolin or cofilin confirmed that ADP-ribosylation modified interactions in a manner that inhibited filament assembly of these complexes. Cofilin formed a 1:1 complex with ADP-rib-actin at pH 8.0 which, unlike complexes with native actin, did not polymerise at pH 6.5. Gelsolin formed complexes with two ADP-rib-actins and cross-linking confirmed that these were in the antiparallel orientation, similar to the behaviour of native actin [18]. Thus modification of Arg177 does not inhibit the formation of these dimers, but their observed stability may account for the lack of the nucleation activity when complexed to gelsolin [24]. In the case of native actin, LD reorients to the upper dimer or dissociates as polymerisation proceeds. In contrast, S1A1 was shown to bind to ADP-rib-actin with high affinity and then to induce slow polymerisation even at low ionic strength and the absence of native actin.

In summary, our data indicate that ADP-ribosylation of actin does not completely eliminate its potential for polymerisation. This is not unexpected since it is unlikely that introduction of a single bulky group at Arg177 could completely

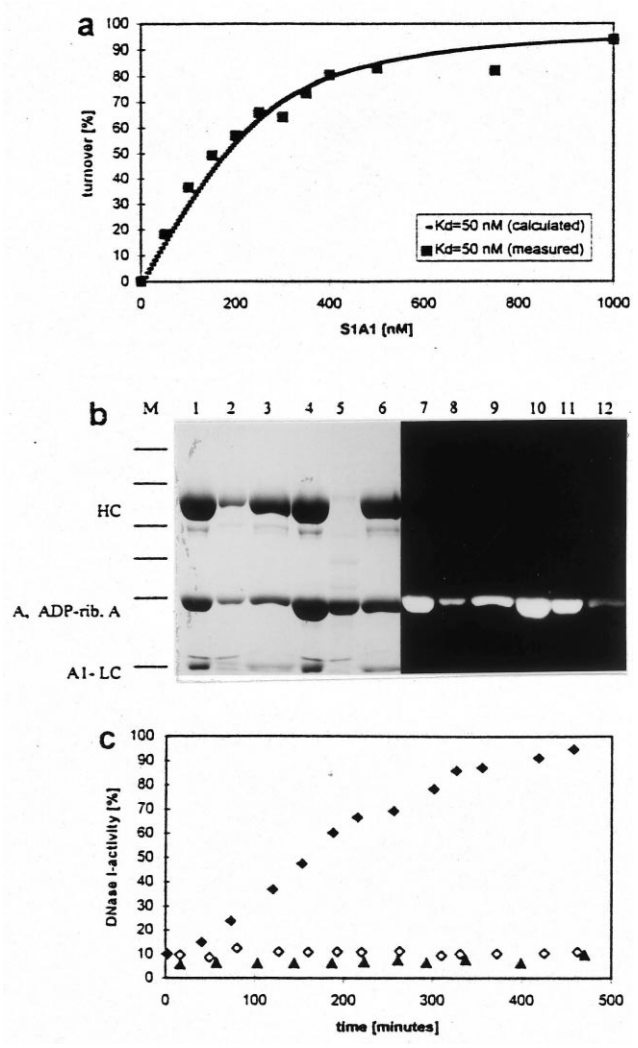

Fig. 8. Analysis of the interaction of ADP-rib-actin with S1A1. a: Titration of 250 nM pyrenyl-ADP-rib-actin with increasing concentration of S1A1. Abscissa gives immediate fluorescence increase in arbitrary units. b: SDS–PAGE analysis (lanes 1–6) of co-sedimentation of an incubation of 10 μM S1A1 with dansyl-labelled ADP-rib- (lanes 1–3; 7–9) and unlabelled native actin both at 10 μM (lanes 4–6; 10–12) in the absence of salt. Lanes 7–12 give the same gel viewed on a fluorescent screen. Lanes: 1, 4, 7 and 10: samples before centrifugation; 2, 5, 8 and 11: supernatant and 3, 6, 9 and 12: pellet after centrifugation. Note in lanes 3 and 9 the presence of S1A1 and ADP-rib-actin in the pellet of lanes 2 and 8 the presence of both S1A1 and ADP-rib-actin in the supernatant. HC and LC: heavy and light chain of S1A1. Bars in (a) and (b) indicate 205, 130, 90, 64, 53, and 37 kDa. c: Time dependent change of DNase I inhibitory capacity of 3 μM ADP-rib-actin incubated with 2.1 μM S1A1 in the absence of salt and ATP (♦), plus 5 mM ATP (◇), ADP-rib-actin on its own (▲).

disrupt the surface contacts between adjacent subunits needed for polymerisation. Nevertheless the equilibrium between monomeric and polymeric forms is greatly changed and in the case of $T\beta_4$, sequestration of the modified actin is improved. Similarly, gelsolin was found to stabilise antiparallel dimers by formation of GA_2 complexes and to inhibit their ability to form filaments. All these effects are consistent with the depolymerisation of actin that is seen when cells are treated with these toxins.

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