Changes in cell morphology and actin organization during heat shock in *Dictyostelium discoideum*: does HSP70 play a role in acquired thermotolerance?

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Abstract

In response to heat shock (34°C, 30 min), cell morphology and actin organization in *Dictyostelium discoideum* are drastically changed. Loss of pseudopodia and disappearance of F-actin-containing structures were observed by using fluorescence microscopy. These changes were paralleled by a rapid decrease of the F-actin content measured by a TRITC-phalloidin binding assay. The effects of heat shock on cell morphology and actin organization are transient: After heat shock (34°C) or during a long-term heat treatment (30°C), cell morphology, F-actin patterns and F-actin content recovered/adapted to a state which is characteristic for untreated cells. Because F-actin may be stabilized by increased amounts of heat shock proteins, their response and interaction with F-actin was analyzed. After a 1 h heat treatment (34°C), the major heat shock protein of *D. discoideum* (HSP70) showed maximally increased synthesis rates and levels. During recovery from a 34°C shock or during a continuous heat treatment at 30°C, the HSP70 content first increased and then declined slowly toward normal levels. Pre-treatment of cells with a short heat shock of 30 min at 34°C stabilized the F-actin content when the cells were exposed to a second heat shock. Furthermore, a transient colocalization of HSP70 and actin was observed at the beginning of heat treatment (30°C) using immunological detection of HSP70 in the cytoskeletal actin fraction. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Heat shock affects many, if not all, functions of a cell including cellular morphology, the cytoskeleton and movement. Cell extensions such as pseudopodia are retracted and the cytoskeleton reorganized (review: [1]). The partial breakdown of the cytoskeleton leads to aggregates around the nucleus (heat shock granules) as observed particularly in plant cells [2]. After a non-lethal heat shock, the initial responses are reversed during a recovery period until a ‘normal’ state is reached. During recovery cells are transiently less sensitive to stress (acquired thermotolerance [3,4]). During moderately elevated temperatures...
a similar reversal of the initial responses of cellular functions is observed even when the stress exposure is continued. Acquired thermotolerance during recovery and adaptation are probably aided, at least in part, by elevated levels of heat shock proteins (chaperones) which stabilize synthesis and conformation of essential cellular proteins [5].

Among the cytoskeletal filaments, actin plays an important role in the determination of cell shape, pseudopodia and cellular movement which has been analyzed intensively in the ameba *Dictyostelium discoideum* [6–10]. We, therefore, chose this organism to study the effects of heat shock on cell morphology, pseudopodia and F-actin formation, recovery from heat shock and adaptation during continued exposure. We focused on F-actin dynamics, because heat shock was shown to cause a rapid loss of stress fibers [11,12] and a disorganization of actin filaments in mammalian cells [13]. The negative effect of heat shock on the F-actin level that we measured in these experiments may be causally related to the observed retraction of pseudopodia and prompted us to analyze possible factors involved in the heat-induced actin depolymerization.

Important factors of F-actin stability are actin binding proteins, some of which were identified as HSPs [14–21]. The role of HSP70 in the balance between polymerization and depolymerization of actin is not quite clear, even though experiments with *D. discoideum* have shown that constitutive members of HSP70 possess capping activity [22] or function as adapters that stimulate the capping activity of Cap32/34 [23].

In a first approach to analyze a possible involvement of HSP70 in F-actin stabilization, we compared the recovery and adaptation kinetics of both F-actin stability and HSP70 level after or during heat shock treatment. For the HSP70 kinetics we confirmed and extended the study of Loomis and Wheeler [24]. In further series of experiments we analyzed the adaptation kinetics of both the HSP70 level and the stability of F-actin after heat shock in heat shock-primed and unprimed cells. We finally analyzed the possible interactions of HSP70 and F-actin during heat shock by determining the cytoskeleton-bound HSP70. The results of these experiments are compatible with the assumption that HSP70 contributes to F-actin stability.

2. Materials and methods

2.1. Cell culture

The *D. discoideum* strain AX2 was grown axenically in HL5 medium in a shaken suspension [25]. Cells were harvested at a concentration of 1–2×10⁶ cells ml⁻¹ and washed by two centrifugations (2 min, 300×g each) in Na/K buffer (17 mM Na₂HPO₄, 17 mM KH₂PO₄, pH 6.4) before use.

2.2. TRITC-conjugated phalloidin staining of F-actin

Quantitations of changes in F-actin content after heat treatments were assessed by measurement of the fluorescence intensity of TRITC-phalloidin-stained cells modified from the method of Hall et al. [7]. 2×10⁶ cells grown at 21°C or heat-shocked cells were fixed by 3.7% (v/v) formaldehyde solution (final concentration) for 20 min on a rotator and then washed with Na/K buffer containing 0.1% (w/v) saponin by two centrifugations (1 min at 12,000 ×g). The cells were stained 1 h with 0.7 μM TRITC-phalloidin (Sigma-Aldrich, Steinheim, Germany) on a rotator and were washed again twice with Na/K buffer as above. Cell pellets were resuspended in 1 ml Na/K buffer; the emitted fluorescence was read by a SPF-500TM ratio spectrofluorometer (American Instrument Company, Rochester, USA) at 570 nm (555 nm excitation). The relative F-actin content is defined as the ratio of the emission of a treated sample divided by that of an untreated sample.

The distribution of intracellular F-actin was visualized by TRITC-phalloidin staining of fixed intact cells. 5×10⁴ cells in Na/K buffer were plated on a washed 18×18 mm glass cover slip. After 30 min adhesion at 21°C cells were incubated at 34°C (heat shock) with the times indicated. The cells were then fixed for 20 min in 3.7% formaldehyde at 21°C. After extensive washing in Na/K buffer, cells were incubated for 30 min at 37°C with 0.7 μM TRITC-phalloidin. Cells were then washed twice and mounted in Na/K buffer, pH 6.4, containing 90% (v/v) glycerol and 2.5% (w/v) NaN₃. Fluorescence micrographs were taken on Kodak TMX-400 film using a Zeiss Standard Fluorescence Microscope with a 100× Neofluar objective (N.A. 1.30) and filter combination BP540, FT580, LP590.
2.3. Immunological detection of HSP70

Cell lysates were prepared by dissolving the cells with Laemmli buffer [26] and heated to 100°C for 5 min. Protein concentration was determined according to Neuhoff et al. [27]. The proteins were separated on 10% sodium dodecylsulfate polyacrylamide gels (SDS-PAGE [26]) and were then transferred to a...
nitrocellulose membrane using a Trans Blot Cell (Bio-Rad Laboratories, Munich, Germany) with a buffer containing 25 mM Tris, 192 mM glycine and 20% (v/v) methanol (pH 8.3) at 400 mA for 4 h (4°C). After blocking with 0.2% Tween-20 in phosphate-buffered saline (PBST) for 30 min, the nitrocellulose membrane was incubated with the primary antibody against HSP/HSC70 (Biomol, Hamburg, Germany, AP 822) diluted in PBST (1:1000) for 1 h and then washed with PBST. Subsequently, the samples were incubated with 1:1000 secondary antibody (anti-mouse IgG alkaline phosphatase conjugate, Sigma-Aldrich, Steinheim, Germany) for 1 h. After washing with PBST, the immuno-complex was detected by nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Boehringer, Mannheim, Germany).

2.4. Extraction of cytoskeletal actin

Cytoskeletal actins were isolated using Triton X-100 according to McRobbie and Newell [28]. 5 x 10^7 cells were incubated with 500 µl Triton solution containing 1% (v/v) Triton X-100, 10 mM potassium chloride, 10 mM imidazole, 10 mM EGTA, 2 mM sodium azide (pH 7.0). The samples were placed on ice for 10 min, then allowed to warm up to room temperature for 10 min with occasional agitation. After centrifugation for 4 min at 8000 g, the pellets were resuspended in 200 µl Triton X-100. The samples were again centrifuged, and the supernatants from each centrifugation collected. The pellets or supernatants were mixed with Laemmli buffer. Gel electrophoresis and immunologic detection of proteins were carried out as above.

3. Results

3.1. Distribution of F-actin in untreated and heat-shocked D. discoideum cells

The D. discoideum cells in our study were slightly less sensitive to heat shock compared to those used by Loomis and Wheeler [24]. We therefore applied slightly higher heat shock temperatures: 34°C for a 30 min pulse (which resulted in maximal HSP induction, data not shown) and 30°C for long-term treatments (which were not lethal in these cells in contrast to those of [24]).

In order to characterize the effect of heat shock on the distribution of F-actin in vivo, we used a fluorescent probe (TRITC-conjugated phalloidin, Sigma) which specifically binds to F-actin. Cells of the axenic strain which had been growing in suspension at
21°C were plated on glass cover slips. After 30 min adhesion the cells were shifted to 34°C for 30 min and thereafter were returned to 21°C. Immediately after heat shock and during the process of recovery, cells were fixed and labelled with TRITC-phalloidin. In untreated cells, an extensive accumulation of F-actin is observed in pseudopodia (see [29]) (Fig. 1A). When cells were analyzed 30 min after heat treatment they showed a spherical form (i.e. no pseudopodia) and exhibited uniform fluorescence when stained for F-actin (Fig. 1C, D). Four h after heat shock cells began to develop morphological asymme-

Fig. 3. Relative F-actin content. A: During recovery from a heat shock (34°C, 30 min); B: During a continuous heat shock at 30°C. The F-actin content of cells was measured by using the TRITC-phalloidin binding assay described in Section 2 and calculated relative to time zero (1.0). Each point represents the average of three experiments. Error bars indicate standard deviations. Ordinates, relative F-actin levels; abscissae: A, recovery time after heat shock (in h), R beginning of recovery; B, time after beginning of exposure to heat shock (in h).
A

Relative HSP70 content vs. time (h)

B

Relative HSP70 content vs. time (h)
tries of actin distribution and increased the intensity of fluorescence staining for F-actin, concomitant with the appearance of filamentous actin in the form of granules or small dots (Fig. 1E, F). When cells were analyzed after 6 h recovery, new pseudopodia were formed and filamentous actin reappeared (Fig. 1G, H). Cell shape and intracellular F-actin pattern after more than 6 h recovery appeared similar to those observed in untreated cells.

When the heat shock-induced changes of cell morphology and number of cells with F-actin spots were quantified the following kinetics of the response was observed: In the untreated controls of the recovery experiment 88% of the cells showed pseudopodia, 85% F-actin spots. Only a few cells (12%) appeared spherical (Fig. 2A). Cells were then incubated for 30 min at 34°C and then shifted to 21°C for recovery. After the first 2 h of recovery only 17% cells showed extended pseudopodia while 83% of the cells were spherical. Only 5% of the cells contained F-actin spots. After 4 h recovery, the number of cells with pseudopodia and spots increased. Cells gradually reached a state close to normal after 6–24 h recovery (Fig. 2A). Similar changes were observed during continuous heat shock treatment at 30°C. In the untreated controls of the adaptation experiment 90% of the cells showed pseudopodia and 70% F-actin spots (Fig. 2B). After 1 h incubation at 30°C a sharp decrease of cells with pseudopodia and actin spots is observed. During the following time the number of cells with pseudopodia and spots increased again. Beginning after about 3 h of heat treatment the cells reached almost their normal appearance after 6–9 h at 30°C.

Inspection of cells during these experiments by a fluorescence microscope revealed a rapid loss of filamentous actin induced by heat shock. During recovery or a moderate, long-term heat treatment the changes of the F-actin pattern associated with cell shape were reversed.

3.2. Effect of heat shock on actin polymerization

In order to better quantify the heat shock effects on actin polymerization, the F-actin content of cells was measured by a TRITC-phalloidin binding assay. In a first series of experiments changes of F-actin content were determined after the cells had been exposed to different temperatures for 30 min. As a result, the relative cellular F-actin decreased with increasing temperatures from 1.0 (at 21°C) to 0.69 (at 40°C) (data not shown). These results confirm the notion that elevated temperatures inhibit actin polymerization in a dose-dependent manner.

In order to determine whether the changes of cell morphology and F-actin organization during recovery from heat shock and during adaptation to 30°C were correlated with changes of the F-actin content, the latter was measured in heat-treated cells. The cells were exposed to 34°C for 30 min and then recovered at 21°C (Fig. 3A). Immediately after heat shock a sharp decrease in F-actin content by approximately 20% is observed as compared to untreated cells. The F-actin content then decreases further when determined after 30 min recovery. Thereafter, an increase in F-actin content occurs reaching the normal level after about 4 h and even higher values thereafter.

When the F-actin content was measured during a continuous heat shock (30°C), an initial drop by approximately 35% is seen 2 h after beginning of the heat treatment (Fig. 3B). Thereafter, the F-actin content increases gradually. After 9–12 h at 30°C the F-actin content reaches approximately the pre-stress level and subsequently even higher levels. This overshoot may in both cases be the result of continuing compensating mechanisms.

During a continuous heat treatment at 34°C the F-actin changes showed similar kinetics, the decrease of F-actin content, however, was much stronger (70%) than at 30°C, and the adaptational increase was de-
The recovery and adaptation kinetics of the changes in the morphology and actin spot formation (Fig. 2) and the respective kinetics of the F-actin content (Fig. 3) have two things in common: A rapid negative change immediately after beginning of HS treatment followed by a slower positive change. An approximately normal state is reached after about 6–9 h.

3.3. Effect of heat shock on the expression of HSP70

If HSP70 were functionally involved in the stabilization of F-actin a first hint for such a relation might be deduced from the HSP70 kinetics under the same heat shock conditions as applied in the previous experiments. HSP70 is the major heat shock protein in *D. discoideum* and was reported to be maximally synthesized in response to a heat shock of 30°C [24]. In order to reanalyze the data of Loomis and Wheeler [24] with respect to this strain and our experimental conditions, the following experiments were performed.

Cells grown in suspension at 21°C were incubated at different temperatures for 1 h. After 1 h exposure to different temperatures cells were labelled with [35S]-methionine (370 kBq ml⁻¹) for 1 h. A stimulation of the synthesis of HSP70 is observed already after a shift to 28°C. The stimulation increased with increasing temperatures. At 34°C the synthesis rate of HSP70 was maximal and about 20 times higher as compared to untreated cells. When the temperature was shifted to values above 34°C, the radioactivity of labelled proteins became very low, showing a strong inhibition of total protein synthesis at these temperatures. These results were confirmed by Western blot analysis using a monoclonal antibody against HSP70 (data not shown). This corresponds in principle to the results of Loomis and Wheeler [24], but showed that our strain was less sensitive to heat shock and responded maximally to 34°C exposure.

In order to determine the kinetics of the HSP70 content during recovery, cells were first shifted to 34°C for 1 h followed by incubation at 21°C. Within the first 3 h of recovery, an about 4-fold increase in the HSP70 content was observed as compared with the cells kept at 21°C (Fig. 4a). Thereafter, the HSP70 content slowly declined. This recovery kinetics in the amount of HSP70 is similar to the recovery kinetics of the synthesis rate as reported by Loomis and Wheeler [24], who observed high syn-
thesis rates for 4 h during recovery and a return to control during another 4 h. The fast decline in the amount of HSP70 may be partially due to degradation [30], but also due to the resumption of cell proliferation during recovery [24] and the resulting dilution of HSP70.

The HSP70 level during continuous heat treatment at 30°C showed an increase during the first 9 h and a subsequent plateau for another 9 h at a level about 6-fold higher than the controls (Fig. 4B). The decrease of the HSP70 content was delayed with respect to the recovery kinetics and began after about 18 h of 30°C exposure. This is again compatible with the data of Loomis and Wheeler [24] who observed...
an increase in the synthesis rate of HSP70 during 30°C exposure up to 5 h. The longer maintenance of high HSP70 levels at 30°C compared to the recovery kinetics may be due to an inhibition of proliferation at this temperature. The HSP70 response kinetics show an inverted type of curve compared to response kinetics of the morphology and F-actin, with a fast positive response shortly after a heat shock and a slower negative change. In all cases the recovery curves show a faster recovery to normal values than the adaptation curves.

3.4. Possible role of HSP70 in F-actin stabilization

Increased levels of HSPs contribute to a state of ‘acquired thermotolerance’ which is characterized by a lower sensitivity to stress [4]. In order to test a possible influence of acquired thermotolerance on F-actin stability or recovery, cells were first exposed to a 30 min heat shock at 34°C, after which HSP70 and the other HSPs are maximally stimulated (data not shown). After 4 h recovery at 21°C, when the HSP70 content is still rather high (see Fig. 4), cells were treated with a second heat shock of 30°C. The amount of HSP70 during this second heat shock was determined for 2 h at 15 min intervals and compared to that during a single heat treatment at 30°C by using Western blot detection of HSP70 (Fig. 5A). During a single heat shock a sharp increase of HSP70 content was observed, beginning after about 15 min and peaking after 45 min of treatment. After 45 min of treatment, the cell content of HSP70 was about 4 times higher than in control cells. When the cells had been pre-treated with a 30 min heat shock at 34°C followed by 4 h recovery, HSP70 already showed a 5 times higher level compared to untreated cells before the application of the second heat shock. During the second heat shock at 30°C (2 h) the HSP70 content maintained this level for the time analyzed (Fig. 5A).

We then determined the kinetics of F-actin content in cells treated with a single or a double heat shock by using the TRITC-phalloidin assay. In the unprimed cells the relative F-actin content decreased with time during a 2 h heat shock at 30°C (Fig. 5B), corresponding to the results in Fig. 3B. When the cells were pre-treated by a 34°C exposure as described above, no significant decrease was observed at the end of the second heat shock as compared to a decrease of 45% at the end of the single heat shock. These results indicate that the acquired thermotolerance state as induced by a priming heat shock stabilizes F-actin during a second heat treatment. Whether this stabilization of F-actin is due to the increased amounts of HSPs in general and HSP70 in particular or other heat-induced changes of cellular functions remains an open question.

In order to test a physical interaction of HSP70 and F-actin during heat shock, cytoskeletal actin was extracted using Triton X-100 according to McRobbie and Newell [28]. Following incubation with a Triton X-100 solution the Triton insoluble cytoskeleton (P: pellet) and the Triton soluble fraction (S: supernatant) were separated by centrifugation. Analysis of the proteins in the two fractions by SDS-PAGE revealed that the proteins in the pellet were mainly actins (Fig. 6A). This Triton insoluble fraction is referred to as cytoskeletal actin [28]. According to Hall et al. [7] the cytoskeletal actin isolated by centrifugation at low g-forces should represent filamentous actin, containing especially the cross-linked filaments of actin.

During a 2 h heat shock at 30°C the Triton X soluble and insoluble fractions were then analyzed as to their HSP70 content by Western blot. In un-stressed cells, HSP70 was mainly observed in the Triton X soluble fraction (S) (Fig. 6B). After application of heat shock, the amount of HSP70 in the cytoskeletal actin fraction increases abruptly to a maximum after 15 min and remains high until 45 min. This plateau is then followed by a rapid decline of the HSP70 content in the cytoskeletal actin-containing fraction to the control value. This apparent colocalization of HSP70 with F-actin in the Triton X-100-extracted cytoskeleton which was observed in three independent experiments suggests a direct binding of HSP70 to F-actin-containing structures during the first 45 min after heat shock.

4. Discussion

Cell morphology and actin organization in D. discoideum are drastically altered by heat shock: pseudopodia are rapidly retracted, and the cells adopt a spherical shape. At the same time, visible actin fila-
ments, frequently in form of spots, are lost (Figs. 1, 2). Determination of the F-actin content by means of TRITC-phalloidin binding assay confirmed the rapid reduction of actin filaments after heat shock (Fig. 3). In fact, the F-actin concentration decreased with increasing temperature of the treatment (data not shown). The transient nature of these changes becomes clear when cells are recovering from heat shock or when they adapt to continuous moderately elevated temperatures (30°C): within a few hours they regain their pseudopodia, actin filaments and spots as well as a higher F-actin content (Figs. 1, 2 and 3). Such observations were not yet reported for D. discoideum, but effects of heat shock on actin organization are known in mammalian cells [11,13,18]. The molecular mechanisms responsible for these changes are, however, little understood.

One possible factor in this mechanism may be heat shock-induced changes in the amount of monomeric G-actin. In D. discoideum, heat shock suppresses several actin genes, but at least one out of 17 actin genes is activated [31]. A transient decrease in the synthesis of actin after heat shock was reported for Drosophila and other organisms (review: [1]) as well as for Dictyostelium [24] (W. Xiang, unpublished experiments). In D. discoideum, however, the synthesis rate of G-actin remains rather constant at moderately elevated temperatures (>32°C, not shown) at which temperatures, however, a significant depolymerization of actin is observed (Fig. 2). This makes the assumption rather unlikely that a lower level of G-actin is involved in the decrease of the F-actin level.

A better explanation for this decrease may be based on heat shock-dependent changes in the activity of actin-associated proteins. There is increasing evidence that various HSPs interact with the cytoskeleton in general, but also with actin and actin-associated factors (review: [1]). It was shown that low molecular mass HSPs and their phosphorylated forms play an important role in the regulation of actin polymerization [17–20,32]. There is also evidence for a role of members of the HSP70 family: constitutive HSC70 was identified in D. discoideum as actin capping protein [16,22] or as an adapter that increases the capping activity and stability of the actin capping protein Cap 32/34 [23].

The F-actin stabilizing effects of HSPs may contribute to the recovery/adaptation kinetics of F-actin in the experiments described above (Figs. 2 and 3). When F-actin polymerization starts again during recovery and adaptation (30 min and 2 h, after HS) an earlier increase in HSP synthesis ([24], our own unpublished results) and HSP70 level is observed (Figs. 4 and 5). The steepest increase of HSP70 occurs within the first 45 min of heat shock (Fig. 5), whereas the beginning of F-actin polymerization during continuous 30°C treatment is observed only after 2 h (Fig. 3B). This result indicates that apart from a possible role of HSPs other factors are required for the resumption of F-actin polymerization. During adaptation to continuous heat exposure, the level of HSP70 decreases but remains higher compared to the controls as long as the heat treatment lasts. This was also observed in Neurospora crassa [33].

A role of HSPs in the stabilization of F-actin can be deduced from the acquired thermotolerance experiments (Fig. 5): after a priming HS the amount of HSP70 (and of low molecular mass HSPs) is already high when the second heat shock is given. This may prevent the subsequent decrease of F-actin. From these experiments, however, it is not clear whether HSP70 or other HSPs are playing a protective role in the stabilization of F-actin (or even other, unknown factors).

In the subsequent series of experiments a colocalization of HSP70 with the Triton insoluble fraction containing mainly filamentous actin was shown (Fig. 6). This association occurs transiently only during the first 45 min after the beginning of heat shock and can thus not been involved in the subsequent recovery or adaptation processes. Instead, the binding of HSP70 to F-actin may stabilize F-actin to some extent and may prevent depolymerization (but also polymerization). The acquired thermotolerance may thus result from the higher content of HSP70 and a subsequent more extensive binding of HSP70 to F-actin in the primed cells.

Our experiments are compatible with the assumption that the stability of F-actin is influenced by the level of HSPs, particularly by the level of HSP70. An immediate but transient binding of HSP70 to actin may be a first protective response, which occurs during the depolymerization phase. During the subsequent recovery/adaptation phase the binding between F-actin and HSP70 is abolished, but HSP70
(and other HSPs) may still interact with actin binding factors [23].

References


