

Combination of Two Activating Mutations in One *HOG1* Gene Forms Hyperactive Enzymes That Induce Growth Arrest

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Mitogen-activated protein kinases (MAPKs) play key roles in differentiation, growth, proliferation, and apoptosis. Although MAPKs have been extensively studied, the precise function, specific substrates, and target genes of each MAPK are not known. These issues could be addressed by sole activation of a given MAPK, e.g., through the use of constitutively active MAPK enzymes. We have recently reported the isolation of eight hyperactive mutants of the *Saccharomyces cerevisiae* MAPK Hog1, each of which bears a distinct single point mutation. These mutants acquired high intrinsic catalytic activity but did not impose the full biological potential of the Hog1 pathway. Here we describe our attempt to obtain a MAPK that is more active than the previous mutants both catalytically and biologically. We combined two different activating point mutations in the same gene and found that two of the resulting double mutants acquired unusual properties. These alleles, *HOG1*^{D170A,F318L} and *HOG1*^{D170A,F318S}, induced a severe growth inhibition and had to be studied through an inducible expression system. This growth inhibition correlated with very high spontaneous (in the absence of any stimulation) catalytic activity and strong induction of Hog1 target genes. Furthermore, analysis of the phosphorylation status of these active alleles shows that their acquired intrinsic activity is independent of either phospho-Thr174 or phospho-Tyr176. Through fluorescence-activated cell sorting analysis, we show that the effect on cell growth inhibition is not a result of cell death. This study provides the first example of a MAPK that is intrinsically activated by mutations and induces a strong biological effect.

Mitogen-activated protein kinase (MAPK) signaling pathways play important roles in differentiation, growth, proliferation, and apoptosis. The MAPK module has been highly conserved throughout evolution and is present in all eukaryotic cells (20, 26, 38, 48, 54). It consists of three kinase proteins: a MAPK kinase kinase (MAPKKK) that activates MAPK kinase (MAPKK), which in turn activates the relevant MAPK by phosphorylating it on both Thr and Tyr residues in an appropriate Thr-X-Tyr motif (7, 17, 27). Phosphorylated activated MAPKs translocate from the cytoplasm into the nucleus, where many of their substrates reside. However, they phosphorylate cytoplasmatic and membrane proteins as well (26, 27).

In mammalian cells, three major families of MAPKs have been identified, the *ERK*, *JNK*, and *p38* families (24, 31, 45). Each family, as well as each member within the family, responds differently to various activating conditions (14, 15, 24, 30, 35). As many MAPKs are activated in response to various external signals, albeit to different degrees, it is rather difficult to study the precise role of a given MAPK.

A direct way to reveal the biochemical and biological activities of a given MAPK would be selective activation of that molecule in vivo. This approach could be applied through expression of constitutively active forms of the MAPK of interest. Strategies to produce such molecules included construction of MAPKK-MAPK fusion genes (46, 56) and a variety of

genetic approaches (3, 5, 6, 21). The MAPKK-MAPK fusion proteins were indeed found to be active, but the genetic screens usually provided MAPK molecules carrying point mutations that manifested only residual activities. A recent study showed that combining some of these mutations into one MAPK protein (ERK2 was used) resulted in a synergism that increased the catalytic activity (16).

Recently, the isolation of eight different hyperactive mutants of the *Saccharomyces cerevisiae* MAPK Hog1 was reported (1). Hog1 is a yeast homolog of the mammalian MAPKs *p38* and *JNK* (18, 22). It is phosphorylated and activated by the MAPKK Pbs2 (4). The Pbs2/Hog1 cascade is activated under osmotic stress and is essential for survival under these conditions (4). The active Hog1 mutants were isolated in a screen planned to identify *HOG1* alleles that allow *pbs2Δ* cells to survive under osmotic stress. Each of these mutants harbors a distinct single point mutation and was shown to possess intrinsic catalytic activity that is independent of dual phosphorylation. Most importantly, a human *p38* protein carrying equivalent mutations also manifested an increased catalytic activity that was independent of upstream regulation (1).

Biochemical analysis of the eight active Hog1 mutants (e.g., level of activity, responsiveness to salt, and phosphorylation level) revealed distinct properties suggesting that the different alleles may be activated through different mechanisms (1).

Although very active, the alleles carrying the single activating mutations do not seem to possess the maximal activity possible. This notion is based on previous observations that hyperactivation of the *HOG1* pathway is lethal to yeast cells (28, 29, 40). As the active Hog1 mutants did not impose lethality, it may be that they do not fully activate Hog1 down-

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stream targets and are therefore not an optimal research tool. In order to develop Hog1 enzymes that fully potentiate the *HOG1* cascade, we combined different activating mutations in the same gene, thereby potentially combining two activating mechanisms. We hoped for an additive if not a synergistic effect. A battery of dually mutated Hog1 molecules was prepared. Two of these double-mutant Hog1 enzymes acquired extremely high catalytic activity, induced elevated expression levels of Hog1 target genes, and imposed a severe growth arrest on yeast cells.

MATERIALS AND METHODS

Construction of six double mutants containing the N-terminal Y68H mutation. A HOG1 5' primer, bearing the Y68H mutation and the most 5' *PstI* site of the *HOG1* gene, and a 3' primer termed BamHI3' HOG1 (see primer sequences below), were used to amplify *HOG1* genes in a series of PCRs. The templates used were *HOG1* clones each bearing a different activating mutation of the six C-terminal mutations (1). The resulting PCR products were fragments containing both Y68H and one of the C-terminal mutations. These were digested with *PstI*, and the fragments obtained (still retaining both mutations) were ligated into a Bluescript SK⁺-HA-*HOG1* plasmid, which was also digested with *PstI*. In turn, each of the resulting HA-*HOG1* double mutants were digested from the SK⁺ plasmid by using *NotI* and *HindIII* and ligated into the PES86⁺ yeast expression vector, which was digested with the same enzymes. PES86⁺ is a derivative of pADNS (13), in which the *BamHI* fragment of pADNS was ligated (following a fill-in Klenow reaction) to the large *PvuII* fragment of pRS426 (11).

Primer sequences used in the PCRs. The following primer sequences were used in the PCRs: 5' HA-*HOG1*: 5'-TCC ACT GCA GTG CTG GCC AAA AGG ACA CAT CGT GAA C-3'; BamHI 3' *HOG1*: 5'-CTA GCC GGA TCC TTA CTG TTG GAA CTC ATT AGC-3'.

Construction of six double mutants containing the N-terminal D170A mutation. The plasmid PES86⁺-HA-*HOG1*^{D170A} (1) was digested with *NotI* and *SalI*, resulting in a backbone bearing the vector fused to the N-terminal half of the *HOG1* gene containing the D170A mutation. The same double digestion was applied to each of the six C-terminal mutants providing six fragments, each harboring a different C-terminal mutation. Each of these fragments was ligated to the PES86⁺-HA-*HOG1*^{D170A} digested with *NotI* and *SalI*.

All twelve dually mutated clones were sequenced to verify the existence of both point mutations and the absence of any other mutation.

Construction of *MET3*-inducible double mutants. *MET3*-inducible vectors were constructed by inserting each of the 12 dually mutated *HOG1* genes, as *HindIII*+*NotI* fragments removed from the PES86⁺-based plasmids, into the Met 425 plasmid, bearing the *MET3* promoter digested with the same enzymes. The plasmid used is a derivative of the p425 *MET25* plasmid in which the *MET25* promoter was replaced with the *MET3* promoter (34). The resulting expression cassettes *MET3*-*HOG1* were transferred as *KpnI*+*SacI* fragments, to pRS426 plasmids digested with the same enzymes.

To construct plasmids bearing the various *HOG1* alleles mutated in one of the phosphoacceptors, we used Bluescript plasmids carrying *HOG1*^{wt} or *HOG1*^{D170A} into which either the T174A or Y176F mutation was previously inserted (information available upon request). These vectors were digested by *HindIII* and *SalI*, resulting in an insert comprising the N terminus of the *HOG1* gene mutated in one of the phosphoacceptors alone or jointly with the proximal D170A mutation. This insert was then ligated into the relevant pRS426-*MET3*-*HOG1* backbones (described above) identically digested.

Construction of integrative *HOG1* alleles. For construction of single-copy integrative plasmids harboring *HOG1* alleles under the control of the endogenous *HOG1* promoter (490 bp), the various HA-*HOG1* fragments were digested out of pBs15 vectors by using *BamHI* and *ClaI* and ligated into the pRS306 vector digested identically. The construction of pBs15 was performed as follows. In order to introduce a *HindIII* restriction site to the 3' end of the *HOG1* promoter, the *HOG1* gene was amplified in a PCR with the following primers: forward primer (Fwd), 5'-CCA TCG ATT GAA GGA AAT AAG AGG-3'; and reverse primer (Rev), 5'-GGC CCA AGC TTT ATT ATA TAC GAT AGT TGT AGT TTT-3'. This PCR fragment was digested with *ClaI* and *HindIII* and ligated into a Bluescript vector that harbors the coding sequence of HA-*HOG1*. This vector was termed pBs14. Next, pBs14 was cut with *ClaI* and *AatII* and ligated into pRS426-*HOG1*, which was digested with the same restriction enzymes. This new vector, which harbors a hemagglutinin (HA)-tagged full-length *HOG1* gene (including promoter and terminator), was termed pRS1. pBs15 was

subsequently constructed by digesting pRS1 with *BamHI* and *ClaI* and ligating the *HOG1* fragment into Bluescript.

Yeast strains and media. The *S. cerevisiae* strains used were the *pbs2Δ* strain MAY1 (*MATa ura3-52 lys2-80Iamber ade2-10Iochre leu2-Δ1 his3-Δ200 pbs2-Δ2::LEU2*) and the *hog1Δ* strain JBY13 (*MATa leu2 ura3 his3 trp1 ade2 lys2 hog1::TRP1*). Both strains were obtained from M. C. Gustin, Rice University. SP1*hog1Δ*, SP1*pbs2Δ*, and SP1*hog1Δpbs2Δ* strains were produced by disrupting the relevant genes in the SP1 strain (13) (*MATa his3 leu2 ura3 trp1 ade8 can'*). A MAY1 strain expressing the kinase dead form of *pbs2* (*PBS2*^{KD}) was created by integrating a pRS303 plasmid bearing the *PBS2*^{K389M} allele into the *HIS3* locus of the genome. The same plasmid was also integrated into the SP1*Δpbs2* strains already carrying the various integrated *HOG1* alleles (as explained above).

Cultures were maintained on YPD (1% yeast extract, 2% Bacto Peptone, 2% glucose), or on the synthetic medium YNB-URA or YNB-LEU [0.17% yeast nitrogen base without amino acids and NH₄(SO₄)₂, 0.5% ammonium sulfate, 2% glucose, and 40 mg of adenine, histidine, tryptophan, lysine, and uracil or leucine per liter, and 160 mg of methionine/liter for noninducing conditions (or without methionine for induction)]. To induce Hog1 expression in liquid media, cultures were grown to logarithmic phase (optical density at 600 nm [OD₆₀₀] of 0.5 to 1.0) at 30°C. Then, cells were split in half and collected by centrifugation followed by resuspension of one half in the same media and the other half in media lacking methionine. Cultures were further grown in media lacking methionine for 2 h.

Transformation and dot plating. DNA was introduced into yeast cells according to the method of Schiestel and Gietz (50). Cells were plated on YNB-URA or YNB-LEU supplemented with 160 mg of methionine per liter. pRS306-based plasmids (single-copy vectors) were linearized with *StuI* prior to transformation to allow integration into the *URA3* locus.

To test the ability of the transformants to grow when Hog1 expression was induced, yeast cultures were grown in noninducing media, washed with sterile double-distilled water (DDW), and resuspended in methionine-free media. The culture was then sequentially diluted and plated onto plates containing or lacking methionine. Cells in which the *HOG1* genes were integrated into the genome were grown in liquid YPD, serially diluted, and plated on YPD plates supplemented with high NaCl concentrations (0.7, 0.8, and 0.9 M).

Preparation of native and denatured cell lysates and Western blotting. Cell cultures were grown to an OD₆₀₀ of 0.5 to 1.0 in media supplemented with 160 mg of methionine per liter. Cultures were collected, split, and resuspended in media containing or lacking methionine. In assays for which salt induction is also indicated, cells were exposed to 1 M NaCl for 10 min 1 h after methionine removal. For lysates, preparation cells were centrifuged at 4°C and the pellet was washed with 30 ml of ice-cold DDW. The pellet was resuspended in 1 to 2 volumes of ice-cold lysis buffer (50 mM Tris-HCl [pH 7.4], 0.25 M NaCl, 0.1% NP-40, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 10 μg of trypsin inhibitor per ml, 10 μg of pepstatin A per ml, 250 μg of benzamide per ml, 1 mM sodium vanadate, 10 mM NaF, 1 mM p-nitro phenyl phosphate [Sigma], 10 mM β-glycerol-P). Six hundred milligrams of glass beads was added and 8 × 1' vortexing was applied. Samples were centrifuged at 800 × g for 5 min, and the supernatant was centrifuged again at 15,000 × g for 15 min at 4°C. Supernatants were aliquoted in small volumes (~200 μl) and frozen immediately in liquid nitrogen. Denatured cell lysates were prepared by using trichloroacetic acid precipitation as described previously (1).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and enhanced chemiluminescence reaction for identification of the HA-Hog1 protein and the phosphorylated Hog1 were performed as described by Sambrook et al. (47) and by Bell et al. (1). The antibody for the HA tag, 12CA5, is a monoclonal antibody derived from mouse. A dilution of 1/1,000 was used. For identification of the dually phosphorylated Hog1, commercial rabbit anti-P-p38 antibody (New England Biolabs) was used (diluted 1/2,500). For identification of threonine or tyrosine phosphorylation, rabbit antiphosphothreonine (Zymed) diluted 1/1,000 or mouse antiphosphotyrosine (4G10) diluted 1/100 was used. All secondary antibodies were diluted 1/10,000.

Kinase assay and quantification. In vitro kinase assays were performed as previously described (1), except that following SDS-PAGE proteins were transferred to nitrocellulose by use of a semidry blotter. The kinase assay blots were quantified by using a FUJIFILM FLA-3000 phosphoimager through Image Gauge V3.45 program. Normalization to protein levels was done by NIH Image 1.62 software.

Northern blot analysis. Total RNA was isolated at the time points indicated in the figure and separated by electrophoresis as described previously (47). Blots were hybridized in a buffer containing 7% SDS, 0.5 M sodium phosphate buffer [pH 7.2], and 1 mM EDTA. The signal was quantified using a phosphoimager (Bio-Rad Molecular Imager FX). The primers used for generating the PCR fragments, which were used as probes, were as follows: *STL1* Fwd, 5'-TAA GCA

TABLE 1. Numbers of transformants obtained with active *HOG1* alleles (under the control of a strong constitutive promoter) and their ability to support growth under osmotic pressure

<i>HOG1</i> allele	<i>hog1Δ</i> cells		<i>pbs2Δ</i> cells	
	Approx no. of colonies/μg of DNA	Growth on 1.3 M NaCl ^b	Approx no. of colonies/μg of DNA	Growth on 1.3 M NaCl ^b
Y68H,A314T	A few thousand	+	A few thousand	+
Y68H,F318L	A few thousand	+	A few thousand	+
Y68H,F318S	A few thousand	+	A few thousand	+
Y68H,W320R	A few thousand	+	A few thousand	+
Y68H,F322L	A few thousand	+	A few thousand	+
Y68H,W332L	A few thousand	+	A few thousand	-
D170A,A314T	Hundreds of tiny colonies	+	Hundreds of tiny colonies	+
D170A,F318L	No colonies ^a	-	Hundreds of tiny colonies	+
D170A,F318S	No colonies ^a	-	No colonies ^a	-
D170A,W320R	Hundreds of tiny colonies	-	Hundreds of tiny colonies	+
D170A,F322L	Hundreds of tiny colonies	-	Hundreds of tiny colonies	+
D170A,W332L	Hundreds of tiny colonies	-	Hundreds of tiny colonies	+

^a In these transformations, 30 to 70 big colonies appeared and were considered a consequence of rearrangement events. Some of these colonies were tested on salt and were found negative.

^b +, growth observed; -, no growth observed.

HOG1^{D170A,F318S} alleles to rescue *hog1Δ* cells is probably a result of their extreme activity that imposes growth arrest under high osmotic stress (see results of catalytic activity measurements below). The fact that these double mutants did not give rise to transformants when expressed at high levels (Table 1) supports this notion.

Inducible expression of Hog1 double mutants severely impedes cell growth. To directly test the link between expression of the double mutants and retardation of colony propagation, we ligated the dually mutated *HOG1* alleles downstream from the *MET3* inducible promoter. This promoter is shut off in the presence of methionine and induced by its removal (9, 10). These constructs were introduced into the *hog1Δ* and *pbs2Δ* yeast strains, and transformants were plated onto YNB (-URA) plates supplemented with 160 mg of methionine per

liter to suppress expression of the Hog1 molecules. Under these conditions, thousands of colonies with normal appearance were obtained with all constructs introduced into both strains.

To test whether expression of the double mutants indeed affects cell growth, cultures harboring the various *MET3*-driven constructs were plated onto YNB plates lacking methionine. On these plates, the growth of *hog1Δ* cells harboring alleles *HOG1*^{D170A,F318L} or *HOG1*^{D170A,F318S} was severely affected (Fig. 3A, plates on the left). In contrast, normal growth was observed for *hog1Δ* cells expressing wild-type Hog1 or the hyperactive mutants Hog1^{D170A}, Hog1^{F318L}, or Hog1^{F318S} under the same conditions (Fig. 3A). *pbs2Δ* cells expressing Hog1^{D170A,F318L} or Hog1^{D170A,F318S} also showed growth retardation on plates lacking methionine, although the effect was

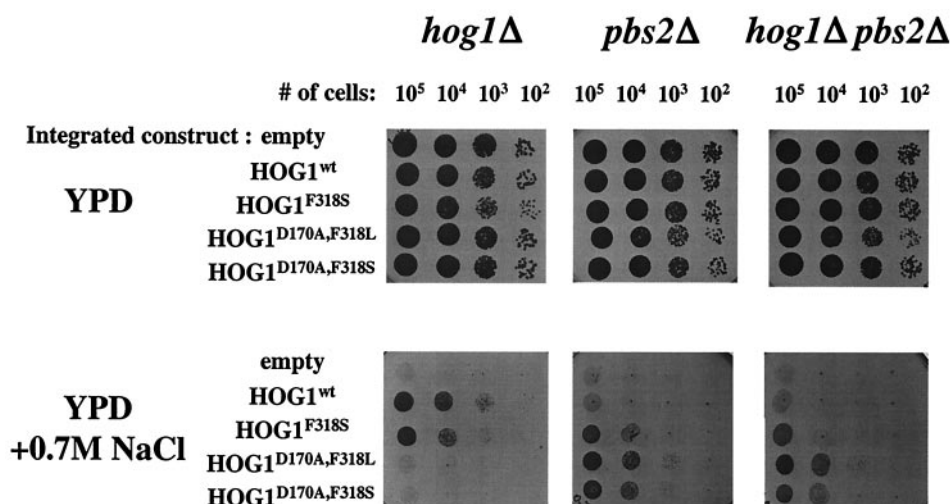
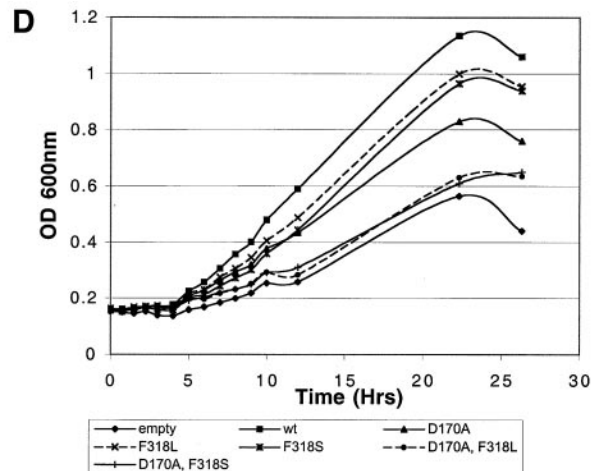
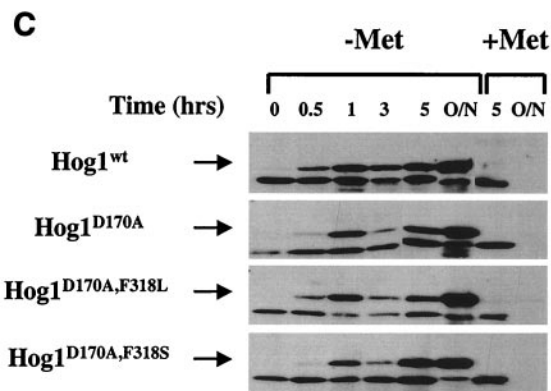
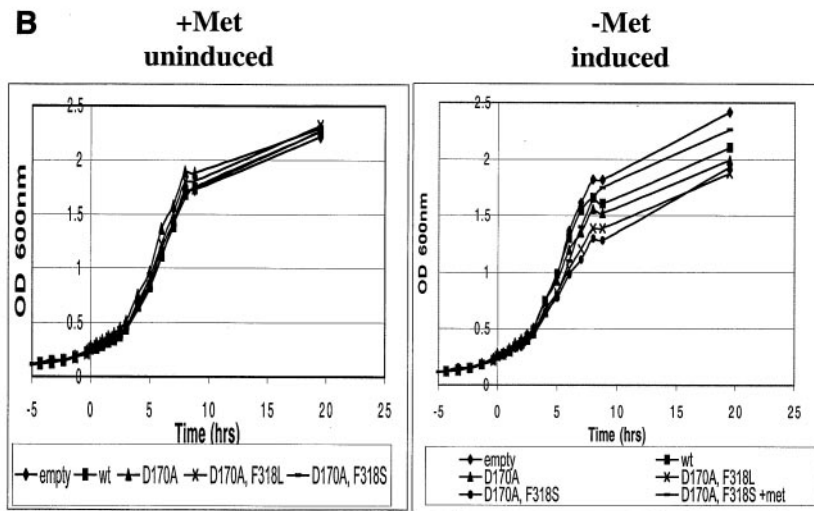
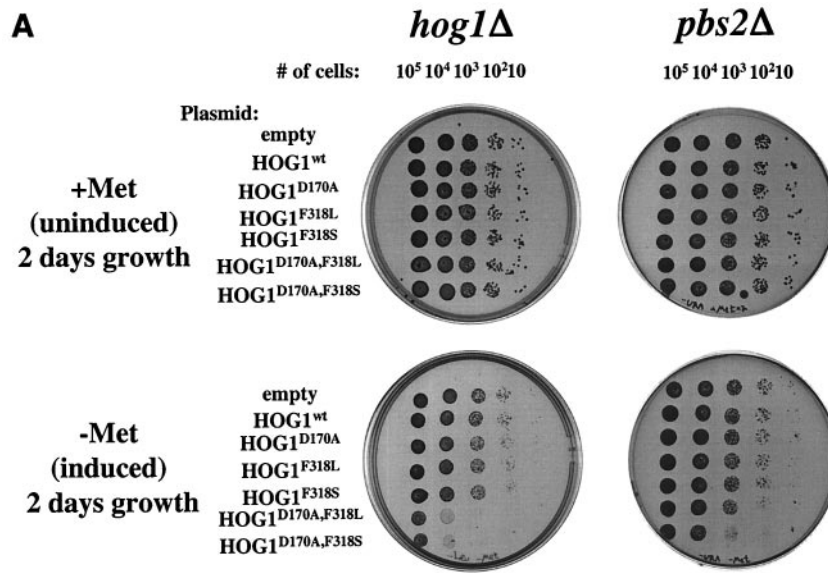


FIG. 2. *HOG1*^{D170A,F318L} and *HOG1*^{D170A,F318S} mutated alleles rescue *pbs2Δ* cells when expressed from the native *HOG1* promoter as single integrated copies. Plasmids bearing wild-type or mutant *HOG1* expressed under the control of the endogenous *HOG1* promoter were integrated as single copies into the genome of SP1*hog1Δ*, SP1*pbs2Δ*, or SP1*hog1Δpbs2Δ* strains. Cells carrying the integrated genes were grown to logarithmic phase on YPD media, serially diluted to the specified numbers, and plated on either YPD plates (upper panel) or YPD plates supplemented with 0.7 M salt (lower panel).



less pronounced (Fig. 3A, plates on the right). Notably, all cultures grew normally on plates supplemented with methionine (Fig. 3A, top plates). In both *hog1Δ* and *pbs2Δ* cells harboring Hog1^{D170A,F318L} or Hog1^{D170A,F318S}, some degree of growth recovery was observed after a prolonged incubation period. Within 4 days, the growth of *pbs2Δ* cells harboring the dually mutated alleles was nearly fully recovered. In *hog1Δ* cells, however, only tiny undeveloped colonies were observed following several days of incubation (data not shown).

The effect of methionine removal (and induction of the different Hog1 molecules) was also tested in liquid media with *hog1Δ* cells (Fig. 3B). When methionine was present in the media, all clones showed very similar growth rates and reached similar cell densities (Fig. 3B, left panel). Upon methionine removal, cells expressing active Hog1 mutants showed a reduction in growth rate (Fig. 3B, right panel). Most significantly affected were *hog1Δ* cells expressing the dually mutated Hog1 proteins. Western blot analysis verified that the *MET3* system provided rapid induction of Hog1 protein expression following methionine removal (Fig. 3C). The effect of active Hog1 on cell growth in liquid medium was transient and was significant between 3 and 9 h after removal of methionine. Ten hours after methionine removal, all cultures manifested similar growth rates. Cells expressing the Hog1^{D170A,F318L} or the Hog1^{D170A,F318S} alleles, however, reached a lower cell density (Fig. 3B). Although similar in principle, the growth inhibition observed in the liquid media (Fig. 3B) is somewhat less severe than that seen on plates (Fig. 3A). Given the known physiological and anatomical differences between cultures grown in liquid and solid media (33, 36, 49), the different effects of the active *HOG1* alleles under different culture conditions is not surprising (see Discussion).

When expressed from single-copy integrated genes, Hog1^{D170A,F318L} and Hog1^{D170A,F318S} induced growth arrest only in the presence of salt (Fig. 2). In the *MET3* system it seems that significant, but not complete growth retardation was caused by their expression alone in the absence of any osmotic stress (Fig. 3A and B). Western blot analysis showed that the *HOG1* alleles are indeed expressed at levels in the *MET3* system that were significantly higher than those of the integrated alleles (data not shown).

To test whether this growth inhibition could be further enhanced by osmotic stress, *hog1Δ* cells carrying the various *MET3*-driven *HOG1* alleles were grown in liquid cultures that were supplemented with 1 M NaCl 2 h after removal of methionine (Fig. 3D). Under these conditions, the growth differ-

ential between the various clones was more pronounced than that observed without salt (compare panels B and D of Fig. 3). Cells expressing Hog1^{wt} overcame the osmotic stress and entered a steep growth phase after approximately 5 h. Similar kinetics were observed with cells expressing the three single mutants tested, although their growth rate was slower. In contrast, *hog1Δ* cells carrying the dually mutated alleles (*HOG1*^{D170A,F318L} or *HOG1*^{D170A,F318S}) grew very poorly, at a rate similar to that of the osmosensitive *hog1Δ* cells carrying a control plasmid (Fig. 3D).

Combining the results obtained with the *MET3*-based inducible constructs (Fig. 3) with those obtained with the constitutive expression vectors (Table 1) and the integrative constructs (Fig. 2), it seems that *HOG1*^{D170A,F318L} and *HOG1*^{D170A,F318S} alleles impose a severe growth retardation. This effect probably stems from intrinsic activity of these molecules, since their expression was sufficient to induce it. The effect could be further enhanced, however, by high salt concentrations.

The catalytic activity of the double mutants is significantly higher than that of the single mutants. The results above demonstrated that the double mutants induced a severe growth inhibition. This effect may stem from strong catalytic activity or from another property of these molecules. To test the catalytic activity of the double mutants, we immunoprecipitated Hog1 proteins from yeast cells before and after methionine removal and monitored their ability to phosphorylate GST-ATF2 *in vitro* (Fig. 4 and 5).

As shown in Fig. 4A, Hog1^{wt} proteins immunoprecipitated from *hogΔ* cells showed no activity. Single Hog1 mutants Hog1^{D170A}, Hog1^{F318L}, and Hog1^{F318S} showed high catalytic activity, as expected (1). Hog1 double mutants immunoprecipitated from *hog1Δ* cells portrayed very high catalytic activity compared to those of the single mutants (see quantification in Fig. 4B). Also, the double mutants exhibited some degree of autophosphorylation in this assay (Fig. 4A). These very high activities of Hog1^{D170A,F318L} and Hog1^{D170A,F318S} proteins may account for their biological effect.

Having observed the autophosphorylation activity of the double mutants, we measured the steady state levels of phosphorylation of the mutants through Western blot analysis (Fig. 4C). As expected, we were unable to detect any phosphorylation of the Hog1^{wt} protein. Strikingly, however, all mutants (single and double) were found to be highly dually phosphorylated even though the cells were not exposed to osmotic stress (Fig. 4C). To determine whether this phosphorylation is a result of autophosphorylation or rather Pbs2 mediated, we

FIG. 3. Expression of dually mutated Hog1 proteins severely impedes yeast growth. (A) Cells used were of the *hog1Δ* (plates on left) and the *pbs2Δ* (plates on right) strains harboring plasmids expressing the indicated HA-tagged Hog1 variants, under the control of the *MET3* promoter. Cultures were grown in the presence of methionine to logarithmic phase, counted, washed with water, resuspended in inducing (methionine-free) media, serially diluted to the specified numbers, and plated onto the indicated plates (+Met or -Met). Plates were scanned 48 h after plating. (B) *hog1Δ* cells harboring the same constructs as above were grown overnight in the presence of methionine and diluted to an OD₆₀₀ of 0.1 in the same media. At an OD₆₀₀ of 0.25 (time zero in the graph), each culture was split in two, washed with DDW, and resuspended in media supplemented (+Met) or not (-Met) with methionine. Similar results were obtained in several independent experiments with a standard of deviation of <10% for all strains. (C) Protein extracts were precipitated by trichloroacetic acid from the cultures shown in Fig. 2B at the indicated time points, separated on SDS-PAGE, blotted, and probed with anti-HA antibodies. (O/N, overnight) (D) *hog1Δ* cells harboring the indicated plasmids were grown overnight in media containing methionine, diluted to an OD₆₀₀ of 0.15 into media lacking methionine, and incubated for 1 h. Then (time zero in the graph), cultures were collected and resuspended in methionine-free media supplemented with 1 M NaCl. Similar results were obtained in three independent experiments.

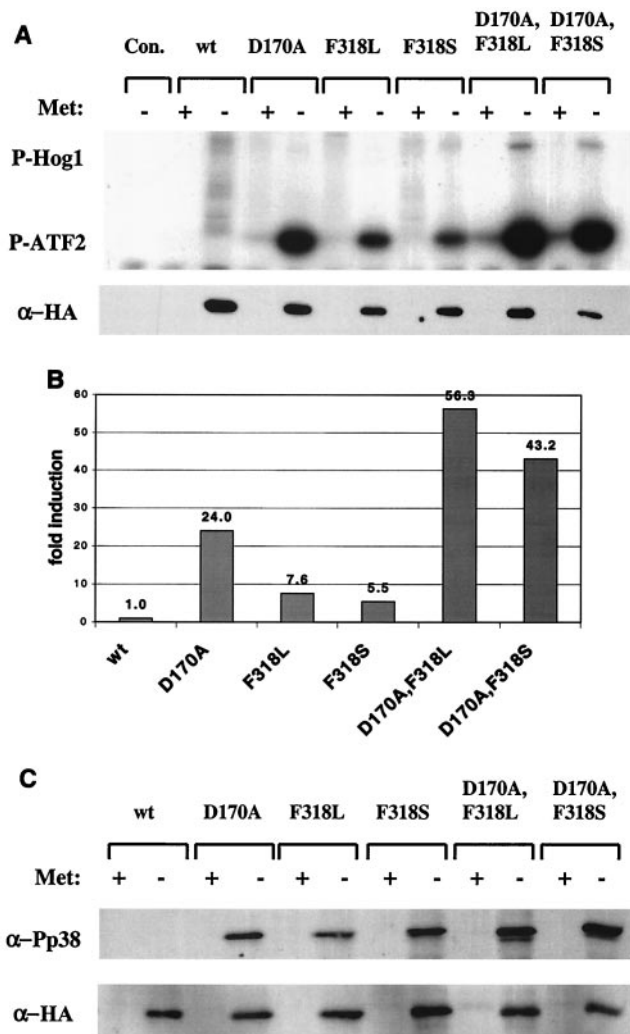


FIG. 4. Hog1 double mutants immunoprecipitated from *hog1Δ* cells are catalytically active in vitro and are dually phosphorylated in the absence of salt. (A) The various Hog1 proteins expressed in *hog1Δ* cells under the *MET3* promoter were immunoprecipitated and assayed in vitro using glutathione *S*-transferase (GST)-ATF2 as a substrate. Immunoprecipitation was performed on lysates prepared from cells grown in the presence (+) or absence (-) of methionine. The upper panel is an autoradiogram of the in vitro kinase assay. The top bands show autophosphorylated Hog1 proteins, and the bottom bands show the phosphorylated GST-ATF2 substrate. The lower panel is a Western blot performed on samples of the immunoprecipitated proteins. (B) Quantification (using a phosphorimager) of the above kinase assay blot. The activity of each sample was normalized to its protein level and is shown as induction compared to Hog1 wild-type activity, which was set as 1 (in the absence of methionine). (C) Phosphorylation state of the various proteins expressed in *hog1Δ* cells under the control of the *MET3* promoter. The upper panel portrays the level of dually phosphorylated Hog1 as measured by a Western blot of whole-cell lysates using anti-phospho-p38 antibodies. The lower panel shows the same blot after stripping and reprobing with anti-HA antibodies.

tested phosphorylation levels of the same Hog1 molecules in *pbs2Δ* cells. As the dual phosphorylation is completely abolished in *pbs2Δ* cells (Fig. 5C), the phosphorylation observed in *hog1Δ* cells must be Pbs2 dependent.

The strong and spontaneous Pbs2-dependent phosphoryla-

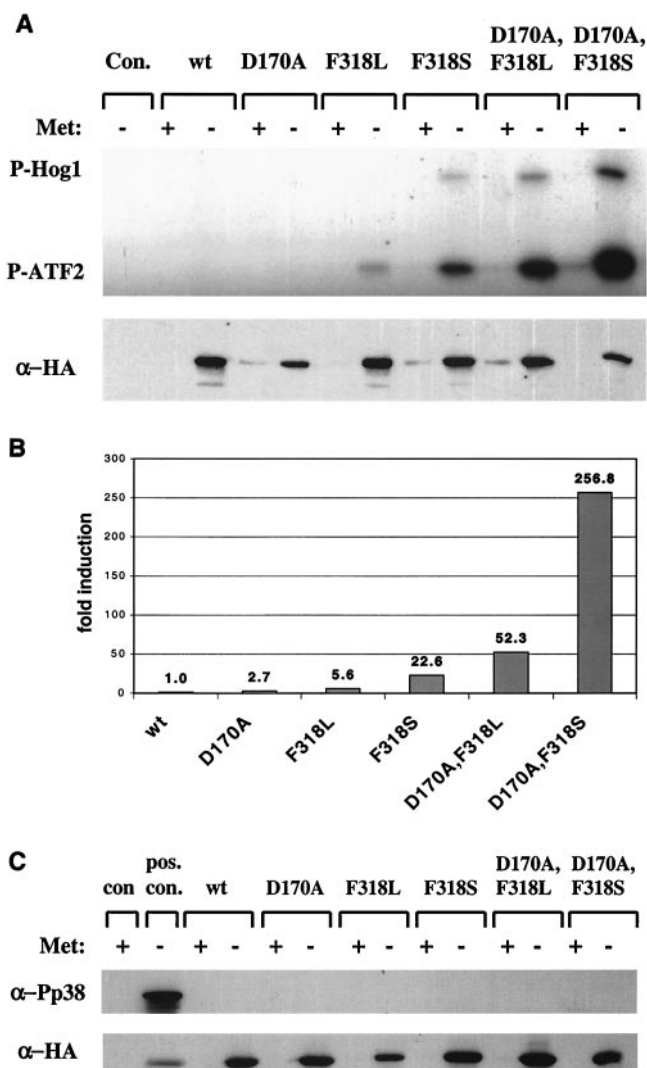


FIG. 5. Hog1 double mutants immunoprecipitated from *pbs2Δ* cells are catalytically active in vitro but are not phosphorylated. The experimental procedures for this figure were identical to those described in the legend of Fig. 4, with the exception that they were done with *pbs2Δ* cells. (A) In vitro kinase assay (upper panel) and Western blot analysis (lower panel) of immunoprecipitated Hog1 proteins. (B) Quantification of above kinase assay. (C) Anti-phospho-p38 and anti-HA Western blot analysis of whole-cell lysates. The positive control (pos. con.) is a lysate extracted from *hog1Δ* cells carrying Hog1 single mutant D170A 10 min after 1 M salt induction.

tion of the double mutants is intriguing because the mutants were originally isolated as Pbs2-independent active molecules. All the mutants are indeed biologically active in the absence of Pbs2 (1) (Fig. 2). The question remains whether the catalytic activity of the mutants is dependent on Pbs2 phosphorylation. To address this matter, we measured the activity of the mutants in *pbs2Δ* cells (Fig. 5).

The activity of the single mutants was low in *pbs2Δ* cells compared to their activity in *hogΔ* cells. Yet, their activity was clearly measurable whereas activity of Hog1^{wt} was not. Most importantly, the activity of the dually mutated proteins expressed in *pbs2Δ* cells was very high (Fig. 5A and B). Given

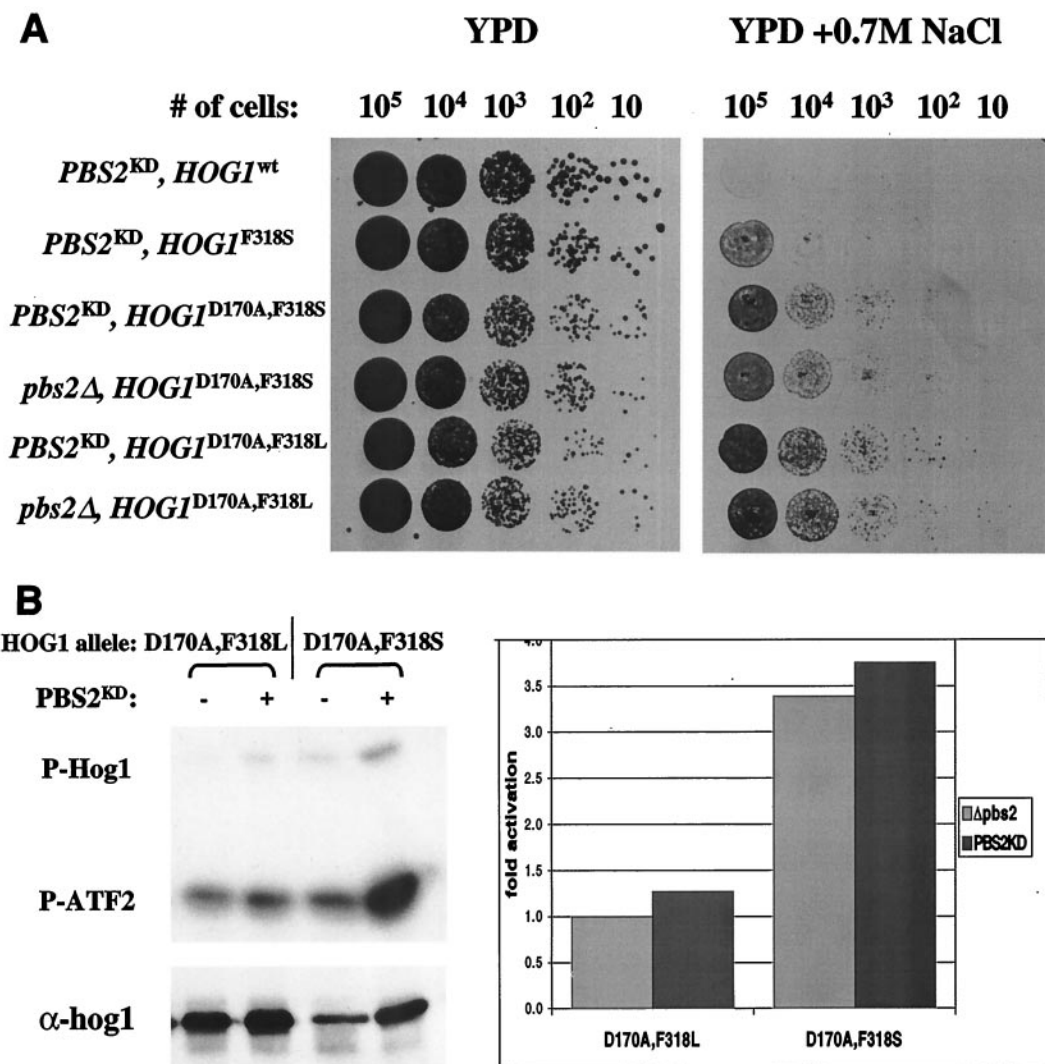


FIG. 6. Pbs2 scaffold function is not required for the double mutants' biological or catalytic activity. (A) Plasmids bearing *HOG1* wild type or mutants expressed under the control of the endogenous *HOG1* promoter were integrated as single copies into the genome of SP1*pbs2Δ* or SP1*pbs2^{KD}* strains. Cells carrying the integrated genes were grown to logarithmic phase on YPD media, serially diluted to the specified numbers and plated onto either YPD plates (left panel) or YPD plates supplemented with 0.7 M salt (right panel). (B) *pbs2Δ* cells of the MAY1 strain, containing or lacking an integrated *PBS2^{KD}* allele, were transformed with *MET3*-based plasmids harboring either *HOG1^{D170A,F318L}* or *HOG1^{D170A,F318S}*. Hog1 proteins were immunoprecipitated and assayed in vitro by using glutathione *S*-transferase (GST)-ATF2 as a substrate. Immunoprecipitation was performed on lysates prepared from cells grown in the absence of methionine. The upper left panel is an autoradiogram of the in vitro kinase assay. The top bands are autophosphorylated Hog1 proteins, and the bottom bands are phosphorylated GST-ATF2 substrate. The lower left panel shows a Western blot performed with the immunoprecipitated proteins. The right hand panel is the quantification (using a phosphorimager) of the kinase assay blot. The activity of each sample was normalized to its protein level and is shown as increase in activation compared to the *HOG1^{D170A,F318S}* activity in *pbs2Δ* cells, which was set as 1.

that none of the mutants were dually phosphorylated in this strain (Fig. 5C), it seems to us that spontaneous phosphorylation of the mutants, observed in *hog1Δ* cells (Fig. 4C), plays no role in establishing the high basal activity of the mutants. This activity is an intrinsic independent property of the active alleles. Pbs2-mediated phosphorylation further enhances this activity, contributing to the differences in activity levels observed when the same alleles are expressed in *hog1Δ* or *pbs2Δ* cells. This notion also coincides with the observations that all effects on growth were more severe in *hog1Δ* cells than in *pbs2Δ* cells.

Since the hyperactive *HOG1* alleles were not phosphory-

lated and still showed significant intrinsic catalytic activity in *pbs2Δ* cells, the possibility remains that their increased activity in *hog1Δ* cells is also phosphorylation independent. Namely, Pbs2 contributes to their activity (in *hog1Δ* cells) as a scaffold protein and not as a catalyst (39). To test this possibility, we expressed a "kinase dead" allele of *PBS2* (*PBS2^{KD}*) in *pbs2Δ* cells that also express the integrated *HOG1^{D170A,F318L}* or *HOG1^{D170A,F318S}* genes. If *PBS2^{KD}* supports an increase in catalytic activity of the double mutants, the relevant clones would not grow under osmotic stress, as was the case for *hog1ΔPBS2* cells expressing these double mutants (Fig. 2). Yet,

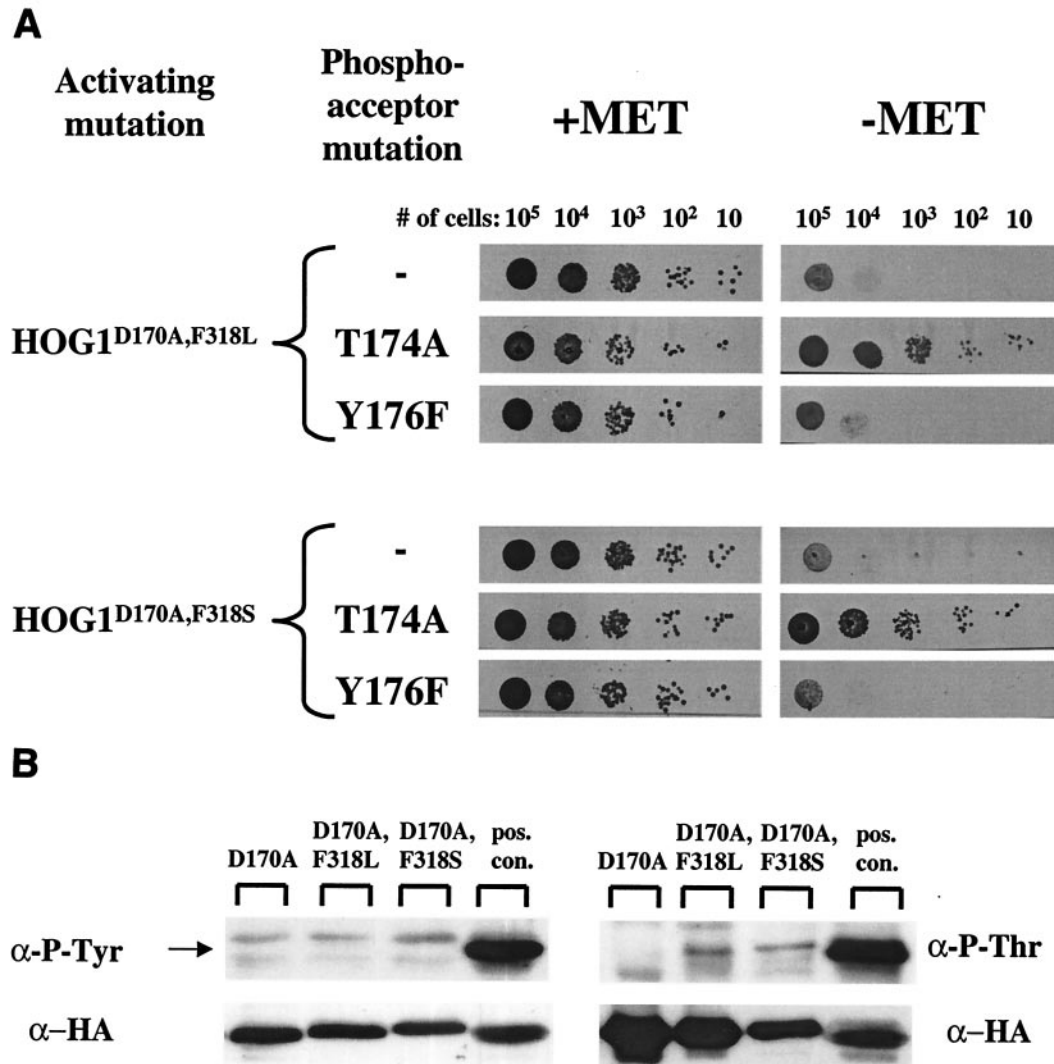


FIG. 7. The double mutants' intrinsic activity is independent of either Thr174 or Tyr176 phosphorylation. (A) Cells of the *hog1Δ* strain harboring *MET3*-based plasmids encoding Hog1 double mutants (activating mutation) with a mutated phosphoacceptor (phosphoacceptor mutation) were used. Cultures were grown in the presence of methionine to logarithmic phase, counted, washed with water, resuspended in inducing (methionine-free) media, serially diluted to the specified numbers, and plated on the indicated plates (+Met or -Met). Plates were scanned 48 h after plating. (B) Western blot analysis of whole-cell lysates using anti-phospho-Tyr (left panel) or of immunoprecipitated Hog1 proteins using anti-phospho-Thr (right panel). The positive control (pos. con.) is a lysate extracted from *hog1Δ* cells carrying Hog1 single mutant D170A 10 min after 1 M salt induction.

as shown in Fig. 6A, strains expressing *PBS2^{KD}* and the various *HOG1* alleles grew under osmotic stress as well as their parental *pbs2Δ* cells. Also, catalytic activity of the two doubly mutated hyperactive Hog1 enzymes was not significantly affected by the presence of kinase-dead *PBS2* allele (Fig. 6B).

In summary, when expressed in either *pbs2Δ* or *hog1Δ* cells, the double mutants (in particular *HOG1^{D170A,F318S}*) are significantly more active than the single mutants. This high catalytic activity is well correlated with the growth retardation induced by the double mutants and not by the single mutants (Fig. 3). Catalytic and biological activities are more pronounced in *hog1Δ* cells, due to further activation by Pbs2, which functions as a catalyst and not as a scaffold protein.

Intrinsic activity of the double mutants is independent of either Thr174 or Tyr176 phosphorylation. The results pre-

sented above show that the double mutants are biologically and catalytically active in *pbs2Δ* cells in which they are not dually phosphorylated (Fig. 5). Current understanding of MAPK activation suggests that dual phosphorylation at the phosphorylation lip is an absolute requirement for stabilizing an active conformation and increasing catalysis (41, 44). We wished to test whether the double mutants may require phosphorylation of at least one of the phosphoacceptors (Thr174 or Tyr176). We therefore produced *HOG1* alleles that carry a mutation in either Thr174 (substituted to Ala) or Tyr176 (changed to Phe) in addition to the two activating mutations.

When expressed in *hog1Δ* cells under the *MET3* promoter, *HOG1^{Y176F,D170A,F318L}* and *HOG1^{Y176F,D170A,F318S}* induced growth inhibition to the same degree as their parental double mutants (Fig. 7A). It therefore appears that the Tyr176 phos-

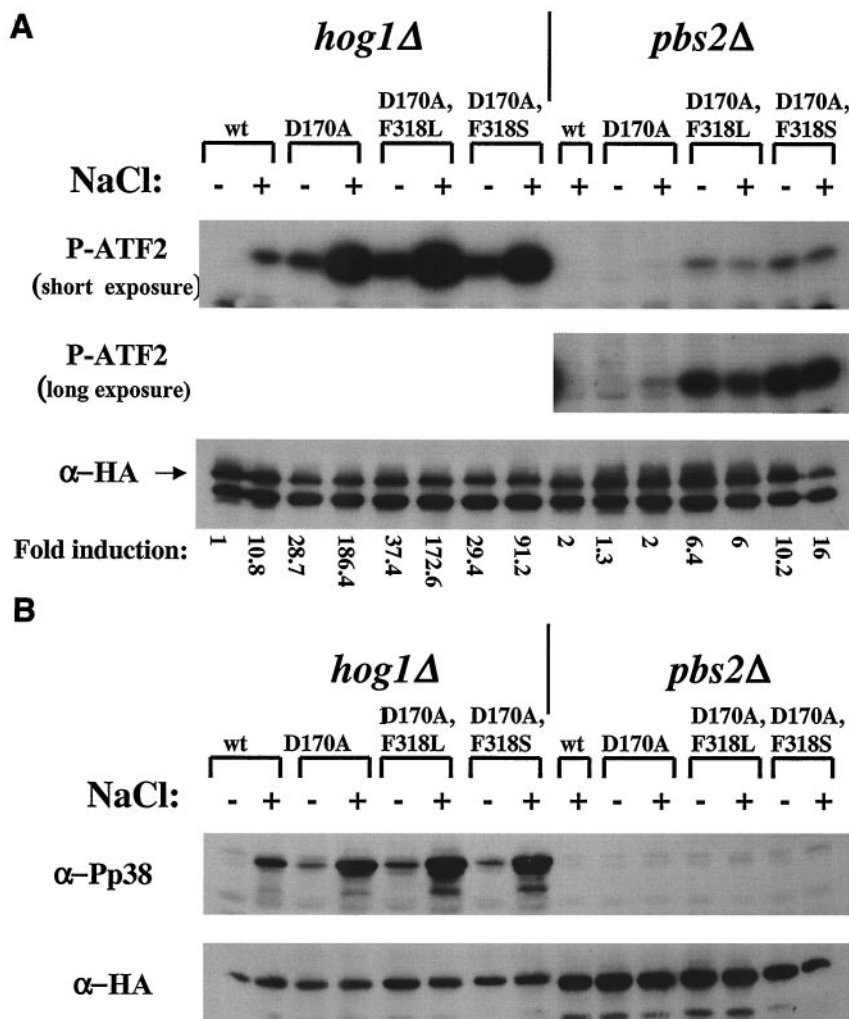


FIG. 8. Activity and phosphorylation of Hog1 double mutants is further enhanced by osmotic stress in *hog1Δ* cells but not in *pbs2Δ* cells. *hog1Δ* or *pbs2Δ* cells expressing the indicated Hog1 molecules were grown in the absence of methionine for 2 h. Half of each culture was subjected for 10 min to 1 M NaCl. (A) The upper panels show two exposures of the same autoradiogram of a kinase assay performed with the various Hog1 proteins immunoprecipitated from cells treated (+) or untreated (-) with 1 M NaCl. The lower panel shows a Western blot of the immunoprecipitated lysates. (B) Western blot analysis of whole-cell lysates prepared from the indicated strains. The upper panel shows the level of dually phosphorylated Hog1 when anti-phospho-p38 antibodies are used. The lower panel shows the total Hog1 protein levels when anti-HA antibodies are used.

phoacceptor does not play a role in the double mutants' biological effect. To further test this idea, tyrosine phosphorylation was directly tested in *HOG1* alleles expressed in *pbs2Δ* cells. All Hog1 proteins tested were not phosphorylated on tyrosine (Fig. 7B, left panel).

Unlike what was observed with the Tyr176 mutants, *HOG1^{T174A,D170A,F318L}* and *HOG1^{T174A,D170A,F318S}* grew normally in the presence or absence of methionine (Fig. 7A), suggesting that the T174A mutation abolished the capability of the double mutants to impair growth. These results strongly suggested that an intact Thr174 is essential for the activity of the mutants and raised the question as to whether this residue is somehow phosphorylated and thereby contributes to the catalytic properties of the mutants. Direct measurement of Thr phosphorylation of the double mutants revealed very low phosphorylation levels (Fig. 7B, right panel). These phosphoryla-

tion levels are particularly low when compared to those measured in Hog1 proteins extracted from osmotically challenged cells (Fig. 7B, pos. con. lanes). It could be that the intrinsic specific activity of the few Thr monophosphorylated molecules is so high that this small fraction could account for the properties portrayed by the double mutants. However, we believe that it is more likely that Thr174 serves as an essential structural motif and not as a phosphoacceptor in the active mutants (see also Discussion).

To verify that the detected phospho-Thr is indeed the Thr174 residue, Hog1 proteins carrying activating mutations in addition to the T174A mutation were probed with α -phospho-Thr. No signal was detected (data not shown).

The catalytic activity of the double mutants is further enhanced by osmotic shock. The observation that addition of salt further enhances the growth retardation effect induced by the

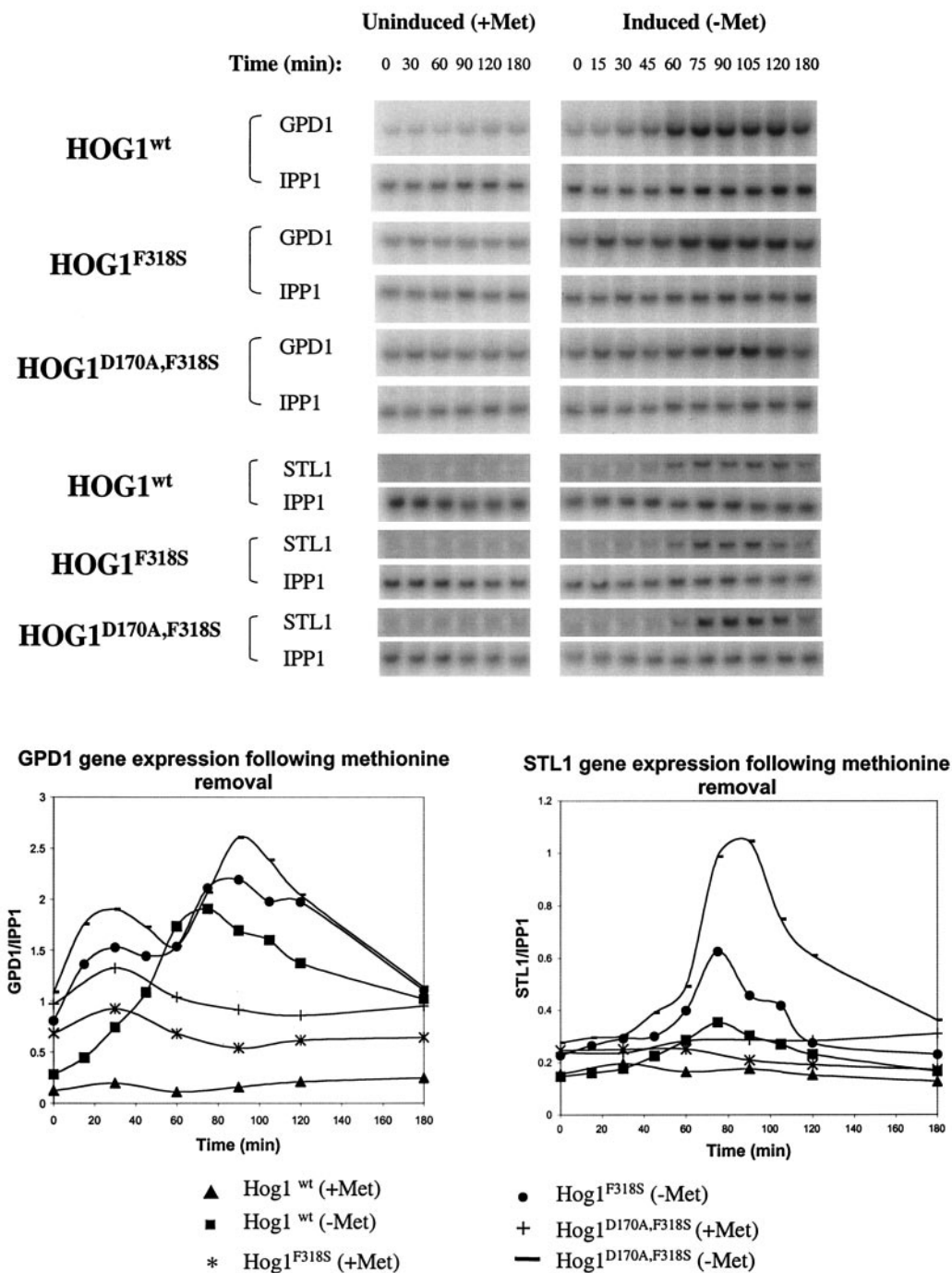


FIG. 9. The Hog1 double mutant induces high expression of target genes in the absence of osmotic stress. *hog1Δ* cells harboring plasmids with the indicated *HOG1* alleles were grown to logarithmic phase in the presence of methionine. Then, cultures were split and methionine was removed from one half (time zero was after the split and methionine removal). Samples for RNA extraction were taken at the indicated time points. Northern blots (upper panels) were hybridized with ³²P-labeled probes corresponding to the Hog1 target genes *GPD1*, *STL1*, or the *IPP1* gene that was used as a loading control. *IPP1* was hybridized to the blots after stripping. Blots were quantified (by phosphorimager) and were normalized to *IPP1* levels and plotted against time (lower panel).

double mutants (Fig. 2 and 3D) suggested that salt may increase the catalytic activity of these molecules. To test this idea, various Hog1 proteins were expressed for 2 h (by removal of methionine) in *hog1Δ* or *pbs2Δ* cells that were subsequently exposed for 10 min to 1 M NaCl. Hog1 proteins were then

immunoprecipitated from cell lysates, and their activity was monitored in vitro (Fig. 8).

The activity of all Hog1 proteins extracted from *hog1Δ* cells was further increased in response to salt (Fig. 8A). In addition, a significant increase in their dual phosphorylation level was

observed following the osmotic stress (Fig. 8B). When Hog1 proteins were isolated from *pbs2Δ* cells, neither enhancement of catalytic activity nor increase in dual phosphorylation was observed (Fig. 8A and B). Thus, Pbs2-dependent phosphorylation is responsible for the enhanced, but not the basal, catalytic activity of the mutants. These results confirm the notion that Pbs2 functions on the mutants as a catalyst.

Hog1 double mutants spontaneously induce high expression of target genes. One of the goals of developing a hyperactive MAPK is the study of its specific downstream targets and biological functions. Therefore, we wished to test whether the active mutants isolated in our studies spontaneously and accurately activate known Hog1 target genes (23, 42, 43). We also wanted to test whether the double mutants induce abnormal levels of these genes in an attempt to explain the growth arrest. To this end, we performed Northern blot analysis, examining the expression of known Hog1 target genes, *STL1* (mnemonic for sugar transporter-like protein) and *GPD1* (mnemonic for glycerol-3-phosphate dehydrogenase). RNA was prepared from *hog1Δ* cells containing the different *HOG1* alleles at different time points after methionine removal.

The kinetics of *STL1* mRNA accumulation following methionine removal showed that expression of the double mutant Hog1^{D170A,F318S} induced very high expression of this gene compared with that of wild-type Hog1 or the single mutant F318S (Fig. 9). Induction of *STL1* in cells expressing Hog1^{wt}, Hog1^{F318S}, and Hog1^{D170A,F318S} peaked at 75 min after methionine removal. *STL1* mRNA levels in cells expressing the single mutant were about twofold higher than the levels measured in cells expressing wild-type Hog1. In cells expressing the double Hog1 mutant *STL1* levels were 3.5-fold higher.

GPD1 mRNA showed somewhat different kinetics, but as in the case of *STL1* mRNA, it was induced to very high levels when the Hog1 double mutant was expressed (Fig. 9). Interestingly, the hyperactive Hog1 molecules induced high expression of *GPD1* in the presence of methionine. This may reflect some leakiness of the methionine-inducible system, the consequence of which can be detected only with the expression of hyperactive Hog1 molecules. The fact that low expression levels of the active Hog1 mutants are sufficient to induce target genes (time zero in Fig. 9) underscores the power of these molecules.

In summary, *HOG1* alleles carrying the two mutations are much more effective than *HOG1*^{wt} or alleles carrying a single mutation in spontaneous induction of Hog1 target genes. These results demonstrate that the intrinsic catalytic activity acquired by the mutants (single and double) is sufficient to activate the entire pathway up to its target genes. Since these events occur in the absence of any osmotic stress, the double mutants could be used to selectively study the biochemistry and biology of Hog1.

The observation that the expression pattern of *STL1* peaked and then declined to basal levels in all strains tested may be related to the ability of cells expressing the double mutants to recover after initial growth arrest (Fig. 3B and data not shown). This observation also suggests that the hyperactive Hog1 mutants are permissive, just like Hog1^{wt}, to regulation by the feedback machinery responsible for compromising the *HOG1* pathway 1 to 2 h after stimulation with salt.

We also examined transcription of *GPD1* in *hog1Δ* cells that

constitutively overexpressed wild-type Hog1 or Hog1^{F318S} from the *ADHI* promoter in a 2 μ m-based plasmid. Double mutants could not be studied using a constitutive promoter, since no colonies were obtained (Table 1). These experiments revealed an unexpected expression pattern. Following exposure to salt, cells expressing wild-type Hog1 manifested the expected fourfold-greater induction of *GPD1* mRNA. Cells expressing Hog1^{F318S}, however, failed to induce a significant increase in *GPD1* expression (data not shown). These results indicate that when constitutively expressed, the effects of the active Hog1 molecules on Hog1 target genes are negative, probably as part of a long-term adaptation response. It seems, therefore, that for accurately studying the short-term functions of Hog1, inducible expression systems (such as the *MET3*) should be used in combination with the active mutants.

The growth inhibition effect of the double mutants is not a result of cell death. The inability of cells expressing the *HOG1*^{D170A,F318L} and *HOG1*^{D170A,F318S} alleles to grow normally (Table 1 and Fig. 2 and 3) could result from deleterious effects on essential cellular components that lead to lethality. The observation that growth inhibition is partially reversible (Fig. 3B and data not shown) challenges this possibility. We wished to test this point in more detail and measured the effect of expression of the active mutants on cell viability.

hog1Δ cells harboring a control plasmid or *MET3*-based plasmids coding for either *HOG1*^{WT} or *HOG1*^{D170A,F318S} were grown in the presence of methionine, and samples were taken at different time points from the cultures and stained with PI. Cells were not fixed or permeabilized prior to staining so that only dead cells would be stained (dye exclusion method [19]). Under these experimental conditions, we observed very low levels of PI staining in all cultures (Table 2). Transferring the cultures to media lacking methionine did not change these levels (Table 2), indicating that the very strong activity of the Hog1 variants did not induce lethality. This was also the case for more-prolonged (up to 18 h) expression of the *HOG1* alleles (data not shown).

DISCUSSION

This paper described the development of hyperactive variants of the MAPK Hog1. These novel molecules were shown to induce high levels of Hog1 target genes and severe growth inhibition when expressed in yeast cells. These effects, which were obtained in the absence of any external stimulation, show that the alleles studied here, *HOG1*^{D170A,F318L} and *HOG1*^{D170A,F318S}, could serve as strong tools for the study of the specific downstream effects of Hog1. Hyperactive variants that induce biological effects have not been described so far for members of the MAPK family. We believe that the knowledge obtained here regarding Hog1 could be applied for producing active forms of other MAPKs (see below).

The extreme hyperactivity of the mutants was obtained through the use of a simple strategy, i.e., combining two mutations previously shown to render Hog1 catalytically active (1) in the same gene. Several of the double mutants produced imposed various degrees of growth defects (Table 1). Some of the double mutants induced growth inhibition only when plated on salt. We believe that these alleles were not intrinsically active at sufficient levels and that further salt induced

TABLE 2. Percentage of dead cells (PI positive) following induced expression of HOG1^{WT} or HOG1^{D170A,F318S}

Plasmid	In presence of methionine	% of dead cells ^a		
		After methionine has been removed for indicated time (h)		
		1.5	3	5
Control	13.3	6.4	2.6	6.1
Coding for HOG1 ^{WT}	8.5	3.2	2.1	3.2
HOG1 ^{D170A,F318S}	13.2	5.5	4.2	4.5

^a Results are the averages from two independent experiments.

activation was necessary to impose growth inhibition. However, the HOG1^{D170A,F318L} and HOG1^{D170A,F318S} alleles manifested the most severe effects. The protein products of these two alleles portrayed intrinsic catalytic activity, sufficient to impose growth inhibition on the cells (Fig. 4 to 6). Both alleles carried mutations in D170 and F318. D170 is located at the phosphorylation lip (7, 53, 55), while F318 is located at the L16 domain (53, 55). Current understanding of the mode of activation of MAPKs suggests that MAPKK phosphorylation induces a significant movement of the phosphorylation lip so that it interacts with the L16 domain (7, 12). This hypothesis is based on the three-dimensional structure of ERK2 (active and nonactive) (7, 25) and p38 (8, 53). This interaction is stabilized mainly by hydrogen and ionic bonds between the phosphothreonine at the phosphorylation lip and various residues in L16. It could be that the activating point mutations identified in Hog1 (1) stabilized to a certain degree some interactions between the phosphorylation lip and L16, thereby mimicking the active conformation. Combining such stabilizing mutations may result in even stronger interactions between those domains, increasing the stability of the active conformation. The catalytic activity of Hog1^{D170A,F318L} and Hog1^{D170A,F318S} is indeed significantly higher than that of Hog1^{D170A}, Hog1^{F318S}, or Hog1^{F318L}. The fact that intact Thr174 (but not Tyr176) is essential for activity of the mutants and probably serves as a structural anchor and not a phosphoacceptor (Fig. 6B) strongly supports the idea that the activating mutations stabilize an active conformer.

The significant difference between the activities of the double mutants and the single mutants is best observed in *pbs2Δ* cells (Fig. 4 and 5). As these cells cannot activate Hog1 alleles, this difference can only stem from an increase in the intrinsic activities of the double mutants. In *hog1Δ* cells, Pbs2-dependent phosphorylation (Fig. 4 and 8) enhances activity of all hyperactive Hog1 alleles, and therefore the difference between the activities of the double and single mutants is less appreciated.

The observation that in *hog1Δ* cells all active mutants (but not Hog1^{WT}) are dually phosphorylated in the absence of external stimulation is unexpected. Under these conditions the mutated Hog1 molecules are active, since they possess intrinsic activity, while Pbs2 is not expected to be active. Hence, it could be that the active Hog1 variants induce a positive feedback loop that culminates in activation of Pbs2.

D170 is a residue conserved in many MAPKs including

mammalian p38s and ERKs. It would be interesting to test the effect of mutating this residue in other MAPKs. Previous attempts to use the information obtained from the activating Hog1 mutations to produce active human p38α were successful and provided p38α variants that are 70- to 100-fold more active than native p38 in the absence of any upstream phosphorylation (1). The specific activity of the mutated p38α molecules, tested as recombinant proteins produced in *Escherichia coli*, is, however, about 25% of that of the dually phosphorylated p38α (data not shown). The experience with Hog1 described here suggests that combining several activating mutations into a single p38α cDNA would create a molecule with stronger specific activity. The combinations used here in HOG1 could not be directly applied to p38, as the Phe residue at position 318 in HOG1 is not conserved in p38. For production of hyperactive p38α mutants, other mutations should be tested in combination with the equivalent of D170. Preliminary catalytic results with doubly mutated p38α are promising (data not shown). Phe318 is conserved in members of the ERK family of MAPKs, including ERK1, ERK2, and ERK5. In these molecules it should be possible to combine mutations equivalent to D170A and F318L or F318S used here.

A combination of other mutations was shown to increase specific activity of ERK2 (16). In this report various mutations identified in several genetic screens were combined, and some of the combinations gave rise to ERK2 molecules, which were active in vitro and in vivo. The most significant combination was that of L73P and S151D. These residues are not part of the domains that we found to be important for MAPK activation. The specific activity of those mutated ERK2 molecules was rather low (16), but the data could support the notion that combinations of more than one mutation may be required to produce hyperactive forms of MAPKs.

The catalytic activity of the singly mutated Hog1 is several-fold higher than that of the phosphorylated Hog1^{WT} (1) (Fig. 6). Yet, this activity, which is sufficient to rescue *pbs2Δ* cells, had only minor effects on cell growth. Only the double mutants that possess even higher activity had a severe effect on growth. It will be interesting to find out whether there are particular substrates or target genes of Hog1 that are responsible for the growth inhibition and whether they are significantly more affected by the double mutants. As Hog1 is activated to different levels and shows different kinetics of activation when exposed to low versus high salt concentrations (51), it could be that the effects of the single and double mutants reflect the two degrees of response.

Growth inhibition following expression of the double mutants is most dramatic on plates and less severe on liquid media (Fig. 2). We believe that the effect on plates is more relevant, because in nature microorganisms usually grow on solid surfaces on which they form multicellular colonies. Several aspects of the physiology, metabolism, and gene expression are different in cells grown on solid surfaces compared to cells grown in liquid (32, 33, 36, 37). On solid media, diffusion of chemicals and movement of cells are limited. As a result cells interact and form colonies with organized anatomy (2, 49, 52). It could be that yeast cells expressing doubly mutated Hog1 produce a growth inhibitory agent (glycerol?) that is less effective if dispersed by shaking and diffusion in liquid media.

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