Protection against osmotic stress by cGMP-mediated myosin phosphorylation
Kuwayama, H; Ecke, M; Gerisch, G; VanHaastert, PJM

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Cells exposed to osmotic stress can avoid dehydrative collapse either by using a cell wall (1) or by increasing the intracellular osmotic potential by biosynthesis of small molecules like glycerol, uptake of ions, or discharge of water (2). Actin and some of its binding proteins are required to resist high osmotic stress in yeast (3). The other major component of the cytoskeleton, myosin II or conventional myosin, exerts motive force by interacting with actin filaments (4). *Dictyostelium discoideum* is a microorganism with a small haploid genome from which mutants altered in cytoskeletal proteins have been isolated (5-7). Studies of mutants lacking myosin II heavy chain (mhc-^AA^) reveal that this form of myosin is essential for cytokinesis, capture of cell surface lectin receptors, and normal cell motility and chemotaxis (5, 8, 9). Here we have used *D. discoideum* to investigate the role of myosin II in protecting amoeboid cells from high osmotic pressure.

Wild-type XP55 cells resisted an osmotic shock of 300 mM glucose for ~30 min; 50% of the cells died after a shock of about 60 min (Fig. 1A). In contrast, mhc-^AA^ cells were very sensitive to osmotic stress with a 50% reduction of cell viability after 5 to 10 min (Fig. 1A). Cells of the mhc-^AA^ mutant that had been transfected with complementary DNA (cDNA) encoding normal myosin II heavy chains (mhc-^AW^) showed a viability comparable to that of the wild-type XP55 (Fig. 1A).

Myosin II filaments bind to actin filaments more effectively than myosin monomers (10, 11). Phosphorylation of myosin II at three threonine residues of its tail region inhibits filament formation (10, 12) and thus weakens the interaction with actin filaments. Mutant mhc-^AAA^ produces a myosin II heavy chain in which the phosphorylatable threonines are replaced by alanine residues. As a consequence of this mutation, myosin II exists predominantly in the filamentous state, and the assembly and disassembly rate are probably strongly reduced (9, 10). These mhc-^AAA^ cells showed the same sensitivity to high concentrations of glucose as mhc-^ cells (Fig. 1A), which suggests that phosphorylation of myosin II at its tail region is required to protect cells against osmotic stress (13).

Guanosine 3',5'-monophosphate (cGMP) acts as a universal second messenger in eukaryotic cells (14). In *D. discoideum*, cGMP levels increase upon stimulation with the chemotactant adenosine 3',5'-monophosphate (cAMP) (15). Intracellular cGMP regulates the assembly and disassembly of myosin filaments by inducing the phosphorylation of the threonine residues (16). Osmotic stress also activates guanylyl cyclase in wild-type *D. discoideum* cells; the cGMP concentration increased after 1 min and reached a peak at ~10 min after the onset of stimulation (17) (Fig. 1B). A transient accumulation of cGMP levels upon addition of 300 mM glucose was also observed in wild-type XP55 and in the mhc-^, mhc-^AA^ and mhc-^AAA^ strains (Fig. 1B). No increase of cGMP levels was found in the nonchomictic mutant KI-8 in which gua-
nnylyl cyclase activity is strongly reduced (18). To investigate the role of cGMP in osmoregulation, we tested the survival of this mutant under conditions of osmotic stress. Mutant KI-8 cells proved to be very sensitive to osmotic stress; survival of these cells was reduced cGMP and was specific for this cell-permeable cGMP analog (19). In addition, 8Br-cGMP in the presence of glucose induced myosin II phosphorylation in KI-8 cells (Fig. 2B). Thus, a cell-permeable cGMP analog restored osmosensitivity in the guanylyl cyclase mutant but not in the myosin mutants.

The distribution of the cytoskeletal proteins actin and myosin was analyzed by confocal fluorescence microscopy with labeled phalloidin to stain actin filaments and monoclonal antibodies to label myosin II (Fig. 3). Control cells were highly motile and elongated with many pseudopodial extensions. Actin filaments were localized primarily in the extending pseudopodia (20), whereas myosin II was found throughout the cytoplasm and enriched in pseudopodia. At 10 min after the osmotic shock with 300 mM glucose, cells shrank by ~50% (21), assuming a rigid state in which a more spherical core region was surrounded by flattened extensions. Filamental actin remained in the extensions. Myosin II moved within 10 min toward the cortex of the core; dual labeling revealed that myosin II was localized primarily in a layer beneath the actin-rich cortex. Thus, we propose the following model: Osmotic stress induces the activation of guanylyl cyclase. The main function of the produced cGMP is to mediate myosin II phosphorylation at three threonine residues on its tail, which enhances the disassembly of myosin II filaments and thereby recruits myosin for deposition below a peripheral layer of actin filaments. Dictyostelium discoideum cells respond chemotactically to cAMP. Guanylyl cyclase and myosin II phosphorylation are essential for both osmo- and chemosensory transduction. Activation of guanylyl cyclase by these signals must use different mechanisms because chemotactic signal transduction is ablated in mutants lacking the heterotrimeric guanosine triphosphate binding protein (G protein) subunits Ga2 (22) or GB (23), which show a normal response to osmotic stress (24). In yeast, osmosensing is mediated by a two-component system composed of a histidine kinase and its receiver domain (25). In the yeast system, the sensor mediates osmoregulation through the production of glycerol. Recently, a gene encoding a putative histidine kinase has been identified in D. discoideum; interestingly, a mutant with a disruption of this gene is sensitive to high osmolarities (26). Possibly, this two-component system is a common constituent of the osmosensory pathways, including the one that controls the actin and myosin system shown here.

REFERENCES AND NOTES

Fig. 1. Time course of (A) survival, (B) cGMP accumulation, and (C) myosin II heavy chain (MHC) phosphorylation in the presence of 300 mM glucose for wild-type XP55 and mutants KI-8, mhc-/-, mhc-/-, and mhc-/- of D. discoideum (30). Survival and myosin II phosphorylation are presented as percent of unstimulated cells. Results in (C) are the means ± SD of three independent experiments with triplicate determinations.

Fig. 2. Restoration of (A) survival and (B) MHC phosphorylation by the cell-permeable cGMP analog 8Br-cGMP (31). Survival and myosin II phosphorylation are presented as percent of unstimulated cells. Solid bar, control; stippled bar, 8Br-cGMP. Statistical analysis: Asterisk indicates P < 0.01, which is significantly lower than in wild-type (WT) cells; double asterisk indicates P < 0.05, which is significantly higher than without 8Br-cGMP. Results in (C) are the means ± SD of three independent experiments with triplicate determinations.

Fig. 3. Cell shape and localization of myosin II (red) and actin filaments (green) in XP55 cells before and after an osmotic shock with 300 mM glucose (32). A confocal section was made through the center of the cell; areas where myosin II and actin filaments colocalized are yellow. Bar, 5 μm.
The transcription of PHO5, which encodes a secreted acid phosphatase, is tightly repressed when Saccharomyces cerevisiae are grown in phosphate-rich medium, and its induction is more than 100-fold increased when yeast are starved for phosphate (1). Transcriptional induction of PHO5 requires the transcription factor PHO4, and mutation analysis suggests that PHO4 activity is negatively regulated by phosphorylation (2). When yeast are grown in phosphate-rich medium, the PHO80-PHO85 cyclin-CDK (cyclin-dependent kinase) complex phosphorylates PHO4 (2) and transcription of PHO5 is repressed. When yeast are starved for phosphate, the kinase activity of the PHO80-PHO85 complex is down-regulated by the CDK inhibitor PHO81 (3). This reduced activity results in the appearance of an underphosphorylated form of PHO4 (2) that, in combination with a second transcription factor, PHOB, binds to the PHO5 promoter and activates PHO5 transcription (4).

We wished to determine how changes in phosphate availability affect PHO4 function. Phosphate starvation does not have a large effect on PHO4 stability; no difference in the amount of PHO4 is observed between yeast grown in low versus high phosphate medium (2). Because PHO4 occupies binding sites in the PHO5 promoter in vivo under inducing, but not repressing, conditions (5), the phosphorylation level of PHO4 may affect the level of DNA binding or some prior step, such as nuclear localization. Preliminary data suggest that phosphorylated and unphosphorylated PHO4 have a similar affinity for DNA (6).

We therefore examined the subcellular localization of PHO4 in wild-type cells grown in low or high phosphate medium. PHO4 is concentrated in the nucleus when yeast are starved for phosphate and is largely cytoplasmic when yeast are grown in phosphate-rich medium. The sites of phosphorylation on PHO4 were identified, and phosphorylation was shown to be required for full repression of PHO5 transcription when yeast were grown in high phosphate. Thus, phosphorylation of PHO4 by PHOB-PHO85 turns off PHO5 transcription by regulating the nuclear localization of PHO4.

Regulation of PHO4 Nuclear Localization by the PHO80-PHO85 Cyclin-CDK Complex

Elizabeth M. O'Neill, Arie Kaffman, Emmitt R. Jolly, Erin K. O'Shea*