

Research article

Hisactophilin is involved in osmoprotection in *Dictyostelium*

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Abstract

Background: *Dictyostelium* cells exhibit an unusual stress response as they protect themselves against hyperosmotic stress. Cytoskeletal proteins are recruited from the cytosolic pool to the cell cortex, thereby reinforcing it. In order to gain more insight into the osmoprotective mechanisms of this amoeba, we used 1-D and 2-D gel electrophoresis to identify new proteins that are translocated during osmotic shock.

Results: We identified hisactophilin as one of the proteins that are enriched in the cytoskeletal fraction during osmotic shock. In mutants lacking hisactophilin, viability is reduced under hyperosmotic stress conditions. In wild type cells, serine phosphorylation of hisactophilin was specifically induced by hypertonicity, but not when other stress conditions were imposed on cells. The phosphorylation kinetics reveals a slow accumulation of phosphorylated hisactophilin from 20–60 min after onset of the hyperosmotic shock condition.

Conclusion: In the present study, we identified hisactophilin as an essential protein for the osmoprotection of *Dictyostelium* cells. The observed phosphorylation kinetics suggest that hisactophilin regulation is involved in long-term osmoprotection and that phosphorylation occurs in parallel with inactivation of the dynamic actin cytoskeleton.

Background

Cells steadily face changes of the external osmolarity, to which they have to adapt. To withstand a steep increase in osmolarity, eukaryotic cells activate responses like "regulatory volume increase", accumulation of compatible osmolytes and stimulated expression of stress proteins [1–4]. Recently, an exception from this scheme has been identified: *Dictyostelium* cells protect themselves against hyperosmolarity by largely rearranging cellular proteins, whereas no "regulatory volume increase", no accumulation of compatible osmolytes and no change of the ex-

pression pattern of the most abundant proteins were observed [5]. Among the translocated proteins identified, cytoskeletal proteins appear to be predominant. In particular, the rearrangement of actin and myosin II to the cell cortex beneath the plasma membrane [6] was shown to constitute a pivotal element of osmoprotection in *Dictyostelium*. These two proteins form the core of a rigid network resembling a shell-like structure [7]. Conversely, DdLIM, a cytoskeletal protein involved in the formation of protrusions [8], is depleted from the cytoskeletal fraction under hypertonic conditions, which is in consistence with the

rounding up and the retraction of protrusions of cells [5]. 30–60 min after onset of the shock condition, progressive actin phosphorylation is observed [9], which presumably leads to the inactivation of the dynamic actin system [10].

The pivotal role of the cytoskeleton in hyperosmotic stress response is also reflected by the fact, that the cytoskeletal proteins β -actinin and "120 kDa gelation factor" are essential to ensure survival under hyperosmotic conditions [11].

However, the signalling pathways mediating protein translocation and actin phosphorylation largely remain to be elucidated. The best understood signalling pathway involves myosin II translocation: upon hyperosmotic stress an increase in cGMP concentration was observed, which is due to guanylyl cyclase activation. This in turn results in the activation of the kinase, which eventually phosphorylates myosin II heavy chain [6,12]. Thereby, disassembly of myosin II filaments is induced as a prerequisite for their reassembly at the cell cortex [13], which is accomplished within 10 min after onset of hyperosmotic shock [6].

In addition, DokA, a homologue of bacterial histidine kinases and cAMP are involved in hyperosmolar signal transduction [14,15], however, molecular targets of these signalling pathways during osmoregulation have not been identified yet.

Hence, the translocation and phosphorylation of cytoskeletal proteins constitute pivotal osmo-responses in *Dictyostelium*. To further elucidate this unusual hyperosmolar stress response we attempted to identify additional proteins, which are translocated under hypertonic conditions and investigated, whether protein phosphorylation of a candidate protein is regulated in response to hyperosmolarity. We could identify hisactophilin as such a protein, which is translocated and phosphorylated during hyperosmotic shock. Hisactophilin is a 15 kDa protein in *Dictyostelium*, that consists of two highly identical isoforms Hisactophilin I and II, which are myristoylated at the N-terminus [17]. The two isoforms exhibit 84% sequence identity and are both myristoylated and distributed between plasma membrane and cytoplasm [17]. Due to this high degree similarity, both isoforms are in this manuscript referred to as hisactophilin, without distinguishing between them.

The biochemical properties of hisactophilin, namely actin- and membrane binding have recently shown to be strongly pH-dependent, which is due to the high content of 26–30% histidine residues [17–19]. Therefore, a role also as pH-sensor was postulated for this protein [26]. In addition to myristoylation, phosphorylation was shown to be a posttranslational modification of hisactophilin

[17]; the physiological role of hisactophilin phosphorylation however is unknown. Hisactophilin appears to play an essential role in osmoprotection, as hisactophilin null cells are osmosensitive.

Results

Hisactophilin is enriched in the cytoskeletal fraction of wild type cells exposed to hyperosmotic shock

To identify proteins, which are translocated to or depleted from the cytoskeleton upon hyperosmotic stress, we used 2-D electrophoresis as a differential method. Wild type cells were subject to hypertonic shock in liquid culture prior to cell lysis. The Triton X-100-insoluble cytoskeletal fraction [16] was isolated and the proteins were separated by 2-D electrophoresis. Samples from three independently grown cell cultures were analyzed in parallel. As control, the same procedure was performed with wild type cells shaken in SPB buffer. Computer analysis of the silver-stained gels with the software package Melani II (Biorad) revealed, that a 15 kDa protein, consisting of several isoforms with a pI of 7.3–7.5, is enriched in the cytoskeletal fraction of hyperosmotically shocked cells compared to control cells (a typical result is shown in Fig. 1A, upper panels). Correspondingly, reduction of the protein amount in the cytosolic pool was observed under hypertonic conditions compared to the control (Fig. 1A, lower panels), suggesting, that the protein is translocated from the cytosol to the cytoskeleton under hypertonic conditions. Search of protein databases for *Dictyostelium* proteins exhibiting a molecular weight of 15 kDa and a pI of 7.3–7.5 resulted in hisactophilin as a candidate cytoskeletal protein, which consists of two isoforms, hisactophilin I and II [26]. The identity of the characterized protein as hisactophilin was demonstrated by immunostaining with a monoclonal α -hisactophilin antibody (Fig. 1B). In addition, analysis of digested peptides of the protein eluted from 2-D gels by mass spectrometry demonstrated the identity of hisactophilin (data not shown). The enrichment of hisactophilin in the cytoskeletal fraction of hyperosmotically shocked cells was also confirmed by analyzing cytoskeletal fractions by 1-D SDS-PAGE followed by immunostaining with an α -hisactophilin antibody (Fig. 1C). As the actin-rich cytoskeleton is primarily found in the cell cortex of hypertonically shocked cells [6], we investigated, whether a hisactophilin II-GFP fusion protein expressed in wild type cells [26] is translocated to the cell cortex after onset of hyperosmotic shock. In fact, the hisactophilin II-GFP fusion protein was found to form a thick layer beneath the plasma membrane of cells exposed to hypertonicity for 10 min, whereas only a weak staining of the plasma membrane was observed in control cells (data not shown).

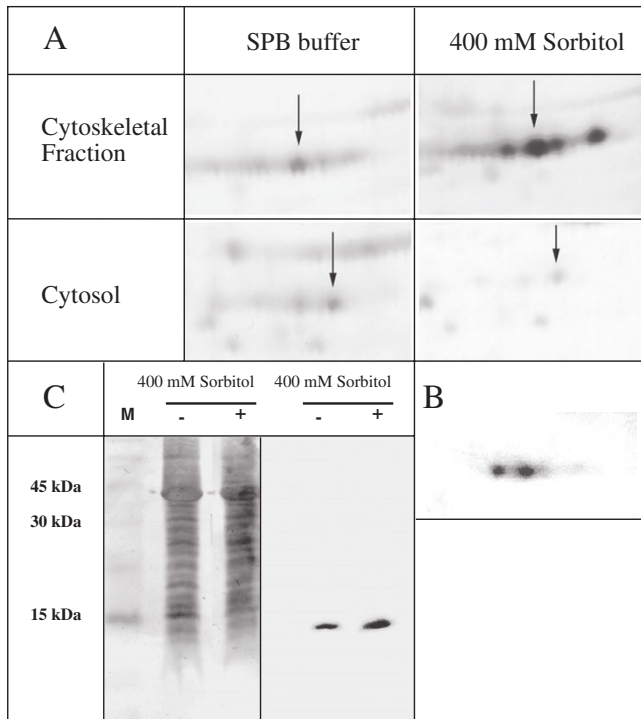


Figure 1
Hisactophilin is enriched in the cytoskeletal fraction of hyperosmotically shocked cells. (A) Hisactophilin is enriched in the cytoskeletal fraction of cells hyperosmotically shocked for 2 h with respect to control cells suspended in SPB buffer for 2 h. (upper frames). Correspondingly, less hisactophilin was found in the cytosol of cells exposed to hypertonicity when compared to cells suspended in SPB buffer (lower frames). Proteins from cytosolic and cytoskeletal fractions were separated by 2-D electrophoresis. The frames show a silver-stained region of the gel corresponding to a molecular weight of 15 kDa and to an IEP of 7.3–7.5. The predominant spot of each panel is indicated with an arrow. (B) Identification of the protein in (A) as hisactophilin by Western blotting of a typical gel of the cytoskeletal fraction and immunostaining of with a α -hisactophilin antibody. (C) The enrichment of hisactophilin in the cytoskeletal fraction of cells exposed to hypertonicity was confirmed by I-D SDS-PAGE. The cytoskeletal fraction was isolated from cells suspended in SPB buffer (-) or in SPB buffer/400 mM sorbitol (+) for 2 h. The proteins were separated by SDS-PAGE and blotted. Immunostaining was performed with an α -hisactophilin antibody (right panel). The membrane was subsequently coomassie-stained (left panel). Abb.: M= marker.

Hisactophilin is phosphorylated upon hyperosmotic stress

As phosphorylation is an important regulatory modification of actin and myosin II under hypertonic conditions, we investigated, whether this also applies for hisactophilin. Wild type cells deprived of phosphate were suspended in Mes buffer and were metabolically labeled with ^{32}P -orthophosphate. After 1 h incubation, hyperosmotic shock

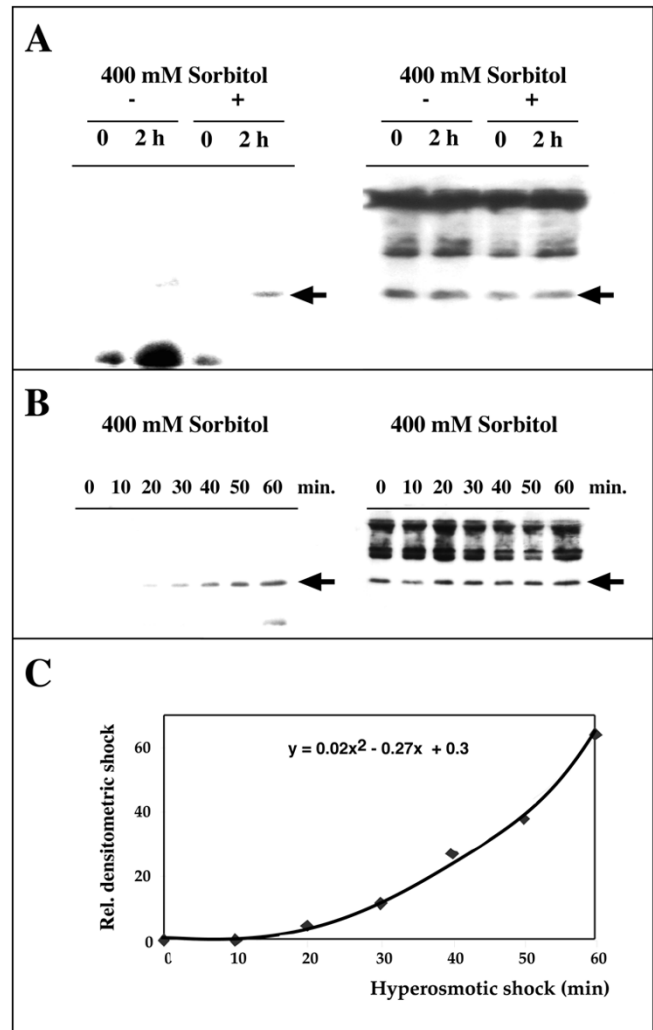


Figure 2
Hisactophilin is phosphorylated in wild type cells exposed to hyperosmotic conditions (A). Cells were metabolically labeled with ^{32}P -orthophosphate prior to hypertonic shock. Hisactophilin was immunoprecipitated from samples withdrawn at T = 0 and T = 2 h. The probe was separated by SDS-PAGE and blotted. Autoradiography (left panels A,B) was performed for 48 h and hisactophilin was subsequently identified by immunostaining (right panels A, B). (+) denotes samples from shocked cells, (-) denotes samples from control cells suspended in SPB buffer. The arrow indicates the position of 15 kDa proteins. (B) The amount of phospho-hisactophilin progressively increased in hyperosmotically shocked cells. The experiment was performed as described in (A). (C) Densitometric analysis of the autoradiography in (B) reveals an exponential increase in phospho-hisactophilin upon hyperosmotic stress: the kinetics can be fitted by a quadratic function displayed in the figure. The densities are given in relative arbitrary units, setting the background as 0.

was initiated and samples were withdrawn at intervals. Hisactophilin was isolated after cell lysis by immunoprecipitation. The samples were analyzed by autoradiography and subsequently by immunostaining with an α -hisactophilin antibody. Hisactophilin, that was isolated from cells shocked for 2 h, was found to be phosphorylated, whereas no detectable hisactophilin phosphorylation was observed in control samples isolated from cells suspended in SPB buffer for 1 h or 3 h (Fig. 2A). Analysis of the kinetics of the phosphorylation reaction revealed, that the phosphorylation does not constitute an acute stress response (Fig 2B). Phospho-hisactophilin progressively accumulated 20–60 min after onset of the shock condition. The result of the densitometric analysis revealed that the kinetics of phospho-hisactophilin can be fitted by a quadratic equation (Fig. 2C).

To test the specificity of hisactophilin phosphorylation as a hyperosmotic stress response, wild type cells were labeled with ^{32}P -orthophosphate and were then exposed to various other stress conditions. Subsequently, samples were withdrawn and hisactophilin phosphorylation was detected as described above. The phosphorylation reaction was found to be specific for hypertonic conditions, as other stress conditions as heat stress, acid stress, oxidative stress and energy depletion did not result in a detectable hisactophilin phosphorylation (Fig. 3A).

To prove that the phosphorylation of hisactophilin is dependent on osmolarity, but not on the osmolyte used, wild type cells were exposed to hypertonic stress conditions, using either NaCl, glucose or sorbitol as osmolyte. The concentrations of the osmolytes were adjusted to 400 mOsm in the case of sorbitol (400 mM) and NaCl (200 mM) and a slightly reduced osmolarity (350 mOsm) was chosen in the case of glucose. Analysis of hisactophilin phosphorylation in response to the hypertonic stress conditions revealed that phosphorylation occurred to the same extent in the presence of all three osmolytes, indicating that hisactophilin phosphorylation is a hyperosmolar stress response (Fig. 3B).

Serine phosphorylation of Hisactophilin

Recently, it has been demonstrated *in vitro* using a crude kinase fraction, that hisactophilin is phosphorylated on threonine (95%) and on serine residue(s) (5%) [17]. We addressed the question, whether the phosphorylated residues are identical under hyperosmolar conditions *in vivo*. We therefore labeled wild type cells with ^{32}P -orthophosphate, exposed them to hypertonic stress and immunoprecipitated hisactophilin. The protein probe was hydrolyzed in the presence of constant boiling HCl and was then separated together with phosphoamino acid standards by 2-D thin layer electrophoresis. ^{32}P -labeled phosphoamino acids originating from phospho-his-

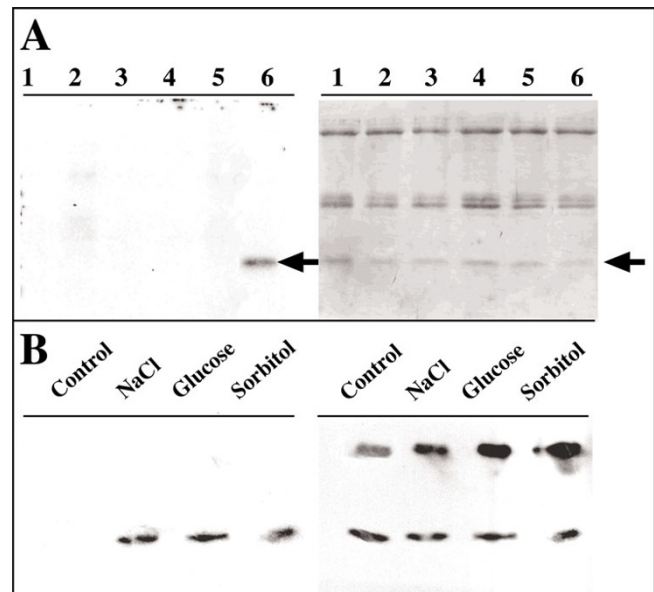


Figure 3
Hisactophilin phosphorylation is not a general stress response. Autoradiographies are shown on the left panels, the corresponding immunostainings are displayed on the right panels. (A) Wild type cells were metabolically labeled with ^{32}P -orthophosphate and were then exposed to various stress conditions. Hisactophilin phosphorylation was detected as described in Fig. 2. 1: Control (cells shaken in SPB buffer). 2: Cellular Acidification. 3: Heat stress. 4: Energy depletion. 5: Oxidative stress. 6: Hyperosmotic stress. (B) Hisactophilin phosphorylation is dependent on osmolarity, but not on the osmolyte. Wild type cells were metabolically labeled with ^{32}P -orthophosphate and were then exposed to either 200 mM NaCl, 350 mM glucose or 400 mM sorbitol for 1 h. As control, cells were shaken in SPB buffer (1). Hisactophilin phosphorylation was detected as described in Fig. 2.

tophilin were detected by autoradiography. Identification of the phosphoamino acid was performed by staining the standards with Ninhydrin and subsequent comparison of the staining pattern with the autoradiography. As shown in Fig. 4, only phospho-serine could be detected under *in vivo* conditions, whereas no phospho-threonine or phospho-tyrosine was observed, indicating that serine phosphorylation is favored over threonine phosphorylation under hyperosmotic stress conditions *in vivo*.

Hisactophilin-deficient cells exhibit reduced viability under hyperosmotic stress conditions

To investigate whether hisactophilin plays a role in osmoregulation, we determined whether survival of *his*-cells was affected under hypertonic conditions.

The viability of *his*-cells was found to be reduced by 75% 2 h after onset of the stress condition, whereas viability of

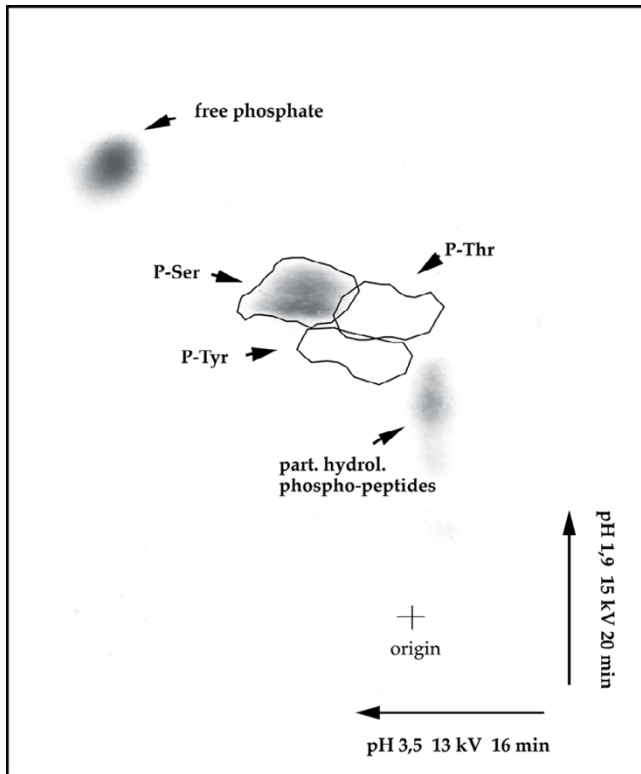


Figure 4
Hisactophilin is phosphorylated on serine residue(s) *in vivo*. Wild type cells were metabolically labeled with ³²P-orthophosphate and were then exposed to 400 mM sorbitol for 2 h. Hisactophilin was isolated by immunoprecipitation and was analyzed by 2-D thin layer electrophoresis. Phosphoamino acid standards were detected by Ninhydrin staining (positions indicated by lines) and were compared to the autoradiography. Abbreviation: part. hydrol.=partially hydrolyzed

the wild type cells was not significantly affected (Fig. 5). Hence, the presence of hisactophilin is essential to ensure viability under hypertonic conditions.

Discussion

Dictyostelium was shown to employ an unusual mechanism to cope with the effects of osmotic stress by rearranging its cellular cortex [5,22]. As the cells round up, actin and myosin moieties are being redistributed underneath the plasma membrane. In this process the distribution of the actin-associated protein Hisactophilin was investigated in order to gain a better insight into osmo-protective mechanism of the cell. Hisactophilin was found to be enriched in the cytoskeletal fraction of wild type cells exposed to hyperosmotic stress (Fig. 1A,1B). In addition, a thick layer of Hisactophilin II-GFP was formed at the cell cortex of cells, that were shocked for 10 minutes (data not shown), which correlates with the observation that actin

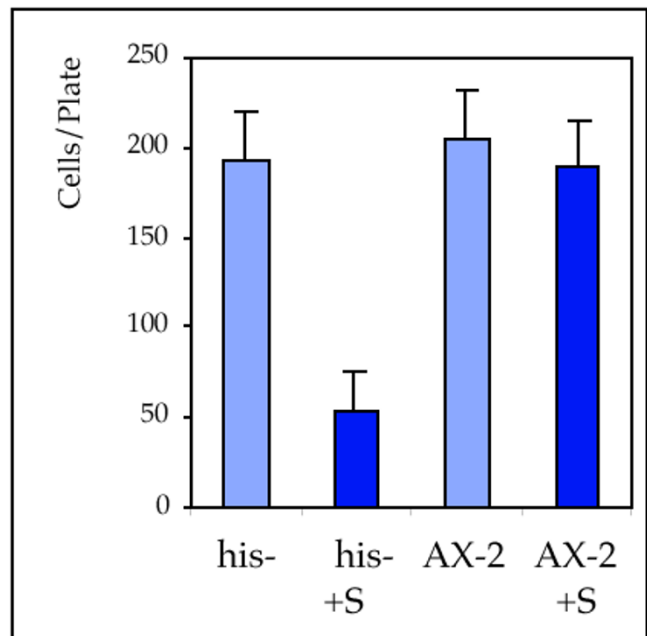


Figure 5
Viability of *his*-cells is reduced under hypertonic conditions. *his*-cells and wild type cells were suspended in either 400 mM sorbitol or SPB buffer for 2 h. Samples were plated together with bacteria and plaques were counted after 2–4 days incubation (n = 5).

and myosin II are translocated to the cell cortex within 10 minutes after onset of the stress condition [6]. As hisactophilin was shown to enhance actin polymerization *in vitro* [20] and as cells overexpressing hisactophilin II exhibit an increased F-actin content [27], this protein could concur in the formation of the rigid filamentous network of actin and myosin II filaments.

The essential role of hisactophilin becomes evident when hisactophilin negative cells are investigated under osmotic stress conditions. They show a markedly reduced survival rate in a test were they are first exposed to high osmolarity for 2 hours and are then plated onto agar plates containing bacteria. This procedure involves a volume reduction and a subsequent volume increase caused by the re-dilution in the plating process. It therefore remains to be investigated whether hisactophilin deploys its function in the process of rearranging the cell's cortex upon volume reduction or is essential for reinforcing the cortex in its rounded up state.

A role comparably to the family of MARCKS (myristoylated alanine-rich protein kinase C substrate) proteins in mammalian cells was postulated recently [19]. These proteins are also myristoylated at the N-terminus and crosslink actin into a rigid meshwork at the membrane.

Upon phosphorylation, MARCKS proteins are rejected from the membrane by electrostatic interactions, which results in the spatial separation of the cytoskeleton from the membrane [21]. In fact, hisactophilin phosphorylation was found to be specifically induced under hypertonic conditions (Fig. 3A, 4). However, the phosphorylation reaction is too slow to account for the translocation of the protein within 10 minutes (Fig. 3B, 3C): phospho-hisactophilin progressively accumulated within 60 min with a kinetics strikingly similar to the kinetics of actin phosphorylation under hyperosmotic conditions [9]. This points out the possibility, that actin and hisactophilin cooperate in two distinct phases of osmoregulation. During the first phase, the proteins are translocated within the first 10 minutes of stress which is accompanied by cortical reinforcement. The second phase is characterized by actin and hisactophilin phosphorylation reinforced after about 30 min of hypertonic shock. Actin phosphorylation was shown to correlate with an inactivation of the dynamic actin system [10]. The identical kinetics suggests a similar role for hisactophilin during this process. In addition, the coinciding phosphorylation kinetics raises the possibility of a common regulation of hisactophilin and actin phosphorylation. The signalling pathways regulating actin and hisactophilin phosphorylation are unknown, however, the second messengers involved in osmoregulation identified so far, namely cGMP and cAMP could be excluded as modulators of hisactophilin phosphorylation, as an increase or decrease in these messengers did not affect hisactophilin phosphorylation (data not shown). This suggests the presence of an additional signalling pathway concomitantly regulating a serine kinase specific for hisactophilin (Fig. 5) and, due to the identical phosphorylation kinetics, a tyrosine kinase specific for actin [5].

A conceivable signalling pathway regulating the hisactophilin translocation is a change in pH, as the biochemical properties of Hisactophilin are strongly pH-dependent: actin-binding is strongly increased *in vitro* when pH is lowered to = 7.0 [20]. In fact, progressive cytosolic acidification was observed during hyperosmotic stress, resulting in a pH drop from 7.5 to 6.8 15 min after onset of the stress condition [22]. Hence, the translocation of hisactophilin to the actin-rich cytoskeleton can be attributed to the cytosolic acidification. Also, the enrichment of the elongation factor 1α in the cytoskeletal fraction of hyperosmotically shocked cells [5] can be explained by cytosolic acidification, as enhanced binding of this protein to F-actin at pH 6.5 *in vitro* has been described recently [23].

These examples are in accordance with the notion of pH changes as "synergistic messenger" [24]. Changes in proton concentration affect the biochemical properties of proteins and can thereby act as signals. However, other

signals have to concur to elicit the osmoprotective responses: the translocation of hisactophilin potentially caused by the drop in cytosolic pH is complemented by the relocation of myosin II mediated by cGMP and of actin [6] by a yet unknown mechanism. Therefore, the role of hisactophilin in osmoprotection also exemplifies the numerous interactions of the signalling pathways that are necessary for osmoprotection in *Dictyostelium*.

Conclusions

We could demonstrate that Hisactophilin is both translocated to the cytoskeleton and phosphorylated during hyperosmotic stress in *Dictyostelium*. The phosphorylation kinetics suggests a slow accumulation of phosphorylated hisactophilin resembling the phosphorylation kinetics for actin, which is known to eventually lead to cytoskeletal inactivation. Second messengers known to play an essential role during osmoprotection are not involved in hisactophilin phosphorylation suggesting a further signalling pathway being involved in the unusual osmoprotection mechanisms in *Dictyostelium*.

Materials and Methods

Growth of *Dictyostelium* cells

D. discoideum strain AX2-214 (AX-2), referred to as wild type, was cultivated axenically in shaken suspension at 21°C [25] and harvested during exponential growth. Clones of HsII-GFP [26] were cultivated as described for AX-2, but under the selection of 20 µg/ml Geneticin (G418). Clones of *his*-cells [27] were cultivated as described for AX-2, but under the selection of 7.5 µg/ml Geneticin.

Hyperosmotic shock in liquid culture

Hyperosmotic shock experiments were performed according to Schuster *et al.* (1996). Axenically grown *Dictyostelium* cells were harvested, washed twice in Soerensen Phosphate Buffer (SPB: 2.0 mM Na₂HPO₄, 14.6 mM KH₂PO₄, pH 6.0) and were then adjusted to 2×10^7 cells/ml with SPB buffer (osmolarity: 34 mOsm). Cell density was 1.6×10^7 cells/ml during the osmoshock, adjusted by the addition of SPB buffer/2 M sorbitol. To the control cells, SPB buffer was added until the same cell density was reached.

The cell suspension was shaken for 1 h. SPB buffer/2 M sorbitol was added to a final concentration of 400 mM sorbitol (osmolarity of the buffer: 434 mOsm). The cell suspension was incubated up to 2 hours according to the experiment. Viability of *Dictyostelium* cells after exposure of the cells to hyperosmotic stress was determined as described [14]. In short, *his*-cells were shaken in SPB buffer for 1 h prior to the hyperosmotic shock. Samples were withdrawn at intervals and cell suspension corresponding to 200 cells was plated on agar plates together with *Kleb-*

siella aerogenes bacteria. Plaques in the bacterial lawn were counted after 2–4 days.

Exposure to stress conditions

To acidify *Dictyostelium* cells, axenically grown wild type cells were washed twice in SPB buffer and were resuspended in SPB buffer/5 mM propionic acid, pH 6.0. To impose oxidative stress, the cells were incubated in SPB buffer/3 mM H₂O₂ for 60 min. To study heat stress, the cells were exposed to 30°C for 30 min. Energy depletion was achieved by incubating the cells in SPB buffer/50 μM 2,4-dinitrophenol for 60 min.

2-D Gel electrophoresis

Preparation of the cytoskeletal fraction from hyperosmotically shocked cells and separation of the proteins by 2-D gel electrophoresis was performed as described recently [5]. The cytoskeletal fraction was obtained by isolating the Triton X-100-insoluble fraction. The corresponding soluble fraction was defined as cytosolic fraction. 100 μg total protein was applied per gel.

Metabolic labeling with ³²P-orthophosphate

50 ml cell suspension of axenically grown wild type cells were transferred to 300 ml phosphate-free medium buffered with 20 mM Mes, pH 6.0. The cells were shaken for 16 h and were subsequently washed twice in ice-cold 20 mM Mes, pH 6.0 (Mes buffer). The cells were adjusted to 2*10⁷ cells/ml with Mes buffer. After incubating the cells for 30 min, ³²P-orthophosphate (Amersham Pharmacia) was added to a final activity of 0.25 mCi/ml in the cell suspension. The cell suspension was shaken for 60 min, before the cells were exposed to hyperosmotic shock. Samples of 1 ml were withdrawn at intervals, the cells were washed twice with Mes buffer and were resuspended in 800 μl RIPA buffer (50 mM Tris/HCl, pH 7.5; 150 mM NaCl, 1% TritonX-100; 1% Natriumdeoxycholate; 0.1% SDS; 1 mM DTT; 1 mM EDTA; 100 μM Na-orthovanadate; Complete Protease Inhibitor Cocktail (Boehringer Mannheim)). The cell lysate was immediately frozen in liquid nitrogen. As control, samples were prepared from a cell suspension to which Mes buffer was added instead of Mes buffer/2 M sorbitol.

Immunoprecipitation of hisactophilin

Thawed cell lysate from ³²P-labeled cells was centrifuged for 20 min at 10000 g (4°C). α-hisactophilin antibody 54-11-10 [28] was added to the cleared lysate and was incubated for 3 h. Immunoprecipitation was performed by adding fixed *Staphylococcus aureus* cells (Pansorbin, Calbiochem) preincubated with rabbit-α-mouse IgG (Sigma). After 45 min incubation, the immunoprecipitate was washed twice with TBS and was analyzed by SDS-PAGE, Western blotting and autoradiography.

Fluorescence microscopy

Axenically grown HisII-GFP cells were shaken in SPB buffer for 1 h at a cell density of 2*10⁷ cells/ml. SPB buffer/2 M sorbitol was added to a final concentration of 400 mM sorbitol (cell density: 1,6*10⁶ cells/ml). An aliquot of the living cells was allowed to adhere to glass cover slips for 10 min. Subsequently, fluorescence microscopic observations were carried out. As control, cells suspended in SPB buffer were analyzed.

The equipment consisted in a Leitz Aristoplan fluorescence microscope, set at 450–490 nm for excitation.

Phosphoamino acid analysis

Phosphoamino acid analysis was performed as described [29]. In short, hisactophilin was immunoprecipitated from ³²P-labeled cells exposed to hyperosmotic stress for 60 min. Acid hydrolysis of the immunoprecipitate was performed, followed by the separation of the phosphoamino acids by electrophoresis in two dimensions on TLC plates together with phosphoamino acid standards. The plates were analyzed by autoradiography and by the detection of amino acids with Ninhydrin.

Miscellaneous

Protein quantification, SDS-PAGE and Western blotting onto PVDF membrane were performed according to the published methods of Bradford [30], Laemmli [31] and Towbin *et al.*[32]. Western blots were treated with the mouse monoclonal α-hisactophilin antibody 54-11-10 [28]. Antibodies were detected using peroxidase-coupled rabbit-α-mouse IgG and the Renaissance® system (Du Pont). Prestained Seebue marker (Novex) was used as a protein molecular weight standard.

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