Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 368 (2008) 50–55
BBRC
www.elsevier.com/locate/ybbrc

Identification of Tup1 and Cyc8 mutations defective in the responses to osmotic stress

Yoshifumi Kobayashi, Tomomi Inai, Masaki Mizunuma *, Ichitaro Okada, Atsunori Shitamukai, Dai Hirata, Tokichi Miyakawa

Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8530, Japan

Received 6 January 2008
Available online 15 January 2008

Abstract

In the yeast *Saccharomyces cerevisiae*, Tup1, in association with Cyc8 (Ssn6), functions as a general transcriptional corepressor. This repression is mediated by recruitment of the Tup1–Cyc8 complex to target promoters through sequence-specific DNA-binding proteins such as Sko1, which mediates the HOG pathway-dependent regulation. We identified *tup1* and *cyc8* mutant alleles as the suppressor of osmo-sensitivity of the *hog1*Δ strain. In these mutants, although the expression of the genes under the control of DNA-binding proteins other than Sko1 was apparently normal, the Sko1-regulated genes *GRE2* and *AHP1* were derepressed under non-stress conditions, suggesting that the Tup1 and Cyc8 mutant proteins were specifically defective in the repression of the Sko1-dependent genes. Chromatin immunoprecipitation analyses of the *GRE2* promoter in the mutants demonstrated that the Sko1–Tup1–Cyc8 complex was localized to the promoter, together with Gcn5/SAGA, suggesting that the erroneous recruitment of SAGA to the promoter led to the derepression.

Keywords: *Saccharomyces cerevisiae*; Tup1; Cyc8; Sko1; SAGA; Hog1; *GRE2*

In the yeast *Saccharomyces cerevisiae*, Tup1, in association with Cyc8 (Ssn6), functions as a general repressor of transcription and regulates a wide variety of processes, such as the responses to osmo-stress [1], glucose utilization, mating type, and other mechanisms (reviewed in [2]). This repression is mediated by recruitment of the Tup1–Cyc8 complex to target promoters by sequence-specific DNA-binding proteins [2]. The DNA-binding proteins utilize different domain of Tup1 and Cyc8 for recruitment of the corepressor complex to target promoters [3,4]. Of these DNA-binding proteins, Sko1, a member of the ATF-CREB family, is a repressor that mediates Hog1 pathway-dependent regulation by binding to cyclic AMP-response elements (CRE) in target promoters [5]. Sko1 is phosphorylated by the Hog1 MAP kinase upon osmo-stress [6], and this phosphorylation is crucial to switch the Sko1–Tup1–Cyc8 complex from a repressor to an activator complex [5]. This repressor/activator switch is achieved by the additional recruitment of the chromatin-modifying complexes SAGA and Swi/Snf, but it does not result in the dissociation of Tup1–Cyc8 [5]. However, the molecular mechanism of the Tup1–Cyc8 mediated interaction with sequence-specific DNA-binding proteins is poorly understood.

We previously reported that the 2 Ca^{2+}-activated pathways, namely the Mpk1 MAP kinase cascade and calcineurin coordinately regulate the G_2/M cell-cycle transition [7,8]. Because calcineurin and the Mpk1 pathway act redundantly in essential cellular events, simultaneous loss of these pathways causes lethality. This lethality is suppressed by a defect in Hog1, suggesting that the Hog1 antagonizes the Ca^{2+}-signaling pathways in growth regulation [9].

To investigate the antagonistic regulation, we performed genetic screening for mutations that suppress osmo-sensitivity

* Corresponding author. Fax: +81 82 424 7765.
E-mail address: mmizu@hiroshima-u.ac.jp (M. Mizunuma).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.01.033

of a *hog1*-null mutant (*hog1*Δ) strain. Genetic analyses of the suppressor mutations identified mutant alleles of the *TUP1*, *CYC8*, and *SKO1* genes. Tup1 (H484Q) and Cyc8 (389 STOP) mutations led to a derepression of Sko1-dependent genes, but not the genes under the control of other DNA-binding proteins. We demonstrate herein that the Sko1–Tup1–Cyc8 repressor complex is localized to the *GRE2* promoter, together with Gcn5, a subunit of the SAGA complex in these mutants under the non-stress conditions, suggesting that an erroneous recruitment of SAGA to the promoter led to the derepression of the Sko1-dependent genes in a Hog1-independent manner.

Materials and methods

Materials. Yeast strains DHT22-1b (*MAT*a *trp1 leu2 ade2 ura3 his3 can1-100*), SOS5 (*MAT*a *hog1*Δ::*TRP1*), YFK100 (*MAT*α *hog1*Δ::*TRP1*), and YFK114 (*MAT*α *SKO1*-HA) were the derivatives of strain W303. The following strains were the derivatives of SOS5 or YFK100: SHI123 (*MAT*a *tup1-484*), SHI124 (*MAT*α *tup1-484*), YFK105 (*MAT*a *sko1-18*), YFK106 (*MAT*α *sko1-18*), YFK109 (*MAT*a *cyc8-389*), YFK110 (*MAT*α *cyc8-389*), YFK107 (*MAT*a *sko1*Δ::*kanMX4*), YFK108 (*MAT*α *sko1*Δ::*kanMX4*), YFK103 (*MAT*a *tup1*Δ::*kanMX4*), YFK104 (*MAT*α *tup1*Δ::*kanMX4*), YFK111 (*MAT*a *cyc8*Δ::*kanMX4*), YFK112 (*MAT*α *cyc8*Δ::*kanMX4*), YFK119 (*MAT*a *SKO1*-HA), YFK127 (*MAT*a *tup1-484 SKO1*-HA), and YFK133 (*MAT*a *cyc8-389 SKO1*-HA). The media used were as described previously [7]. The oligonucleotide primer sequences used in this study are available on request.

Mutant isolation. Spontaneous Ca^{2+} -resistant mutants (about 13,000) were isolated from the *hog1*Δ parental strain on YPD-agar plate containing 150 mM $CaCl_2$. The mutants that exhibited normal morphology in the presence of exogenous $CaCl_2$ were selected after inspection under a microscope. Mutants with a dominant mutation and those with multiple mutations were eliminated and the remaining 75 mutants were further subjected to complementation analysis.

Plasmids. The full-length *TUP1* gene and *CYC8* gene were amplified from genomic DNA by PCR. Each product was cloned into T7 Blue T-Vector (Novagen) to make T-Tup1 and T-Cyc8. The pRS315-Tup1 plasmid was constructed by ligation of the PstI–HindIII fragment of T-Tup1 with PstI- and HindIII-digested pRS315; and the pRS316-Cyc8 plasmid by ligation of the BamHI–SalI fragment of T-Cyc8 with BamHI- and SalI-digested pRS316.

Site-directed mutagenesis. pRS315-Tup1-484 and pRS316-Cyc8-389 were constructed by using a QuikChange XL Site-Directed Mutagenesis Kit (STRATAGENE). The mutations were confirmed by DNA sequencing.

Gene disruption and strain construction. The *tup1*Δ, *sko1*Δ, and *cyc8*Δ strains were constructed by gene replacement. Genomic DNA was isolated from the *tup1::kanMX4*, *sko1::kanMX4*, and *cyc8::kanMX4* strains on a BY4741 background (Invitrogen). The amplified fragment was used to transform the DHT22-1b strain.

RNA isolation and Northern blot analysis. RNA isolation and Northern blot analysis were performed as described previously [7]. The *GRE2*, *AHP1*, *SUC2*, *RNR3*, *MFA2*, and *ACT1* probes were amplified from genomic DNA by PCR.

Sko1-tagging. Sko1 at its original chromosomal locus was tagged at its C-terminus with 3-HA according to Longtine et al. [10].

Chromatin immunoprecipitation (ChIP). Yeast cells were grown in YPD medium. To impose osmo-stress, KCl was added to a final concentration of 0.4 M. Chromatin preparation and immunoprecipitation (IP) were performed as described previously [11]. Quantitative PCR analyses were performed in real time by using a Light Cycler version 3.0 (Roche), with the *POL1* coding sequence as a negative control in all experiment. Each IP was performed at least three times with different chromatin samples. The following antibodies were used: anti-HA.11 (Covance), anti-Tup1 [12], anti-Cyc8 [12] and anti-yeast Gcn5 (Santa Cruz Biotechnology). The PCR primers were designed to amplify the promoters or ORF regions: from −303 to +1 of the *GRE2* gene and from +2436 to +2718 of the *POL1* gene.

Results and discussion

Isolation and characterization of mutants that suppress Ca^{2+} sensitivity of hog1-null strain

The growth of *hog1*Δ strain is compromised on solid medium containing 150 mM $CaCl_2$, exhibiting hyperpolarized bud growth and G_2 cell-cycle delay [9]. These defects are restored by deletion of the components of the Ca^{2+}-mediated cell-cycle regulation (e.g., *cnb1*- and *swe1*-null mutations), suggesting that these events are mediated by the activation of calcineurin and Swe1 [9]. To identify unknown factors involved in this regulatory mechanism, we screened for the mutations that exhibited a phenotype similar to that of *cnb1*Δ *hog1*Δ and *swe1*Δ *hog1*Δ double-deletion strains. The recessive, Ca^{2+}-resistant mutants exhibiting normal morphology in the medium containing $CaCl_2$ were selected and they were classified by genetic crosses into 6 complementation groups designated *sgh1–6* (for suppressors of Ca^{2+}-induced growth phenotypes of *hog1*). As expected, we identified the mutants of calcineurin (e.g., Cnb1) and Swe1 as the *sgh2* and *sgh1* loci, respectively, confirming the validity of the screening procedure. We focused on characterization of the mutants of the complementation group *sgh3, 4, 5,* and *6*.

Identification of tup1, cyc8, and sko1 mutant alleles in sgh mutations

Compromised cell growth of the *hog1*Δ strain in the medium containing 150 mM $CaCl_2$ was restored by an additional mutation of any of the complementation groups *sgh3, 4, 5,* and *6* (Fig. 1A). These double-mutants were also more resistant to high-osmolarity than the *hog1*Δ single mutant (Fig. 1A, 0.5 M KCl). In this regard, the phenotypic effect of these mutations was distinct from that of the *sgh1* and *sgh2,* which failed to confer osmo-resistance (Fig. 1A, strains *swe1*Δ *hog1*Δ and *cnb1*Δ *hog1*Δ). These data suggest that the *sgh3–6* mutations may have caused an alteration in the response to high-osmolarity, but not to the Ca^{2+} signal, due to external $CaCl_2$.

To identify the genes for the *sgh* loci, we first characterized the *sgh3-1* mutant. A centromeric genomic library of a WT yeast was introduced into the *sgh3-1 hog1*Δ strain and the plasmids that restored the osmo-resistance to a level of the *hog1*Δ strain were recovered. *TUP1* gene, encoding the Tup1 component of the Tup1–Cyc8 (Ssn6) corepressor complex, was identified as the gene responsible for the phenotype [2]. DNA sequence analysis of the mutant gene revealed a point mutation at position +1452 of the *TUP1* gene from cytosine to adenine, which resulted in an amino acid substitution at position 484 from histidine (CAC) to

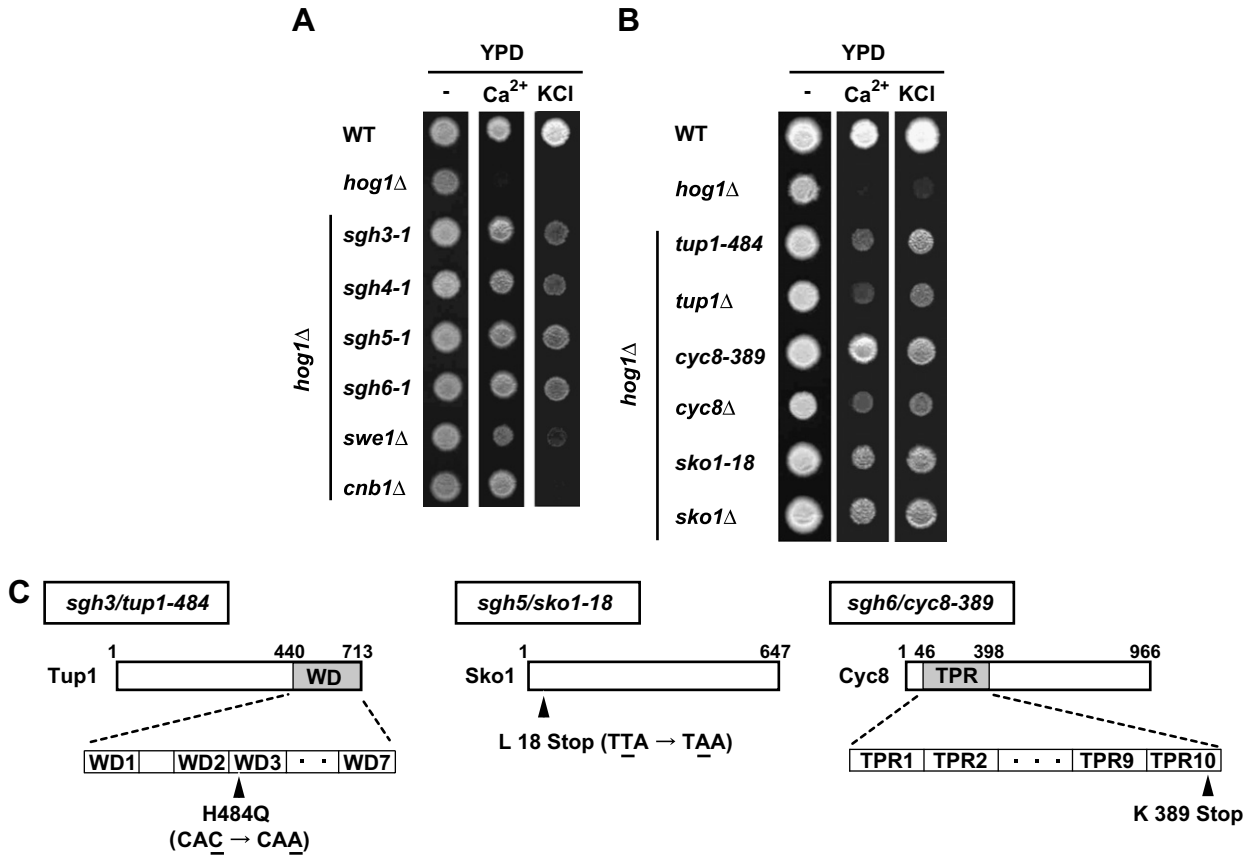

Fig. 1. The mutants of *sgh* complementation groups exhibit the resistance to $CaCl_2$ and osmotic stress. (A) Cell of WT, *hog1*Δ, *sgh3-1 hog1*Δ, *sgh4-1 hog1*Δ, *sgh5-1 hog1*Δ, *sgh6-1 hog1*Δ, *swe1*Δ *hog1*Δ, and *cnb1*Δ *hog1*Δ strains were spotted on YPD solid medium containing 150 mM $CaCl_2$ or 0.5 M KCl at 30 °C for 3 days. (B) Cell of WT, *hog1*Δ, *tup1-484 hog1*Δ, *tup*Δ *hog1*Δ, *cyc8-389 hog1*Δ, *cyc8*Δ *hog1*Δ, *sko1-18 hog1*Δ, or *sko1*Δ *hog1*Δ strains were spotted on YPD solid medium containing 0.2 M $CaCl_2$ or 0.5 M KCl at 30 °C for 4 days. (C) Diagrams of Sko1, Tup1, and Cyc8 proteins showing the mutation site.

glutamine (CAA). The mutation was situated within the third repeat of 7 copies of the WD40 repeats located toward the C-terminus of Tup1 (Fig. 1C). The osmo-resistance level of the *hog1*Δ *tup1*Δ strain bearing the mutant plasmid was similar to that of the *sgh3-1 hog1*Δ strain (data not shown). Based on these results, we concluded that the osmo-resistance of the *sgh3-1 hog1*Δ strain was due to an amino acid substitution (H484Q) in Tup1. Hereafter, we shall refer to this *sgh3-1* mutation as the *tup1-484* mutation.

The Tup1 protein forms a general transcriptional repression complex with Cyc8 protein [2]. In the HOG pathway-dependent regulation, Sko1 protein is required to repress the gene expression by recruiting the Tup1–Cyc8 corepressor complex to the promoters by binding to CRE sites in target promoters [5]. Therefore, we expected that *sko1* and *cyc8* mutations would be contained among the *sgh* mutations. To examine this possibility, we first constructed *sko1*Δ *hog1*Δ and *cyc8*Δ *hog1*Δ double-deletion strains and compared the osmo-resistance of these strains to that of the *hog1*Δ strain. As expected, the double-deletion strains exhibited an increased osmo-resistance similar to that of the *sgh* mutants (Fig. 1B). To examine if the mutations of the *sgh4, 5,* and *6* complementation groups can be assigned to the *SKO1* and *CYC8* loci, the mutant strains *sgh4-1 hog1*Δ, *sgh5-1 hog1*Δ, and *sgh6-1 hog1*Δ were chosen as a representative of each complementation group; and each of these strains was crossed to the *sko1*Δ *hog1*Δ and *cyc8*Δ *hog1*Δ double-deletion strains. The diploid strains thus constructed were sporulated and subjected to tetrad analysis. In all 8 tetrads derived from the diploid strains *sgh5-1 hog1*Δ/*sko1*Δ*::KanMX4 hog1*Δ and *sgh6-1 hog1*Δ/*cyc8*Δ*::KanMX4 hog1*Δ, exhibited osmo-resistance, suggesting that the *sgh5-1* and *sgh6-1* mutations were situated at or closely linked to the *SKO1* and *CYC8* loci, respectively.

DNA sequence analysis of the *sgh5-1* mutant gene revealed a point mutation in the *SKO1* gene at position +53 from thymine to adenine, which changed leucine to a stop codon at amino acid position 18, most probably producing a nonfunctional Sko1 peptide (Fig. 1C). Similarly, the *sgh6-1* mutant gene was revealed to have a single-base insertion + thymine at position 1159 of the coding region, resulting in a frame-shift to yield a stop codon at amino acid position 389 of Cyc8 (Fig. 1C). Cyc8 contains 10 tetratricopeptide repeats (TPR) located toward its N-terminus

[4]. The Cyc8-389 mutant protein contained first 9 repeats of TPR motif and a truncated 10th repeat, lacking the C-terminal 390–966 residues (Fig. 1C). The *cyc8*Δ *hog1*Δ double-deletion strain transformed with the plasmid containing the PCR-created *cyc8* mutation according to the *sgh6-1* mutation showed osmo-resistance like the *sgh6-1 hog1*Δ strain, confirming that the resistance phenotype was caused by the *sgh6-1* mutation (data not shown). We noted that the *tup1-484* and *cyc8-389* mutants grew better than their corresponding deletion strains under stressed and non-stressed conditions (Fig. 1B). Since these genes have been described previously, we shall hereafter refer to the *sgh5-1* mutation as *sko1-18,* and the *sgh6-1* one as *cyc8-389*. The unassigned mutation *sgh4-1*, which is involved in a distinct mechanism operative in cell-cycle regulation, will be described elsewhere.

Effects of tup1-484 and cyc8-389 mutations on the expression of various genes under the control of the Tup1-Cyc8 corepressor complex

The Sko1 repressor regulates the expression of the HOG pathway-dependent stress-defense genes such as *GRE2* and *AHP1* by recruiting the Tup1–Cyc8 co-repressor complex to the promoter [1,5]. Upon osmotic stress, the Sko1–Tup1–Cyc8 complex is inactivated in a HOG pathway-dependent manner [1]. The *GRE2* gene which encodes NADPH-dependent methylglyoxal reductase is the best characterized gene with respect to the expression patterns upon high osmotic stress. Since *GRE2* transcription in *sko1*Δ strain is derepressed under non-stress conditions [6], we expected that the *GRE2* mRNA levels of these mutants would be elevated. We compared the effect of the *sko1*, *tup1*, and *cyc8* mutations on *GRE2* mRNA levels on the *hog1*Δ background under normal growth conditions. As expected, the *GRE2* gene was expressed at higher levels in these mutants compared with the expression in the *hog1*Δ strain, except that the *GRE2* expression was partially repressed in the *tup1-484* mutant compared with that in the *tup1*Δ strain (Fig. 2). The mRNA of *AHP1,* another Sko1-target gene, was also expressed at a higher level in the *tup1-484* mutant (Fig. 2).

The specificity of repression is determined by the sequence-specific DNA-binding repressors, which recruit Tup1–Cyc8 to turn off subsets of genes. Besides Sko1, the repressors include Mig1, which regulates glucose-repressible genes; Crt1, which regulates DNA damage response genes; α2, which represses **a**-cell-specific genes, and the flocculence-related genes [2]. We examined the expression levels of these genes in each of the *tup1-484*, *tup1*Δ, *cyc8-389, cyc8*Δ, *sko1-18*Δ, and *sko1*Δ mutant strains on the *hog1*Δ background (Fig. 2). Although the expression of glucose-repressible gene *SUC2*, DNA damage-response gene *RNR3*, and **a** cell-specific gene *MFA2* was derepressed in the *tup1*Δ and *cyc8*Δ strains, the expression of these genes in the *tup1-484* and *cyc8-389* mutants remained repressed. The expression of these genes was unaffected in *sko1-18* and *sko1*Δ mutants. The result of the Northern blot analysis indicated that the Tup1-484 and Cyc8-389 mutant proteins were specifically defective in the regulation of the Sko1-mediated gene expression. The *tup1-484 hog1*Δ, and *cyc8-389 hog1*Δ cells in liquid culture did not show flocculence; whereas the cells of *tup1*Δ and *cyc8*Δ strains showed flocculence, suggesting that the mutant proteins were functional in regulating flocculation, as well (data not shown).

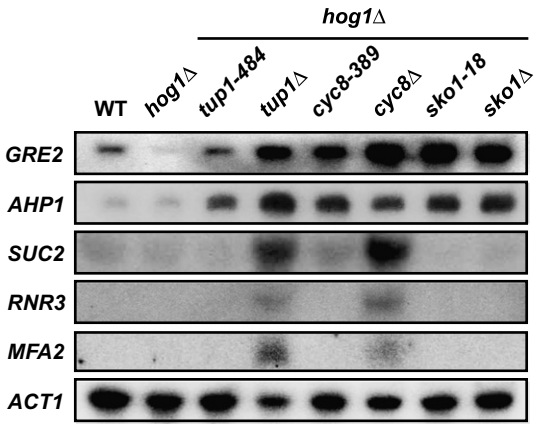

Fig. 2. The expression of the Sko1-mediated osmo-responsive genes is specifically derepressed in *sko1*, *tup1,* and *cyc8* mutants, but not in the genes mediated by the other DNA-binding proteins. The *GRE2*, *AHP1*, *SUC2*, *RNR3*, *MFA2,* and *ACT1* (as a loading control) mRNA levels in WT, *hog1*Δ, *tup1-484 hog1*Δ, *tup*Δ *hog1*Δ, *cyc8-389 hog1*Δ, *cyc8*Δ *hog1*Δ, *sko1-18 hog1*Δ, and *sko1*Δ *hog1*Δ strains were determined by Northern blotting.

Sko1 localization to the GRE2 promoter in tup1-484 and cyc8-389 strains

The question arises as to why the Sko1-mediated transcription became deregulated in the *tup1-484* and *cyc8-389* mutants under the non-stress conditions? Sko1 binds to the promoter of the osmo-stress-regulated genes irrespectively of stress [5], and this is important for repression of the *GRE2* gene in the absence of osmo-stress [6]. To examine if the derepressed gene expression was caused by the failure of Sko1 localization to the *GRE2* promoter, we determined Sko1 localization by performing the chromatin immunoprecipitation (ChIP) assay by using *hog1*Δ strains carrying a chromosomally integrated construct for HA-epitope-tagged Sko1 at the C-terminus under the control of its own promoter. These strains did not show increased resistance to KCl compared with the *hog1*Δ strain, suggesting that the tagged version of Sko1 was functional *in vivo*. Sko1-HA on its natural locus of chromatin was immunoprecipitated with antibodies against the HA-epitope, and analyzed by quantitative PCR in real time by using primers spanning the *GRE2* promoter regions. In WT and *hog1*Δ strains, Sko1 was found to be localized to *GRE2* promoter, as previously reported [5]. In the *tup1-484 hog1*Δ and *cyc8-389 hog1*Δ mutants, Sko1 was similarly localized to the *GRE2* promoter as in the *hog1*Δ strain

(Fig. 3A), suggesting that the deregulated expression was caused by a defect at a step after the recruitment of Sko1 to the promoter.

Tup1 and Cyc8 localization to the GRE2 promoter in tup1-484 and cyc8-389 strains

To analyze the localization of Tup1 and Cyc8 (WT and mutant proteins) to the *GRE2* promoter, we first compared the cellular levels of these proteins in the cell extract of WT and mutant strains by Western blot analysis with anti-Tup1 and anti-Cyc8 antibodies. The expression levels of these proteins in the *cyc8-389 hog1*Δ and *tup1-484 hog1*Δ strains were comparable to those in the *hog1*Δ cells, suggesting that the normal amounts of Tup1, Tup1-484, and Cyc8 proteins were produced in the mutant cells (data not shown). However, Cyc8-389 mutant protein was not detectable by Western blotting in the extract of the *cyc8-389 hog1*Δ strain. Nonetheless, it should be noted that the *SUC2, RNR3,* and *MFA2* genes were still repressible in the *cyc8-389 hog1*Δ strain (Fig. 2), indicating that the mutant protein functions in the regulation of these genes. Presumably, the truncated mutant protein is either unstable *in vitro* or it was not recognizable by the antibody.

Next, we examined by the ChIP assay if the derepressed *GRE2* expression was caused by impaired recruitment of the Tup1–Cyc8 complex to the *GRE2* promoter. In the *tup1-484 hog1*Δ strain, both Tup1-484 and Cyc8 proteins were localized to the *GRE2* promoter, similarly as Tup1 and Cyc8 WT proteins in the *hog1*Δ strain (Figs. 3B and C). Likewise, although the localization of Cyc8-389 could not be determined by this procedure, Tup1 was localized to the promoter in the *cyc8-389 hog1*Δ strain (Fig. 3B). These results suggested that the derepression of the osmo-stress genes was not due to impaired recruitment of the Sko1–Tup1–Cyc8 repressor complex to the promoter, but rather due to some defects after the formation of the regulatory complex on the promoter.

Gcn5 localization to the GRE2 promoter in tup1-484 and cyc8-389 strains

The Tup1–Hog1 complex is important in the recruitment of the SAGA complex to the *GRE2* promoter for the activation of gene expression in response to osmotic stress [5].The *GRE2* derepression could have been due to the recruitment of the SAGA complex to the mutant repressor complex localized to the promoters. To examine this possibility, we assessed the recruitment of SAGA complex to the *GRE2* promoter by conducting a ChIP assay with an antibody against Gcn5, which is a subunit protein of the SAGA complex. The Gcn5 protein in the WT strain was localized to the *GRE2* promoter in response to osmo-stress as previously described [13]. In the *tup1-484 hog1*Δ and *cyc8-389 hog1*Δ mutants, Gcn5 was recruited to the *GRE2* promoter of the *tup1-484 hog1*Δ and *cyc8-389 hog1*Δ

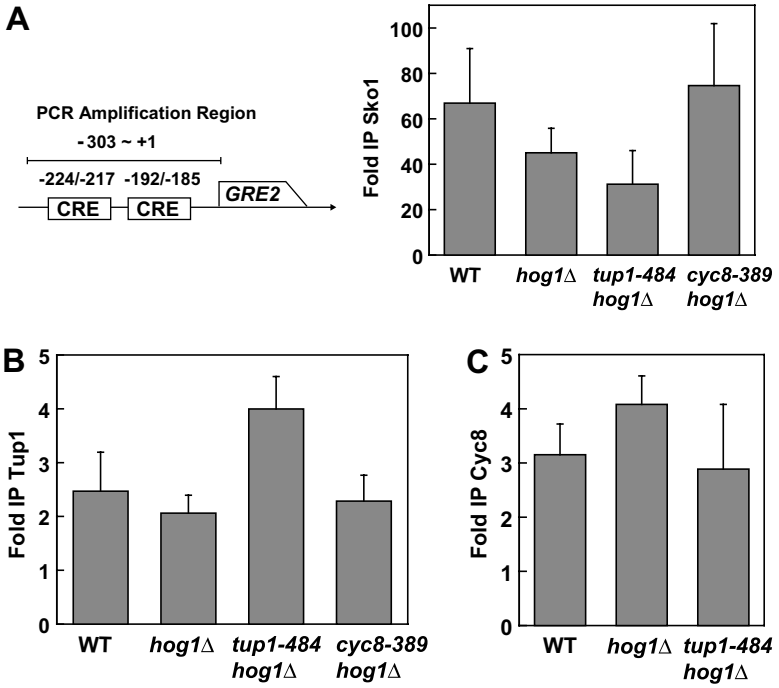

Fig. 3. Recruitment of WT and mutant proteins of Sko1, Tup1 and Cyc8 to the *GRE2* promoter in *tup1-484 hog1*Δ and *cyc8-389 hog1*Δ mutants. (A) ChIP analysis of the localization of Sko1 to the *GRE2* promoter in WT, *hog1*Δ, *tup1-484 hog1*Δ, and *cyc8-389 hog1*Δ cells expressing Sko1-3HA. (B) ChIP analysis of Tup1 localization to the *GRE2* promoter in WT, *hog1*Δ, *tup1-484 hog1*Δ, and *cyc8-389 hog1*Δ cells. (C) ChIP analysis of the Cyc8 localization to the *GRE2* promoter in WT, *hog1*Δ, and *tup1-484 hog1*Δ strains. Immunoprecipitation efficiencies for (A–C) are expressed as the fold over the *POL1* coding sequence control. The values were calculated from at least three independent experiments and represent means ± SD.

mutants at a level higher than that in the case of the *hog1*Δ strain, and the localization occurred irrespective of osmo-stress (Fig. 4). These results suggest that an erroneous recruitment of SAGA to the repressor complex occurred in these mutants in a Hog1-independent manner, and led to the derepression of gene expression.

In conclusion, the genetic analyses of the suppressor mutants of the osmo-sensitivity of the *hog1*Δ strain led to the identification of peculiar mutations of the Tup1 and Cyc8 components of the general corepressor. The mutations caused a defect specifically in the repression of the Sko1-regulated genes, but not in that of the genes regulated by the other DNA-binding proteins. Of the 10 tandem copies of the TPR motif of the Cyc8 protein, the first 3 copies are involved in the binding to Tup1, whereas the other TPR repeats in combination mediate the interaction with certain pathway-specific repressor proteins, such as Mig1 and Rox1 [4]. The Cyc8-389 mutant protein contains the first 9 repeats of TPR but lacks the C-terminal 390–966 residues including the 10th repeat (Fig. 1C). The Tup1 protein contains 7 repeats of WD40 domains in the C-terminal portion, which fold into a 7-bladed propeller structure that interacts with the α2 repressor [3,14]. The substitution mutation of Tup1-484 was situated within the third repeat of the WD40 (Fig. 1C). Specific combinations of the Cyc8 TPR motifs and the Tup1 WD domains possess distinct protein–protein interaction specificities [3,4]. The functional defect of the mutations was observed specifically in the Sko1-mediated gene expression. ChIP analyses of the *GRE2* promoter demonstrated that the Sko1–Tup1–Cyc8 repressor complexes of the mutants were recruited to the promoter (Fig. 3). Consistently with the derepression of the *GRE2* gene, further ChIP analyses have demonstrated the recruitment of the Gcn5, a component of the SAGA complex, to the *GRE2* promoter, irrespectively of osmo-stress. Hence, the corepressor complex containing either of the Cyc8-389 or Tup1-484 mutant protein, in contrast to the WT complex, appeared to have gained a function to recruit the co-activator complexes, thus turning on the activator switch. Further studies of these peculiar properties of the Tup1 and Cyc8 mutations will provide us insights on the coordination of the global regulation of distinct biological pathways by the Tup1–Cyc8 corepressor complex.

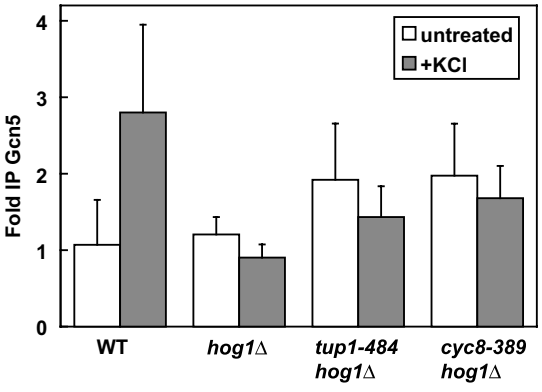

Fig. 4. Recruitment of Gcn5 to the *GRE2* promoter in *hog1*Δ *tup1-484* and *hog1*Δ *cyc8-389* mutants. Analysis of the localization of Gcn5 to the *GRE2* promoter in WT, *hog1*Δ, *tup1-484 hog1*Δ, and *cyc8-389 hog1*Δ cells. The cells were osmotically stressed for 5 min by 0.4 M KCl. Immunoprecipitation efficiencies are expressed as the fold over the *POL1* coding sequence control. The values were calculated from three independent experiments and represent means ± SD.

Acknowledgments

We thank S.Y. Dent and Y. Mukai for antibodies against Tup1 and Cyc8. This work was supported in part by grant-in-aid for scientific research from the Ministry of Education, Science, Culture, Arts, and Sports of Japan to M.M. (18688004) and T.M. (17208009).

References

[1] J. Márquez, A. Pascual-Ahuir, M. Proft, R. Serrano, The Ssn6-Tup1 repressor complex of *Saccharomyces cerevisiae* is involved in the osmotic induction of HOG-dependent and -independent genes, EMBO J. 17 (1998) 2543–2553.
[2] R. Smith, A. Johnson, Turning genes off by Ssn6-Tup1: a conserved system of transcriptional repression in eukaryotes, Trends Biochem. Sci. 25 (2000) 325–330.
[3] K. Komachi, M. Redd, A. Johnson, The WD repeats of Tup1 interact with the homeo domain protein alpha 2, Genes Dev. 8 (1994) 2857–2867.
[4] D. Tzamarias, K. Struhl, Distinct TPR motifs of Cyc8 are involved in recruiting the Cyc8–Tup1 corepressor complex to differentially regulated promoters, Genes Dev. 9 (1995) 821–831.
[5] M. Proft, K. Struhl, Hog1 kinase converts the Sko1–Cyc8–Tup1 repressor complex into an activator that recruits SAGA and SWI/SNF in response to osmotic stress, Mol. Cell 9 (2002) 1307–1317.
[6] M. Proft, A. Pascual-Ahuir, E. de Nadal, J. Ariño, R. Serrano, F. Posas, Regulation of the Sko1 transcriptional repressor by the Hog1 MAP kinase in response to osmotic stress, EMBO J. 20 (2001) 1123–1133.
[7] M. Mizunuma, D. Hirata, K. Miyahara, E. Tsuchiya, T. Miyakawa, Role of calcineurin and Mpk1 in regulating the onset of mitosis in budding yeast, Nature 392 (1998) 303–306.
[8] T. Miyakawa, M. Mizunuma, Physiological roles of calcineurin in *Saccharomyces cerevisiae* with special emphasis on its roles in G2/M cell-cycle regulation, Biosci. Biotechnol. Biochem. 71 (2007) 633–645.
[9] A. Shitamukai, D. Hirata, S. Sonobe, T. Miyakawa, Evidence for antagonistic regulation of cell growth by the calcineurin and high osmolarity glycerol pathways in *Saccharomyces cerevisiae*, J. Biol. Chem. 279 (2004) 3651–3661.
[10] M. Longtine, A.r. McKenzie, D. Demarini, N. Shah, A. Wach, A. Brachat, P. Philippsen, J. Pringle, Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*, Yeast 14 (1998) 953–961.
[11] T. Inai, M. Yukawa, E. Tsuchiya, Interplay between chromatin and trans-acting factors on the *IME2* promoter upon induction of the gene at the onset of meiosis, Mol. Cell. Biol. 27 (2007) 1254–1263.
[12] A. Watson, D. Edmondson, J. Bone, Y. Mukai, Y. Yu, W. Du, D. Stillman, S. Roth, Ssn6–Tup1 interacts with class I histone deacetylases required for repression, Genes Dev. 14 (2000) 2737–2744.
[13] N. Guha, P. Desai, A. Vancura, Plc1p is required for SAGA recruitment and derepression of Sko1p-regulated genes, Mol. Biol. Cell 18 (2007) 2419–2428.
[14] K. Komachi, A. Johnson, Residues in the WD repeats of Tup1 required for interaction with alpha2, Mol. Cell. Biol. 17 (1997) 6023–6028.