Role of a Mitogen-Activated Protein Kinase Cascade in Ion Flux-Mediated Turgor Regulation in Fungi

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Fungi normally maintain a high internal hydrostatic pressure (turgor) of about 500 kPa. In response to hyperosmotic shock, there are immediate electrical changes: a transient depolarization (1 to 2 min) followed by a sustained hyperpolarization (5 to 10 min) prior to turgor recovery (10 to 60 min). Using ion-selective vibrating probes, we established that the transient depolarization is due to Ca\(^{2+}\) influx and the sustained hyperpolarization is due to H\(^+\) efflux by activation of the plasma membrane H\(^+\)-ATPase. Protein synthesis is not required for H\(^+\)-ATPase activation. Net K\(^+\) and Cl\(^-\) uptake occurs at the same time as turgor recovery.

Osmotic shock is a perilous condition for any organism. The shock can be due to either hyperosmotic or hypoosmotic changes in the external environment. Unlike unwalled cells (mostly animal), which must remain isotonic to the external environment, organisms having walled cells can utilize a high internal hydrostatic pressure (turgor) created by internal hydrostatic pressure (turgor) created by osmotic and/or turgor regulation. In yeast, hyperosmotic treatment: a hyperpolarization that is probably transient and a sustained depolarization (5 to 10 min) prior to turgor recovery (10 to 60 min). Using ion-selective vibrating probes, we established that the transient depolarization is due to Ca\(^{2+}\) influx and the sustained hyperpolarization is due to H\(^+\) efflux by activation of the plasma membrane H\(^+\)-ATPase. Protein synthesis is not required for H\(^+\)-ATPase activation. Net K\(^+\) and Cl\(^-\) uptake occurs at the same time as turgor recovery.

MATERIALS AND METHODS

Strains. Stock cultures of the wild type (strain 74-OR23-1A, FGSC no. 987), os\(^{-}\)- (allele UCLA80, FGSC no. 2238), and cut (allele LLM1, FGSC no. 2385) were obtained from the Fungal Genetics Stock...
Center (School of Biological Sciences, University of Missouri, Kansas City, Missouri) (17). The stock cultures were maintained on Vogel’s plus 1.5% (wt/vol) sucrose and 2.0% (wt/vol) agar (medium) (VM) (27) and stored at 4°C in petri dishes sealed with Parafilm.

The os-1 B135 allele encodes a single amino acid substitution in the protein kinase (31). To confirm that the os-1 mutant exhibited the correct phenotype and had not reverted to wild type, an agar plug of mycelium grown on VM was placed in the center of a 10-cm petri dish containing either VM, VM plus 4% (wt/vol) NaCl or VM plus the dicarboximide fungicide vinclozolin (50 μg/ml; Supelco, Bellafonte, PA). Growth at 28°C was quantified every few hours by measuring colony diameter. Growth rates of os-1 were about 70% those of the wild type in VM. Unlike the wild type, the os-1 mutant did not grow in 4% NaCl and was insensitive to vinclozolin, confirming the mutant phenotype (18). The os-2 UCLAL80 allele encodes a stop to trp codon mutation in the protein kinase domain and is expected to be nonfunctional (31). The os-2 mutant phenotype was tested for growth from an agar plug of mycelium placed in the center of a 10-cm petri dish containing either VM, VM plus 4% (wt/vol) NaCl, or VM plus the phenylpyruvyl fungicide fluconidoxil (80 μM; Sigma-Aldrich, Oakville, Ontario). The wild type and os-2 had similar growth rates in VM. Unlike the wild type, the os-2 mutant was unable to grow in 4% NaCl and was insensitive to fluconidoxil, confirming the mutant phenotype (31).

Culture preparation for experiments. Cultures used for experiments were grown overnight from 3- to 5-mm agar plugs excised from the stock culture and placed on strips (2.5 by 6 cm) of dialysis tubing stretched with fine sandpaper that overlay the VM in petri dishes. The cultures were incubated at 28°C or at room temperature (21 to 24°C) overnight. Ideally, the mycelium had grown about 3 cm. Just before an experiment, the cellophane strip was cut with scissors or a razor blade to a size of about 1 by 3 cm, which included the growing edge of the colony. Care was taken when moving the cellophane to avoid damaging the hyphae. The cellophane strip with mycelium was placed inside the cover of a 30-mm petri dish, immobilized on the bottom with sealing tape or a Plexiglas frame, and flooded with 3 ml of buffer solution (BS) containing (mM concentrations indicated in parentheses) KCl (10), CaCl2 (10), MgCl2 (1), sucrose (133), and Mes (10), with pH adjusted to 5.8 to KOH. The dish cover was transferred to the microscope, and a hypha was chosen from the growing edge which included the growing edge of the colony. Care was taken to avoid damaging the hyphae. After positioning near the hypha, the media were mixed to assure rapid and complete equilibration. However, after addition of the hyperosmotic solution, the media were recommenced 2 to 5 min after hyperosmotic treatment. The modified BS plus 1,000 mM sucrose was poorly mixed with the BS and took time to equilibrate. In addition to the refractive wave observed as hyperosmotic solution entered the field of view, the decrease in pressure required to bring the os-micropipette tip confirmed that the hypha was being subjected to high external osmolarity.

If the tip of the pressure probe became irreversibly plugged during recording, it was withdrawn from the cell and a new probe was used to impale a new cell compartment. Every attempt was made to impale the same hypha, a few compartments away from the first hypha measured. In some cases, damage caused by withdrawal of the micropipette did not result in cytoplasm loss from adjacent compartments. Otherwise, a different hypha was used.

Electrophysiological Measurements. As for pressure measurements, large-trunk hyphae, usually about 0.5 cm behind the growing edge, were selected for impalements. Double-barrel micropipettes (12, 13) were used to allow simultaneous current injection and potential monitoring for current-voltage measurements. Voltage clamping was performed using an operational amplifier configured as a current-voltage converter, controlled by a computer program via a data acquisition hardware unit (Labmaster DMA, Scientific Solutions, Inc., Solon, Ohio). A voltage range of −300 to 0 mV was clamped using a bipolar step protocol of alternating positive- and negative-going voltages to avoid membrane hysteresis. Clamping currents were not corrected for the cable properties of the hypha (7). We measured the cable length constant (20) along the hypha and across one septal pore to be about 200 μm. Cable-corrected currents should be added to the current of the measurements. However, this correction requires multiple impalements into adjacent hyphal compartments, very technically difficult when hyperosmotic treatments may cause small movements of the hypha, dislodging the micropipette(s).

After impalement and recording of a stable potential for about 4 min from the hypha in 3 ml of BS, 0.5 ml of BS plus 1,000 mM sucrose was added dropwise in a circle surrounding the objective. Observation of a refractive wave soon after addition confirmed the arrival of the hyperosmotic solution. If the hyphal surface in the circle was not reverted to wild type, an agar plug of mycelium grown on BS was placed in the center of a 10-cm petri dish containing either BS, BS plus 4% (wt/vol) NaCl, or BS plus 1,000 mM sucrose to the 3 ml BS as described above.

Ion flux measurements. Noninvasive ion-selective microelectrodes were used to measure the diffusive ion gradients at the surface of the hypha, from which the ion flux across the cell membrane can be calculated (19, 26). The instrumentation (the MIFE technique) was developed at the University of Tasmania (Hobart, Australia) (19, 23). As for electrical and pressure measurements, large-trunk hyphae were selected. Care was taken to assure that there was no obstruction access to the hypha, with no other hyphae in the vicinity, to avoid interfering ion experiments. The BS was modified by adding 1.0% (vol/vol) agar, dislodging the micropipette(s).

Cycloheximide treatments. To test whether hyperosmotically induced hyper-polarization was due to de novo synthesis of plasma membrane H+-ATPase, hyphae were pretreated for 13.5 to 22.2 min by adding a final concentration of 100 μM cycloheximide (Sigma-Aldrich, Oakville, Ontario) from a 20 mM stock (dissolved in 95% [vol/vol] ethanol, then diluted to 17% ethanol with distilled H2O). The hyperosmotic treatment was given by adding 0.5 ml of BS plus 1,000 mM sucrose to the 3 ml BS as described above.
BS osmolality was in the range of 140 to 170 mosmol kg\(^{-1}\), and the osmolality of the BS plus 1,000 mM sucrose was adjusted to 1,000 to 1,100 mosmol kg\(^{-1}\). The final osmolality was measured after experiments and was between 320 and 370 mosmol kg\(^{-1}\), so the net osmotic change was about 190 mosmol kg\(^{-1}\).

In initial experiments, there was a background K\(^+\) efflux (300 nmol m\(^{-2}\) s\(^{-1}\)) from the tape used to hold the cellophane securely in the dish. Initial net K\(^+\) fluxes were zero when the hyphae were immobilized with a Plexiglas frame. Some of the wild-type data had to be corrected for the background flux that occurred when tape was used.

Reverse transcription (RT)-PCR measurement of gene expression. Cellophane strips overlaid with mycelium were transferred to an empty petri dish and flooded with 15 ml of BS. After 10 min of preconditioning, 2.5 ml of BS plus 1,000 mM sucrose was added to the dish. Mycelia were harvested after 0, 2, 10, 20, and 60 min and ground to a fine powder in liquid nitrogen. In all cases, 300 \(\mu\)g of the 

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Reverse transcription (RT)-PCR kit (Hilden, Germany) was used with 6 ng/reaction mixture of mRNA, and mRNA enriched with the PolyATract mRNA isolation system IV (Promega, Madison, Wis.). Gene-specific primers Os1-2fwd (ATCACAAATGCAGCAT), Os2-2rev (AATCGAACTTCTCCTCGACAGACGGG), Os1-2r (AACTTCTCGTTTGCCTTGACGGC), Os2-2fwd (AATACGTTCACTCCGCCGGTG), and Os2-2rev (AATCGAACTTCTCCTCGACAGACGGG) were designed to produce fragments of 452 bp (os-2), 302 bp (os-1), and 302 bp (wild type). All of the oligonucleotide primers used in this study were purchased from Operon Biotechnologies, Inc. (Chicago, IL).

The band sizes for the os-1, os-2, and tubulin bands calculated from molecular markers (0.41, 0.56, and 0.69 kb, respectively) were very similar to the predicted sizes. Quantitation was performed by measuring integrated density of the bands using the public domain ImageJ program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/ij/).

Statistical analysis. Data are shown as means ± standard deviations (sample size) unless stated otherwise. Independent two-tailed \(t\) tests were performed in either SYSTAT (Systat, Inc.) or Excel (Microsoft).

RESULTS

Initial turgor is different in the os mutants. After flooding the mycelium with BS and growth resumption, the three strains had significantly different turgors. The wild type had the highest turgor (496 ± 71 kPa, \(n = 26\)), os-1 had the lowest turgor (302 ± 71 kPa, \(n = 26\)), and the os-2 turgor was intermediate (422 ± 64 kPa, \(n = 32\)) (Fig. 1). Since the os mutants are unable to grow at high osmolarities, we examined turgor regulation after hyperosmotic treatment.

The os mutants exhibit turgor recovery. Even though initial turgors were different in os-1 and os-2 compared to the wild type, both of the osmotic mutants did exhibit turgor recovery after hyperosmotic treatment with sucrose (Fig. 2B and C), similar to the wild type (Fig. 2A) (15). The recovery time was similar in all strains; turgor recovered to near initial level within 60 min. Since most phenotype analyses of osmotic mu-
tants have used NaCl to cause hyperosmotic shock, we performed preliminary experiments to show that turgor recovery also occurs after NaCl treatment in the wild type and os-1 (Fig. 2D). We used BS plus 70 mM NaCl (final concentration), which should have an osmolality increase of about 140 mOsM/kg H2O, similar in magnitude to the hyperosmotic treatment with sucrose. Wild-type turgor recovery was faster in NaCl compared to sucrose, consistent with uptake of the ions from the extracellular medium to counter the high external osmolarity. The time course of os-1 turgor recovery after NaCl treatment was similar to the time course after sucrose treatment.

Rather than taking up sucrose directly, N. crassa relies upon an extracellular invertase and diffusional hexose uptake through a neutral carrier or active uptake through a H+/hexose symport (2). The mechanism underlying turgor recovery after sucrose addition could involve ion transport or osmotic production. If ionic influxes are required, it is expected they will affect the electrical properties of the hyphae, both potential and conductance.

Wild-type hyperpolarization is absent in the os mutants. The electrical differences between the wild type, os-1, and os-2 are shown in Fig. 3 and 4. In all three strains, hyperosmotic treatment caused a transient depolarization. In the wild type, the transient depolarization was followed by a prolonged hyperpolarization in the wild type (A). In os-1 (B), the transient depolarization occurs, but the prolonged hyperpolarization does not. os-2 (C) exhibits a response intermediate between those of os-1 and the wild type. (Right panels) Current-voltage relations are shown for the WT (average of 16 experiments) and os-1 (9 experiments) and os-2 (9 experiments) mutants. The curves are the averages of current-voltage measurements (shown by vertical bars on the membrane potential trace) prior to the hyperosmotic treatment (initial), during the transient depolarized state, and during the final repolarized state, as marked. During the transient depolarization, the current-voltage relation shifts to depolarized potentials during the transient depolarization, but conductance remains the same. During repolarization, the WT conductance decreased at negative potentials and recovered completely at positive voltages. The recovery of the outward current at positive voltages, indicative of positive ion flux out of the cell, is consistent with activation of the plasma membrane proton pump. These changes during repolarization are not observed in os-1, which exhibited a lower conductance at positive voltages compared to the initial current-voltage relation. os-2 had consistently high conductances initially, during the transient depolarization, and during the repolarization.
and os-2 mutants. Hyperosmotically induced conductance changes were similar between the wild type and os-1, with the exception of a large outward current in the wild type at positive potentials during hyperpolarization which was consistent with increased H⁺-ATPase activity (Fig. 3A, B). The os-2 conductances before and after hyperosmotic treatment were much larger than those of either the wild type or os-1 (Fig. 3C).

To ensure that the absence of hyperpolarization in the os-1 and os-2 mutants was due to a nonfunctional MAP kinase cascade rather than a pleiotropic consequence of osmotic sensitivity, we compared the electrical responses of the wild type with another osmotic mutant, cut (29). Like the os mutants, the cut mutant is unable to grow on VM plus 4% NaCl (data not shown) (29), but it is not a member of the MAP kinase cascade family (29). In an experimental run comparing the wild type and cut, the cut mutant exhibited electrical responses to hyperosmotic treatment that were very similar to those of the wild type (both the transient depolarization and the sustained hyperpolarization). The hyperpolarization observed in cut (-13 ± 28 mV, n = 11) was statistically the same as that in the wild type (-15 ± 35 mV, n = 11) (P = 0.926). Therefore, the hyperpolarization induced by hyperosmotic treatment appears to be mediated by the MAP kinase cascade, either by activating the H⁺-ATPase directly or by inducing expression of the H⁺-ATPase gene.

**Protein synthesis is not required for the wild-type hyperpolarization.** To determine whether the hyperpolarization caused by hyperosmotic treatment in the wild type was due to de novo
The net changes in K⁺ (400 nmol m⁻² s⁻¹) and Cl⁻ (600 nmol m⁻² s⁻¹) uptake are in the range appropriate for osmotic adjustment required to recover initial turgor. Typical dimensions of a hyphal compartment are 15-μm diameter and 100-μm length, so the surface area is 5.1 × 10⁻⁹ m⁻² and the volume is 1.8 × 10⁻¹¹ liters. If the net uptake of K⁺ and Cl⁻ across the plasma membrane is about 1 μmol m⁻² s⁻¹, K⁺ and Cl⁻ accumulation in the cell will cause a concentration increase of 17.2 mM min⁻¹, or as much as 1,000 mM in the 60 min required for complete turgor recovery. Thus, ion uptake alone is more than sufficient to account for turgor recovery in the wild type but does not account for turgor recovery in os-1, which does not exhibit K⁺ influx and must rely upon an alternative pathway.
motic, a net increase of 155 mosmol kg
mycelia were preincubated in 15 ml BS and then treated by adding
0- to 60-min time courses.
slightly (ranging from 1.11- to 2.41-fold), suggesting that an alternative
expression decreased ( 1
expression in the wild type and the os
modest increase in expression in the os
between the wild type and the os
(Lewis et al. 2015), the wild type (and the osmotic
and
-1
-2
mutant strains. The
osmitic treatment, gene expression of os
and os
, as well as that of beta-tubulin, exhibited a modest increase in expression in the os
-1 and os
mutants but not in the wild type (Fig. 6).

**DISCUSSION**

Walled cells such as fungi normally maintain a high internal hydrostatic pressure that can be used to drive cell expansion (15). Direct turgor measurements reveal a significant difference between the wild type and the os
-1 and os
-2 mutants that lack a complete MAP kinase cascade pathway. The wild-type turgor measurements, 496 ± 87 kPa (n = 26), are very similar to turgors measured by Lew et al. (15): 476 ± 124 kPa (n = 65).

In the two osmotic mutants, turgor is significantly lower, and os
-1 turgor is significantly lower than the turgor of os
-2 (Fig. 1). Even though the os
-1 and os
-2 mutants are unable to grow at high osmolarity, they are still able to regulate turgor. Therefore, in the absence of a functional MAP kinase cascade, the turgor poise is lower and may be insufficient to maintain turgor when the mutants are subjected to high external osmolarity. The results are consistent with a MAP kinase cascade regulating turgor, but other signal transduction systems must also contribute to turgor regulation. A similar conclusion was reached by Furukawa et al. (6) in an analysis of the *Aspergillus nidulans* HOG pathway. In *N. crassa*, in addition to glycerol production as in yeast (3, 5), the MAP kinase cascade regulates turgor (Fig. 2) by activating ion transport, based on our electrical (Fig. 3, 4) and ion flux measurements (Fig. 5).

As reported previously (15), the wild type (and the osmotic mutant cut, which is unrelated to the MAP kinase cascade) exhibits a transient depolarization followed by a sustained hyperpolarization. The transient depolarization was observed in both os mutants, but the hyperpolarization was not (Fig. 3). Unlike os
-1, os
-2 exhibited an intermediate return to the initial potential, an intermediate response similar to its intermediate turgor magnitude in BS. It is possible that there are multiple pathways after the os
-1 step. That is, MAPKK and MAPK kinases may activate other targets besides MAP kinase (the OS-2 protein). This would explain the intermediate response of the os
-2 mutant between those of the wild type and the os
-1 mutant. On the basis of ion flux measurements, the transient depolarization observed in the wild type and the os mutants can be attributed to Ca
2
+ influx into the cell (Fig. 5). Stretch-activated Ca
2
+-permeable channels have been identified in *N. crassa* (10), although one would expect them to be activated by a hyposmotic shock that would swell the hypha rather than a hyperosmotic shock that causes cell shrinkage unless they are mechano-sensitive, responding to either tensile or compressive forces on the membrane. Other Ca
2
+ channels have not been characterized for *N. crassa*, although genomic analysis does identify a number of putative Ca
2
+ channels (30). *N. crassa* has homologs of the yeast Cch1p, Mid1p, and Yvc1p Ca
2
+-permeable channels (30) and two IP
3
-activated Ca
2
+ channels (25). Thus, the cause of the hyperosmotically induced transient Ca
2
+ influx is not known, but it is probably due to a Ca
2
+ channel in the plasma membrane.

The hyperpolarization implicates the H
+ -ATPase. This is corroborated directly by the hyperosmotically induced H
+ influx, which has a time course very similar to the hyperpolarization (Fig. 2, 5). Inhibiting protein synthesis does not affect the hyperpolarization, so the H
+ -ATPase is activated directly, probably via phosphorylation. Elevated cytoplasmic Ca
2
+ is also reported to cause hyperpolarization in *N. crassa* by activating the H
+ -ATPase (11), but Ca
2
+ influx cannot be implicated in the electrical response to hyperosmotic shock because the os
-1 has a transient Ca
2
+ influx but does not hyperpolarize.

Turgor recovery can be explained completely by the changes in net ion flux in the wild type: a MAP kinase cascade mediates turgor regulation by regulating ion fluxes, including the activity of the plasma membrane H
+ -ATPase and K
+ uptake. The fact that turgor recovery is also observed in the two os mutants, although they maintain a lower turgor than the wild type, is a clear indication that other signaling pathways are present, acting in concert to maintain turgor during cellular growth. This conclusion is corroborated by the fact that os
-1 and os
-2 gene expression is stimulated by osmotic treatment only in the os
-1 and os
-2 mutants, consistent with regulation by alternative osmoreponse pathway(s) (Fig. 6). The absence of osmotically induced expression of os
-1 and os
-2 was noted by Youssar et al. (29). Expression of cut, which encodes a member of the halocid dehydrogenase family that may function as a phosphatase, is induced by hyperosmotic treatment (29). Alternative osmoreponse pathway(s) may involve Ca
2
+, given the presence of transient Ca
2
+ influx in the wild type and the os
-1 mutant, but this possibility awaits further research.

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