Human Mitogen-activated Protein Kinase CSBP1, but Not CSBP2, Complements a hog1 Deletion in Yeast*

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CSBP1 and CSBP2 are human homologues of the Saccharomyces cerevisiae Hog1 mitogen-activated protein kinase which is required for growth in high osmolarity media. Expression of CSBP1, but not CSBP2, complemented a hog1Δ phenotype. A CSBP2 mutant (A34V) that complements hog1Δ was isolated and found to have ~3-fold lower kinase activity than the wild-type CSBP2. Further analysis revealed that both the kinase activity and tyrosine phosphorylation of CSBP1 and CSBP2 (A34V) is regulated by salt. In contrast, wild-type CSBP2 is constitutively active but dependent on the upstream kinase, Pbs2. Mutagenesis studies showed that reduction or elimination of CSBP2 kinase activity restores salt responsiveness as measured by tyrosine phosphorylation suggesting that too high a level of kinase activity can result in desensitization of the host cell and inability to grow in high salt.

We recently reported the cloning of a pair of closely related novel MAPK kinase homologues, CSBP1 and CSBP2 (1). These kinases were identified as a target of a series of pyridinyl imidazoles, which inhibited cytokine production from human monocytes (1). The two proteins are splice variants and differ only in an internal 25-amino acid sequence. The murine (p38) and Xenopus (Mpk2) homologues of CSBP2 also have been identified and cloned (2, 3).

At least three distinct MAP kinase pathways exist in mammalian cells, as exemplified by the extracellular signal-regulated kinases (ERKs), the c-jun amino terminus kinases (J Nks), and the CSBPs/p38/Mpk2 (4). Each of these kinases define a distinct signal transduction pathway and are characterized by the presence of a regulatory TXY (Thr-Xaa-Tyr, where X is any amino acid) motif. Phosphorylation of both the threonine and the tyrosine by a dual specificity kinase(s) is essential for activation of MAP kinase activity. They can be grouped according to X in the TXY motif: glutamic acid in ERKs, proline in J Nks, and glycine in CSBP/p38/Mpk2s (4). The J NK and CSBP protein kinases are activated in response to inflammatory agents and environmental stress, whereas ERKs are stimulated primarily by growth factors and tumor promoters (4, 5). Further functional separation of these kinases is illustrated by their distinct activators; SEK or MKKs for J Nks/CSBPs and MEKs for ERKs (6–9). While there is some overlap in the activating enzymes and in vitro substrates for J Nks and CSBPs, there are also some distinctions (8, 9). Since there has been no comparative study, it is not known if there are any differences between CSBP1 and -2.

The CSBPs are human homologues of Saccharomyces cerevisiae Hog1 (10), a MAP kinase required for growth under high osmolarity and other environmental stress (11) suggesting that stress response pathway may be conserved across species, and that CSBP might be an active kinase in yeast. In support of this conservation, it has been reported that murine p38 can partially complement a hog1 deletion in yeast (3). Neither CSBP1 nor CSBP2 has been tested in this system, nor have they been individually compared. Furthermore, most studies of p38 in mammalian cells have used polyonal antipeptide antibodies which would be expected to immunoprecipitate both CSBP1 and CSBP2 (11, 12). As a means to provide rapid structure-function information on CSBP1 and CSBP2, we used site-directed mutagenesis to alter key residues and analyzed the expressed mutant proteins for functional complementation in yeast, as well as for tyrosine phosphorylation and kinase activity. We have found that CSBP1 and CSBP2 differ in their ability to complement hog1Δ and are differentially activated by salt.

MATERIALS AND METHODS

Expression of Recombinant CSBPs in S. cerevisiae—A construct for the constitutive expression of CSBP2 was created as follows. An 898-bp BglII-XhoI fragment (3′-polylinker) from pBS-CSBP2 (1) was subcloned into the BglII and SalI polylinker of p137NBU, destroying the XhoI site. p137NBU is a modified version of p138NBU (13) where the TRP1-selectable marker and the copper-inducible CUP1 promoter were replaced by the URA3-selectable marker (p138NBU) and the strong constitutive TDH3 promoter (p137NBU). The plasmid contains a partial 2μ sequence for maintenance at high copy number. The resulting plasmid was then digested with Xhol (polylinker site) and BglII and ligated with a 314-bp Xhol-BglII PCR fragment engineered to contain a Xhol site at the initiating methionine of CSBP2 creating p137NBU-CSBP2. The amino terminus of CSBP2 was re-engineered to contain the 1IB FLAG (Eastman Kodak) sequence to aid in immunoprecipitation. A BamHl-PstI linker (5′-GATCCTACATGAGATTATAAAGATGACGA-TGAAATCTCAGGAAAGCCGCGTTCGGTGTCGCTGCA-3′ and its complement, synthesized to contain sticky ends) was ligated into the unique BamHI and PstI sites of pBS (Stratagene). The resulting plasmid was digested with AgeI and PstI and ligated to the 1.7 kb BsrFI-PstI fragment of the CSBP1(1) DNA. The NarI-KpnI region of this plasmid was replaced with the 1.1-kb NarI-KpnI (3′-polylinker site of pBS) of the CSBP2 (DNA, creating pBS/FLAG-CSBP2. The ORF-encoding FLAG-CSBP2 was then isolated as a 1.6-kb XhoI fragment and subcloned into the same site of p138NBU, creating p138NBU/FLAG-CSBP2. The FLAG-CSBP2 expression is driven by the copper-inducible CUP1 promoter. A similar construct was created for CSBP1 by replacing a 407-bp PvuII-BstXI fragment of p138NBU/FLAG-CSBP2 with a fragment with the same sites from the original CSBP1 clone; this resulted in switching the alternatively spliced region. For mutagenesis, the 1.6-kb BamHI-KpnI fragment of this plasmid was subcloned into the same sites of pALTER1 and site-directed mutagenesis

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The abbreviations used are: MAP, mitogen-activated protein; J NK, c-jun amino-terminal kinase; ERK, extracellular signal-regulated kinase; HOg, high osmolarity glycerol response; MBP, myelin basic protein; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s); bp, base pair(s).
was performed using the Altered Sites system (Promega, Madison, WI). The DNA sequence was altered to encode the amino acid changes indicated (for the mutants) and/or to add a 5’ XhoI site in the polynucleotide.

Plasmids were introduced into S. cerevisiae strains YPH499 (MAT a, ura3-52, lys2-801 his3D100 trp1-101 his3A200, ade2-101 his3A200, leu2Δ1), YPH102 (MAT a, ura3-52, leu2Δ1, his3A200 lys2-801 his3A200, ade2-101 trp1-101 his3A200, leu2Δ1), JBY10 (YPH 499 + his3A200, trp1-101, or MAY1 (YPH102 + PBS2::LEU2)](10) using the lithium acetate method (15). Ura⁻ pbs2Δ::LEU2 plus or minus 0.9M KCl. Plates were incubated at 30°C for 5 days. Phenylmethylsulfonylfluoride, 20mM NaF, and 2mM Na3VO4. Extracts indicated (for the mutants) and/or to add a 5’ XhoI site in the polynucleotide.

Complementation Assay—For complementation studies, cells were grown in SC-Ura as above, harvested, resuspended at 1 × 10⁸ cells/ml, and a 1:10 dilution series of 5-µl spots was plated on SC-Ura, 50 µM CuSO4, plus or minus 0.9 µM KCl. Plates were incubated at 30°C for 5 days.

Kinase Assay—The CSBPs were immunoprecipitated from yeast cell lysates with an anti-FLAG antibody M2 conjugated to agarose (IBI, Kodak). After washing the agarose beads with lysis buffer three times, an immune complex kinase assay was performed in a 20-µl reaction containing 25 µl Hapes, pH 7.4, 10 mM MgCl2, 20 µM [γ-32P]ATP (10 Ci/mmol), and 5 µg of myelin basic protein (MBP, Life Technologies, Inc.). After 10 min at 30°C, SDS-PAGE buffer was added and samples were boiled for 2 min, and phosphorylated MBP was resolved by SDS-PAGE and visualized by autoradiography. The radioactivity in each band was quantitated in a PhosphorImager (Molecular Dynamics).

Western Blotting—Immunoprecipitated CSBPs were resolved in SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, immunoblotted with 1 µg/ml antiphosphotyrosine monoclonal antibody PY69 (Santa Cruz Biotechnology), and developed with ECL (Amersham). Blots were stripped in 62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 10 mM β-mercaptoethanol for 30 min at 50°C, washed extensively in phosphate-buffered saline containing 0.1% Tween 20, and then reprobed with a polyclonal rabbit anti-CSBP2 antibody (1:2000).

RESULTS

Complementation of the hog1 Phenotype by CSBPs—The human MAP kinases, CSBP1 and CSBP2, are most homologous to the HOG1 protein of yeast. p38, the murine homologue of human CSBP2, was reported to partially complement the hog1Δ phenotype, i.e. the inability to grow on high osmolarity media, when expressed under the control of the strong constitutive TDI promoter (3). We were, however, unable to obtain any transformants when we attempted to express CSBP2 under control of the constitutive TDI promoter, using a high copy number plasmid (Fig. 1). This suggested that CSBP2 is toxic to yeast cells when constitutively expressed. This toxicity was dependent upon the presence of active Pbs2, the activating kinase of HOG1. The toxicity, therefore, was due to the kinase activity of CSBP2, since transformants could be obtained using a pbs2Δ strain (Fig. 1). In contrast, we were able to express both CSBPs under the control of the copper-inducible CUP1 promoter and used this system to test the ability of induced CSBP1 and -2 to complement the yeast hog1Δ phenotype. As shown in Fig. 2, expression of both Hog1 and CSBP1 were able to complement the hog1Δ phenotype under high osmolarity conditions (0.9 µM KCl), although CSBP1 was much weaker at complementation than Hog1. We tested two independent clones of CSBP2 generated by polymerase chain reaction. Only one clone exhibited complementation of the hog1Δ phenotype. Sequence analysis revealed the noncomplementing clone to be the wild-type CSBP2, whereas the complementing clone turned out to have a single point mutation that resulted in an alanine to valine substitution at position 34 (referred to as CSBP2(A34V)). This mutation lies in the ATP-binding motif in domain I of CSBP and in all MAP kinases. This result was confirmed by generating the alanine 34 to valine mutation in wild-type CSBP2 by site-directed mutagenesis.

Activation of CSBP by Pbs2-dependent—Functional complementation by the CSBPs suggests that the yeast HOG MAP kinase pathway is able to activate at least CSBP1 and the A34V mutant of CSBP2. To further explore the relationship between Pbs2 and the CSBPs, the activity and tyrosine phosphorylation of the CSBPs were examined in pbs2Δ and wild-type yeast strains. Both the tyrosine phosphorylation and the activity of CSBP1, CSBP2, and CSBP(A34V) were found to be Pbs2-dependent (Fig. 3, left panel, lanes 2, 3, and 10). No tyrosine phosphorylation or little or no kinase activity of CSBP was seen in the absence of Pbs2 (Fig. 3, right panel, lanes 2, 3, and 10). This suggests that Pbs2 can phosphorylate and activate both CSBP isoforms.

Thr180 and Tyr182 Are Required for Activation by Pbs2—For the MAP kinases, it is known that their activity depends upon dual phosphorylation on both Thr and Tyr in the regulatory TXY motif (4). We next tested whether the Thr180 and Tyr182 residues in the putative regulatory domain of CSBP are essential for Pbs2-mediated phosphorylation and subsequent activation. Mutation of either Thr180 (T180E, T180A) or Tyr182 (Y182F) abrogated the Pbs2-mediated activation of CSBP2, as did the double mutation (T180E/Y182E) (Fig. 3, lanes 6–9), as illustrated by the kinase assay. However, distinct Tyrine phosphorylation was seen in the case of T180E and, to a much lower extent, in the case of T180A. This suggests that the tyrosine phosphorylation alone is not sufficient for kinase activity, and that both Thr and Tyr residues need to be phosphorylated, similar to what has been observed with the ERKs (5). As expected, substitution of the catalytic residues Lys53 with Arg (K53R, Fig. 3, lane 4) and Asp168 to Ala (D168A, Fig. 3, lane

**FIG. 2.** Complementation of *Saccharomyces cerevisiae* hog1Δ by CSBP1, CSBP2, CSBP2(A34V), and Hog1 under normal (KCI) or high salt (+KCl) conditions. All cDNAs were expressed under the control of the copper-inducible CUP1 promoter. Spots assay (increasing 1:10 dilution from left to right) were performed as described under “Materials and Methods.” Growth after 5 days is shown.

**FIG. 3.** Characterization of CSBP1 and -2 in Yeast

Complementation of the *Saccharomyces cerevisiae* hog1Δ (Y182F) abrogated the Pbs2-mediated activation of CSBP2, as did the double mutation (T180E/Y182E) (Fig. 3, lanes 6–9), as illustrated by the kinase assay. However, distinct Tyrine phosphorylation was seen in the case of T180E and, to a much lower extent, in the case of T180A. This suggests that the tyrosine phosphorylation alone is not sufficient for kinase activity, and that both Thr and Tyr residues need to be phosphorylated, similar to what has been observed with the ERKs (5). As expected, substitution of the catalytic residues Lys53 with Arg (K53R, Fig. 3, lane 4) and Asp168 to Ala (D168A, Fig. 3, lane
Fig. 3. Kinase activity and immunoblotting of CSBPs and various CSBP2 mutants expressed in PBS2+ and pbs2- yeast cells. Kinase assays were performed using myelin basic protein (MBP) as the substrate. Western blotting was carried out using a monoclonal anti-phosphotyrosine (Anti-PY) and a polyclonal antibody generated against recombinant CSBP2 (Anti-CSBP). The first lane in each panel represents results from yeast containing control vector.

11) also resulted in a loss of kinase activity. However, in both cases, tyrosine phosphorylation was seen (Fig. 3, lanes 4 and 11). An unrelated mutation (T175A) had no effect on either the kinase activity or tyrosine phosphorylation, and the mutant protein behaved identically with wild-type CSBP2 (Fig. 3, lane 5).

CSBP2(A34V) Mutant Exhibits Reduced Kinase Activity Compared to the Wild-type CSBPs—Because the CSBP2(A34V) expressed in hog1Δ yeast would be expected to have reduced kinase activity, we suspected that complementation may be linked to the varying capacity of CSBP kinase to be activated in response to osmolarity changes. We examined the effect of salt treatment by analyzing CSBPs for Tyr phosphorylation and kinase activity. However, the expression levels of CSBP1, CSBP2, and CSBP2(A34V) differed quite considerably (see Fig. 3), making a direct comparison with respect to kinase activity or tyrosine phosphorylation difficult. Therefore, we first adjusted the amount of protein in each yeast lysate such that equal amounts of CSBP1, CSBP2, and CSBP2(A34V) would be analyzed. As shown in Fig. 4A, the basal kinase level of CSBP1 and CSBP2(A34V) is 3-fold or more lower than that of CSBP2 (lanes 3, 5, and 7), and the kinase activity of both CSBP1 and CSBP2(A34V), but not CSBP2, could be further activated by salt (Fig. 4, lanes 3–8). As expected, the kinase activity correlated with the increased Tyr phosphorylation of these proteins. The induced kinase activity of CSBP1 and -2 are roughly the same and 3-fold higher than that of CSBP2(A34V) (Fig. 4B). While salt distinctly affects both tyrosine phosphorylation and kinase activity of CSBP2, it did not have any effect on CSBP protein expression. Reduction of the expression level by expression of CSBP2 on a yeast centromere-based single copy plasmid did not lead to complementation or a restoration of the induction of tyrosine phosphorylation or kinase activity with salt (data not shown).

Mutations That Reduce Basal CSBP Kinase Activity Restore Its Salt-induced Tyrosine Phosphorylation—if a high basal kinase activity renders CSBP2 unresponsive to salt, then a reduction or elimination of kinase activity should result in the restoration of the salt response. This hypothesis is partially fulfilled by CSBP2(A34V) which exhibits reduced kinase activity and is regulated by salt (Fig. 4). We next tested if the CSBP2 mutants D168A, K53R, and T180E could be induced to be tyrosine-phosphorylated in response to salt. Consistent with this hypothesis, all three mutants were able to respond to salt as measured by an increase in tyrosine phosphorylation (Fig. 4, lanes 9–14) and yet were completely devoid of any kinase activity. Even the T180E mutant did not show any kinase activity, even in presence of salt, suggesting that the threonine phosphorylation is absolutely required for kinase activity (Fig. 4, lane 11). This suggestion was consistent with the observation that a high basal kinase activity renders CSBP2 unresponsive to salt, suggesting that the threonine phosphorylation is absolutely required for kinase activity.
kinase activity of CSBP1, both of which were dependent on the presence of the activating kinase Pbs2. In contrast, CSBP2 expressed similarly was constitutively active and tyrosine-phosphorylated both in the presence and absence of hyperosmolarity despite its failure to complement. Both activity and tyrosine phosphorylation, however, were dependent on Pbs2, suggesting that constitutive expression of active CSBP2 may be desensitizing the host toward a further response to high salt. In support of this, mutants of CSBP2 with reduced (A34V) or absent (K53R,D168A) kinase activity had restored salt responsiveness with respect to tyrosine phosphorylation. Furthermore, reduction of the intrinsic kinase activity of CSBP2(A34V) led to a mutant which was now able to complement the hog1Δ phenotype. However, catalytically inactive CSBP2 mutants (T180E, T180A, T180E/Y182E, Y182F, K53R, and D168A) failed to complement the hog1Δ phenotype. The ability to complement in yeast, therefore, is in part a function of the absolute kinase activity of the expressed CSBP prior to salt treatment.

Differences in basal kinase activity may also explain the different complementation potential seen with CSBP1 and CSBP2. We have shown that this is not due to differences in expression levels (Fig. 4); rather, it is more likely to be due to differences in the extent of activation by Pbs2, sensitivity to phosphatases, or differences in substrate specificity. These differences may also explain the ability of the murine CSBP2 homologue, p38, which differs in only two amino acids from the human protein, to complement hog1Δ, although we cannot rule out differences in expression levels in this case (3). However, we did show that expression of human CSBP2 using the same constitutive promoter was toxic to yeast. Differences in kinase activity may also be the basis for the ability of JNK1, but not JNK2, to complement hog1Δ. JNK1 is known to have a 10-fold lower activity toward one of its known substrates, c-Jun (18).

These results emphasize that while some elements of the stress-activated kinase cascade have been conserved between yeast and mammals, others may not. The results suggest that for the stress-activated pathway to function with heterologous kinases in yeast, the basal kinase activity must be tightly regulated to keep it below a certain threshold. Above that threshold, it leads to a desensitization of the host toward extracellular stimulation, presumably due to interference with some upstream component(s) of the signaling pathway leading to CSBP activation. Since CSBP2 and CSBP2(A34V) are not likely to differ in recognizing the substrate(s), and yet only CSBP2(A34V) complements, it is likely that the high basal kinase activity of CSBP2 affects more than the pathway leading directly to CSBP2, and perhaps includes other pathway(s) that are required for complementation. Whether this desensitization is a general feature of stress-activated kinases or an artifact of heterologous expression is not clear.

Hog1 expressed in the same vector system was able to complement as well as the endogenous Hog1. Furthermore, differences in substrate specificity may also be important as evidenced by the failure of Hog1 to phosphorylate the CSBP substrate MBP.

The expression of active CSBP2 in yeast also allowed us to dissect the role of the TXY regulatory loop in CSBP2. As expected from other MAP kinases and previous reports with p38 (4, 11, 12), mutations in either Thr180 or Tyr182 resulted in loss of kinase activity. Unlike ERK2, however, mutation of Thr180 to Glu, which can sometimes mimic a phosphorylated threonine (17), did not lead to a partially active kinase, even when it was phosphorylated on Tyr182 in response to salt. This suggests that phosphorylation on both Thr180 and Tyr182 is absolutely required for activity. Furthermore, it suggests that tyrosine phosphorylation can occur in the absence of threonine phosphorylation.

The differing ability of CSBP1 and CSBP2 to complement the hog1Δ phenotype suggests that these two kinases may have different properties and roles in mammalian cells as well. Use of yeast-expressed CSBP may enable us to further understand differences between the two in parallel with further work to dissect the role of these kinases in mammalian cells.

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