Stress-induced tyrosine phosphorylation of actin in *Dictyostelium* cells and localization of the phosphorylation site to tyrosine-53 adjacent to the DNase I binding loop

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Abstract **Actin is known to be phosphorylated at tyrosine, serine, or threonine residues in various cells. In cells of *Dictyostelium discoideum*, a rise in the tyrosine phosphorylation of actin is observed in response to ATP depletion. An actin fraction rich in phosphotyrosine was obtained by chromatography on the weak anion exchanger Mono-P. Mass spectrometry and amino acid sequencing of protease cleavage products indicated that a single tyrosine residue was phosphorylated. Localization of this residue to position 53 of the actin sequence attributed the modification to a site that is critical for the capability of actin to polymerize. Induction of the tyrosine phosphorylation by heat shock and Cd^{2+} ions indicates that this modification of actin is implicated in the response of *Dictyostelium* cells to stress.**

Key words: Actin; Tyrosine phosphorylation; *Dictyostelium*; Anoxia

1. Introduction

Actin polymerization and crosslinkage is known to be regulated by non-covalent binding of regulatory proteins and ligands of low molecular weight, particularly ATP, ADP, Ca^{2+} or Mg^{2+}. In addition, actin functions can be controlled by covalent modifications. Liver and platelet actin is ADP-ribosylated by *Clostridium botulinum* C2-toxin and this modification impairs polymerization of the actin [1]. *Physarum* actin is phosphorylated in vitro at threonine residues if it is in a 1:1 complex with fragmin [2]. The phosphorylation prevents formation of a 2:1 actin fragmin complex that is capable of nucleating filament formation [3]. Mammalian actin is phosphorylated in vitro at tyrosine residues [4] and in vivo at serine residues upon stimulation with epidermal growth factor [5].

In cells of *Dictyostelium discoideum* actin can be phosphorylated at serine [6] and tyrosine residues [6,7,8]. Tyrosine phosphorylation of *Dictyostelium* actin has been observed in response to various environmental conditions. Transfer of starving cells to nutrient medium causes a transient rise of tyrosine phosphorylation with a peak at 20 min upon transfer. On a similar time scale, tyrosine phosphorylation of actin increases when ATP is depleted in the cells as a result of anoxia or treatment with the uncoupler 2,4-dinitrophenol. After restoration of oxidative phosphorylation, the content in tyrosine-phosphorylated actin declines within 30 min to the low, hardly detectable levels of normal cells.

For studies on structural and functional consequences of the tyrosine phosporylation it is important to efficiently separate the modified actin from the non-modified one, and to locate the phosphorylated residue(s) to specific sites in the monomeric or filamentous actin. In this paper we describe the enrichment of tyrosine phosphorylated actin under non-denaturing conditions and identify the tyrosine residue that is phosphorylated in response to a block in respiration.

2. Materials and methods

2.1. *Culture and azide treatment of* D. discoideum *cells*

Cells of *D. discoideum* strain AX2-214 were cultivated axenically in shaken suspension at 23°C up to a density of not more than 4×10^6/ml. Tyrosine phosphorylation of actin was induced in these cells by the addition of sodium azide to a final concentration of 1 mM and incubation with the drug for 60 min. To induce stress responses, cells were incubated in nutrient medium on a rotary shaker at 33°C without additions or at 23°C with 100 μM $CdCl_2$.

2.2. *Preparation of tyrosine phosphorylated actin from Triton X-100 insoluble cytoskeletons*

7×10^9 azide treated cells were washed at 23°C in 400 ml of 17 mM Na/K-phosphate buffer, pH 6.0, containing 1 mM sodium azide and lysed at room temperature for 2 min in 15 ml lysis buffer (10 mM PIPES, pH 7.0, 20 mM KCl, 10 mM EGTA, 2 mM $MgSO_4$, 1% Triton X-100, 30% glycerol, 0.2 mM ATP, 4 mM $MgCl_2$, 0.5 mM DTT, 100 μM sodium orthovanadate, 1 mM PMSF, 200 units/ml of aprotinin, bestatin (0.5 μg/ml), and antipain, leupeptin, pepstatin A (each 1 μg/ml; Sigma), The lysate was centrifuged for 3 min at $10,000 \times g$ at 4°C and the pellet washed twice in 7–10 ml lysis buffer without detergent. After the final centrifugation step, the pellet was resuspended and incubated for 1 h on ice in depolymerization buffer: 10 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.1 mM DTT, 100 μM orthovanadate, 200 units/ml of aprotinin, bestatin (0.5 μg/ml), antipain, leupeptin, pepstatin A (each 1 μg/ml; Sigma). After centrifugation at $100,000 \times g$ for 25 min the supernatant was loaded on an FPLC-Mono-Q column (Pharmacia), and bound proteins were separated in 10 mM Tris-HCl, pH 8.0, in a 0 to 400 mM linear NaCl gradient. The major actin fraction eluted at 300 mM NaCl. Fractions of 1 ml were collected and the pooled fractions 14–17, which contained only actin and α-actinin, were subjected to isocratic separation at 350 mM NaCl in 10 mM Tris-HCl, pH 6.8, on an FPLC-Mono-P column (Pharmacia).

2.3. *Protein analysis and protease cleavage*

SDS-polyacrylamide electrophoresis in 10% gels, Coomassie blue staining, and immunoblotting with phosphotyrosine specific antibody 5E2 were performed as described [8]. For proteolysis, 200 μg of actin peaks I and II from the Mono-P column were dialysed and concentrated to 100 μl in 25 mM Tris-HCl, pH 8.5, containing 1 mM EDTA in a centricon 10 tube and cleaved with 4 μg of sequencing grade LysC endoprotease (Boehringer-Mannheim) for 14 h at 35°C.

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2.4. Protein and peptide analysis by HPLC, mass spectrometry, and Edman degradation

Fractions of the two actin peaks eluting from the Mono-P column were subjected separately to reversed phase HPLC coupled on-line to an atmospheric pressure ionization source fitted to the tandem quadrupole instrument API III (Sciex, Thornhill, Ont., Canada), and separated on a YMC C18 column (5 μm, 10 × 2 mm) at a flow rate of 15 μl/min, in a linear gradient of 1% per min of buffer B (0.1% TFA in acetonitrile) in buffer A (0.1% TFA in water).

Peptides obtained by LysC cleavage were separated on a supersphere 60 RP-selectB column (5 μm, 10 × 2 mm; Merck) at a flow rate of 300 μl/min in the same gradient as above and subsequently delivered to the sprayer by a syringe infusion pump (Model 22, Harvard Apparatus, South Natick, USA). The liquid flow rate was set at 5 μl/min for sample

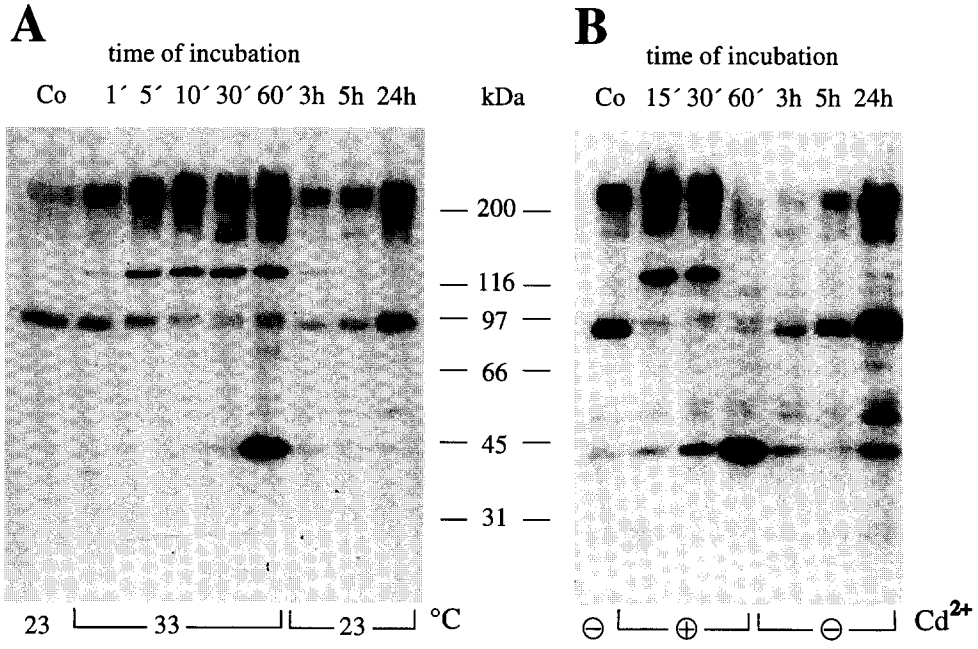

Fig. 1. Tyrosine phosphorylation of actin in response to heat and heavy metal stress. (A) Immunoblot labelled with phosphotyrosine-specific antibody of cell lysates before, during and after heat shock at 33°C. (B) Similar immunoblot before (−), during (+), and after incubation of cells with 100 μM $CdCl_2$. A tyrosine-phosphorylated band at 120 kDa is possibly related to heat shock transcription factors (HSTF) of yeast and *Drosophila* [15,16].

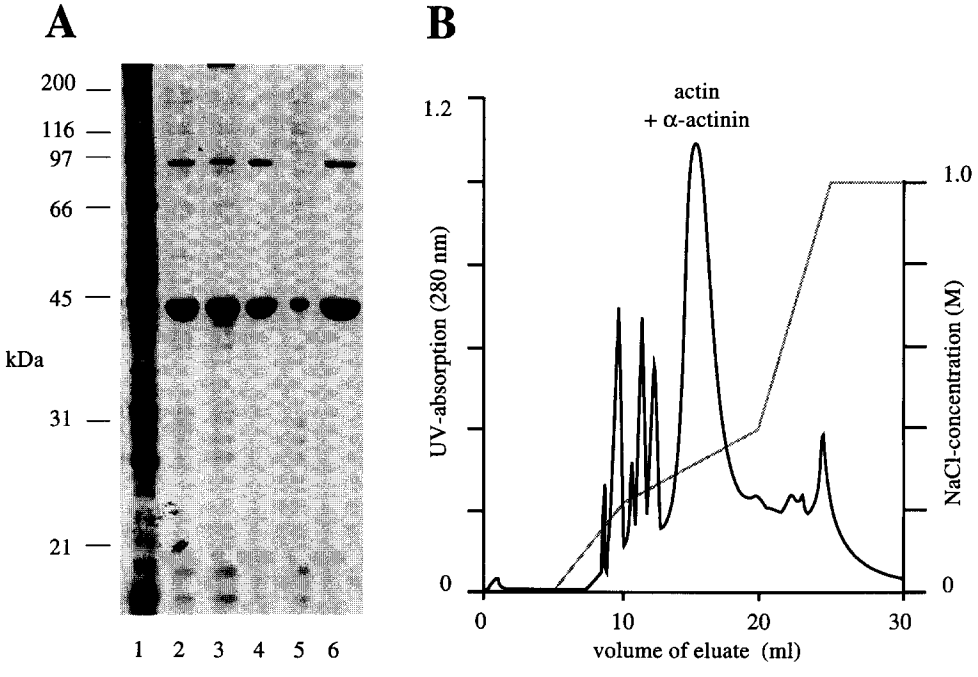

Fig. 2. Purification of total actin from azide-treated *D. discoideum* cells. (A) Coomassie blue stained SDS-polyacrylamide gel showing supernatant (lane 1) and pellet (lane 2) of Triton X-100 treated cell lysate; the same pellet fraction after depolymerization of actin for 1 h (lane 3); 100,000 × *g* supernatant (lane 4) and pellet (lane 5) of the depolymerized actin fraction; and actin from a Mono-Q column (lane 6). (B) Fractionation of the 100,000 × *g* supernatant (lane 4 of A) by FPLC on a Mono-Q column. Fractions 14–17 containing actin contaminated by α-actinin, as shown in lane 6 of A, were subsequently used for the enrichment of tyrosine-phosphorylated actin.

introduction. The m/z scale of the API III instrument was calibrated with the ammonium adduct ions of polypropylene glycol. The average molecular mass values of the proteins were calculated from the m/z peaks in the charge distribution profiles of the multiply charged ions [9,10].

Peptides were sequenced using a pulsed liquid phase sequencer 492 (Applied Biosystems).

3. Results

3.1. Increase in tyrosine phosphorylation of actin upon various stress conditions

Previously we have reduced the ATP-concentration in *D. discoideum* cells with 2,4-dinitrophenol in order to increase the phosphotyrosine content of actin [8]. Here we have used 1 mM sodium azide for the same purpose. Like 2,4-dinitrophenol, the azide completely rounds up the cells [11]. A similar increase of tyrosine phosphorylation was obtained by other stress conditions. Fig. 1 shows that tyrosine phosphorylation of actin is reversibly induced by heat shock and exposure to heavy metal ions.

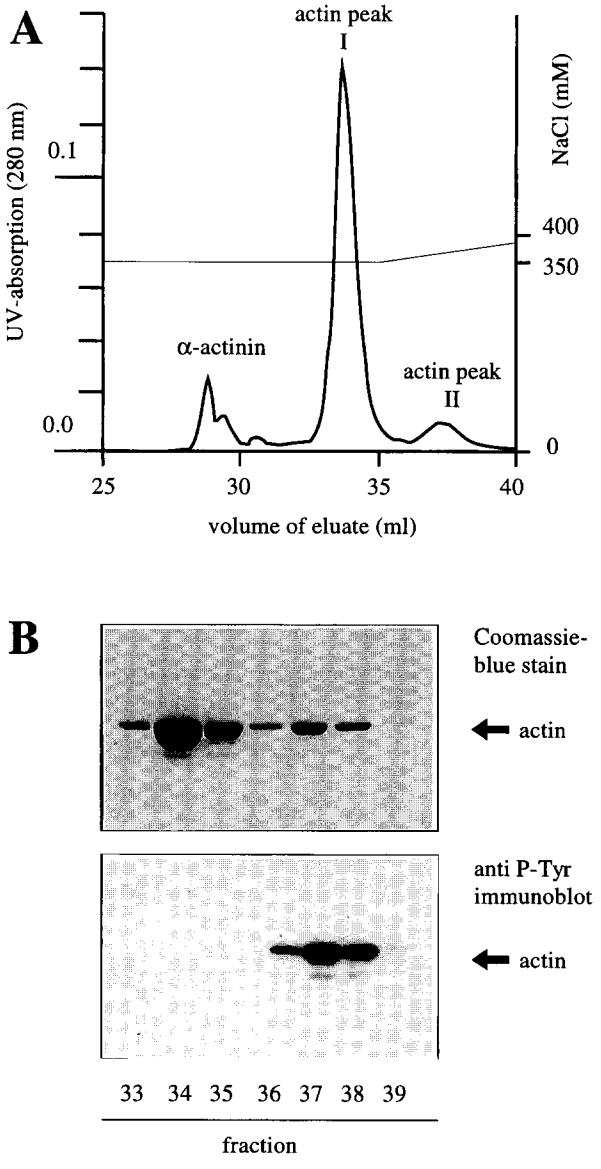

Fig. 3. Enrichment of tyrosine-phosphorylated actin on a Mono-P column. (A) Separation of actin into a major and a minor peak, and removal of α-actinin. (B) SDS-polyacrylamide gel electrophoresis of the actin containing fractions followed by Coomassie blue staining (top panel) or immunoblotting with anti-phosphotyrosine antibody (bottom panel).

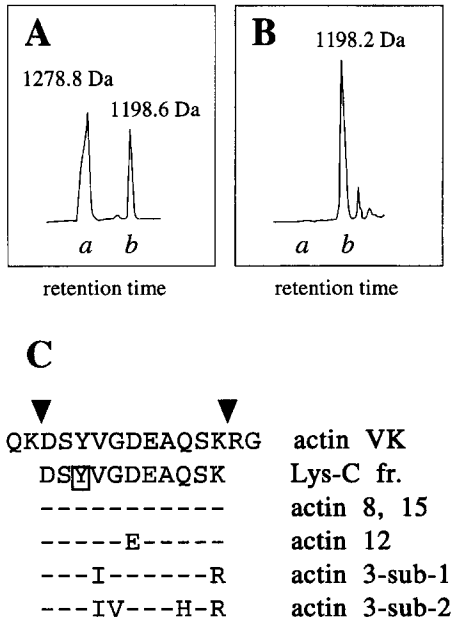

Fig. 4. Localization of phosphorylated tyrosine to a LysC cleavage product of actin. (A) Two peptides separated by reversed phase HPLC from the digest of peak II actin as shown in Fig. 3A. (B) One peptide separated in parallel from the digest of peak I actin. The masses on top of the peaks indicate that peptide *a* differs from peptides *b* of both runs by an additional mass of 80 Da. (C) Comparison of amino acid sequences of peptides *a* and *b* with a predicted LysC cleavage product of the major actin and DNA-derived actin sequences of *D. discoideum*. VK = protein sequence of actin according to Vandekerckhove and Weber [12]; LysC fr. = fragments *a* and *b* from actin peaks I and II. The boxed Y in position 3 was only detectable in peptide *b*. Actin numbers refer to genomic sequences according to Romans and Firtel [17].

3.2. Separation of tyrosine-phosphorylated actin from the major actin fraction

A Triton X-100 insoluble cytoskeleton fraction was prepared from azide-treated cells, and actin extracted from this fraction under depolymerizing conditions was subjected to a Mono-Q column (Fig. 2). The actin-enriched fraction was separated into two peaks on the weak anion exchanger Mono-P (Fig. 3). Most of the actin eluted in peak I, about 10% in peak II (Fig. 3A). Mass spectrometry analysis of the peak I fractions revealed a uniform mass of 41,664 ± 2 Da. In peak II two masses were found, one of 41,744 ± 2 Da, the other identical with that of peak I. The mass difference of 80 Da suggests that peak II contains a single phosphate group that is lacking in peak I actin. This was proven by immunoblotting peak I and peak II fractions with the phosphotyrosine-specific antibody 5E2 (Fig. 3B). Only actin of peak II was labelled, which demonstrates that the phosphate which distinguishes peak II actin from peak I actin is bound to a tyrosine residue.

Assuming N-terminal acetylation and the presence of *N*-methyl-histidine in position 73, the measured mass of peak I actin differs from the calculated mass of *D. discoideum* major

actin by only 5 Da. Therefore we infer that the actin found in peak I and peak II is identical with the established major actin [12].

3.3. Identification of the phosphorylated tyrosine residue

The peak I and II actin fractions eluted from the Mono-P column were digested with LysC endoprotease, and the peptides obtained from each fraction were separated by reversed phase HPLC. Peptides of potential interest were analysed by Edman degradation and ESI-MS. The digest from peak II contained a peptide that was not found in the digest from peak I (Fig. 4A,B). This peptide eluted in an acetonitrile gradient in front of the other peptides, indicating that it is highly hydrophilic. The mass of this peptide, 1278 Da, did not fit to any of the LysC cleavage products predicted from the published sequence of *D. discoideum* actin [12]. However, this mass coincided with the sum of the masses of a predicted peptide, 1198 Da, and a phosphate group of 80 Da. The peptide in question comprises residues 51–61 of the actin sequence, and contains a single tyrosine residue in position 3. Amino acid sequence analysis identified the peptide *a* as the predicted cleavage product of the major actin of *D. discoideum* with a gap in position 3 (Fig. 4C). This gap is in accord with the tyrosine to be modified by phosphorylation.

Peptide *b*, that eluted next behind the phosphorylated peptide *a* (Fig. 4A,B), had a mass of 1198 Da, suggesting that *a* and *b* represent the same actin fragment in its phosphorylated and unphosphorylated state, respectively. This was established by complete sequencing of peptide *b* from actin peaks I and II of Fig. 3A. The sequences proved to be identical with that shown in the first line of Fig. 4C; tyrosine in position 3 was recognized as an unmodified residue in peptides *b* from both actin peaks.

4. Discussion

Localization of the single phosphorylatable tyrosine residue to position 53 of the amino acid sequence attributes the phosphorylation site to the vicinity of the DNase I binding loop comprising residues 40–50 in subdomain 2 of actin. Reduced affinity of the tyrosine-phosphorylated actin to a DNase I column [6] suggests an involvement of phosphorylation at tyrosine 53 in DNase I binding. According to the atomic model of polymerized actin, Tyr-53 is not part of the actin–actin interface in the microfilament, but it appears to stabilize the adjacent 40–50 loop [13].

Evidence for a role of Tyr-53 in actin assembly has been provided by the finding that modification of this residue by 5-diazonium-(1*H*)tetrazole blocks the polymerization of rabbit skeletal muscle actin [14]. Preliminary results suggest that the tyrosine phosphorylated fraction of *Dictyostelium* actin polymerizes less efficiently than the unphosphorylated one (A. Jungbluth, unpublished).

The phosphorylation at Tyr-53 of actin appears to be of biological interest for three reasons. This modification has been shown to occur in vivo, it is induced upon re-initiation of growth, ATP-depletion or other stresses, and it is reversible when the cells recover to continue growth or development. A similar response in tyrosine phosphorylation has been found to osmotic shock conditions (Stefan Schuster, Martinsried, personal communication). From these results it appears that tyrosine phosphorylation of actin is a common denominator of stress responses in *D. discoideum.*

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