Rearrangement of cortex proteins constitutes an osmoprotective mechanism in *Dictyostelium*

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***Dictyostelium* responds to hyperosmotic stress of 400 mOsm by a rapid reduction of its cell volume to 50%. The reduced cell volume is maintained as long as these osmotic conditions prevail. *Dictyostelium* does not accumulate compatible osmolytes to counteract the osmotic pressure applied. Using two-dimensional gel electrophoresis, we demonstrate that during the osmotic shock the protein pattern remains unaltered in whole-cell extracts. However, when cells were fractionated into membrane and cytoskeletal fractions, alterations of specific proteins could be demonstrated. In the crude membrane fraction, a 3-fold increase in the amount of protein was measured upon hyperosmotic stress. In the cytoskeletal fraction, the proteins DdLIM and the regulatory myosin light chain (RMLC) were shown to be regulated in the osmotic stress response. The elongation factors eEF1 α (ABP50) and eEF1 β were found to increase in the cytoskeletal fraction, suggesting a translational arrest upon hyperosmotic stress. Furthermore, the two main components of the cytoskeleton, actin and myosin II, are phosphorylated as a consequence of the osmotic shock, with a tyrosine residue as the phosphorylation site on actin and three threonines in the case of the myosin II heavy chain.**

Keywords: cytoskeleton/*Dictyostelium discoideum*/osmotic stress/two-dimensional electrophoresis

Introduction

During evolution, cells developed a number of strategies to adapt to high or low osmotic conditions (Yancey *et al.*, 1982). Cells respond to osmotic changes in their environment by either shrinking or swelling. Organisms without a cell wall activate mechanisms that regulate cell volume, termed regulatory volume increase (RVI) and regulatory volume decrease (RVD) (Kwon and Handler, 1995). Under hyperosmotic conditions, these mechanisms involve the pumping of ions into the cytosol in the acute phase of the osmotic stress and the accumulation of compatible osmolytes on a larger time scale. Organic compounds such as polyols (e.g. glycerol), amino acids (e.g. glutamine, glutamate and proline), urea or carbohy-

drates (e.g. trehalose) are known as compatible osmolytes. These solutes allow the cell to decrease its internal electrolyte concentration as high intracellular concentrations of ions affect the activity of many enzymes (Yancey *et al.*, 1982). Whereas polyols are accumulated mainly in fungi, algae or insects (Yancey *et al.*, 1982; Blomberg and Adler, 1992; Mager and Varela, 1993), bacteria use amino acids as compatible osmolytes. In eukaryotic cells, several transporters for betaine, taurine or *myo*-inositol have been described (Kwon and Handler, 1995).

Upon osmotic stress, most cells begin to change their gene transcription in order to step up defensive mechanisms that counteract the external forces (Burg *et al.*, 1996). Known examples are the outer membrane porins OmpF and OmpC in *Escherichia coli*. These proteins are regulated contrarily upon high osmolarity, with a decreased transcription of *ompF* and an increased transcription of *ompC*. Their expression is regulated by the EnvZ–OmpR two-component system (Pratt and Silhavy, 1995). Furthermore, a three-component K⁺-ATPase that actively transports K⁺ ions into the cell under hyperosmotic conditions is known to be expressed in *E.coli* upon osmotic stress (Altendorf *et al.*, 1994). The resultant transport of K⁺ is accompanied by the osmotic uptake of water, which restores volume and turgor pressure in the cell. This K⁺-ATPase complex is encoded by the *kdpFABC* operon and its transcription was also shown to be regulated by a two-component system, KdpD–KdpE. In *Saccharomyces cerevisiae*, glycerol is the major organic compatible osmolyte accumulated in response to hypertonicity. The synthesis of glycerol from dihydroxyacetone phosphate is mediated by the glycerol-3-phosphate dehydrogenase. This enzyme is encoded by the *GPD1* gene, and hypertonicity greatly increases *GPD1* transcription (Albertyn *et al.*, 1994; Wurgler-Murphy and Saito, 1997).

Amoebae such as *Dictyostelium discoideum* encounter rapidly changing osmotic conditions in their soil habitat, and have developed successful means to avoid the harmful consequences thereof. Recent investigations (Heuser *et al.*, 1993) have demonstrated that *Dictyostelium* responds to a decreasing tonicity mainly via the contractile vacuole in order to maintain osmohomeostasis. In this process, water leaking through the membranes is pumped into the contractile vacuole together with neutral amino acids (Steck *et al.*, 1997). In contrast to the described effects towards decreasing tonicity, *Dictyostelium* cells shrink spontaneously upon increasing tonicity, followed by rearrangement of cytoskeletal proteins (Kuwayama *et al.*, 1996). The complex effects of hyperosmotic stress on the several cytoskeletal components in *Dictyostelium* recently have attracted interest. For example, disassembly of myosin II filaments is an essential part of the hyperosmotic stress response in *Dictyostelium* (Kuwayama *et al.*, 1996). Besides, *Dictyostelium* double mutants which lack the two

F-actin cross-linking proteins α -actinin and gelation factor were shown to be sensitive to hyperosmotic stress (Rivero *et al.*, 1996).

Here we report the absence of regulation of compatible osmolytes in *Dictyostelium* and an increase in protein degradation in response to hyperosmotic stress. We investigated changes in the protein expression caused by hyperosmotic stress, using two-dimensional gel electrophoresis (2-D electrophoresis) as a differential method. This method enabled us to demonstrate that the complex protein patterns of whole-cell extracts did not change, whereas various alterations were found in subcellular compartments. Seven proteins were identified that are regulated by hyperosmotic stress.

Results

Hyperosmotic stress induces cell volume reduction

Like most cells without a cell wall, *Dictyostelium* changes its cell volume in response to osmotic stress within 2–3 min, as observed by light microscopy (Figure 1A). We investigated the osmotic behavior of *Dictyostelium* cells (strain AX-2) under conditions of either low (34 mOsm) or high (434 mOsm) osmolarity, as it was shown previously that *Dictyostelium* cells are capable of surviving an osmotic shock of 434 mOsm for up to 2 h with almost no reduction of viability (Schuster *et al.*, 1996). Using centrifugation in graded glass tubes, we investigated the cell volume changes of *Dictyostelium* under these conditions over a period of up to 2 h. Upon exposure to the stress, *Dictyostelium* decreased its cell volume to ~50% of its original value and maintained this state as long as the hyperosmotic conditions prevailed (Figure 1B).

***Dictyostelium* does not produce compatible osmolytes upon hyperosmotic shock**

Although *Dictyostelium* cells do not manage to regain their cell volume under an osmotic pressure of 434 mOsm, we investigated whether vital defense mechanisms still involve the accumulation of compatible osmolytes. We therefore measured the contribution of the cell's cytosol to the total osmolarity with and without hyperosmotic shock. The osmolarity of the cleared cell lysate was measured in an osmometer by freezing point determination. A relative osmolarity of 100% was defined as the osmolarity of the corresponding buffer plus the total solutes of the cells (1×10^8 cells/ml), corrected for the volume of the cell debris. These measurements (Figure 2) demonstrated that intracellular osmolarity does not increase within 1 h after the shock relative to the control cells. Since the cells shrink to about half their starting volume while retaining the same amount of osmolytes, the resulting intracellular osmolarity doubles to ~230 mOsm.

After 60 min, an increase of intracellular solute concentration from 130% relative osmolarity to 170% was observed in osmotically shocked cells. This corresponds to an intracellular osmolarity of 330 mOsm. This increase, however, is still not sufficient to counteract the external forces applied (434 mOsm), a finding which is consistent with the fact that no volume increase was observed.

Fig. 1. Cell volume changes of *D. discoideum* upon hyperosmotic stress. (A) Phase-contrast photographs of *Dictyostelium* AX-2 cells in low (panel I, phosphate buffer, 34 mOsm) and high (panel II, 400 mM sorbitol in phosphate buffer, 434 mOsm) osmolarity buffer. *Dictyostelium* cells were shaken in Soerensen phosphate buffer for 1 h and shocked osmotically by adding 400 mM sorbitol. AX-2 cells shrink and round up within 2–3 min. Bar = 2 μ m. (B) Relative changes of cell volume during osmotic shock. The volume of cell pellets was determined by centrifugation of 3×10^7 cells into graded glass capillaries. At different time points, samples were taken as indicated. Under high osmolarity conditions (434 mOsm), the relative cell volume decreases to <50% of the original value and remains in this state as long as the stress condition prevails.

Hyperosmotic stress induces ubiquitination of cellular proteins

We further investigated the issue of the increase of intracellular solutes after a prolonged stress (60–120 min) by probing the degradative activity of hyperosmotically stressed cells. Ubiquitin has been shown to be a central element in protein degradation in eukaryotic cells (for a review, see Hochstrasser, 1996). Therefore, the hyperosmotically stressed cells were probed for free available ubiquitin as well as for the ubiquitination of cellular proteins. Cells were hyperosmotically stressed as described above and subsequently lysed. The whole-cell extract was analyzed by SDS-PAGE and Western blotting. The resulting membrane was probed with a monoclonal anti-ubiquitin antibody. The ubiquitin signal was reduced significantly after 2 h at high osmolarity (Figure 3A).

Fig. 2. The intracellular osmolarity of *Dictyostelium* cell lysates does not increase within the first hour of osmotic shock relative to the control cells and increases only slightly thereafter. *Dictyostelium* cells were osmotically shocked and at different time points samples were taken and subsequently lysed. The osmolarity of the cell lysates was measured by freezing point determination to examine the accumulation of osmotically active substances during hyperosmotic shock.

Fig. 3. Hyperosmotic stress causes a decrease in free ubiquitin which is paralleled by an increase in protein ubiquitination. *Dictyostelium* cells were osmotically shocked for 2 h, lysed and separated by SDS-PAGE. Western blot analysis was performed with a monoclonal anti-ubiquitin antibody. Control cells were incubated at low osmolarity and subsequently treated in the same way. (A) Decrease of free ubiquitin upon hyperosmotic shock after 2 h (arrow). (B) Increase of ubiquitinated proteins of a whole-cell lysate after 2 h.

This decrease was paralleled by a strong increase of ubiquitination of cellular proteins (whole-cell lysate) after 2 h, with no apparent specificity for distinct proteins (Figure 3B). It is therefore reasonable to assume that the increase in intracellular tonicity after 60 min results from an increase in degradation products.

Hyperosmotic stress induces changes at the protein level of the cytoskeleton and the crude membrane fraction but not of the whole-cell protein composition

To monitor global changes of the cell's expression pattern in response to an osmotic shock, we used 2-D electrophoresis as a differential method. In a series of experiments, cells were exposed to different osmotic conditions prior to lysis. In each set, three independently grown batches of cells were analyzed in parallel. Typical silver-stained gels from whole-cell extracts of cells exposed to

low and high osmolarity are shown in Figure 4A and B. The analysis of the protein patterns from whole-cell extracts resulted in ~3200 protein spots, as detected by the software package Melanie II (Bio-Rad), in the molecular mass range from 5 to 220 kDa and in a pI range from 3 to 10. Interestingly, computer analysis of the expression patterns of shocked and unshocked cells revealed no obvious differences. We therefore conclude that *Dictyostelium* does not specifically regulate the overall gene expression in response to an osmotic shock (i.e. of the 2000 most abundant proteins). However, if cells were subfractionated into crude membrane and cytoskeletal fractions, differences at the protein level could be seen in an osmotic stress-dependent fashion (Figure 4C and D, and E and F, respectively). Differences occurring between the shocked and the unshocked fractions can be categorized into three classes: an increase, a decrease in the amount of protein, as well as protein modifications making the protein more acidic, suggesting a post-translational modification such as phosphorylation. Further investigation of the cytoskeleton and the cytosol revealed that several proteins, which were altered in amount upon hyperosmotic shock, redistribute between these compartments. These results demonstrate that neither up- or downregulation of gene products, but rather relocation from the cytosol to the cytoskeleton, and vice versa, constitute the response system of *Dictyostelium* to hyperosmotic stress. Furthermore, *Dictyostelium* seems to reinforce its cortex by bringing cellular proteins to the membrane: crude membrane fractions from cells that were exposed to high osmolarity showed an ~3-fold increase in total protein amount ($207.1 \pm 79.6 \mu\text{g protein}/10^7 \text{ cells}$; $n = 13$) when compared with crude membrane fractions from control cell suspensions that were exposed to phosphate buffer only ($64.7 \pm 12.6 \mu\text{g protein}/10^7 \text{ cells}$; $n = 13$).

Identification of proteins regulated by hyperosmotic stress

To identify the proteins which are regulated in response to the hyperosmotic shock, 2-D electrophoresis of the cytoskeletal and the crude membrane fractions of hyperosmotically shocked cells was performed with preparative amounts of protein (1–3 mg). The obtained gels were stained with Coomassie Blue to allow a linear comparison of the protein quantity. Gel images were again analyzed for proteins that were regulated in their amount or altered in their isoelectric point under the two osmotic conditions examined. Among the most abundant proteins, six proteins were found to be regulated in the cytoskeletal fraction and four in the crude membrane fraction. Proteins thus identified were submitted to an in-gel protease digestion. Peptides retrieved from the gel slices were separated by reversed phase HPLC and analyzed by Edman degradation. The obtained protein sequences led to the identification of seven proteins (Figures 5A–G). As an additional validation, the estimated molecular mass and the pI of the identified proteins was compared with database values and found to be in good agreement (unless indicated).

The protein indicated in Figure 5A was identified as DdLIM by three internal peptide sequences (amino acids 35–45 STCNSTLNVKT, amino acids 53–60 LYCPVHTP, amino acids 83–94 VAEGLGNAHRGL). DdLIM was found to be present in substantial amounts in the cyto-

Fig. 4. Hyperosmotic stress induces changes at the protein level of the crude membrane fraction and the cytoskeleton, but not in whole-cell extracts. Two-dimensional gel electrophoresis was performed as described. The left side shows typical protein patterns of whole-cell extracts and subcellular compartments obtained from *Dictyostelium* cells incubated for 2 h at low osmolarity, and the right side shows patterns of the corresponding fractions incubated for 2 h at high osmolarity. The protein patterns of the whole-cell extracts do not show significant changes upon hyperosmotic stress (A and B). The protein patterns of the crude membrane fraction (C and D) and of the cytoskeleton (E and F) show distinct alterations (indicated by arrows) upon hyperosmotic stress. Images of these gels at their original size can be viewed under <http://oeserverg3.biochem.mpg.de/osmo/osmstress.html>.

skeletal fraction under low osmotic conditions, and decreased strongly under hypertonic stress. These results are in agreement with the recent observation that DdLIM is present in the filopodia and pseudopodia of *Dictyostelium* (Prassler *et al.*, 1998). These cellular extrusions are retracted under osmotic stress (Kuwayama *et al.*, 1996).

The arrow in Figure 5B indicates an 18 kDa protein that increases in amount in the cytoskeletal fraction upon hyperosmotic shock and was identified by the peptide sequence DRTGFIK, which accounts for amino acids 35–

41 of the protein sequence of the regulatory myosin light chain (RMLC) (Tafari *et al.*, 1989).

The protein shown in Figure 5C decreases in amount in the cytoskeletal fraction under hypertonic conditions. The peptide sequences obtained after protease treatment of this protein showed high homology to an actin-like protein of *Dictyostelium* (FSYNVDDTIQS, sequence identity at position 300–306) (Murgia *et al.*, 1995) and to the actin-related protein 87C (ARP87C) of *Drosophila melanogaster* (TALEGDIDNSEK, sequence identity at

Fig. 5. Identified proteins that change in amount under hyperosmotic stress conditions. Panels show areas of Coomassie-stained protein patterns obtained from subcellular compartments after low (left side) and high (right side) osmolarity incubation. Arrows indicate the proteins that were identified by Edman degradation.

position 51–56) (Fyrberg *et al.*, 1994). These homologies indicate that this is a new member of the growing ARP family.

Two proteins that increase in amount upon hyperosmotic stress in the cytoskeletal fraction were identified as the actin-binding protein 50 (ABP50) or eukaryotic elongation factor eEF1 α (amino acids 356–363 KFTEIVDK; Figure 5D), and as the elongation factor eEF1 β (amino acids 4–15 ADLTTENGLVEL and amino acids 131–136 ILLDVK; Figure 5E) (Chae and Maeda, 1998).

Calreticulin (Figure 5F) was identified by the peptide sequence VETDQLTHQYTLV (amino acids 163–175). This protein, highly abundant in the endoplasmic reticulum (Coppolino and Dedhar, 1998), increases in amount in the crude membrane fraction under hyperosmotic stress.

Fig. 6. Hyperosmotic stress causes changes in the amount of the identified proteins. *Dictyostelium* cells were osmotically shocked for 2 h (+), lysed and the subcellular components were isolated as described. After separation by SDS-PAGE, Western blot analysis was performed with antibodies against the proteins indicated. Control preparations (–) were made from cells incubated at low osmolarity and subsequently treated in the same way. While the amount of DdLIM decreases upon hyperosmotic shock, the proteins RMLC, MHC and eEF1 α (indicated by arrows) increase upon hyperosmotic shock after 2 h in the cytoskeletal fraction. In the crude membrane fraction, an increase of calreticulin was observed. Equal amounts of protein were applied.

The vacuolar H⁺-ATPase (Figure 5G) was identified through the peptide sequences LGFITGVMNTDK (amino acids 182–193) and DARIEEEIIDPQTG (amino acids 213–226). In this case, the observed apparent molecular mass of 40 kDa did not fit the predicted value of 100 kDa (Liu and Clarke, 1996). Whether this is due to limited proteolysis remains to be investigated.

We reconfirmed the results obtained from 2-D electrophoresis by Western analysis for the proteins DdLIM, RMLC, eEF1 α and calreticulin (Figure 6). Again, it could be demonstrated that the identified proteins were regulated in their amount in the cellular subfractions (cytoskeletal and crude membrane fraction). In the course of these experiments, we also found a 2-fold increase in the amount of myosin II heavy chain (MHC) in the cytoskeletal fraction.

Actin and myosin are phosphorylated under hyperosmotic conditions

Since it became apparent that the cytoskeleton is the main response system for osmotic stress in *Dictyostelium*, we concentrated on the two main components of the cytoskeleton, actin and myosin. Hyperosmotically shocked and unshocked *Dictyostelium* cells were lysed, and whole-cell extracts were analyzed by 2-D electrophoresis and Western blotting. The resulting membranes were probed with an anti-actin antibody. Almost identical amounts of actin (1.6-fold increase) were found to be present in the cells that were exposed to hyperosmotic conditions in comparison with cells that were suspended in phosphate buffer, as measured by densitometric analysis (Figure 7A). Probing of identical membranes with an anti-phosphotyrosine antibody showed, however, a 32-fold increase in the phosphorylation of actin in hyperosmotically treated cells (Figure 7B). Thus, hypertonic conditions lead to a highly increased phosphorylation of actin.

It was shown recently that MHC is phosphorylated *in vivo* during hyperosmotic stress (Kuwayama *et al.*,

Fig. 7. Actin and myosin II heavy chain (MHC) are phosphorylated upon hyperosmotic conditions. Hyperosmotically shocked (right panels) and unshocked (left panels) *Dictyostelium* cells were lysed and whole-cell extracts were analyzed by 2-D electrophoresis and Western blotting (A and B). The resulting membranes were probed with an anti-actin antibody (A) and an anti-phosphotyrosine antibody (B). Whereas almost identical amounts of actin were found on both blots, the phosphorylation of actin increased significantly upon hyperosmotic stress. (C) Phosphorylation of MHC. Cleared *Dictyostelium* cell lysates were subjected to an *in vitro* phosphorylation assay with [γ - 32 P]ATP as described. MHC was immunoprecipitated and the precipitate was analyzed by SDS-PAGE and autoradiography. At low osmolarity, there is little phosphorylation of MHC from *Dictyostelium* wild-type cells (AX-2). The phosphorylation increases upon hyperosmotic stress. The *in vitro* phosphorylated and immunoprecipitated MHC from the mutant strain HG 1555 showed no phosphorylation.

1996). The mutant strain HG 1555 (Lück-Vielmetter *et al.*, 1990) that carries threonine to alanine mutations in MHC at amino acid positions 1823, 1833 and 2029, respectively, is sensitive to hypertonic stress (Kuwayama *et al.*, 1996). Therefore, it was proposed that osmotic stress induces phosphorylation of MHC at these three threonine residues. An *in vitro* phosphorylation assay was performed with the cleared lysate of hyperosmotically shocked *Dictyostelium* cells and subsequently MHC was immunoprecipitated. As expected, MHC showed increased phosphorylation in the hyperosmotic shock situation (Figure 7C, AX-2). In contrast, MHC precipitated from the mutant strain HG 1555 after hyperosmotic stress showed no phosphorylation (Figure 7C, HG 1555). Thus we conclude that

the phosphorylation site involves at least one of these three threonine residues.

Discussion

Dictyostelium does not undergo a regulatory volume increase upon hyperosmotic stress

To investigate the volume changes that *Dictyostelium* cells undergo upon hyperosmotic stress, we measured the cell volume by centrifugation into graded glass capillaries. We found that the cells shrink under hyperosmotic conditions of 434 mOsm to 50% of their original volume and that they do not regain their volume as long as the hyperosmotic conditions prevail (Figure 1A and B). This is in good agreement with the results obtained by Oyama (1996), who measured the volume of the extracellular medium of an osmotically treated *Dictyostelium* NC4 cell suspension. Thus, *Dictyostelium* does not actively up-regulate the cell volume under the conditions investigated. Since several cellular mechanisms of counteracting an osmotic pressure are known, such as the production of compatible osmolytes and the pumping of ions, we measured the contribution of the cell's cytosol to the total osmolarity. There was no difference in relative osmolarity between hyperosmotically shocked and unshocked cells after 1 h of hyperosmotic stress. The increase during the first hour of incubation to a relative osmolarity of 130% in both shocked and control cell suspensions can be explained by increased catabolism of polymeric intracellular energy sources (e.g. glycogen, see Loomis, 1975, and references therein) due to starvation. Furthermore, a modest cell lysis could occur in both cell suspensions with a subsequent release of degraded cellular compounds into the medium.

An increase in intracellular solute concentration due to hyperosmotic conditions was observed after prolonged stress (Figure 2). This increase in intracellular osmotically active particles could be attributed to a degradative process, as the amount of free ubiquitin declines significantly after 2 h of hyperosmotic stress and cellular proteins were found to be ubiquitinated (Figure 3). It is therefore likely that the increase of solutes is due to proteolytic activity in the shrunk cells. However, this increase was not sufficient to counteract the external forces applied.

Hyperosmotic stress causes relocation of proteins to subcellular compartments

Since it became apparent that protein degradation is involved in the hyperosmotic stress response in *Dictyostelium*, the question arose of whether this is reflected by an altered protein expression. By using 2-D electrophoresis, we showed that the protein pattern at the level of whole-cell extracts remained unchanged during hyperosmotic stress (Figure 4A and B). This is in contrast to the observations in *S.cerevisiae*, where up to 200 transient changes in the protein pattern were detected at high osmolarity (Varela *et al.*, 1992; Blomberg, 1995). However, if cells were subfractionated into a crude membrane and a cytoskeletal fraction, differences at the protein level could be seen in an osmotic stress-dependent fashion. While the overall protein patterns were very similar, we found distinct changes in the composition of the crude membrane fraction (Figure 4C and D) and the cytoskeleton (Figure 4E and F). This indicates that during hyperosmotic

stress specific proteins are relocated from the cytosol to the various cellular compartments, or vice versa.

Dictyostelium reinforces the cell cortex upon hyperosmotic stress

Upon hyperosmotic stress, the total amount of protein in the crude membrane fraction increased ~3-fold. We identified two proteins that increase in relative amount in the crude membrane fraction upon hyperosmotic stress (Figure 5F and G): calreticulin, a protein that is highly abundant in the endoplasmic reticulum (Coppolino and Dedhar, 1998) and the vacuolar H⁺-ATPase, a protein that is localized primarily in membranes of the contractile vacuole complex of *Dictyostelium* (Heuser *et al.*, 1993). Since both proteins belong to membranous compartments of the cell, it is likely that specific organelles contribute to the large amount of protein isolated with the crude membrane fraction under osmotic stress. Goodloe-Holland and Luna (1987) found by electron microscopy that the isolated plasma membranes contain remnants of intracellular organelles, but it was unclear from which organelles these contaminants arose. We conclude, from our results, that the co-isolated organelles are parts of the endoplasmic reticulum and the contractile vacuole complex. Since these organelles are connected to cytoskeletal components, it is likely that their array is perturbed, as the cytoskeleton rearranges.

Hyperosmotic stress affects the Dictyostelium cytoskeleton

Many distinct changes in the protein pattern of the cytoskeleton were found (Figure 4E and F). Among other regulated proteins, actin and myosin II, the two main components of the cytoskeleton, are affected by hyperosmotic stress. By immunostaining, we observed a strongly increased phosphorylation of actin on a tyrosine residue upon hyperosmotic stress (Figure 7B). Tyrosine phosphorylation of actin was reported in several cellular events in *Dictyostelium* (Schweiger *et al.*, 1992), such as upon inhibition of oxidative phosphorylation (Jungbluth *et al.*, 1994) and associated with morphological cell shape changes (Howard *et al.*, 1993) or, recently, in the dormancy state of *Dictyostelium* spores (Kishi *et al.*, 1998). Howard *et al.* (1993) suggested a regulation of filamentous actin through phosphorylation. These authors demonstrated a positive correlation between tyrosine phosphorylation of actin and the rounding up of developing *Dictyostelium* cells. In contrast, Kishi and co-workers found that tyrosine phosphorylation did not affect the polymerizability of actin *in vitro*; however, dephosphorylation of actin leads to a 'reactivated dynamic actin system' during the spore germination process. We did not observe a significant phosphorylation until 15 min after the hyperosmotic shock (data not shown). At this time, the morphological changes of the cell have already occurred. Therefore, our results indicate that the observed actin phosphorylation during hyperosmotic stress leads rather to a 'deactivated actin system', resembling the situation in the dormancy state. We also showed that DdLIM, a protein associated with the actin cytoskeleton (Prassler *et al.*, 1998), decreases in amount in the cytoskeletal fraction isolated from osmotically shocked cells (Figure 5A). This protein localizes in pseudopodia and at sites of membrane ruffling (Prassler

et al., 1998). Since high osmolarity causes a rounding up of cells and retraction of cellular protrusions (Kuwayama *et al.*, 1996), the decrease of DdLIM in the cytoskeletal fraction gives further evidence for its role in the construction of cellular protrusions.

We observed an increase of RMLC (Figures 5B and 6) and MHC (Figure 6) in the cytoskeletal fraction under hyperosmotic conditions, suggesting a reinforcement of the cell cortex. This result is in agreement with the observations of Kuwayama *et al.* (1996), who found that myosin from the cytoplasm and pseudopodia moved towards the actin-rich cortex of the cells within 10 min of hyperosmotic shock.

Also, we and others observed phosphorylation of the MHC in the cleared lysate during hyperosmotic stress (Kuwayama *et al.* 1996; Figure 7C, AX-2). The phosphorylation site was shown to be located on at least one of three threonine residues at the C-terminus of the protein (Figure 7C, HG 1555). It has been suggested that phosphorylation of the MHC is the molecular mechanism in the disassembly process of filamentous myosin (Egelhoff *et al.*, 1993). It is therefore reasonable to assume that the increased phosphorylation is indicative of an assembly/disassembly process in the course of the cell shrinkage. Future experiments need to address the question of whether one or more phosphorylation events may differentially regulate the localization and assembly state of the MHC, since phosphorylated myosin was found in the cytosol, as well as trapped in the cytoskeletal fraction (Egelhoff *et al.*, 1993).

Hyperosmotic stress causes a sequestration of translational factors to the cytoskeleton

The amounts of two elongation factors eEF1 α (ABP50) and eEF1 β also increased in the cytoskeleton under hyperosmotic stress (Figure 5D and E). ABP50 was found to associate with actin (Edmonds, 1993). The fact that eEF1 α increases in the cytoskeletal fraction suggests a sequestration of the translation factor to this compartment. In the current model of the elongation cycle of eukaryotic protein translation, eEF1 α is involved in transporting aminoacyl-tRNA to the ribosome during protein synthesis. Edmonds *et al.* (1998) could show that binding of eEF1 α -GTP to F-actin causes a rate of GTP hydrolysis that is too slow to account for *in vivo* protein synthesis. Thus, eEF1 α bound to F-actin is inactive with respect to protein translation. Furthermore, Liu *et al.* (1996) demonstrated the ability of eEF1 α to bind either F-actin or aminoacyl-tRNA in competition binding experiments. This means that the binding of eEF1 α to F-actin or aminoacyl-tRNA is mutually exclusive. Thus, an increased sequestration of eEF1 α to the cytoskeleton would lower the amount of eEF1 α that is capable of binding to aminoacyl-tRNA to such an extent that a pause in translation could occur. We also found an increase of eEF1 β in the cytoskeletal fraction upon hyperosmotic stress, suggesting that this other translation factor also binds to actin. From the increase in the amount of the two elongation factors in the cytoskeleton, we conclude that translational control plays a crucial role in the osmotic stress response. Binding of these elongation factors to the cytoskeleton decreases their availability for cytoplasmic peptide elongation and their free concentration could become rate limiting.

In summary, our findings show that upon hyperosmotic stress *Dictyostelium* activates a variety of mechanisms, such as protein rearrangements, post-translational modifications of major cytoskeletal components, protein degradation and a translational arrest, suggesting a resting state of the cells.

Materials and methods

Growth of *Dictyostelium* cells

Cells of *D. discoideum* [strain AX-2(214)] were axenically cultivated in shaken suspension at 21°C to a density of $3\text{--}5 \times 10^6$ cells/ml (Watts and Ashworth, 1970).

Hyperosmotic treatment of *Dictyostelium* cells

Axenically grown *Dictyostelium* cells were washed twice in Soerensen phosphate buffer (SPB: 2.0 mM Na_2HPO_4 , 14.6 mM KH_2PO_4 , pH 6.0). Cells were resuspended to a density of 3×10^7 cells/ml and shaken in this buffer at 150 r.p.m. After 1 h, 2 M sorbitol in SPB was added to a final concentration of 400 mM (434 mOsm = high osmolarity condition); the same amount of pure SPB (34 mOsm = low osmolarity condition) was added to the control. After 2 h at 21°C, cells were pelleted for 3 min at 4°C and 500 g.

Preparation of crude membrane fraction

The crude membrane fraction was prepared according to Goodloe-Holland and Luna (1987) with slight modifications: hyperosmotically shocked *Dictyostelium* cells were resuspended as described in ice-cold sucrose lysis buffer supplemented with protease inhibitor cocktail Complete (Boehringer Mannheim) and immediately frozen in liquid nitrogen. After thawing on ice, crude membranes were collected from this lysate as described and applied on a sucrose step gradient. Ultracentrifugation was performed overnight at 2°C and 150 000 g. The interphase was collected, washed and resuspended in protease/phosphatase inhibitor solution (containing 100 μM sodium vanadate, 5 mM EGTA, 5 mM benzamidine and protease inhibitor cocktail Complete).

Preparation of the cytoskeletal fraction

The cytoskeletal fraction was prepared according to Chia *et al.* (1993) with the following modifications: axenically grown *Dictyostelium* cells were treated as described in the hyperosmotic shock procedure and washed with 20 mM sodium phosphate buffer, pH 6.8 (PB) supplemented with 400 mM sorbitol, whereas the control cells were washed with PB only. The cytoskeletal fractions were isolated as described in an isolation buffer containing protease and phosphatase inhibitors and subsequently precipitated (10 min, 11 000 g).

Measurement of the cell volume

The volume of *Dictyostelium* cell pellets was determined by centrifugation of 3×10^7 cells into a graded glass capillary before and after the osmotic shock according to Maniak *et al.* (1995).

Determination of the relative osmolarity

Dictyostelium cells at a density of 1×10^8 cells/ml were shocked osmotically as described. Samples from suspensions of low and high osmolarity were taken at different time points. Cell lysates were prepared by boiling 0.5 ml of the cell suspension for 2 min, followed by sonification of the lysate in a cooled test tube for 30 s (Branson sonifier). Cell membranes were removed by centrifugation at 20 800 g for 30 min. The osmolarity of buffers and cell lysates was measured with a cryoscopic osmometer (Osmomat 030, Gonotec). The relative osmolarity of the lysate was corrected with respect to the osmolarity of buffers and to the volume of the cell membrane pellet.

Quantification of free ubiquitin and determination of protein ubiquitination

Dictyostelium cells at a density of 1×10^7 /ml were osmotically shocked and resuspended in SPB containing protease inhibitors (Complete, Boehringer) and 5 mM *N*-ethylmaleimide. The cells were pelleted and frozen in liquid nitrogen. The thawed cell lysate was centrifuged at 10 000 g for 30 min at 4°C. The supernatant was analyzed by SDS-PAGE and immunoblotting to a PVDF membrane. Immunostaining was performed with a monoclonal anti-rat ubiquitin antibody, a gift of Dr S. Wolf.

2-D electrophoresis

All samples (i.e. cells lysed with liquid N_2 , crude membrane fractions and cytoskeletal fractions) were lyophilized. Samples (75 μg of total protein content) were applied to isoelectric focusing strips pH 3–10 as published previously (Görg, 1993; Rabilloud *et al.*, 1997). Isoelectric focusing was performed in a Dry-Strip kit on a Multiphor II according to the manufacturer's instructions (Pharmacia Biotech). The second dimension was performed at 40 mA/gel in a Bio-Rad Protean II xi multi-cell according to the manufacturer's protocol. Silver staining was performed in a Hoefer automated gel stainer (Pharmacia Biotech).

For preparative gels (1–3 mg protein content), the focusing time was increased 1.5-fold and gels were stained with Coomassie R-250.

In vitro labeling and immunoprecipitation of myosin II

Osmotically shocked *Dictyostelium* cells (10^7 cells) were resuspended in 100 μl of reaction solution [20 mM HEPES, pH 7.4, 10 mM MgCl_2 , 0.2% (v/v) Triton X-100, protease inhibitors (Complete, Boehringer)] and immediately frozen in liquid nitrogen. The cleared lysate was incubated with 100 μl of 50 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 30 min at room temperature. Immunoprecipitation was performed with a monoclonal anti-MHC antibody (mAb 56-396-5, specific for filamentous and monomeric myosin) and fixed *Staphylococcus aureus* cells (Pansorbin, Calbiochem) according to the manufacturer's protocol. The precipitate was analyzed by SDS-PAGE and autoradiography.

Miscellaneous

Protein quantification, SDS-PAGE and Western blotting were performed according to the published methods of Bradford (1976), Laemmli (1970) and Towbin *et al.* (1979), respectively. Immunostaining of proteins was performed with various antibodies: actin (mAb 236), MHC (mAb 56-396-5 and mAb 21-96-3), calreticulin (mAb 252-234-3), all a kind gift of Dr G. Gerisch; DdLIM (mAb 284-213-1), a gift of Dr G. Marriott; RMLC (anti-RMLC), a gift of Dr R. Chisholm; and eEF1 α (#05:235, Upstate Biotechnology, Lake Placid, NY). For detection of phosphotyrosine, blots were probed with an anti-phosphotyrosine mAb (mAb 5E2), a gift of Dr A. Ullrich. The detection of proteins after blotting onto PVDF was performed using the ECL detection system (NEN).

Acknowledgements

We would like to thank Drs G. Gerisch, A. Ullrich, S. Wolf, R. Chisholm and G. Marriott for the gift of various antibodies. This work was supported by grants of the Boehringer Ingelheim Fonds to T.P. and of the Deutsche Forschungsgemeinschaft to S.C.S. (Schu778/3-1, Schu778/3-2, Schu778/5-1).

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Received March 10, 1999; revised and accepted June 7, 1999