The Serine/Threonine Kinase Cmk2 Is Required for Oxidative Stress Response in Fission Yeast*

Maribel Sánchez-Piris‡§, Francesc Posas¶, Vicenç Alemany‡, Ingeborg Winge‡‡, Elena Hidalgo¶, Oriol Bachs‡, and Rosa Aligue‡‡**

From the *Department of Cell Biology, Institut de Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Universitat de Barcelona, E-08036 Barcelona and ¶Cell Signalling Unit, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra (UPF), E-08003 Barcelona, Spain

Cmk2, a fission yeast Ser/Thr protein kinase homologous to mammalian calmodulin kinases, is essential for oxidative stress response. Cells lacking cmk2 gene were specifically sensitive to oxidative stress conditions. Upon stress, Cmk2 was phosphorylated in vivo, and this phosphorylation was dependent on the stress-activated MAPK Sty1/Spc1. Co-precipitation assays demonstrated that Cmk2 binds Sty1. Furthermore, in vivo or in vitro activated Sty1 was able to phosphorylate Cmk2, and the phosphorylation occurred at the C-terminal regulatory domain at Thr-411. Cell lethality caused by overexpression of Wis1 MAPK kinase was abolished by deletion of cmk2 or by mutation of Thr-411 of Cmk2. Taken together, our data suggest that Cmk2 acts downstream of Sty1 and is an essential kinase for oxidative stress responses.

Stress-activated protein kinases (SAPKs)1 are a conserved subfamily of MAPKs responsive to diverse environmental stress stimuli rather than to growth factors or other mitogenic stimuli (1, 2). SAPKs in mammals and the fission yeast *Schizosaccharomyces pombe* are activated by various forms of stress (for review, see Ref. 3). In *S. pombe*, the SAPK Sty1/Spc1/Phl1 is activated by high osmolarity, oxidative stress, and heat shock (4–6). Sty1 is activated through phosphorylation by the MAPK kinase (MEK), Wis1. Osmostress and oxidative stress are transmitted to Wis1 MEK by two MEK kinases (MEKKs), and the phosphorylation occurred at the C-terminal regulatory domain at Thr-411. Cell lethality caused by overexpression of Wis1 MAPK kinase was abolished by deletion of cmk2 or by mutation of Thr-411 of Cmk2. Taken together, our data suggest that Cmk2 acts downstream of Sty1 and is an essential kinase for oxidative stress responses.

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The activation of Sty1 in response to stress stimulates gene expression via the Atf1 and Pap1 transcription factors, homologues of human ATF2 and c-Jun, respectively (19–25). Atf1 is phosphorylated by Sty1 in vivo and in vitro (22), and both Δsty1 and Δatf1 mutants are defective in osmotic stress (22, 26). Pap1 and Atf1 are required for the induction of *ctl1* and other genes in response to oxidative stress. *Ctl1* encodes cytoplasmic catalase, which decomposes hydrogen peroxide (H₂O₂) and protects cells from oxidative stress (27). Oxidative stress brings accumulation of Pap1 to the nucleus in a Sty1-dependent manner (24). However, the reason why Sty1 is required for nuclear translocation of Pap1 is not known, since Pap1 is not a substrate of Sty1 (26).

Although the Atf1 and Pap1 transcription factors are key components of the fission yeast SAPK pathway, they are not the only targets for Sty1. Cells lacking Sty1 are delayed in the timing of mitotic initiation, whereas cells lacking both Atf1 and Pap1 are not (28). Here, we describe Cmk2 as a component of the fission yeast SAPK pathway. *cmk2* was isolated by its sequence similarity to the yeast and mammalian calmodulin kinases.2 It has a high degree of homology to budding yeast RCK2, previously isolated by virtue of its sequence similarity to mammalian calmodulin kinases (30). It has also been described as a suppressor of fission yeast checkpoint mutants (31) and a substrate of Hog1, the MAPK in budding yeast responsive only to osmolarity stress (32).

MATERIALS AND METHODS

Fission Yeast Strains, Media, and General Techniques—The strains used in this study are listed in Table I. The rich medium used was YES, and the selective medium was Edinburgh synthetic minimal medium supplemented with 225 mg/liter of the required amino acids (33). Yeast growth was at 30 °C. Standard techniques for fission yeast genetics were used following Moreno et al. (33). Plasmid DNA was transformed by lithium acetate as described elsewhere (33). Standard molecular biology techniques were applied (34). Restriction enzymes were used as recommended by their suppliers (New England Biolabs or MBI Fermentas). Recovery of DNA fragments from agarose gels was performed with a CLONTECH Advantage PCR pure kit, following the manufacturer instructions.

Drug Sensitivity Assay—The *S. pombe* strains to be assayed for sensitivity to various toxic compounds were first grown on fresh YES agar plates for 48 h. The plates were then flooded with the compound of interest at appropriate concentrations, and the resistance of the cultures to the compound was assessed after 24 h of incubation at 30 °C.

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1 The abbreviations used are: SAPK, stress-activated protein kinase; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAP kinase/extracellular signal-regulated kinase; MEKK, MEK kinase; HA, hemagglutinin; GST, glutathione S-transferase; YES, yeast extract medium.
plates, after which the cells were streaked on YES plates containing the specific compound at the indicated concentration (sodium arsenite 0.4 mM, calcium chloride 300 mM, hydrogen peroxide 0.6 mM) and incubated at 30 °C for 3 days.

cmk2 Gene Disruption—The cmk2::ura4" disruptant mutant was generated by inserting a 1.8-kilobase fragment encoding the ura4" gene between the BglII-HindIII sites of cmk2 from plasmid pVA21 (plasmid pBluescript containing the chromosomal PstI-XhoI fragment from cmk2).2

The ura4" gene was amplified from pURA4 plasmid using VAv6 and T7 oligonucleotides. VA6 is essentially the standard T3 promoter oligonucleotide with an added BglII site (underlined), gccacaagacttaacctcactaaag. The amplified fragment was digested with BglII and HindIII and ligated to pVA21, creating plasmid pVA24. The fragment PstI-BamHI isolated from plasmid pVA24 was used to transform the wild-type strain. Stable ura4− transformants were confirmed by PCR and Southern blotting.

Chromosomal Integration—To tag genomic cmk2 with two copies of the HA epitope and hexahistidine, plasmid pREP1-cmk2 was digested with PstI and SacI, releasing a ~3-kilobase fragment that contained the full nmt1-cmk2 expression cassette and was cloned into p Bluescript SK− (Stratagene) digested with the same enzymes. The resulting plasmid was digested with HindIII, which released the full nmt1 promoter and the first 215 amino acids of cmk2, and ligated to leu2" from pREP1 plasmid digested with HindIII. The resulting construction was linearized with SacI, transformed into the appropriate S. pombe strains (See Table I), and selected for Leu− transformants. Plates were replicated to obtain two YES media for selection on Edinburgh synthetic minimal medium plates lacking leucine. Colonies that grew on selective media were screened for HA integration by immunoblotting with a specific anti-hemagglutinin (anti-HA) epitope antibody.

Cells containing cmk2-His6Ha did not show any phenotype compared with wild-type cells (i.e. oxidative stress). To replace the endogenous cmk2 gene by a cmk2 with a point mutation on Thr-411 to Ala, the cmk2-T411A from pGEX-KG-cmk2T411A plasmid (described in next paragraph) was digested with SnaBI and NotI, releasing a fragment that contained the cmk2T411A, and ligated to plasmid pBluescript (described in the previous paragraph) with the same enzymes, and the construction was integrated as described above.

cmk2 Truncations and Point Mutation—The bacterial expression plasmid pGEX-KG allows the expression of GST-fused proteins in Echerichia coli. cmk2 from pREP1-cmk2 plasmid was digested with NdeI and NotI and cloned into the pGEX-KG plasmid. Mutagenesis of cmk2 to create cmk2K94A, cmk2T411A, cmk2K94AT411A was achieved by PCR using overlapping oligonucleotides at the site of the mutation and verified by DNA sequencing (QuikChange site-directed mutagenesis kit, Stratagene). The truncation of cmk2 was made by PCR and cloned into pGEX-KG with NdeI/NotI. Truncation cmk2C23 (341 amino acids) was made using primers Cmk2-5′, cacacacacacatgtgcatacctaggggtt, and Cmk2-3′, cacacacacacacacacgcagcagagtcgtgt. Truncation cmk2C48 (402 amino acids) was made using primers Cmk2-5′ and Cmk2C36, cacacacacacacacacacacgcagcagagtcgtgt. Truncation cmk2C87 (102 amino acids) was made using primers Cmk2C37, cacacacacacacacacacagcagagtcgtgt. cmk2−3′, cacacacacacacacacacgcagcagagtcgtgt.

Expression and Purification of Epitope-tagged Proteins—The GST fusion proteins were expressed in E. coli, and pellets were lysed in NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 20 μg/ml aprotinin, 40 μg/ml leupeptin, 20 μg/ml pepstatin A, and 1 mM phenylmethylsulfonfyl fluoride). GST proteins were purified by affinity chromatography on glutathione Sepharose 4B (Sigma-Aldrich, Inter-Science), and after subsequent washing in NETN buffer, they were eluted with the elution buffer (50 mM Tris, pH 8.5, 100 mM NaCl, 10 mM glutathione, 2 mM diithiothreitol).

The Sty1 protein was purified from cells expressing Sty1 fused to an HA peptide epitope and a His6 in the C-terminal tail. Pelleted cells were lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% IGEVA (octylphenoxypolyethoxyethanol), 0.5% Triton X-100, 10% glycerol, 50 mM NaF, 1 mM phenylmethylsulfonfyl fluoride, 1 mM NaVO4, 5 μg/ml aprotinin, 5 μg/ml leupeptin), and purification was carried out by immunoprecipitation with anti-HA monoclonal antibody 12CA5 (Roche Molecular Biochemicals) and protein A-Sepharose beads (Immunus Purine Immobilized Protein A, Pierce). Beads were washed extensively with lysis buffer and resuspended in kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 2 mM diithiothreitol).

In Vivo Coprecipitation Assay—Wild-type cells were transformed with pREP41-sty1−myc or pREP42-cmk2-HA6His or both plasmids. Cells were grown in minimal medium for 20 h in the absence of thiamine. Pelleted cells were lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.1% Triton X-100, 10% glycerol, 1 mM sodium arsenite, and 1 mM phenylmethylsulfonfyl fluoride, 1 mM NaVO4, 5 μg/ml aprotinin, 5 μg/ml leupeptin) and purified by immunoprecipitation with anti-HA monoclonal antibody 12CA5 (Roche Molecular Biochemicals) and protein A-Sepharose beads or with anti-Myc and protein G-Sepharose (Sigma). Beads were washed with lysis buffer three times and resuspended with 35 μl of 4 × SDS loading buffer. Proteins were resolved by SDS-PAGE, and co-purification was monitored by Western blotting with anti-HA monoclonal antibody 12CA5 (Roche Molecular Biochemicals) or anti-Myc.

In Vivo Kinase Assay—Phosphorylation of Cmk2 protein was monitored by Western blot analysis of HA-tagged Cmk2. The MB260, MB269, and MB264 strains (Table I) were grown in the presence of 1 mM sodium arsenite for 4, 15, and 30 min to induce Sty1 activity by vortexing with glass beads in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM imidazole, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na2VO4, 10 mM NaF, 5 μg/ml aprotinin, 5 μg/ml leupeptin). The samples (15 μg protein/ml) were dephosphorylated by treatment with λ phosphatase (400 units/ml, Calbiochem) for 60 min at 30 °C. Cell extracts containing 100 μg of total protein were run on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Immobilon, Micro- pore). Membranes were probed with a monoclonal antibody to the HA epitope (12CA5, Roche Molecular Biochemicals). Phosphorylated and activated Sty1 protein was detected by Western blotting with anti-phospho-p38 MAPK antibody (New England Biolabs).

In Vitro Kinase Assay—Phosphorylation of Cmk2 by Sty1 activated in vitro. The Sty1-HA6His protein was purified by immunoprecipitation with anti-HA monoclonal antibody 12CA5 (Roche Molecular Biochemicals) from yeast cells treated or not with 1 mM H2O2 for 10 min in wild type or Δwis1 background. 5 μg of GST-Cmk2KA or 5 μg of the different Cmk2 forms fused to GST protein purified from E. coli were added to the purified Sty1-HA6His protein activated in vivo together with 20 μM ATP and or [γ-32P]ATP (0.1 μCi/μl). The mixture was then incubated for 20 min at 30 °C, and the reactions were terminated by addition of SDS loading buffer. Labeled proteins were resolved by SDS-PAGE and detected by autoradiography using dried gels.
S. pombe ura4 cmk2 examine the cellular function of Cmk2, gene disruption of does not bind calmodulin. present in Cmk2. Rck2p/Clk1p has also been described in bud-chk1 (31). They have a long glycine-rich insert between conserved domains VIb and VII of protein kinases, which is also present in Cmk2. Rck2p/Clk1p has also been described in budding yeast as a calmodulin kinase-like protein (30), although it does not bind calmodulin.

Cells Lacking cmk2 Are Sensitive to Oxidative Stress—To examine the cellular function of Cmk2, gene disruption of cmk2 was performed. A construct in which cmk2 was replaced by the S. pombe ura4+ gene was generated (see “Materials and Methods”). This construct was used to replace the genomic copy of cmk2 in a ura4-D18 strain. The correct integration of the construct in the resulting strain was confirmed by Southern hybridization analysis (data not shown). The cmk2::ura4+ strain was viable, and it presented no morphological abnormalities.

To examine the role of Cmk2 in the stress response, Δcmk2 cells or cells lacking various components of the Sty1 MAP kinase pathway were grown on rich medium in the presence of osmotic (300 mM NaCl) or oxidative stress (0.6 mM H2O2 or 0.4 mM sodium arsenite). We would like to highlight the fact that, like cells lacking either sty1 or pop1, Δcmk2 cells did not grow in conditions of oxidative stress caused by 0.6 mM hydrogen peroxide or 0.4 mM sodium arsenite (Fig. 1, B and C). In contrast to Δsty1 and Δatf1 cells, Δcmk2 cells proliferated in the presence of 300 mM CaCl2 (Fig. 1A) or 1 mM KCl (data not shown). Thus, cmk2 is required for oxidative stress response.

FIG. 1. Cells lacking cmk2 are sensitive to oxidative stress. Wild-type (wt), cmk2::ura4 (Δcmk2), sty1::ura4 (Δsty1), pop1::ura4 (Δpop1), per1::ura4 (Δper1), and atf1::ura4 (Δatf1) cells were grown on YES medium at 30 °C and then streaked to the same medium containing 300 mM CaCl2 (A), 0.6 mM H2O2 (B), or 0.4 mM sodium arsenite (C) and incubated for 3 days.

three exons encoding a putative Ser-Thr protein kinase of 504 amino acids with a predicted molecular mass of 57 kDa. A computer-based amino acid sequence homology search for known proteins revealed that the greatest degree of amino acid sequence identity was shared with budding yeast RCK1 and RCK2 (CLK1) kinases (42 and 43% identity, respectively) and to calmodulin-dependent kinases (CaMKs) (40% identity to CaMK1 and 35% to rat CaMKII). RCK1 and RCK2 were first described as suppressors of radiation sensitivity of fission yeast CaMKI and 35% to rat CaMKII. RCK2 (CLK1) kinases (42 and 43% identity, respectively) and the previous result shows that Cmk2 is a component of the oxidative stress response. Because Sty1 is rapidly phosphorylated and activated by Wis1 after oxidative stress, we next determined whether Cmk2 is also phosphorylated after oxidative stress. Wild-type and Δsty1 strains were subjected to a brief oxidative stress, and endogenous expression of HA-tagged Cmk2 protein was monitored by Western blotting using anti-HA antibodies.

Under oxidative stress, Cmk2 showed slower mobility bands in addition to the main Cmk2 band (Fig. 2A, upper panel). The slow mobility bands of Cmk2 observed at 15 and 30 min after oxidative stress were paralleled by the activation of Sty1 MAPK, as shown by Western blotting of the same samples using monoclonal antibody against phosphorylated p38 SAPK (human Sty1 homologue) (Fig. 2A, lower panel). The slower migrating bands of Cmk2 appeared due to phosphorylation, since the altered mobility pattern was reversed on treating extracts from stressed cells with λ phosphatase (Fig. 2A, upper panel). In addition, when Cmk2 phosphorylation was studied upon oxidative stress in a mutant deficient in the SAPK pathway, Δsty1 strain, no slow mobility bands of Cmk2 were observed after oxidative stress compared with the wild-type strain (Fig. 2A). Therefore, Cmk2 is phosphorylated after oxidative stress in a Sty1-dependent manner.

Cmk2 Is Phosphorylated in Vivo after Oxidative Stress in a SAPK-dependent Manner—The previous result shows that Cmk2 is a component of the oxidative stress response. Because Sty1 is rapidly phosphorylated and activated by Wis1 after oxidative stress, we next determined whether Cmk2 is also phosphorylated after oxidative stress. Wild-type and Δsty1 strains were subjected to a brief oxidative stress, and endogenous expression of HA-tagged Cmk2 protein was monitored by Western blotting using anti-HA antibodies.

FIG. 2. In vivo phosphorylation of Cmk2 during oxidative stress. A, wild-type (wt) and Δsty1 cells containing cmk2 HA-tagged (cmk2-HA6His) were grown and exposed to 1 mM sodium arsenite. Cells were taken at various intervals of oxidative stress, and cell extracts were prepared to detect Cmk2 by immunoblot analysis using anti-HA monoclonal antibody. The Cmk2 phosphorylation state was monitored by the appearance of slow mobility bands of the protein in wild-type cells (upper panel, first three lanes, 0°, 15°, and 30°) and in sty1-deleted cells (upper panel, lane marked Δsty1). Cell extract from 15-min stressed cells was treated with λ phosphatase (upper panel, lane marked 15° + λ), and Cmk2 was detected as described before. Activation of Sty1 by phosphorylation was detected from the same extracts by immunoblot analysis using anti-phospho p38 antibody (lower panel). B, wild-type cells containing a point mutation in Thr-411 to Ala of Cmk2 were subjected to 1 mM sodium arsenite. Cells were taken at various intervals of stress treatment, and cell extracts were prepared to detect Cmk2 as in A.
of Sty1-9myc in the precipitates was revealed with an anti-Myc antibody (Fig. 3, Cmk2 IP, upper panel). As shown in Fig. 3 (Cmk2 IP, lane 3), Cmk2 coprecipitated Sty1. Conversely, when Sty1-9myc was immunoprecipitated using monoclonal antibodies against Myc (Fig. 3, Sty1 IP, lower panel), the presence of Cmk2-HA in the precipitates was determined with specific anti-HA antibodies (Fig. 3, Sty1 IP, upper panel). Cmk2 co-precipitated with Sty1-9myc was immunoprecipitated from cells extracts (Cmk2 IP, lower panel) and the presence of Sty1-9myc in the precipitates was detected (Sty1 IP, upper panel).

Cmk2 Is Phosphorylated by in Vivo and in Vitro Activated Sty1 at Thr-411—We then tested whether activated Sty1 was able to phosphorylate Cmk2. Wild-type cells or wis1-deleted cells expressing HA-tagged Sty1 were exposed to oxidative stress for 10 min, and Sty1-HA was then immunoprecipitated by using monoclonal anti-HA antibodies and protein A-Sepharose beads. The activation of Sty1-HA was assessed by Western blotting using a monoclonal antibody against phosphorylated p38 SAPK (Fig. 4A). Immunoprecipitated Sty1 was incubated in the presence of [γ-32P]ATP and a catalytically inactive GST-Cmk2, named GST-Cmk2KA, which contains Lys-94 mutated to Ala. The use of a kinase-deficient Cmk2 was necessary to avoid autophosphorylation. As shown in Fig. 4B, Cmk2KA was significantly phosphorylated when the protein was incubated with activated Sty1. In contrast, no Sty1-dependent phosphorylation was detected when the protein was incubated with inactive Sty1 from wis1-deleted cells.

To map the phosphorylation site for Sty1 in Cmk2, we created several truncated Cmk2 alleles. Cmk2 contains four putative MAP kinase phosphorylation sites at the C-terminal domain (Thr-370, Thr-393, Thr-411, Ser-436). We generated truncated versions of Cmk2 containing different domains, one containing Thr-370 and Thr-393 (Cmk2Δ3KA, Fig. 5A) and the other containing Thr-411 and Ser-436 (Cmk2Δ7, Fig. 5A). The two Cmk2Δ3KA and Cmk2Δ7 alleles together with the kinase domain (Cmk2Δ3, Fig. 5A) and full-length Cmk2 were expressed as GST-fused proteins in E. coli and subjected to in vitro phosphorylation by Sty1 activated in vivo. Cmk2-truncated forms of Cmk2 contained the Lys-94 mutated to Ala (referred to KA) to create catalytically-deficient enzymes. The C-terminal-truncated forms containing the catalytic domain of Cmk2, Cmk2Δ3KA (from amino acids 1 to 341), and Cmk2Δ4KA (from amino acids 1 to 402) were not phosphorylated by Sty1 compared with the full-length protein (Fig. 5B). In contrast, Cmk2Δ7, which contains the last 100-residues of the C terminus, was phosphorylated by Sty1 as efficiently as the full-length, suggesting that the C-terminal regulatory domain of Cmk2 is the target of Sty1 phosphorylation and that phosphorylation is restricted to Thr-411 or Ser-436.

We created a point mutation version to replace Thr-411 by Ala of the Cmk2Δ7 truncation (Cmk2Δ7T411A, Fig. 5A) and tested it for phosphorylation by Sty1. As shown in Fig. 5B, phosphorylation of Cmk2 by Sty1 was mainly abolished in the mutated version.

We then attempted to determine whether Sty1 directly phosphorylates Cmk2 using purified proteins in an in vitro kinase assay. For this purpose, Sty1 was purified as a GST fusion protein from E. coli (see “Material and Methods”). Purified Sty1 was incubated with a constitutively activated version of the S. cerevisiae Wis1-related kinase Pbs2 (32). In the first step of the reaction, Sty1 was activated by phosphorylation in the presence of purified Pbs2(EE) and ATP. Thereafter, Cmk2 fragments, purified from E. coli as GST fusion proteins, were added to the reaction together with [γ-32P]ATP. Pbs2 did not phosphorylate Cmk2 (data do not shown). As shown in Fig. 5C, lane 1, full-length Cmk2 was phosphorylated directly by the Sty1 kinase. Removal of the C terminus of the protein abolished Cmk2 phosphorylation (Fig. 5C, lanes 3 and 4). Moreover, when a C-terminal polypeptide Cmk2Δ7 (amino acids 402–505) was tested, it was phosphorylated by Sty1, suggesting that the C-terminal region was indeed the main target for Sty1 phosphorylation. Interestingly, mutation of Thr-411 to Ala completely abolished phosphorylation of the C-terminal region (Fig. 5C, lane 6). Mutation of T411A in the full-length protein dramatically reduced its phosphorylation by Sty1 (Fig. 5C, lane 2). All these results indicate that Cmk2 is directly phosphorylated by Sty1 and that phosphorylation occurs mainly in the regulatory domain of Cmk2.

We also attempted to determine whether the in vivo phosphorylation of Cmk2 in response to oxidative stress was abol-
Fission yeast Cmk2 is homologous to budding yeast Rck2, which is a direct substrate of Hog1 MAPK (32, 36). Hog1 is the Hog1 kinase in budding yeast. Although Rck2 binds and is phosphorylated by Hog1 MAPK, it is not the direct substrate of Hog1. Hog1 phosphorylates Rck2, but Rck2 phosphorylation alone is not sufficient to activate Rck2. Instead, Rck2 phosphorylation is necessary for the activation of the downstream element, Sty1. Sty1 is a direct substrate of Hog1 MAPK and is activated by hyperactivation of Wis1 MAPKK. Sty1 activation is required for the activation of downstream elements like the Sty1 MAPK and is necessary for the activation of downstream elements like the Sty1 MAPK.

We have identified Cmk2 kinase as a new component of the fission yeast oxidative stress-activated Sty1 MAP kinase response. One central observation is that Cmk2 kinase is essential for oxidative stress responses. Cmk2-deleted cells are sensitive to oxidative stress but not to osmotic, pH, or temperature stress. Furthermore, Cmk2 is a substrate of Sty1 MAPK. Cmk2 binds Sty1, which phosphorylates it in vivo and in vitro after oxidative stress activation. In addition, the biochemical and physiological level of Cmk2 phosphorylation by Sty1 depends on a single phosphorylation site. Finally, cell lethality caused by overexpression of Wis1 and the phosphorylation site mutant cmk2T411A was partially suppressed by cmk2T411A. As shown in Fig. 6, both deletion of the cmk2 gene (Fig. 6A) or mutation of the Sty1 phosphorylation site of Cmk2 (Fig. 6B) partially suppressed cell lethality caused by overexpression of Wis1, further supporting Cmk2 as a direct element of the Sty1 pathway that acts downstream of the Sty1 MAPK.

DISCUSSION

We have identified Cmk2 kinase as a new component of the fission yeast oxidative stress-activated Sty1 MAP kinase response. One central observation is that Cmk2 kinase is essential for oxidative stress responses. Cmk2-deleted cells are sensitive to oxidative stress but not to osmotic, pH, or temperature stress. Furthermore, Cmk2 is a substrate of Sty1 MAPK. Cmk2 binds Sty1, which phosphorylates it in vivo and in vitro after oxidative stress activation. In addition, the biochemical and physiological level of Cmk2 phosphorylation by Sty1 depends on a single phosphorylation site. Finally, cell lethality caused by hyperactivation of Wis1 MAPKK can be suppressed by deletion of cmk2 or by mutation of the Sty1 phosphorylation site of Cmk2. This suggests that Cmk2 and Cmk2 phosphorylation by Sty1 are necessary for the MAPK response.

Fission yeast Cmk2 is homologous to budding yeast Rck2, which is a direct substrate of Hog1 MAPK (32, 36). Hog1 is specific for the cellular response of osmotic stress in budding yeast. Although Rck2 binds and is phosphorylated by Hog1 MAPK, Rck2-deleted cells do not show increased sensitivity to osmotic stress (32, 36). In contrast to the Hog1 kinase in the budding yeast oxidative stress-activated Sty1 MAP kinase response.
ding yeast, Sty1 is activated by multiple environmental stresses including osmotic stress, heat shock, H₂O₂, UV light, certain DNA-damaging agents, and the protein synthesis inhibitor anisomycin (5, 8, 37). Like Rck2, Cmk2 is a substrate of Sty1 MAPK and in fission yeast Cmk2 is required for the cellular response to oxidative stress, as illustrated by the fact that cells lacking Cmk2 proliferate under oxidative stress.

Which is the role of Cmk2 in the oxidative stress response? In fission yeast, the Pap1 transcription factor is a target of Sty1 MAPK in oxidative stress conditions (24, 25). Pap1 is required for the induction of catalase (ctt1), thioredoxin reductase (trr1), and other genes in response to oxidative stress. In addition, oxidative stress brings about nuclear accumulation of Pap1 in a Sty1-dependent manner (24). However, Pap1 is not a substrate of the Sty1 MAPK (26). Thus, the regulation of Pap1 by Sty1 is not understood. We investigated whether Cmk2 is involved in the regulation of Pap1 transcription activation or nuclear localization, but these are not affected by Cmk2. Loss of Cmk2 did not block the nuclear accumulation of ectopically expressed green fluorescent protein-Pap1 fusion protein (data not shown). Furthermore, Pap1 was not phosphorylated in vitro by Cmk2, and we have also confirmed that Pap1 is not phosphorylated by purified in vitro activated Sty1 (data not shown). Thus, Cmk2 is not required for the induction of Pap1-dependent gene transcription or Pap1 cellular localization.

In addition to the phosphorylation of transcription factors, MAP kinases are known to activate downstream protein kinases involved in several cellular processes. These include kinases such as MAP kinase-activated protein kinases (MAPKAP-K2 and MAPKAP-K3), MAP kinase signal-integrating kinase (MNK), p38 regulated-activated kinase (PRAK), and mitogen- and stress-activated kinase (MSK) (29, 38, 40). Their activation results in the phosphorylation of both the transcrip-

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REFERENCES

1. Waskiewicz, A. J. & Cooper, J. A. (1995) Curr. Opin. Cell Biol. 7, 798–805
2. Ip, Y. T. & Davis, R. J. (1998) Curr. Opin. Cell Biol. 10, 205–219
3. Bannett, F. (1998) Microbiol. Mol. Biol. Rev. 62, 249–274
4. Millar, J. B., Buck, V. & Wilkinson, M. G. (1995) Genes Dev. 9, 2117–2130
5. Shiozaki, K. & Russell, P. (1995) Nature 378, 739–743
6. Kato, T. J., Okazaki, K., Murakami, H., Stettler, S., Fantes, P. A. & Okayama, H. (1996) FEBS Lett. 378, 207–212
7. Samejima, I., Mackie, S. & Fantes, P. A. (1997) EMBO J. 16, 6162–6170
8. Si, J., Wilkinson, M. G., Buck, V., Morgan, B. A., Makino, K. & Millar, J. B. (1997) Genes Dev. 11, 1008–1022
9. Shiozaki, K., Shiozaki, M. & Russell, P. (1997) Mol. Cell. Biol. 8, 409–419
10. Teige, M., Scheikl, E., Reiser, V., Ruis, H. & Ammerer, G. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 1022–1027
11. Degols, G., Shiozaki, K. & Russell, P. (1996) Science 271, 2807–2813
12. Ito, Y. T. & Davis, R. J. (1998) Curr. Opin. Cell Biol. 10, 205–219
13. Sambrook, J., Fritsch, E. & Maniatis, T. (1989) Molecular Cloning, A Laboratory Manual.
14. Buck, V., Quinn, J., Soto, P. T., Martin, H., Saldanha, J., Makino, K., Morgan, B. A. & Millar, J. B. (1998) Mol. Biol. Cell 9, 2325–2335
15. Wilkinson, M. G., Pino, T. S., Tournier, S., Buck, V., Martin, H., Christiansen, N., Baxter, S., Gurtler, E., Stettler, S., Fantes, P. A. & Okayama, H. (1996) Nature 384, 242–245
16. Nakagawa, C. W., Mutoh, N. & Hayashi, Y. (1995) J. Biochem. 118, 109–116
17. Wilkinson, M. G., Fino, T. S., Tournier, S., Buck, V., Martin, H., Christiansen, J., Wilkinson, D. G. & Millar, J. B. (1999) EMBO J. 18, 4210–4221
18. Toone, W. M., Kuge, S., Samuels, M., Salarthan, J., Yanagida, M. & Jones, N. (1995) EMBO J. 14, 6193–6208
19. Wilkinson, D. G. & Millar, J. B. (1999) J. Biol. Chem. 274, 29958–29968
20. Pulido, F. J., Watanabe, Y., Ohsugi, M., Iino, Y. & Yamamoto, M. (1996) Genes Cells 1, 391–408
21. Wilkinson, D. G. & Millar, J. B. (1996) Genes Dev. 10, 1169–1181
22. Buck, V., Quinn, J., Soto, P. T., Martin, H., Saldanha, J., Makino, K., Morgan, B. A. & Millar, J. B. (1998) Mol. Biol. Cell 9, 407–419
23. Toda, T., Shimanaiki, M. & Yanagida, M. (1995) Genes Dev. 9, 60–73
24. Kruse, T., Kominami, K., Kohnou, A., Yanagida, M. & Jones, N. (1996) EMBO J. 15, 1180–1184
25. Toone, W. M., Kuge, S., Samuels, M., Morgan, B. A., Toda, T. & Jones, N. (1998) Gene Dev. 12, 1453–1463
26. Wilkinson, M. G. & Millar, J. B. (1998) Genes Dev. 12, 1391–1397
27. Nakagawa, C. W., Mutoh, N. & Hayashi, Y. (1995) J. Biochem. 118, 109–116
28. Wilkinson, M. G., Fino, T. S., Tournier, S., Buck, V., Martin, H., Christiansen, J., Wilkinson, D. G. & Millar, J. B. (1999) EMBO J. 18, 4210–4221
29. Thomson, S., Clayton, A. L., Hazzalin, C. A., Rose, S., Barratt, C. M. & Mahadevan, L. C. (1999) EMBO J. 18, 4779–4783
30. Melcher, M. L. & Thouver, J. (1996) J. Biol. Chem. 271, 29958–29968
31. Dahlkvist, A., Kanter-Smol, G. & Sunnerhagen, P. (1995) Mol. Gen. Genet. 246, 316–326
32. Billsland-Marshals, E., Arinso, J., Saito, H., Sunnerhagen, P. & Posas, F. (2000) Mol. Cell. Biol. 20, 3887–3895
33. Moreno, S., Klar, A. & Nurse, P. (1991) Methods Enzymol. 194, 795–823
34. Sambrook, J., Fritsch, E. & Maniatis, T. (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
35. Shieh, J. C., Martin, H. & Millar, J. B. (1998) J. Cell Sci. 111, 2799–2807
36. Teige, M., Scheikl, E., Reiser, V., Ruis, H. & Ammerer, G. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5625–5630
37. Degols, G., Shiozaki, K. & Russell, P. (1996) Mol. Cell. Biol. 16, 2870–2877
38. New, L., Jiang, Y., Zhao, M., Liu, K., Zhu, W., Flood, L. J., Kato, Y., Parry, G. C. & Han, J. (1998) EMBO J. 17, 3372–3384
39. Arthur, J. S. & Cohen, P. (2000) FEBS Lett. 482, 44–48
40. Cohen, P. (1997) Trends Cell Biol. 7, 353–361
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