Protection Against Osmotic Stress by cGMP-Mediated Myosin Phosphorylation

Hidekazu Kuwajama, Maria Ecke, Günther Gerisch, Peter J. M. Van Haastert*

Conventional myosin functions universally as a generator of motive force in eukaryotic cells. Analysis of mutants of the microorganism Dictyostelium discoideum revealed that myosin also provides resistance against high external osmolarities. An osmo-induced increase of intracellular guanosine 3',5'-monophosphate was shown to mediate phosphorylation of three threonine residues on the myosin tail, which caused a relocation of myosin required to resist osmotic stress. This redistribution of myosin allowed cells to adopt a spherical shape and may provide physical strength to withstand extensive cell shrinkage in high osmolarities.

C. Cells exposed to osmotic stress can avoid dehydrative collapse either by using a cell wall (1) or by increasing the intracellular osmotic potential by synthesis 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−) revealed that this form of myosin is essential for cytokinesis, clamping 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− 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− mutant that had been transfected with complementary DNA (cDNA) encoding normal myosin II heavy chains (mhc+) 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−AA 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 was required to protect cells against osmotic stress (13).

Guanosine 3',5'-monophosphate (cGMP) acts as a universal secondary 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−, and mhc−AAA strains (Fig. 1B). No increase of cGMP levels was found in the nonchomotropic mutant K1-8 in which guar
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, mhcα+, mhc−, and mhcAAA 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.

- **A**
  - Survival (%)
  - (30 min after osmotic shock)
  - WT
  - KI-8
  - mhcα+
  - mhc−
  - mhcAAA

- **B**
  - MHC phosphorylation (%)
  - (30 min after osmotic shock)
  - WT
  - KI-8
  - mhcα+
  - mhc−
  - mhcAAA

- **C**
  - pmol GMP/106 cells
  - Time (min)
  - WT
  - KI-8
  - mhcα+
  - mhc−
  - mhcAAA

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 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 colocalized are yellow. Bar, 5 μm.
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*

PHO4, a transcription factor required for induction of the PHO5 gene in response to phosphate starvation, is phosphorylated by the PHO80-PHO85 cyclin-CDK (cyclin-dependent kinase) complex when yeast are grown in phosphate-rich medium. PHO4 was shown to be concentrated in the nucleus when yeast were starved for phosphate and was predominantly cytoplasmic when yeast were 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 PHO80-PHO85 turns off PHO5 transcription by regulating the nuclear localization of PHO4.

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 PHO4 requires the transcription factor PHO4, and preliminary data suggest 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, PHO2, 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 status of PHO4 affects 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 (Fig. 1A to D). In pho80Δ and pho85Δ strains, in which PHO4 is not phosphorylated (2) and which express PHO5 constitutively (1), PHO4 was concentrated in the nucleus, even when the strains were grown in phosphate-rich medium (6). In contrast, PHO4 was predominantly cytoplasmic in a pho81Δ strain grown in low or high phosphate conditions (6). In this strain, PHO4 is phosphorylated even when yeast are starved for phosphate (2),

Department of Biochemistry and Biophysics, University of California at San Francisco, School of Medicine, San Francisco, CA 94143-0448, USA.

*To whom correspondence should be addressed.