Defects in glycosylphosphatidylinositol (GPI) anchor synthesis activate Hog1 kinase and confer copper-resistance in *Saccharomyces cerevisiae*

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(Received 17 October 2001, accepted 16 November 2001)

Las21/Gpi7 contains a heavy-metal-associated motif at its N-terminus. When this motif was disrupted by amino acid substitution, the cells acquired weak copper-resistance. We found that the previously isolated *las21* mutants were strongly resistant to copper. Metallothionein is necessary for the expression of the copper-resistance of the *las21* mutants. However, hyper-production of metallothionein is unlikely to be the cause of copper-resistance of the *las21* mutants. Copper-sensitive mutants (collectively called Cus mutants) were isolated from the *las21*Δ and characterized. One of the Cus genes was found to be *PBS2*, which encodes Hog1 MAP kinase kinase, indicating that the Hog1 MAP kinase pathway is needed for the expression of copper-resistance of the *las21* mutants. As expected, the *las21*Δ *hog1*Δ strain was no longer copper-resistant. We found that Hog1 was constitutively activated in *las21*Δ cells and in *ssk1*Δ *las21*Δ cells but not in *sho1*Δ *las21*Δ cells. Inactivation of either *FSR2/MCD4* or *MPC1/GPI13*, both of which are involved in GPI anchor synthesis, like *LAS21*, caused a similar level of constitutive activation of Hog1 kinase and copper-resistance as found in the *las21*Δ strain. The constitutive activation was canceled by introducing the *ssk1* mutation, but not the *sho1* mutation, in each GPI anchor mutant tested, suggesting that the defect in GPI anchor synthesis specifically affects the Sln1 branch of the MAP kinase pathway. Since the wild-type cells grown in YPD containing 0.5 M NaCl do not show copper-resistance, mere activation of Hog1 is not sufficient for expression of copper-resistance. We propose that a defect in GPI anchor synthesis has multiple consequences, including activation of the Hog1 MAP kinase cascade and conferring copper-resistance.

INTRODUCTION

The *LAS21* gene has been identified as one of the responsible genes in mutants that are sensitive to a local anesthetic, tetracaine, and that at the same time show temperature-sensitive growth (Toh-e and Oguchi, 1998). This gene was also identified as the *GPI7* gene by a different strategy in which mutants defective in glycosylphosphatidylinositol (GPI) anchor synthesis were screened (Benachour et al., 1999). Biochemical analysis of the function of Gpi7/Las21 indicated that this protein is involved in the addition of the side chain phosphoethanolamine to the second mannose of the GPI core (Benachour et al., 1999). There are two genes showing significant homology to *LAS21* (Nelissen et al., 1997): *MCD4/FSR2* (Matsumoto et al., 1986; Mondesert et al., 1997; Packeiser et al., 1999) and *MPC1/GPI13/YLL031C* (Toh-e and Oguchi, 2000; Flury et al., 2000; Taron et al., 2000). Proteins encoded by these genes have multiple membrane-spanning domains in their C-terminal half and the homology is limited to the N-terminal half. Mcd4 was suggested to add the side chain phosphoethanolamine to the GPI core (Gaynor et al., 1999), and this was supported by Hong et al. (1999) who showed that a mammalian homologue of Mcd4, Pig-n, transfers phosphoethanolamine to the first mannose of the GPI core. *GPI13* or *YLL031C*, identical to *MPC1* (Toh-e and Oguchi, 2000), was shown to be involved in transferring phosphoethanolamine to the third mannose of the GPI core (Flury et al., 2000; Taron et al., 2000). In contrast to the fact that *LAS21* is not an essential gene, *MCD4/FSR2* and *MPC1* are essential. Interestingly, *mpc1* mutants show sensitivity to tetracaine (Toh-e and Oguchi, 2000).
Las21 has a heavy-metal-associated domain that is not found in either Fsr2/Mcd4 or Mpc1/Gpi13. Two other yeast proteins have such a motif, Ccc2 (Fu et al., 1995; Yuan et al., 1995) and Atx1 (Lin et al., 1997), both of which are involved in copper/iron metabolism (Pufahl et al., 1997). This fact suggests that Las21 has a function in copper metabolism in addition to a function in GPI anchor synthesis. Copper is an essential metal, but an excess of it injures cells due to its oxidative activity; therefore, the level of copper in cells must be maintained at an appropriate level. To cope with this paradoxical requirement, yeast cells have developed mechanisms for copper homeostasis. Yeast cells have high affinity copper transporters Ctr1 (Dancis et al., 1994) and Ctr3 (Knight et al., 1996) that are repressed by a metal-binding regulator Mac1 (Labbe et al., 1997) at high concentrations of copper. In the presence of an excess of copper, Ctr1 (Ooi et al., 1996) and Mac1 (Zhu et al., 1998) are degraded by proteolysis, so that cells can reduce their incorporation of copper quickly. Yeast has another less characterized copper transporter encoded by CTR2 (Kampfenkel et al., 1995). The expression of CTR2 is not repressed by copper, and Ctr2 seems to be a low affinity copper transporter. Copper is reduced by reductases, Fre1 and Fre2, at the time of incorporation into the cells (Georgatsou et al., 1997). Since free copper in the cytoplasm is toxic, incorporated copper (Cu(I)) is sequestered by binding to metallothionein encoded by CUP1 (Butt et al., 1984a;1984b) which plays a major role in copper-tolerance and CRS5 (Culotta et al., 1994) in S. cerevisiae. One molecule of CUP1 metallothionein binds 8 Cu(I) (Winge et al., 1985), while one molecule of CRS5 metallothionein binds 12 Cu(I), and the binding of the latter is weaker than that of the former. The expression of the CUP1 gene, and that of the CRS5 gene as well, is induced by copper binding activator Ace1 (Hamer et al., 1985). Copper can be detoxified by sequestration into vacuoles (Pearce and Sherman, 1999).

Yeast cells respond to various stresses, including osmotic stress and oxidative stress, etc., in the process of adapting to a new environment. Each stress is sensed by a sensor and the signal is transmitted to nucleus to change gene expression. Among the mechanisms of such signal transduction, the Hog1 MAP kinase pathway has been the most extensively studied (Banuett, 1998). The Hog1 MAP kinase pathway is activated as a response to high osmolarity in the medium, and results in the induction of glycerol synthesis to compensate for the elevated external osmolarity (Hog1 kinase is activated by MAPKK, Pbs2, which is, in turn, activated by two MAP kinase kinase kinases (MAPKKKs), each of which is controlled by a different signaling branch: one is the His-Asp phospho-relay branch consisting of Sln1,Ypd1, and Ssk1 (the Sln1 branch) and the other is the Sho1 branch (Maeda et al., 1994, 1995; Posas et al., 1996). The MAPKKKs belong-
Table 1. The principal yeast strains used in this study

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(A) Toh-e and Oguchi, 1998; (B) Toh-e and Oguchi, 2000.

Table 2. The list of plasmids

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<td>Sikorski and Hieter, 1989</td>
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locus (~916 ~ +1020) was amplified by two successive amplifications by polymerase chain reaction (PCR). The first PCR amplified the DNA segment (~916 ~ +43) by using a forward primer (5'-GGCGTTGACACTCTAGATCAC-3') and a mutagenic primer (5'-GGCGTTGACACTCTAGATCACGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGT
nal eight codons of the CUP1 gene was amplified by PCR using a pair of primers, 5′-GGAAGCCGCCGCAACGGA-
AGTTTCAATCTATTTAAAGG-3′ and 5′-GGAAGGATC-
CAAGTTAATTTCTGCAGCACATTATG-3′, and gen-
omic DNA from W303D as template. The amplified DNA fragments were digested with NotI and BamHI and inserted between the NotI – BamHI gap of pRS306. The resultant plasmid was digested with BamHI and SmaI, and ligated with the lacZ gene (Casadaban et al., 1980) excised from pAT196 (Imura et al., 1989) by digesting with BamHI and NruI, to generate Top812. Top812 was linearized at the unique XbaI site in the promoter region of the CUP1 gene and targeted to the CUP1 locus of W303-
1B. The Pcup1-lacZ gene was transferred to the indicated genetic background by genetic crosses.

Top752 (cup1::URA3): The CUP1 locus consists of two tandem repeats of the CUP1 gene associated with an ORF of unknown function. Two unique regions (approx. 1 kb each) flanking the CUP1 locus were amplified by PCR. The upstream unique region (~838 – +11) was amplified by using a pair of primers, 5′-GGAAGCGGCCGCAACGGGAGTTTCAATCTATTTAAAGG-3′ and 5′-GGAAGGATCCAGTTAATTTCTGCAGCACATTATG-3′, and genomic DNA from W303D cells as template. The upstream segment was digested with NotI and EcoRI, the downstream segment digested with HindIII and EcoRI, and pRS306 digested with NotI and HindIII, were ligated to obtain Top752. For disruption of the CUP1 locus, Top752 was linearized with EcoRI and introduced into W303-1B by selecting Ura+ strains. The result of the trans-
formation was substitution of the DNA region flanked by the upstream and downstream DNA segments amplified by PCR with the sequence of pRS306.

Top843 (ssk1::HIS3): The upstream 961bp of the SSK1 gene were amplified by PCR using a pair of primers SSK1-1 (5′-GGAAGGATCCATCTTGTTGCTAACTTAGTCTC-3′) and SSK1-2 (5′-GGAAGCACCAGAATTGAGCA-
TTTATCTTGTGCTC-3′) and genomic DNA prepared from W303D. The downstream 1055bp of the SSK1 gene were amplified by PCR using a pair of primers SSK1-3 (5′-
GGAAGGATCCATCTTGTTGCTAACTTAGTCTC-3′) and SSK1-4 (5′-GGAAGGATCCATCTTGTTGCTAACTTAGTCTC-3′) and genomic DNA from W303D. The upstream segments were digested with BamHI and NotI, and the downstream segments were digested with BamHI and SalI. The DNA fragments flanking the SSK1 gene thus prepared were li-
gated with pRS303 (Sikorski and Hieter, 1989) digested with NotI and SalI to generate Top843. Top843 was cut with BamHI and used as donor DNA to transform W303-
1B to His+.

Disruption of SSK1 was confirmed by PCR.

Mutagenesis. Yeast strains to be mutagenized with ethylmethanesulfonate (Eastman Kodak, USA) were pro-
cessed as described by Lindegren et al. (1965). Poly-
merase chain reaction-mediated site-directed mutagenesis was carried out as described previously (Toh-e and Oguchi, 2000).

DNA manipulations. Yeast DNA was extracted by the rapid method described by Hoffman and Winston (1987). Agarose gel electrophoresis, restriction endonuclease treatment, and elution of DNA fragments from agarose gels were performed as described by Sambrook et al. (1989). DNA sequences were determined by the dideoxy chain termination method described by Sanger et al. (1977) using an automated DNA sequencer (ABI 370A). The adenine residue of the putative translation initiation codon ATG is defined as the +1 nucleotide. Restriction endonucleases and DNA modifying enzymes were pur-
chased from Takara Shuzo (Kyoto, Japan) and Toyobo Biochem. (Kyoto, Japan).

β-galactosidase assay. β-galactosidase produced by the strains containing the Pcup1-lacZ fusion gene was assayed as described (Toh-e and Oguchi, 1999). The activity was expressed as 1.7 × OD600nm / 0.0045 × [protein (mg/ml)] × reaction time (minutes). Protein concent-
tration was determined using a BCA kit purchased from Pierce (Rockford, IL, USA) with bovine serum albumin as a standard.

Detection of phosphorylated Hog1. Preparation of protein samples for electrophoresis was performed as described by O’Rourke and Herskowitz (1998). The cells to be tested were grown to logarithmic phase (OD600nm = ca.1.5) and the cell density was adjusted to OD600nm = 1. NaCl (final concentration 0.5 M) was added to 1 ml of culture when needed and the mixture was kept at room temperature for 5 minutes. Cells were pelleted by cen-
trifugation at 5000 rpm for 1 minute (Tomy MR15 centri-
fuge) and culture fluid was removed by aspiration. Cells were suspended in 100 µl of water and then 100 µl of 0.2 N NaOH was added. After 10 minutes in an ice bath, cells were collected by centrifugation and the pellet was mixed with 50 µl of sample buffer (Laemmli, 1970) and proteins were solubilized by boiling for 3 minutes. Ten microliters of extract was loaded on an SDS-10% polyacrylamide gel and the proteins were separated by electrophoresis (Laemmli, 1970) at 20 mA for 1.5 hours. The separated proteins were electrotransferred to nitrocellulose mem-
brane followed by Western blotting using anti-phosphop-
38 rabbit antibody (Cell Signalling Technol., MA USA) as primary antibody and anti-rabbit IgG goat antibody conjugated with horseradish peroxidase (Promega Co., WI, USA) as secondary antibody.
RESULTS

**las21 mutants are resistant to copper.** Las21/Gpi7, Fsr2/Med4, and Mpc1/Gpi13 are structurally and functionally related to each other and participate in glycosylphosphatidylinositol (GPI) anchor synthesis (Benachour et al., 1999; Gaynor et al., 1999; Flury et al., 2000; Taron et al., 2000). The unique feature of Las21 is that it has a heavy-metal-associated motif at its N-terminus that is included in a putative signal peptide sequence. Although this motif might be removed by processing, in order to examine whether the motif has some role(s) in the function of Las21, two cysteine residues in the motif were substituted with alanine (Fig. 1A) and the resultant mutant gene (*las21- AA*) was used to replace the wild type counterpart. The *las21- AA* strain grew on YPD, like the wild type strain, even at 37°C. Next we tested the effects of metals (Mn, Zn, Fe, and Cu) on the growth of the mutant. Among the metals tested, only copper gave rise to a subtle effect on the growth. The *las21- AA* strain displayed weak copper-resistance (Fig. 1B). Next, the *las21* mutants described previously (Toh-e and Oguchi, 1998) were tested for their copper-resistance. The wild-type, *las21-1*, *las21-2*, and *las21Δ* cells were streaked across YPD medium containing the indicated amount of CuSO₄. As seen in Fig. 1C, the *las21* mutants grew on YPD containing up to 15 mM CuSO₄. To confirm that the copper-resistance is due to the *las21* mutations, pLAS21 plasmid and the vector were separately introduced into the *las21Δ* strain, and a representative transformant of each transformation experiment was tested for its growth on SC-URA containing CuSO₄. The copper-resistance was abolished in the transformant carrying the pLAS21 plasmid (Fig. 1D), indicating that the *las21* mutation is responsible for the expression of copper-resistance.

**Effect of the deletion of CUP1 on the copper-resistance of las21Δ.** The major player in the expression of copper-resistance in *S. cerevisiae* is Cup1 (metallothionein), which binds copper (Cu(I)). Intracellular copper binds the transcription factor Ace1 to activate the transcription of the CUP1 gene (Thiele, 1988) on the one hand, and binds another transcription factor, Mac1, to repress the transcription of CTR1 and CTR3, which encode high affinity copper transporters, and FRE1, which encodes a reductase. Cells become sensitive to copper when the CUP1 gene is deleted. To examine whether the copper-resistance resulted from the *las21* mutation is dependent on CUP1, *cup1Δ las21Δ* strains were constructed by crossing the *las21Δ* strain and the *cup1Δ* strain, and tested for their copper-sensitivity (Fig. 2). The copper-resistance was abolished by the deletion of the CUP1 gene from the *las21Δ* strain. This result, along with the fact that CUP1 over-expression confers copper-resistance on the cells (Hamer et al., 1985), suggests that the copper-resistance of the *las21Δ* strain was caused by overproduction of Cup1 due to the *las21* mutation. To examine this possibility, the wild-type and the *las21Δ* strains carrying the integrated *Pcup1-lacZ* fusion gene at the CUP1 locus were grown in YPD or YPD containing the indicated amount of CuSO₄. After 3 hours of incubation, the cells were harvested and assayed for β-galactosidase activity. In both the wild-type strain and the *las21Δ* strain, 10 μM CuSO₄ induced the production of β-galactosidase (Fig. 3). In this experiment, the maximum amount of β-galactosidase produced was higher in the wild-type strain than in the *las21Δ* strain, indicating that the copper-resistance of the *las21Δ* strain was not caused by higher expression of the CUP1 gene in the *las21Δ* mutant. Since the expression of CUP1 began at a similar concentration of CuSO₄ in both strains, it is likely that the *las21* mutant cells transport copper as efficiently as the wild type cells. The results described above indicate that the CUP1 gene was induced normally in the *las21Δ* mutant. Copper-resistance can be caused by the amplification of the CUP1 locus (Welch et al., 1983); however, we found by genomic Southern hybridization analysis that the copy number of the CUP1 gene is not increased in the *las21* strain (data not shown).

A defect in either MCD4/FSR2 or MPC1/GPI13 confers copper-resistance Since the features of the MCD4/FSR2 and MPC1/GPI13 genes resemble those of LAS21/GPI7, we examined whether the *fsr2-1* and *mpc1* mutants were copper-resistant. The freshly grown cells of each strain were suspended in water at OD₆₀₀nm=1.0 and 10-fold dilutions of each suspension were spotted on YPD and YPD containing the indicated concentration of CuSO₄. The plates were incubated at 25°C for 3 ~ 4 days (Fig. 4), and the growth characteristics indicated that the *fsr2-1* and *mpc1* mutants were copper-resistant.

Isolation of copper-sensitive mutants from the las21Δ strain. To explore the factors involved in the expression of the copper-resistance of the *las21Δ* strain, mutants showing sensitivity to copper were screened after mutagenesis of the *las21::LEU2* strain with EMS as described in Materials and Methods. The mutagenized culture was spread on YPD plates after appropriate dilution and the plates were incubated at 25°C for 3 days. Colonies that appeared on the plates were replica-plated onto YPD containing 15 mM CuSO₄ and the plates were incubated at 25°C for 3 days. Copper-sensitive colonies were isolated and crossed with the *las21::URA3* strain with the opposite mating type, and the resultant heterozygous diploids were dissected with caution as described previously (Toh-e and Oguchi, 1998); *las21Δ/las21Δ* diploids produce spores sensitive to lytic enzyme (zymolyase). Mutants whose copper-sensitivity segregated to 2+ : 2− were collected and designated Cus mutants (Cu(II) sensi-
Fig. 1. The las21 mutations cause copper-resistance. (A) The heavy-metal-associated motif of Las21. The conserved amino acid residues of this motif are shown as bold face letters. The las21-1 allele has two amino acid substitutions, C8A and C11A. (B) Copper-resistance shown by the las21-AA mutant. Overnight cultures of the wild-type and the las21-AA strains (YAT2988) were diluted and spread on YPD, YPD with 10 mM CuSO4, or YPD with 15 mM CuSO4 and the plates were incubated at 25°C for 3 (YPD) or 5 (YPD with copper) days. (C) Copper-resistance shown by the las21 mutants. The wild-type strain (W303-1B), the las21-1 strain (YAT1856), the las21-2 strain (YAT1865), and the las21Δ strain (YAT2290) were streaked across YPD or YPD with 15 mM CuSO4 and both types of plates were incubated at 25°C for 3 days. (D) The las21 mutation is responsible for the copper-resistance. YCp50 and pLAS21 were separately introduced into the las21Δ strain (YAT2290) and the representative Ura+ transformant from each transformation experiment was streaked across SC-URA with or without 5 mM CuSO4 and both types of plates were incubated at 25°C for 3 days.
Fig. 2. Metallothionein is needed for the expression of copper-resistance of the las21Δ mutant. The diploid cells (W1940; cup1::URA3/ + las21::LEU2/+) were sporulated and dissected. Two sets of the tetrad clones (2A, 2B, 2C, 2D, 4A, 4B, 4C, and 4D) were streaked across YPD plates containing the indicated amount of CuSO₄. The plates were incubated at 25°C for 3 days. cup1 and las21 indicate cup1::URA3 and las21::LEU2, respectively.

The extent of copper-sensitivity differs from one strain to another, and some mutants are more sensitive than the wild-type strain. A YCp50-based genomic library was introduced into each of the Cus mutant strains, and Ura⁺ copper-resistant transformants were selected. After confirmation of the plasmid-dependence of the cop-
per-resistent phenotype by re-transformation experiments, the nucleotide sequence at the cloning junctions of each clone was determined. By making deletions in or subcloning of the DNA fragments derived from the yeast genome, the responsible ORF of each Cus mutant was determined (Fig.5 and Table 3). No genes related to copper metabolism (Gross et al., 2000) has been identified hitherto. Fig. 5 shows the delimitation of the genes responsible for the Cus mutations.

The DNA segment complementing the Cus1 and Cus6 mutants was subjected to deletion analysis. The shortest segment with the complementation activity contained the SSN8 gene encoding cyclin for Ssn3 Cdk (cyclin-dependent kinase) (Liao et al., 1995). Disruption of SSN8 resulted in a copper-sensitive phenotype and, the ssn8Δ las21Δ strain did not complement the copper-sensitive phenotype of the Cus1 mutant (data not shown), indicating that the gene responsible for Cus1 is SSN8. To examine whether Ssn3/Ssn8 Cdk is needed for copper-resistance, the SSN3 gene was disrupted and the disruptant was tested for its copper-sensitivity. The ssn3Δ strain was found to be sensitive to copper to a similar degree as the ssn8Δ strain (data not shown), indicating that Cdk activity is needed for the expression of copper-resistance of the las21Δ mutant.

A DNA segment complementing the Cus2 and Cus11 mutants was subjected to deletion analysis. The most probable candidate gene thereby identified was YMR315W.

Fig. 3. Induction of the expression of the CUP1 gene in the wild type and las21Δ strains. The Pcup1-lacZ strain (YAT2514) and the las21Δ Pcup1-lacZ strain (YAT2513) were grown in YPD overnight at 25°C. Overnight cultures were refreshed by a 20-fold dilution in YPD, and after the cultures were shaken for 3 hours at 25°C, CuSO₄ was added to each culture at the indicated concentration. After 3 hours of incubation, β-galactosidase activity was assayed. The data shown are from one of two independent experiments.
containing an uncharacterized ORF. Disruption of YMR315W produced cells that were viable and sensitive to copper.

By using Cus10 mutant cells as the host, we cloned an 11-kb DNA segment from chromosome 7. The delimitation analysis of the complementing activity showed that the gene responsible for the Cus10 mutant is HIP1, encoding histidine permease.

The 8-kb DNA fragment containing PBS2 complemented the Cus3 mutation, and delimitation experiments indicated that a likely candidate for the gene responsible for the Cus3 mutation was PBS2. As expected, Cus3 strain did not grow on YPD supplemented with 1 M sorbitol. To confirm that PBS2 is the responsible gene, a las21Δ pbs2Δ double mutant was constructed. The double mutant was copper-sensitive, and a diploid constructed by crossing the double mutant with the Cus3 mutant was sensitive to copper, proving that the gene responsible for the Cus3 mutation is PBS2.

Although the hunting down of the gene responsible for the Cus4 mutation shown in Fig. 5 has not yet been completed, the results obtained thus far suggest that UBA1 is a likely candidate for the gene responsible for the Cus4 mutation.

We are interested in Cus3, which has a defect in PBS2 encoding Hog1 MAP kinase kinase and therefore we further examined the role of the Hog1 MAP kinase pathway in the expression of copper-resistance.

**Contribution of the Hog1 MAP kinase pathway to copper-resistance.** To confirm that the Hog1MAP kinase pathway (Fig. 6A) is involved in the expression of copper-resistance of the las21Δ mutant, we tested whether members of this pathway other than Pbs2 were required for copper-resistance. First, we constructed a hog1Δ las21Δ double mutant by genetic crossing between the hog1Δ strain and the las21Δ strain. The results of the spot testing (Fig. 6B) indicated that the segregants containing the hog1Δ mutation showed sensitivity to copper. The Hog1 MAP kinase pathway has two upstream branches, the Sho1 branch and the Sln1 branch (Fig. 6A). We therefore next examined on which pathway the copper-resistance of the las21Δ mutant depends. The sho1Δ las21Δ and ssk1Δ las21Δ double mutants were constructed and tested for copper-resistance (Fig. 6B). The results indicated that both the sho1Δ las21Δ strain and the ssk1Δ las21Δ strain are more sensitive than the las21Δ strain but more resistant than the hog1Δ or hog1Δ las21Δ strain. It should be noted that the ssk1Δ las21Δ strain was slightly more sensitive than the sho1Δ las21Δ strain.

**Hog1 is constitutively activated in the las21Δ strain.** In the previous section, it was shown that the copper-resistance of the las21Δ strain is dependent on the Hog1 MAP kinase pathway. Next we examined whether Hog1 was activated in the las21Δ strain. Cells of logarithmic-phase cultures of the wild-type, las21Δ and hog1Δ or hog1Δ las21Δ strains were harvested and proteins were extracted by the alkali extraction method described by O’Rourke and Herskowitz (2000). The proteins were separated by electrophoresis on a 10% SDS-polyacrylamide gel followed by Western blotting using anti-phospho-p38 rabbit antibody as primary antibody. In extract prepared from the cells treated with 0.5 M NaCl, phospho-Hog1 was detected in the wild-type, las21Δ and hog1Δ strains. The proteins were separated by electrophoresis on a 10% SDS-polyacrylamide gel followed by Western blotting using anti-phospho-p38 rabbit antibody as primary antibody. In extract prepared from the cells treated with 0.5 M NaCl, phospho-Hog1 was detected in the wild-type, las21Δ and hog1Δ strains. In contrast, the phospho-Hog1 band was detected in the extract of las21Δ cells not treated with NaCl, but not in the extract similarly prepared from wild-type cells. This result indicates that Hog1 is constitutively activated, although at a low level, in the las21Δ cells (Fig. 7A).

**Western blot analysis.** The functional similarity of the gene products of Las21/Gpi7, Fsr2/Mcd4, and Mpc1/Gpi13 prompted us to examine Hog1 kinase activation in the fsr2-1 and mpc1 mutants. As shown in Fig. 7A, we found that the fsr2-1 and mpc1 mutant strains produced phospho-Hog1 kinase constitutively.

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**Fig. 4.** The fsr2-1 and mpc1 mutants exhibit copper-resistance. W303-1B (wild type), YAT2626 (las21Δ), YAT2547 (fsr2-1), YAT2572 (mpc1-3), YAT2573 (mpc1-4), and YAT2574 (mpc1-5) were grown on YPD plate and fresh cells were suspended in water at OD600nm=1.0. 3 μl of ten-fold serial dilutions of each strain was spotted on the indicated medium. YPD plate was incubated at 25°C for 3 days, and other plates were incubated at 25°C for 4 days.
Copper-resistance of the \textit{las21} and related mutants

Fig. 5. Genes responsible for the Cus mutations. Open boxes indicate DNA segments derived from the yeast genome. Arrows indicate ORFs. The thin horizontal lines at both ends of the boxes indicate the DNA segments derived from the cloning vector, YCp50. The nucleotide sequences at both cloning junctions were determined by the dideoxy chain termination method (Sanger et al., 1977) and the nucleotide sequence flanked by the two sequences was retrieved from SGD (Stanford Genome Database). Subcloning and deletion analysis were conducted, and the complementation activity of each DNA segment was examined by introducing each plasmid into the appropriate host. Dashed lines indicate nucleotide sequences that were deleted. Symbols: A, \textit{Afl}II; B, \textit{Bam}HI; C, \textit{Cla}I; G, \textit{Bgl}II; E, \textit{Eco}RI; H, \textit{Hind}III; N, \textit{Nru}I; P, \textit{Pst}I; Pv, \textit{Pvu}II; S, \textit{Sal}I; Sc, \textit{Sac}I; Sp, \textit{Spe}I; X, \textit{Xba}I.

Table 3. Responsible genes of the Cus mutants.

<table>
<thead>
<tr>
<th>Mutant*</th>
<th>Candidate of the responsible gene</th>
<th>Gene product</th>
</tr>
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<tbody>
<tr>
<td>Cus1/Cus6</td>
<td>\textit{SSN8} (=\textit{SRB11})</td>
<td>cyclin of Ssn3 kinase</td>
</tr>
<tr>
<td>Cus2/Cus11</td>
<td>\textit{YMR315W}</td>
<td>unknown</td>
</tr>
<tr>
<td>Cus3</td>
<td>\textit{PBS2}</td>
<td>MAPKK of Hog1 MAP kinase</td>
</tr>
<tr>
<td>Cus4</td>
<td>\textit{UBAI}</td>
<td>ubiquitin activating enzyme</td>
</tr>
<tr>
<td>Cus10</td>
<td>\textit{HIP1}</td>
<td>histidine permease</td>
</tr>
</tbody>
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* Cus1 and Cus6 are allelic, and are Cus2 and Cus11. Cus5, Cus7, Cus8, and Cus9 are not cloned yet.

respectively, like the \textit{las21Δ} cells.

To examine which upstream branch of the Hog1 MAP kinase pathway is used in this activation of Hog1, phospho-Hog1 was detected by Western blotting of extracts prepared from \textit{sho1Δ las21Δ} and \textit{ssk1Δ las21Δ} cells. The results showed that the \textit{ssk1Δ las21Δ} cells lost ...
The Hog1 MAP kinase pathway is required for the expression of copper-resistance of the las21Δ cells. (A) Hog1 MAP kinase cascade. (B) Copper-resistance expressed by the double mutants containing las21Δ and one of Hog1 MAP kinase pathway genes. The fresh cultures of the wild type strain (W303-1B), las21Δ (YAT2626), hog1Δ (YAT2333), las21Δ hog1Δ (YAT2331), ssk1Δ (YAT2632), las21Δ ssk1Δ (YAT2639), sho1Δ (YAT2627), and las21Δ sho1Δ (YAT2637) were suspended in water at OD600nm=1.0. 3 µl of ten-fold serial dilutions was spotted on YPD and YPD with 15 mM CuSO4. The plates were incubated at 25°C for 3 days.

The state of activation of Hog1 kinase in the Cus mutants was examined by Western blotting using anti-phospho-p38 antibody as described above. As expected, the Cus3 mutant (=pbs2 mutant) did not produce phospho-Hog1, and Cus5 and Cus8 mutants did not either (Fig. 7B). Since these mutants were found to be insensitive to 1 M sorbitol, it is unlikely that they are mutants of HOG1 or PBS2.
Fig. 7. Hog1 is constitutively activated in the mutants defective in GPI anchor synthesis. (A) Detection of phospho-Hog1 kinase. Overnight cultures of the indicated yeast strains were diluted in YPD and incubated at 25°C until the OD$_{600nm}$ reached around 1.5. The cells were harvested and processed according to the alkali extraction method described in Materials and Methods. The extracted proteins were separated by 10% SDS-PAGE followed by Western blotting using anti-phospho-p38 as the primary antibody. Samples prepared from the wild-type (W303-1B) denoted as + and hog1Δ (YAT2333) cells treated with 0.5 M NaCl for 5 minutes before protein extraction were included to show the position of phospho-Hog1. Two different las21Δ strains were analyzed. (B) Detection of phospho-Hog1 kinase in the Cus mutants. Extracts were prepared from the cultures of the hog1Δ (YAT2333), las21Δ (YAT2626) and Cus mutants and processed as described above to detect phospho-Hog1 kinase. Extract prepared from the wild-type strain (W303-1B) treated with 0.5 M NaCl was included to provide a size marker for phospho-Hog1.
Fig. 8. The las21Δ, fsr2-1, and mpc1 mutations activate the Hog1 pathway via the Sln1 branch. (A) Activation by las21Δ. The las21Δ (YAT2626), ssk1Δ (YAT2632), las21Δ ssk1Δ (YAT2639), sho1Δ (YAT2627), and las21Δ sho1Δ (YAT2637) cells were grown to OD_{600nm}= approx. 1.5. Proteins were extracted as described above, resolved by 10% SDS-PAGE, and subjected to Western blotting using anti-phospho-p38 antibody. Wild-type (W303-1B) and hog1Δ (YAT2333) cells treated with 0.5 M NaCl were analyzed in parallel. (B) Hog1 activation by the mpc1-3 mutation. (C) Hog1 activation by the fsr2-1 mutation. The blocking buffer used for Western blotting contained 5% skim-milk instead of bovine serum albumin.
As described above, the Hog1 MAP kinase should be activated when cells express copper-resistance. We examined whether copper would activate Hog1 kinase and found that 5 mM CuSO$_4$ did not activate Hog1 kinase (data not shown). We also examined copper-resistance under conditions of high osmolarity. The wild-type cells did not express copper-resistance in the presence of 1 M sorbitol or 0.5 M NaCl. A similar experiment shown in Fig. 8A was carried out using the $fsr2$, $fsr2$ $ssk1D$, $fsr2$ $sho1\Delta$, $mcp1$ $ssk1\Delta$, and $mcp1$ $sho1\Delta$ strains. We found that the $ssk1\Delta$ $fsr2$ or $ssk1\Delta$ $mcp1$ double mutants had lost the phosphorylation of Hog1, whereas the $sho1\Delta$ $fsr2$ and the $sho1\Delta$ $mcp1$ double mutant cells produced phospho-Hog1 (Fig. 8B,C). These results indicate that a defect in GPI anchor synthesis causes activation of Hog1 kinase by way of the Sln1 branch of the Hog1 MAP kinase pathway.

**DISCUSSION**

**Copper-resistance in yeast** In this study, we found that $las21$ mutations caused copper-resistance. Although the copper-resistance of the $las21$ mutants required an intact $CUP1$, it is unlikely that the overproduction of Cup1 metallothionein was the cause of the copper-resistance in these mutants. In medium containing a non-fermentable carbon source, such as lactate, another system of copper-resistance is activated. Knight et al. (1994) found that strains without metallothionein ($cup1\Delta$ $ace1\Delta$) showed resistance to copper in medium containing lactate as the sole carbon source. They isolated copper-sensitive mutants from the $cup1\Delta$ $ace1\Delta$ strain in lactate medium and found that $CUP9$ which encodes a protein containing a homeodomain was the gene responsible for the mutation. This result indicates that the strain with the intact $CUP9$ gene was resistant to copper irrespective of the expression of the $CUP1$ gene in lactate medium. In the same study, it was shown that $CUP9$ is transcribed in lactate medium but not in glucose medium. Since Cup9 is a transcription regulator, it is important to know what genes related to copper-resistance are under its control. Byrd et al. (1998) demonstrated that Cup9 is a repressor for the $PTR2$ gene, which encodes a peptide transporter. Supposing that Cup9 acts as a repressor in copper metabolism to repress a certain gene and thereby confer copper-resistance on the cells in lactate medium, the expression of that gene of interest must make cells sensitive to copper. To examine whether $CUP9$ plays some role in Las21-mediated copper-resistance, we constructed the $cup9\Delta$ $las21\Delta$ double-mutant strain and tested its copper-resistance. We found that the $cup9\Delta$ mutation did not affect the copper-resistance of the $las21\Delta$ strain, indicating that there is no functional connection between Cup9 and Las21.

Different types of yeast have different mechanisms for developing copper-resistance. In *Candida albicans*, a P-type ATPase encoded by the $CRD1$ gene plays a major role in copper-resistance: excessive copper in the cytoplasm is exported from the cells by this enzyme (Riggle and Kumamoto, 2000; Weissman et al., 2000). The E. coli homologue of this type of ATPase, copA, was shown to pump Cu(I) out of cells (Rensing et al., 2000). It was reported that overexpression of the $CRD1$ gene conferred copper-resistance on *S. cerevisiae* cells (Riggle and Kumamoto, 2000; Weissman et al., 2000). In *S. cerevisiae*, there is a structurally similar P-type ATPase encoded by $PCA1$ (Rad et al., 1994). A $PCA1$ mutant allele, named $CAD2$, was identified as a gene responsible for cadmium-resistance in *S. cerevisiae* (Shiraishi et al., 2000). The $CAD2$ P-type ATPase is likely to pump cadmium ions out of the cells. Regarding the function of Pca1 in the copper-resistance of *S. cerevisiae*, Rad et al. (1994) showed that Pca1 has a subtle effect on the copper metabolism: the $pca1\Delta$ cells stop growing at a lower cell density in medium containing 1 mM CuSO$_4$, than wild-type cells, and over-expression of $PCA1$ inhibits cell growth. We constructed a $pca1\Delta$ $las21\Delta$ strain and examined the copper-resistance of the double-mutant strain. The double-mutant and the $las21\Delta$ strain showed similar copper-resistance (data not shown), indicating that Pca1 does not play a major role in the expression of copper-resistance in $las21\Delta$ cells. Furthermore, we constructed a $GAL1$ driven $PCA1$ gene in the wild-type and in the $las21\Delta$ backgrounds and found that induction of $PCA1$ did not result in a change in the responses of these cells to copper.

Copper-resistance can be mediated by several distinct mechanisms: a defect in the import of copper (Kampfenkel et al., 1995), hyper-production of metallothionein (Karim et al., 1984), or hyper-activity of a transporter such as the P-type-ATPase that pumps copper out of the *C. albicans* cells (Riggle and Kumamoto, 2000; Weissman et al., 2000). Which mechanism accounts for the copper-resistance of the $las21$ mutants? The $FCUP1$-lacZ gene integrated at the $CUP1$ locus of the $las21\Delta$ cells was induced by as low a concentration of CuSO$_4$ as that inducing $CUP1$ in the wild-type cells, and the copy number of the $CUP1$ gene was not changed in the $las21\Delta$ cells, indicating that it is unlikely that the copper-resistance displayed by the $las21$ mutants is due to hyper-production of Cup1 metallothionein.

Some toxic metals are detoxified by sequestering into vacuoles: cadmium, for example, is transported into vacuoles as bis(glutathionato)cadmium by Ycf1 (Li et al., 1997) although cadmium can also be detoxified by binding metallothionein. Szczypka et al. (1994) reported that vacuole malfunction resulted in copper-sensitivity and Pearce and Sherman (1999) proposed the idea that histidine accumulated in vacuoles may sequester metals, including copper, leading to tolerance to a high concentration of these metals. In this context, it is of interest that we isolated a $hip1$ mutant as a copper-sensitive mutant.
from the las21Δ (Table 3). It should be noted, however, that the las21 mutants are copper-resistant irrespective of whether they are His⁺ or His⁻ under our experimental conditions.

One intriguing possible cause of copper-resistance of the las21 mutants is a defect in the uptake of copper. S. cerevisiae has two high-affinity copper transporters, encoded by CTR1 and CTR3. The las21 mutants seem to have a normal high affinity copper transporters, judging from the results shown in Fig. 3. Yeast also has another copper transporter, Ctr2, that may be a low-affinity copper transporter. In the presence of a large amount of copper, cells may take up copper via this transporter. It was reported that the ctr2Δ strain was resistant to high concentrations of copper, and that over-expression of CTR2 made the cell sensitive to copper (Kampfenkel et al., 1995). These genetic behaviors displayed by the CTR2 gene are consistent with the interpretation that copper is taken up by Ctr2 at a high concentration of copper. However, in our strain background, no difference was found in copper-resistance between the las21Δ strain and the las21Δ ctr2Δ strain. Furthermore, the GALI-driven CTR2 did not confer copper-sensitivity on the wild-type strain or the las21Δ strain. Therefore, the contribution, if any, of the CTR2 gene to copper metabolism in our experimental system is negligible.

The Hog1 MAP kinase pathway is necessary, but not sufficient, for the expression of copper-resistance of the las21Δ strain To explore the mechanism of the copper-resistance shown by the las21 mutants, we isolated copper-sensitive mutants (Cus mutants) from the las21Δ strain. Some mutants showed higher sensitivity to copper than the wild-type cells. The responsible genes cloned by functional complementation using the Cus mutants as the hosts were, SSN8, PBS2, UBA1, HIP1 and an uncharacterized gene, YMR315W. It should be noted that none of the Cus mutants so far identified was a member of the genes under the control of the copper regulon (Gross et al., 2000). It will be of interest to elucidate the function of each gene involved in copper metabolism. Among the Cus mutants, we further characterized Cus3, in which the defect was found to be in the PBS2 gene encoding Hog1 MAP kinase kinase. Consistent with this, combining the hog1Δ mutation with the las21Δ mutation greatly reduced the copper-resistance (Fig. 6). In accordance with this, we found that Hog1 was constitutively activated in the las21Δ cells through the Snl1 branch of the Hog1 MAP kinase pathway. Furthermore, as shown in Fig. 7A, the fsr2-1 and mpc1 mutants produced phospho-Hog1 constitutively and showed a copper-resistant phenotype (Fig. 4). Taken together, these findings indicate that GPI anchor deficiency seems to inhibit Snl1, thereby activating Hog1 kinase, and at the same time this deficiency confers copper-resistance. However, the wild-type cells grown in medium containing 0.5 M NaCl showed activation of the Hog1 pathway but not copper-resistance, indicating that mere activation of the Hog1 MAP kinase pathway is not sufficient to confer copper-resistance.

Copper-resistance caused by a defect in GPI anchor synthesis The N-terminal amino acid sequence of Las21 contains a putative signal sequence encompassing the heavy-metal-associated motif. Is the putative signal peptide of Las21 cleaved off? To answer this question, it will be necessary in future experiments to analyze the N-terminal amino acid sequence of mature Las21. However, our site-directed mutagenesis toward the heavy-metal-associated motif suggests that this N-terminal sequence remains in the mature form of Las21, because the las21-ΔAA mutant displayed a phenotypic change. If the putative signal sequence containing the heavy-metal-associated motif was cleaved off during targeting to the membrane, no phenotypic change should have been caused by the las21-ΔAA mutation, because the mutated site should not have remained on Las21, although there is a possibility that the las21-ΔAA mutation would change the structure of the putative signal peptide so that it could not be cleaved off. Our findings suggest that the putative signal peptide sequence remains in at least some molecules of Las21. At present, we do not have direct evidence showing that the heavy-metal-associated motif has an activity to bind copper, although the las21-ΔAA mutant displayed a phenotype consistent with this idea. Further study is needed to settle the question whether the heavy-metal-associated motif participates in copper transport or not.

The toxicity of copper arises when the amount of copper begins to exceed the cellular capacity to sequester the copper. The fact that the loss-of-function-type mutations in genes participating in GPI anchor synthesis conferred copper-resistance suggests that a membrane defect may block the copper-flow into a putative copper-sensitive site. This copper-sensitive site is likely to be a membranous compartment such as the ER, Golgi, or nucleus.

Mutations defective in GPI anchor synthesis, such as las21, fsr2/med4, and mpc1 exhibit dual consequences: one is Hog1 activation via the Snl1 branch and the other is an event that induces copper-resistance. It is likely that perturbation in GPI anchor synthesis inhibits Snl1, thereby causing Hog1 activation. The second event may be directly connected with copper-resistance. It is obvious that Hog1 kinase is necessary for the copper-resistance of the las21 mutants, however, the role of Hog1 kinase is not well understood. Our favored interpretation of the participation of the Hog1 pathway in copper-resistance is that it is most likely that a defect in GPI anchor synthesis protects the copper-sensitive site from excess copper by inhibiting the movement of copper from the cytoplasm to the copper-sensitive site, and that the Hog1 pathway is necessary to cope with the higher level of cop-
per in the cytoplasm that results from the blockage of copper movement due to the mutation.

We would like to express our thanks to Tatsuya Maeda (Institute of Molecular and Cellular Biosciences, The University of Tokyo) and Ichiro Yamashita (Center for Gene Science, Hiroshima University) for plasmids.

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