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The unique characteristics of HOG pathway MAPKs in the extremely halotolerant Hortaea werneckii

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One sentence summary: Unique characteristics of the key MAP kinases of the HOG pathway help the fungus Hortaea werneckii to survive and proliferate in hyperosmolar environments, like solar salterns.

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ABSTRACT

HwHog1A/B, Hortaea werneckii homologues of the MAP kinase Hog1 from Saccharomyces cerevisiae, are vital for the extreme halotolerance of H. werneckii. In mesophilic S. cerevisiae, Hog1 is phosphorylated already at low osmolyte concentrations, and regulates expression of a similar set of genes independent of osmolyte type. To understand how HwHog1 kinases activity is regulated in H. werneckii, we studied HwHog1A/B activation in vivo, by following phosphorylation of HwHog1A/B in H. werneckii exposed to various osmolytes, and in vitro, by measuring kinase activities of recombinant HwHog1A, HwHog1B and Hog1/Delta1C. To this end, highly pure and soluble recombinant Hog1 homologues were isolated from insect cells. Our results demonstrate that HwHog1A/B are, in general, transiently phosphorylated in cells shocked with ≥3 M osmolyte, yet constitutive phosphorylation is observed at extreme NaCl and KCl concentrations. Importantly, phosphorylation profiles differ depending on the osmolyte type. Additionally, phosphorylated recombinant HwHog1A/B show lower specific kinase activities compared to Hog1/DeltaC. In summary, HOG pathway MAPKs in the extremely halotolerant H. werneckii show unique characteristics compared to S. cerevisiae homologues. The reported findings contribute to defining the key determinants of H. werneckii osmotolerance, which is important for its potential transfer to economically relevant microorganisms and crops.

Keywords: Hortaea werneckii; HOG pathway; MAP kinase; recombinant Hog1; kinase activity; osmotolerance

INTRODUCTION

The mitogen-activated protein kinase (MAPK) Hog1 from Saccharomyces cerevisiae (Saito and Posas 2012) and its homologues from fungi (Turk and Plemenitaš 2002; Bahn et al. 2005; Krantz, Becit and Hohmann 2006) to human (MAPK p38; Han et al. 1994) are highly conserved for sequence, domain structure and pattern of activation. In the unphosphorylated/ inactive p38, the N-terminal and C-terminal domains are misaligned, and the active site is blocked by the surface loop (TxY motif; Wilson et al. 1996). For maximum enzymatic activity, the TxY motif of the MAPKs must be phosphorylated by the upstream MAPK kinase (MAPKK; Pbs2 in S. cerevisiae) on threonine (Thr), to stabilize the active catalytic conformation, and on tyrosine (Tyr), to further amplify the basal kinase activity (Bell and Engelberg 2003). In S. cerevisiae, the MAPK cascade is part of the high osmolarity glycerol (HOG)
signalling pathway that responds to changes in extracellular osmolarity (Saito and Posas 2012; Fig. 1A). Activated p38 has intrinsic ATCase activity, which is coupled to its kinase activity in the presence of p38 protein substrates (Chen et al. 2000). In the absence of its substrates, purified hyperactive mutants of p38 can undergo autophosphorylation (Bell et al. 2001), as do mammalian MAPKs and the C-terminal-deleted Hog1 when they are expressed in un-stressed S. cerevisiae with deleted Pbs2 (Levin-Salomon et al. 2009; Maayan et al. 2012). The p38 kinase activity is inhibited by the pyridyl imidazole inhibitor SB203580, which competes with ATP for binding and thereby inhibits p38 ATCase activity (Cuenda et al. 1995; Chen et al. 2000). Similarly, Hog1 kinase activity is inhibited by SB203580 and the triazole inhibitor BPTIP in vitro, and the latter also inhibits Hog1 in vivo (Diner et al. 2011; Veide Vilg et al. 2014).

We have used BPTIP in vivo to study the role of the Hog1 kinase homologues, HwHog1A and HwHog1B (HwHog1A/B), in the extremely halotolerant Hortaea werneckii (Kejžár et al. 2015). H. werneckii was isolated from eutrophic solar salterns, and can withstand and adapt to life in an environment with up to almost saturated NaCl solution (5.5 M), while also thriving in environments without NaCl (Gostincár et al. 2011). Kinases HwHog1A/B can both rescue the osmosensitive phenotype of the S. cerevisiae hog1Δ strain, due to their high amino-acid-sequence conservation (Turk and Plemenitaš 2002; Lenassi et al. 2007; Kejžár et al. 2015). In H. werneckii, the activities of HwHog1A/B are regulated by proteins homologous to the components in the S. cerevisiae HOG pathway and by additional unique histidine kinases (Fig. 1A; Gostincár et al. 2011; Kejžár et al. 2015). In contrast to Hog1, which is phosphorylated already at low osmolyte concentrations (Saito and Posas 2012), HwHog1A/B are fully active only at salinities ≥ 3.0 M NaCl (Turk and Plemenitaš 2002). Besides post-translational regulation by phosphorylation, levels of HwHOG1A/B transcripts are also regulated by NaCl, but not sorbitol fluctuations (Lenassi et al. 2007; Kejžár et al. 2015). Active HwHog1A/B are translocated into the nucleus, where they regulate expression of a different set of genes in response to NaCl, compared to sorbitol (Vaupotić and Plemenitaš 2007; Vaupotić et al. 2008). Inhibition of the HwHog1A/B kinases activity with BPTIP restricts H. werneckii colony growth at ≥ 3 M NaCl, KCl or sorbitol, most likely due to inhibition or delay in cell division, but has no effect at lower osmolyte concentrations (Kejžár et al. 2015). The HwHog1 kinase also is important for induction of gene transcription with high NaCl, for regulation of specific genes with high sorbitol, and has no role in gene regulation in KCl stressed cells (Kejžár et al. 2015).

To understand how HwHog1A/B kinases activity is regulated in extremely halotolerant H. werneckii and how this differs from mesophilic fungi, we studied HwHog1A/B activation in vivo, by following phosphorylation of HwHog1A/B in H. werneckii exposed to various osmolytes, and in vitro, by measuring kinase activities of recombinant HwHog1A, HwHog1B and Hog1ΔC. This will help us to define the key determinants of H. werneckii osmotolerance for its potential transfer to economically important microorganisms and crops (Gašparič et al. 2013).

**MATERIALS AND METHODS**

**Protein expression and purification**

Hortaea werneckii HwHog1A/B and C-terminally truncated S. cerevisiae Hog1 (Hog1ΔC; Fig. 2A) were expressed in S99 insect cells using the ‘Bac-to-Bac’ baculovirus expression system (Life Technologies), as described previously (Wilson et al. 1996). The coding sequences of HwHOG1A (GenBank, AF516914.2), HwHOG1B (GenBank, KJ841888.1) and HOG1 (1-1095 nucleotides; NCBI, NM001182000) were C-terminally tagged with polyhistidine (His-tag) and cloned into the pFastBac1 plasmid. The recombinant expression bacmids were generated by site-specific transposition of the plasmids into Escherichia coli DH10Bac cells, which were later transfected into the insect cells using the Cellfectine II reagent, to generate high titres of pure recombinant baculovirus particles.

For purification of His-tagged Hog1 homologues, the insect cells were pelleted by centrifugation at 6000 × g for 15 min, resuspended in lysis buffer (30 mM HEPES, 500 mM NaCl, 5 mM MgCl₂, 10 mM imidazol, 10% glycerol, 0.5% Tween 20, 10 mM β-mercaptoethanol, pH 7.5) and lysed by three cycles of freezing and thawing, and sonication. After centrifugation at 40 000 × g for 45 min, the clear supernatants contained the recombinant proteins, which were applied to HisTrap HP nickel-chelating columns, and the eluted protein fractions were additionally purified on Superdex 200 HiLoad 16/60 gel-filtration columns (both GE Healthcare). Fractions containing more than 95% of the pure HwHog1A, HwHog1B and Hog1ΔC proteins (estimated by SDS-PAGE) were pooled, concentrated in kinase buffer (30 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, pH 7.5) and stored at ~70°C.

The GST-tagged constitutively active Pbs2 mutant (Pbs2EE) was expressed in E. coli from the pGEX-4T1 plasmid (Amerham), and later purified using glutathione Sepharose 4B (GE Life Sciences), with the elution buffer of 10 mM glutathione, 30 mM Tris, 0.2 M NaCl (pH 8.0), as described previously (Posas et al. 1996). The pGEX-PBS2EE plasmid was kindly provided by Prof. Markus J. Tamás.

**Immunoblotting**

For HwHog1A/B phosphorylation analysis, total proteins were extracted from H. werneckii cells (strain EXF 225), exposed (0 min, 15 min, 30 min and 24 h) or adapted (several days) to the indicated concentrations of NaCl, KCl or sorbitol, as described previously (Kejžár et al. 2015). Thirty micrograms of extracted protein per sample was separated by SDS-PAGE, blotted onto PVDF membranes, and probed for immunodetection of total HwHog1A/B using an anti-Hog1 antibody (y-215; Santa Cruz Biotechnology), and of the dually phosphorylated HwHog1A/B using anti-phospho-p38 antibodies (Cell Signalling Technology), combined with HRP-conjugated anti-rabbit IgG antibodies.

To determine the identity and phosphorylation status, 1.0 μg or 20 ng recombinant His-tagged HwHog1A, HwHog1B and Hog1ΔC were separated, blotted and processed for immunodetection using anti-His (ab9108; Abcam), anti-Hog1 or anti-phospho-p38 antibodies, as above. For monitoring the purity, 1.0 μg of the recombinant proteins was separated by SDS-PAGE and revealed by Coomassie Blue staining.

**In vitro kinase assay**

Kinase activities were determined by continuously monitoring the fluorescence (590 nm, every 10 min) generated by the released ADP, when detected with the ADP QuantiFast assay (Discov-eRx), on a Synergy 2 plate reader (BioTek). These assays were performed with ultralow-binding, 384-well black plates (Greiner), using purified Pbs2EE or the activated Hog1 homologues as the enzymes, and the Hog1 homologues or myelin basic protein (MBP) as substrates, respectively. The kinase reaction was as
Figure 1. HwHog1A/B phosphorylation profiles are dependent on osmolyte concentration and type in H. werneckii. (a) Schematic representation of the H. werneckii HOG pathway. In H. werneckii, changes in the environmental osmolarity are detected by the HwSho1A/B or putative histidine kinase osmosensors, and the signal is transferred intracellularly to the MAPKKKs HwSte11A/B and HwSsk2A/B, which activate the MAPKKs HwPbs2A/B1/B2, which are responsible for phosphorylation of the MAPKs HwHog1A/B. Activated HwHog1A/B regulate the activities of cellular and nuclear targets, and mediate osmoadaptation. White text, experimentally characterized proteins; black text, in silico identified proteins. Putative orthologues of the MAPK module (i.e. MAPKK kinase [MAPKKK], MAPK kinase [MAPKK], MAPK) are red; of the histidine kinases are green, and of the MAPK phosphatases are orange. The phosphorelay module is indicated by the lightgreen background. (b–d) HwHog1A/B phosphorylation in H. werneckii exposed to the indicated concentrations of NaCl (b), KCl (c) and sorbitol (d). Exponentially growing H. werneckii cells were harvested and resuspended in YNB supplemented with either 1.8, 3.0 or 4.5 M NaCl or KCl or 1.0, 2.0 or 3.0 M sorbitol, and aliquots were collected after the indicated times (NaCl/KCl/sorbitol shock). Alternatively, H. werneckii cells growing in YNB supplemented with the above osmolyte concentrations were collected in exponential phase (NaCl/KCl/sorbitol adaptation). The total protein extracts were analysed for HwHog1 dual phosphorylation with anti-phospho-p38 antibodies (p-HwHog1) and for total HwHog1A/B amounts with anti-Hog1 (y-215) antibodies (HwHog1). Experiments were carried out as three repetitions and representative results are shown.
Figure 2. Purification of recombinant HwHog1A, HwHog1B and S. cerevisiae Hog1/Δ1C expressed in Sf9 insect cells. (a) Schematic representation of kinases HwHog1A, HwHog1B and Hog1. Yellow box, domain important for biological activity (A, active site aspartate; TGY, phosphorylation motif threonine-glycine-tyrosine); blue box, seven-amino-acid domain essential for Pbs2-independent autoactivation of Hog1 in S. cerevisiae (Maayan et al. 2012); blue arrow, C-terminal tail of Hog1, truncated in the recombinant kinase (Hog1/Δ1C). The amino-acid alignments of the conserved regions that mediate the MAPK interactions with the upstream MAPKK Pbs2 (Murakami et al. 2008) are enlarged. Asterisk, a conserved amino-acid residue among homologues. (b) Highly purified recombinant HwHog1A, HwHog1B and Hog1/Δ1C produced in Sf9 insect cells. One microgram of each MAPK sample was separated by SDS-PAGE and visualized by Coomassie Blue staining. M, protein molecular weight standards. (c) Immunoblotting of recombinant HwHog1A, HwHog1B and Hog1/Δ1C. One microgram of each MAPK was immunoblotted with antibodies against the Histag (anti-His), against the 215- amino-acid region of Hog1 (anti-Hog1), and against phosphorylated Thr and Tyr in the TxY motif (anti-phospho(p38)).

RESULTS AND DISCUSSION

The HwHog1A/B phosphorylation profiles are dependent on osmolyte concentration and type in H. werneckii

To understand how HwHog1 kinases activity is regulated in extremely halotolerant H. werneckii, we systematically monitored the phosphorylation status of HwHog1A/B in H. werneckii exposed to different concentrations and types of osmolytes, using immunoblotting (Fig. 1B–D). The HwHog1A/B phosphorylation is dependent on osmolyte concentration, as only low basal phosphorylation of HwHog1A/B were observed when cells were shocked with ≥3 M osmolytes. When
comparing HwHog1A/B phosphorylation in cells stressed with 1.8 M NaCl or KCl with that of 3.0 M sorbitol, or 4.5 M NaCl with that of 4.5 M KCl, it seems the HwHog1A/B phosphorylation profiles are also dependent on osmolyte type. This is supported by the osmolyte-type-specific HwHog1A/B-regulated transcription of a selected group of genes (HwSTL1, HwGUT2, HwOP3, HwGDI1, HwUGP1, HwGPD1; Vaupotič and Plemenitaš 2007; Vaupotič et al. 2008; Kejžar et al. 2015), but should be further analysed on the transcriptome level. In S. cerevisiae, Hog1 phosphorylation is observed already at low osmolyte concentrations (Saito and Posas 2012), and gene induction as a result of NaCl stress (Posas et al. 2000; Rep et al. 2000; Yale and Bohnert 2001) agrees with the observed sorbitol and KCl responses (O’Rourke and Herskowitz 2004).

HwHog1A/B phosphorylation was generally transient, yet constitutive phosphorylation was observed in H. werneckii shocked with or adapted to 4.5 M NaCl, and in cells adapted to 4.5 M KCl (Fig. 1B–D), which suggested important roles for phosphorylated HwHog1A/B in the adaptive mechanisms of H. werneckii to extreme osmolarities. Importantly, H. werneckii cells were viable despite constitutive phosphorylation of HwHog1A/B, similar to what has been observed for Cryptococcus neoformans serotype A cells (Bahn et al. 2005), whereas this has been shown to be lethal for S. cerevisiae (Wurgler-Murphy et al. 1997).

Taken together, these data show that HwHog1A/B phosphorylation is dependent on osmolyte concentration and type, which implies that HOG pathway in H. werneckii senses the osmolyte type, possibly by the unique putative histidine kinases (Fig. 1A), or that the negative regulation of HwHog1A/B by phosphatases (Fig. 1A) is an osmolyte-type dependent process.

Purification of recombinant HwHog1A, HwHog1B and Hog1ΔC expressed in insect cells

Next, we characterized the HwHog1A/B kinase activities in vitro, and compared these to that of Hog1. To this end, we cloned full-length HwHog1A and HwHog1B, and a C-terminally truncated S. cerevisiae Hog1 (Hog1ΔC; Fig. 2) into a baculovirus shuttle vector for expression in the Sf9 insect cells, a system previously used for expression of p38 (Wilson et al. 1996). This enabled us to produce milligram quantities of fungal recombinant kinases, production of which was unsuccessful (data not shown) when using the previously reported E. coli expression system (Posas et al. 1996; Diner et al. 2011). For Hog1ΔC, the sequence after alanine 365 was removed to increase protein solubility, but the domains responsible for its biological activity remained intact (Maayan et al. 2012; Fig. 2A). The high purity of the His-tagged MAPKs was confirmed by the Coomassie staining after separation on SDS-PAGE (Fig. 2B), which showed mostly bands of the expected protein sizes (around 42 kDa). The identities of recombinant proteins were confirmed by immunoblotting using antibodies against the His-tag or against the C-terminal part of Hog1 (Fig. 2C). The latter antibody binds to HwHog1 orthologues specifically (Fig. 1B), although much less efficiently compared to Hog1, due to the differences in the amino-acid sequences. Using antibodies against the phosphorylated (Thr180/Tyr182) p38 that also recognize homologous MAPKs, we showed that HwHog1A, HwHog1B and Hog1ΔC are not dually phosphorylated in insect cells (Fig. 2C), despite some reports that have shown spontaneous phosphorylation when the MAPKs are expressed heterologously (Friedmann et al. 2006; Levin-Salomon et al. 2009).

Altogether, we successfully produced highly pure and soluble recombinant HwHog1A, HwHog1B and Hog1ΔC.

MAPKs HwHog1A, HwHog1B and Hog1ΔC are phosphorylated on Thr and Tyr by the MAPKK Pbs2EE in vitro

In S. cerevisiae, the MAPK Hog1 is phosphorylated/activated by the HOG pathway-activated MAPKK Pbs2 (Bell and Engelberg 2003), whereas in vitro, Hog1 phosphorylation is catalysed by a constitutively active Pbs2 mutant (Pbs2EE; Veide Vilg et al. 2014). We expressed and purified the GST-tagged Pbs2EE (Posas et al. 1996) and used it to phosphorylate HwHog1A, HwHog1B and Hog1ΔC in vitro. HwHog1A/B have 79% identity of amino-acid sequences with Hog1ΔC, with high conservation of the common-docking domain and the Pbs2-binding domain (Fig. 2A), which are important for interactions with the upstream MAPKs (Murakami, Tatebayashi and Saito 2008). HwHog1A/B both complement the osmosensitivity of the S. cerevisiae strain with deleted Hog1 (Lenassi et al. 2007; Kejžar et al. 2015) which indicated that they can interact with and be activated by the S. cerevisiae MAPKK Pbs2.

To optimize the phosphorylation of the recombinant MAPKs (Fig. 3A), we incubated 0.5 or 1.0 μg Pbs2EE with 1.0 μg HwHog1A, HwHog1B or Hog1ΔC in the presence of ATP, and monitored phosphorylation by measuring ADP production through increase in fluorescence signal (Fig. 3B–D) and with immunoblotting (Fig. 3E–G). During the 2-h incubation, there were continuous increases in the fluorescence for the HwHog1A and HwHog1B reaction mixtures, with a steeper slope for 1.0 μg Pbs2EE (Fig. 3B and C). These data corresponded to the signals for the dually phosphorylated HwHog1A and HwHog1B that were detected with antibodies against phospho-p38 (Fig. 3E and F). The activation of Hog1ΔC with 0.5 μg Pbs2EE was approximately 4-fold more efficient than that for HwHog1A/B, as determined by comparing the ADP-dependent fluorescence signals at the 2-h time point (Fig. 3B–D), and intensities of the phospho-MAPK bands at the 1-h time point (Fig. 3E–G). This was as expected, as Hog1 is the natural substrate for Pbs2 (Saito and Posas 2012). The phosphorylation of these recombinant MAPKs is a Pbs2EE-mediated process, as without Pbs2EE, only very low fluorescence (Fig. 3B–D) and no signals after immunodetection (Fig. 3E–G) were observed after 2 h for the respective reaction mixtures.

Still, after 24 h in the absence of Pbs2EE, Hog1ΔC autophosphorylates on Thr and Tyr as shown by immunodetection (Fig. 3G), supporting the previous data on importance of the Hog1 C-terminal region for the restriction of the basal kinase activity (Maayan et al. 2012). This C-terminal region is absent in filamentous fungi (Krantz, Becit and Hohmann 2006; Kejžar et al. 2015) and p38 (Han et al. 1994), which indicates an alternative mechanism of autophosphorylation inhibition (Levin-Salomon et al. 2009). Importantly, there appear to be little or no autophosphorylation activities of HwHog1A and HwHog1B, as only very weak bands were observed after 24 h (Fig. 3E and F).

In summary, HwHog1A/B and Hog1ΔC were efficiently phosphorylated on Thr and Tyr in the TxY motif by the upstream kinase Pbs2EE, with autophosphorylation contributing only minimally over the time-frame studied. To optimally activate these MAPKs in the following in vitro kinase assays, we added 1.0 μg Pbs2EE for an incubation of 1 h.

Dually phosphorylated HwHog1A, HwHog1B and Hog1ΔC are active MAP kinases, and are inhibited by BPTIP and SB203580

To monitor the kinase activities of the dually phosphorylated HwHog1A/B and Hog1ΔC on MBP (Fig. 4A), we measured the
Figure 3. The MAPKs HwHog1A, HwHog1B and S. cerevisiae Hog1ΔC are phosphorylated on Thr and Tyr by the MAPKK Pbs2EE in vitro. (a) Schematic representation of the in vitro Hog1 homologue activation. The MAPKK Pbs2EE catalyses the transfer of the γ-phosphate from ATP to its substrate MAPKs HwHog1A/B. The released ADP is transformed to a fluorescent compound resorufin fluor by a series of enzymatic reactions. (b–d) Measurements of the MAPKs activation using the fluorescence coupled kinase assay. Two microgram HwHog1A (b), HwHog1B (c) or 1 μg Hog1ΔC (d) was incubated without (autophos.) or with the indicated amounts of Pbs2EE in the presence of ATP, and the fluorescence was measured continuously for 2 h. Data are means (±SD) of three replicates from a representative experiment. Experiments were carried out as three repetitions. RFU, relative fluorescence units; *, limiting RFU of the test. (e–g) Measurements of the MAPKs activation using dual phosphorylation of TxY with immunoblotting. One microgram HwHog1A (e), HwHog1B (f) or Hog1ΔC (g) was incubated for 0.5, 1 or 2 h without (autophos.) or with the indicated amounts of Pbs2EE in the presence of ATP, and the levels of the dually phosphorylated MAPKs were determined with anti-phospho-p38 antibodies (p-HwHog1A; p-HwHog1B; p-Hog1ΔC) and the total MAPKs with anti-Hog1 antibodies (HwHog1A; HwHog1B; Hog1ΔC). Experiments were carried out as three repetitions and representative results are shown. Control 1, reaction mixture without added Pbs2EE, control 2, reaction mixture without added ATP.

release of ADP through increase in fluorescence signal. MBP is a general kinase substrate that has already been used for studies of the HwHog1 (Turk and Plemenitaš 2002) and Hog1 (Winkler et al. 2002) activities using radioactive in vitro kinase assays. HwHog1A and HwHog1B and Hog1ΔC recombinant MAPKs were all activated when dually phosphorylated by Pbs2EE, as the fluorescence of their reaction mixtures increased over time (Fig. 4B–D). The fluorescence signal was low in samples where only Pbs2EE and MBP were present, which showed that MBP is not a substrate for Pbs2EE (Fig. 4B–D). Importantly, the specific kinase activity of HwHog1A and HwHog1B was around 3 nmol (mg min)⁻¹, which is much lower compared to that of the Hog1ΔC (29.5 nmol (mg min)⁻¹). When also taking into account the efficiency of MAPKs phosphorylation by Pbs2EE (Fig. 3E–G), the Hog1ΔC kinase activity on MBP is still 2.5-fold greater than for HwHog1A and HwHog1B. This implies certain differences exist in intrinsic properties of HwHog1A/B and Hog1 kinases, which could affect the downstream kinase functions and thereby osmoadaptation of H. werneckii and S. cerevisiae.

Finally, we tested the inhibitor activity of the Hog1 inhibitor BPTIP (Diner et al. 2011; Veide Vilg et al. 2014; Kejžar et al. 2015) and the p38 inhibitor SB203580 (Cuenda et al. 1995) on these recombinant MAPKs (Fig. 4E–G). When the HwHog1A, HwHog1B and Hog1ΔC kinase activities on MBP were measured in the presence of BPTIP or SB203580, these decreased with increasing inhibitor concentrations, to approximately 40% remaining activity for HwHog1A, 30% for HwHog1B and 10% for Hog1ΔC at 10 μM BPTIP or SB203580 (Fig. 4E–G). Although BPTIP is triazole-based and SB203580 is pyridinyl imidazole-based, they both function as competitive inhibitors of the ATP binding (Cuenda et al. 1995; Diner et al. 2011), and showed similar inhibition of recombinant MAPK activities in our assay (Fig. 4E–G). According to Veide Vilg et al. (2014), the Hog1 activity on the substrate Sic1 decreased to 0 or 10% remaining activity with 1 μM BPTIP or SB203580, respectively; whereas in our assay Hog1 activity decreased to 20% in the presence of 1 μM inhibitors (Fig. 4G). This difference in inhibitor efficiencies might reflect the difference in the kinase assay read-outs. Veide Vilg et al. (2014) measured the transfer of the radioactive γ-phosphate to the Hog1 substrate, whereas
Figure 4. Dually phosphorylated HwHog1A, HwHog1B and S. cerevisiae Hog1 are active MAPKs and are inhibited by BPTIP and SB203580. (a) Schematic representation of the in vitro Hog1 homologue kinase reaction. The dually phosphorylated MAPKs HwHog1A and HwHog1B catalyse the transfer of the γ-phosphate from ATP to their substrate MBP. The released ADP is transformed to a fluorescent compound resorufin fluor by a series of enzymatic reactions. The Hog1 inhibitor BPTIP and the p38 inhibitor SB203580 are ATP analogues that prevent ATP binding to the MAPK active site. (b–d) Measurements of the MAPK activities using the fluorescence coupled kinase assay. The indicated amounts of pre-activated HwHog1A (HwHog1A/Pbs2EE) (b), HwHog1B (HwHog1B/Pbs2EE) (c), or Hog1ΔC (Hog1ΔC/Pbs2EE) (d) or non-activated Hog1 homologues HwHog1A (b), HwHog1B (c), or Hog1ΔC (d) were incubated with the substrate MBP in the presence of 100 μM ATP, and the fluorescence was measured continuously for 4 h. Hog1 homologues were absent in the Pbs2EE control reaction mixtures. RFU, relative fluorescence units; * limiting RFU of the test. Data are means (±SD) of three replicates from a representative experiment. Experiments were carried out as three repetitions. (e–g) Effects of the inhibitors BPTIP and SB203580 on the MAPK activities. Ten-fold serial dilutions of the inhibitors BPTIP and SB203580, or the inhibitor solvent DMSO (reference sample), were added to the kinase reaction mixtures prepared as described for (b–d). The data are presented as percentages of the remaining HwHog1A (e), HwHog1B (f) or Hog1ΔC (g) kinase activities after 2.5 h of incubation, according to the reference sample, the enzymatic activity of which was set at 100%. Data are means (±SD) of three replicates from a representative experiment. Experiments were carried out as three repetitions.

we measured the ATPase activity of Hog1 by detecting the ADP product (Fig. 4A). Additionally, they assayed the inhibitor activity on full-length Hog1 tagged with GST (Veide Vilg et al. 2014), whereas we used C-terminally truncated Hog1 tagged with His (Fig. 2A). Interestingly, although the HwHog1A/B activities were not completely abolished by 10 μM BPTIP or SB203580 in these in vitro kinase assays (Fig. 4E–G), BPTIP inhibited colony growth of H. werneckii exposed to ≥3 M NaCl, KCl or sorbitol (Kejžar et al. 2015), which suggests that the HwHog1A/B biological activity was suppressed. As certain Hog1 functions in S. cerevisiae are dependent on the phosphorylation status but independent of the kinase activity, like stress-induced translocation into the nucleus (Ferrigno et al. 1998) or stress-induced change in the nucleosome positioning mediated by physical interaction of Hog1 with the chromatin remodelling complex RSC (Mas et al. 2009), deletion of both HwHOG1A/B genes would be required to fully
understand the role of HwHog1A/B in H. werneckii. However, no genetic tools are yet available for preparation of gene knockouts in H. werneckii.

Altogether, the recombinant MAPKs are activated by dual phosphorylation mediated by Pbs2EE, with Hog1A/C showing a 2.5-fold higher specific kinase activity than HwHog1A or HwHog1B. The ATP analogues BTPIP and SB203580 both inhibit their kinase activities, but to different extents.

In conclusion, HOG pathway MAPKs in the extremely halotolerant H. werneckii show unique characteristics compared to S. cerevisiae homologues, as HwHog1A/B phosphorylation is dependent on osmolyte concentration and type, and the phosphorylated recombinant HwHog1A/B show lower specific kinase activities.

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