Phosphorylation of Tyr-176 of the Yeast MAPK Hog1/p38 Is Not Vital for Hog1 Biological Activity*‡[S]

Mitogen-activated protein kinases (MAPKs); ERK, p38, and c-Jun N-terminal kinase) play vital roles in determining the cell program (1–4). Despite the significant progress in our understanding of MAPK activation and catalysis (5–8) these issues are not fully revealed. MAPKs possess a phosphorylation motif that comprises a Thr-X-Tyr sequence. Upon activation of the relevant pathway this motif is dually phosphorylated, leading to structural changes and a dramatic increase in specific activity (7, 9, 10). Current models of MAPK activation suggest that phosphorylation of both Thr and Tyr at the phosphorylation motif is an absolute requirement for activation. Substitution of any one of these phosphoacceptors diminishes the kinase activity as detected by in vitro kinase assays (9–11).

Although both Thr and Tyr seem to be equally important for catalysis, the three-dimensional structures of phosphorylated ERK2 and p38 suggest that the Thr-183 residue contributes more significantly to stabilization of the active form (5, 7). Upon phosphorylation, Thr-183 forms ionic hydrogen bonds with the N-terminal domain, thereby promoting domain closure. Tyr-185 is positioned to participate in substrate recognition.

Recently we reported the isolation of MAPK kinase-independent hyperactive MAPK mutants of both the yeast Hog1 and the human p38α (12). These MAPKs were rendered intrinsically active by point mutations in either the L16 domain (mutations F318L, F318S, F322L, W320R, and W332R in Hog1) or the phosphorylation lip (D170A in Hog1) and were shown to rescue pbe2Δ cells from high osmolarity (12). Although manifested very high basal activities, the catalytic activity of the mutants was further increased when cells were exposed to osmotic stress.

The goal of this work was to examine the exact role of Thr-176 or Thr-174 phosphorylation in Hog1 catalytic and biological activity. We show that in the hyperactive mutants Thr-176 is required mainly for enhancing catalytic activity following osmostress, whereas Thr-174 is essential for catalytic and catalytic activity although not necessarily as a phosphoacceptor. Unexpectedly, when Tyr-176 was replaced with Phe in the wild type Hog1 enzyme, most of its catalytic activity was abolished, but its biological activity was maintained.

We suggest that Thr phosphorylation stabilizes an active catalytic conformation that is independent of Tyr phosphorylation. Tyr phosphorylation serves to further amplify the basal activity in response to external signals.

MITERALS AND METHODS

**Yeast Strains and Media**—Strains used in this study were the pbe2Δ strain MAY1 and the hog1Δ strain JBY13 (12). Growth conditions were described previously (12).

**Plasmids**—T174A or Y176F mutations were inserted into plasmids pES86-HA-HOG1 (harboring an HA-tagged HOG1 coding sequence under the ADH1 promoter) and pRS1 (harboring the full-length HOG1 sequence with its native promoter and an HA tag at the N terminus). Construction details will be provided upon request.

**Preparation of Native Cell Lysates, Western Blots, in Vitro Kinase Assay, and Detection of Thr Phosphorylation**—Cell lysate preparations and kinase assays were described previously (12). Detection of Thr phosphorylation was performed by immunoprecipitation of HA-Hog1 as described previously (12) followed by Western blot analysis using rabbit anti-phosphothreonine antibodies (Zymed Laboratories Inc.). Hog1 protein levels in the same blots were detected by stripping the blot and re-incubation with monoclonal anti-HA antibodies (Zymed Laboratories Inc.).

**RNA Preparation and Analysis**— Cultures were grown to $A_{600}$ = 0.4–0.5. Next cells were split in half, collected by centrifugation, and resuspended in the same medium or in medium containing 1 M NaCl. 20-ml samples were removed at the indicated time points for RNA isolation. RNA was analyzed by the S1 method (13).
Tyr-176 Phosphorylation Is Not Essential for Hog1 Activity

Thus, Tyr-176 phosphoacceptor is dispensable for catalytic activity of the hyperactive Hog1 mutants, but Thr-174 is essential. These results fully correlate with the biological assay (Fig. 1 of this study and Fig. 7 in Bell et al. (12)).

Tyr-176 Is Important for Increasing the Catalytic Activity of Hog1 Hyperactive Mutants in Response to Salt Induction—Most of the hyperactive Hog1 mutants acquired very high catalytic activity that is independent of salt induction. Yet this activity was further enhanced when cells were exposed to salt (see Fig. 4 in Ref. 12). The results shown in Fig. 2 suggest that when mutated in Tyr-176 the basal catalytic activity of the mutants was not lost, but their ability to further enhance activity in response to salt induction was compromised. To verify this point we measured kinase activity of the Hog1D170A, Hog1Y176F, and Hog1F318L, Y176F derivatives. The results clearly show that the basal catalytic activity of the hyperactive mutants carrying the native position 176 was similar to that of the active mutants carrying the native Tyr-176 (Fig. 3). However, whereas the activity of the Hog1 hyperactive molecules increased upon exposure to salt, molecules mutated in Tyr-176 were not as responsive to salt (Fig. 3). These results suggest that the catalytic activity of the hyperactive Hog1 alleles could be divided into two levels: 1) an intrinsic activity, acquired through the activating mutations, that is Pbs2-dependent, salt-independent, and Tyr-176-independent; and 2) an enhanced activity that is salt-dependent. In most mutants an intact Tyr-176 is important for the enhanced activity and is dispensable for the intrinsic activity. Intact Thr-174 is essential for all levels of activity of the wild type and the hyperactive Hog1 mutants (Fig. 2).

Thr-174 Is Not Required as a Phosphoacceptor for Active Hog1 Activity—It seems that Thr-174 is essential for catalytic and biological activity of the hyperactive mutants. The question remains whether this residue is required as a phosphoacceptor or is essential due to conformational reasons. To address this question we analyzed the phosphorylation state of Thr-174 in some of the active mutants using α-phospho-Thr antibodies (Fig. 4). The results show that when expressed in pbs2Δ cells the active mutants manifested either barely or no detectable Thr phosphorylation (Fig. 4, right panel). When expressed in hog1Δ cells Thr(P) was clearly detected in all hyperactive Hog1 molecules (Fig. 4, left panel). In fact, Thr-174 in the hyperactive Hog1 alleles seemed to have elevated basal phosphorylation levels when Tyr-176 was mutated. Since the active mutants manifested clear catalytic activity in pbs2Δ cells (Fig. 3B) but were not significantly phosphorylated on any Thr residue in this strain (Fig. 4), we conclude that Thr-174 is not required for...
kinase activity as a phosphoacceptor but rather as an essential structural component.

Tyr-176 Is Not Essential for Wild Type Hog1 Biological Activity—As expected (9, 10), when we mutated each of the phosphoacceptors in wild type Hog1 we could not measure any kinase activity (Fig. 2, lanes 5–8). We expected that these mutants would not show any biological activity either (16). Surprisingly, when overexpressed, Hog1T174F was able to rescue hog1Δ cells from osmotic shock (Fig. 5). In contrast, Thr-174 was found to be essential for biological activity as Hog1T174A or even Hog1T174E did not rescue hog1Δ cells (Fig. 5 and Supplemental Fig. 3S).

Our inability to detect any catalytic activity of Hog1T174F on one hand (Fig. 2) and the fact that Hog1T174F is biologically active on the other hand (Fig. 5) led us to test whether Hog1T174F supports growth on salt by activating the authentic downstream targets of the Hog1 pathway. To this end we

analyzed RNA levels of GDP1, GPP2, and STL1. In hog1Δ cells these genes did not show significant increase in RNA levels following exposure to salt (Fig. 6, lanes 1–7). In contrast, when Hog1 or Hog1T174F were expressed and separated by SDS-PAGE, Western blot analysis was performed with α-phospho-p38 antibodies (upper panel) followed by stripping and reincubation with α-HA antibodies. Proteins were expressed under the ADH1 promoter. P-Hog1, phosphorylated Hog1.

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FIG. 5. Hog1 mutated at Tyr-176 is biologically active. Hog1WT, Hog1T174A, or Hog1T174F were expressed in hog1Δ cells. Cells were grown to ρ00 = 0.4 when each culture was diluted, and the indicated number of cells was plated on a YNB-URA plate (left) and a YPD + 1.1 M NaCl plate (right). In Supplemental Fig. 3S, hog1Δ cells harboring the plasmid pBS86, Hog1WT, or Hog1T174A were plated on a YNB-URA plate (left) and on a YPD + 0.9 M NaCl plate (right). Proteins were overexpressed using the ADH1 promoter.

FIG. 6. Hog1T174F is capable of inducing Hog1 target genes in hog1Δ cells. Hog1WT or Hog1T174F was subcloned into the pBS426 plasmid and expressed under the native HOG1 promoter in hog1Δ cells. Cells were exposed or not to 1 M NaCl at the indicated time points. RNA levels were monitored by S1 analysis. HAL3 was used as a loading control.

FIG. 7. Hog1T174F is phosphorylated on Thr-174 in hog1Δ cells. Hog1WT, Hog1T174A, or Hog1T174F was expressed in hog1Δ cells that were exposed or not to 1 M NaCl for 10 min. Proteins were extracted and separated by SDS-PAGE. Western blot analysis was performed with α-phospho-p38 antibodies (upper panel) followed by stripping and reincubation with α-HA antibodies. Proteins were expressed under the ADH1 promoter. P-Hog1, phosphorylated Hog1.
intact Hog1 (23). As shown in Supplemental Fig. 4S, Pbs2\textsuperscript{2D\textsuperscript{3D}} induced growth arrest of cells expressing Hog1\textsuperscript{WT} as expected. Cells expressing Pbs2\textsuperscript{2D} and Hog1\textsuperscript{Y176F} were able to grow (Supplemental Fig. 4S) on galactose. Thus, Hog1\textsuperscript{Y176F} is capable of executing important functions of Hog1 (Figs. 5 and 6) but is probably not maximally activated (Supplemental Fig. 4S).

The ability of Hog1\textsuperscript{Y176F} to efficiently induce gene expression suggests that although the catalytic activity of Hog1\textsuperscript{Y176F} was below the threshold of our in vitro assay the enzyme was activated in the cell. We could not obtain an indication that this is the case because α-phospho-Thr antibodies did not react with Hog1\textsuperscript{Y176F} (Fig. 4, lanes 5 and 6). Although this result may suggest that Hog1\textsuperscript{Y176F} is not phosphorylated on Thr-174, we decided to further explore the issue through the use of antibodies against the dually phosphorylated p38. We speculated that these antibodies might recognize determinants of the active conformation of the phosphorylation motif and not merely the phosphorylated residues. This idea was based on the results of Bardwell et al. (14) who showed that α-phospho-ERK antibodies react with Thr-183 phosphorylated Kss1\textsuperscript{Y185F}. We found (lane 6 in Fig. 7) that α-phospho-p38 reacted with Hog1\textsuperscript{Y176F} after salt induction. This result supports the notion that Hog1\textsuperscript{Y176F} was activated in vivo to some level. We believe that this low activity was responsible for the induction of gene expression shown in Fig. 6, which enabled growth of hog1Δ cells on hyperosmotic medium (Fig. 5).

DISCUSSION

Many enzymes, receptors, and transcription factors are regulated through phosphorylation (18–21). MAPKs are considered unusual as their activation requires concomitant dual phosphorylation of neighboring Thr and Tyr residues. This report provides evidence that at least for the yeast MAPK Hog1 this dogma only partially holds. With respect to biological activity Tyr phosphorylation plays a partial role. Mutating Tyr-176 to Phe in the hyperactive Hog1 alleles revealed that this residue functions in enhancing catalytic activity of these molecules by Pbs2 but has no role in the elevated intrinsic activity of those alleles (Fig. 3). Furthermore it appears that Tyr-176 might have an inhibitory effect on the basal activity of the hyperactive mutants as Hog1\textsuperscript{F138L,Y176F} shows a higher catalytic activity in comparison with Hog1\textsuperscript{F138L} in pbs2Δ cells (Fig. 3B, right panel, lanes 7–10). It must be noted that these roles of Tyr-176 may be specific to the hyperactive mutants. However, the results obtained with Hog1\textsuperscript{WT} mutated in Tyr-176 suggested that these roles might be relevant to the native protein as well.

In wild type Hog1, mutating Tyr-176 resulted in a dramatic decrease of catalytic activity below our detection level (Fig. 2). However, Hog1\textsuperscript{Y176F}, unlike Hog1\textsuperscript{T174A} or even Hog1\textsuperscript{T174F}, was probably catalytically active at a low level in vivo, a level sufficient for induction of target genes (Fig. 6) and for rescuing hog1Δ cells from hyperosmotic shock (Fig. 5). It was not sufficient, however, to mediate Pbs2\textsuperscript{2D}\textsuperscript{3D}-induced growth arrest (Supplemental Fig. 4S) suggesting that Tyr-176 phosphorylation is required to obtain some further increase in activity, a case similar to that observed in the hyperactive mutants (Fig. 3).

The unexpected capabilities of Hog1\textsuperscript{Y176F} led us to carefully inspect previous studies in which MAPKs carrying similar mutations were used. Schüller et al. (16) suggested that Hog1\textsuperscript{T174A} and Hog1\textsuperscript{Y176F} cannot support growth of hog1Δ cells on hyperosmotic media. However, careful inspection of their data reveals that Hog1\textsuperscript{Y176F}-expressing cells (but not cells expressing Hog1\textsuperscript{T174A}) did grow on 0.4 M KCl but grew very poorly on 0.9 M KCl (16). Tyr phosphorylation may be required for extreme conditions. The notion that Hog1\textsuperscript{Y176F} is biologically active was also raised by Warkma et al. (22).

How crucial is tyrosine phosphorylation for the biological activity of MAPKs other than Hog1? In the case of Kss1, mutating Tyr-185 to Phe did not abolish biological activity completely as cells expressing Kss1\textsuperscript{Y185F} were capable of inducing invasive growth to some extent (14). Gartner et al. (15) reported that in Pus3 dual phosphorylation is essential for biological activity. Importantly none of these studies provided sufficient quantitative information regarding the catalytic and biological activities of the mutated MAPK. Based on the available data, we believe that the case of Hog1 analyzed here reflects a general situation in MAPK activation. Namely for many MAPK molecules Tyr phosphorylation may not be as vital as Thr phosphorylation for biological activity.

Structural studies revealed that in both ERK2 and p38 phospho-Thr forms important networks of interactions that appear to be critical for stabilizing the active conformation of the enzyme (5, 7). This information coincides with our results, which show that Thr-174 is essential for both biological and catalytic activity (Bell et al. (12) and Fig. 2). However, it appears that in the hyperactive alleles Thr-174 is important mainly for structural reasons and not as a phosphoacceptor (Fig. 4). One may speculate that the activating mutations maneuver Thr-174 toward the L16 domain and stabilize an active conformation that is not phosphorylated. Upon phosphorylation Thr-174 forms stronger interactions with residues in L16 resulting in a more active conformation.

Phospho-Tyr appears to be involved in changing the conformation of the substrate (P + 1) recognition site (7, 9) but may affect catalysis as well (9). Taking advantage of the hyperactive alleles, it was possible to obtain a more detailed insight into the role of Tyr-176 in Hog1 catalysis and to reveal that it is not essential as a stabilizer of the active conformation but is more important as an amplifier of enzyme activity.

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