1999). Each plasmid was reintroduced into W303B cells to confirm the phenotype and the plasmid that conferred the strongest resistance to methylmercury was selected. The nucleotide sequence of the genomic insert in the selected plasmid was determined with an automated DNA sequencer (LI-COR, Lincoln, NE). After mapping, the genomic insert was excised and subcloned as DNA fragments (F1, F2, and F3) in the pRS425 vector (Miura et al., 1999). Each subclone was introduced into W303B yeast cells and the sensitivity of each resultant line of cells to methylmercury was determined. The gene responsible for resistance to methylmercury was identified as *CDC34*.

Quantitation of Growth Inhibition by Metal Compounds. Suspensions of yeast cells that harbored pRS425-CDC34 or pRS425 were cultured (1×10^4 cells/200 μl) in SD (−Leu) medium that contained various concentrations of the indicated metal compound in 96-well plates. For assays of cells that harbored pYES2-based expression plasmids, cells were grown in synthetic galactose medium (SG medium) (−Ura), that contained 2% galactose and 4% raffinose as sources of carbon. After 48 h, the absorbance at 620 nm (A_{620}) was determined spectrophotometrically to quantify the growth of each lines of cells (Furuchi et al., 2001).

Construction of Vectors for Expression of Various Yeast Genes. Yeast genes were cloned by PCR with yeast chromosomal DNA as the template. The following oligonucleotides were used as primers: 5′-CATACATAAACAAGCATCCAA-3′ and 5′-GCTTCTCTTTTTCAGCTGAG-3′ for amplification of *UBC4*; 5′-TCATTTTCTGCTCACCACCCT-3′ and 5′-CACAATTTATCCGTTAGCCCA-3′ for *UBC5*; 5′-GTAATTTGGAAGGGCATAGC-3′ and 5′-TCATTAACCTGCTACCTGCT for *UBC7*; and 5′-ACCAAACAAGGAAAAAAGAAC-3′ and 5′-TTGTCTTCTTTCTTACTGTTC-3′ for *CDC34* The products of PCR were ligated into the pTargeT vector (Promega, Madison, WI). Each insert was digested with restriction endonucleases, as follows: *Bam*HI and *Not*I for *UBC7*; and *Kpn*I and *Xho*I for *CDC34*, *UBC4* and *UBC5* The resultant fragments were ligated into the pYES2 expression vector (Invitrogen, Carlsbad, CA).

Northern Blotting Analysis. For preparation of RNA, cells (5×10^6/ml) were cultured in 40 ml of yeast extract-peptone-dextrose medium that contained various concentrations of methylmercury chloride. After 90 min, total RNA was prepared as described elsewhere (Hoffman, 1993). The probes for the *CDC34(UBC3)*, *UBC4*, *UBC5*, and *UBC7* genes were obtained by PCR from the yeast genome using gene-specific oligonucleotides. Northern blotting was performed using the digoxigenin system from Roche Applied Science (Indianapolis, IN) according to the manufacturer's instructions.

Results and Discussion

We introduced a yeast genomic DNA library in the vector YEp13 into yeast strain W303B in an attempt to identify novel genes that confer resistance to methylmercury. Transformants were cultured in SD (−Leu) medium containing methylmercury (0.4 μM) for 24 h. Under these conditions, yeast cells harboring the YEp13 vector grew only very slowly and formation of cell aggregates was very limited during the 24-h incubation. Other yeast cells grew rapidly and precipitated as aggregates even in the presence of methylmercury. The aggregates of such yeast cells were collected and plated on agar-solidified SD (−Leu) medium for formation of colonies. We selected several colonies at random and determined their resistance to various concentrations of methylmercury. From these colonies, we selected the eight clones with the highest resistance to methylmercury. Plasmids were rescued from these yeast cells and reintroduced into the parent strain W303B. All yeast cells transformed with such plasmids were resistant to methylmercury.

We sequenced the genomic DNA fragment (1P1) that had been inserted in the plasmid from the clone with the highest resistance to methylmercury and then we mapped the fragment using the *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces/). We found that 1P1 was derived from yeast chromosome IV. The region corresponding to 1P1 contains four open reading frames (ORFs): *YDR057w*, *YDR056c*, *PST1*, and *CDC34* (Fig. 1). To identify the gene involved in resistance to methylmercury, 1P1 was excised and subcloned as DNA fragments (F1, F2, and F3) in the multicopy plasmid pRS425. The gene responsible for resistance to methylmercury was found in subclone F2, which contained a single ORF, *CDC34* (Figs. 1 and 2).

The *CDC34* gene encodes a ubiquitin-conjugating enzyme (E2). This enzyme, Cdc34 (also called Ubc3) (Goebl et al., 1988), is involved in ubiquitin-dependent proteolysis. In this proteolytic pathway, the covalent attachment of ubiquitin to a target protein destines the protein for proteasome-mediated degradation (Deshaies, 1999; Tyers and Jorgensen, 2000). Ubiquitin-conjugating enzymes, such as Cdc34, use ubiquitin that has been activated by a ubiquitin-activating enzyme (E1), and then they catalyze the ubiquitination of substrate proteins, acting alone or in conjunction with a ubiquitin-ligase (E3). Many ubiquitin-conjugating enzymes have been recognized in yeast and mammals (Hochstrasser, 1996; Hershko and Ciechanover, 1998). Mutations in many of these enzymes result in distinct phenotypes, indicating that each ubiquitin-conjugating enzyme has different functions and, presumably, different substrate specificities (Hochstrasser, 1996; Hershko and Ciechanover, 1998). Cdc34 is essential for progression of the cell cycle from the G_1 to the S phase (Goebl et al., 1988), and its catalyzes the ubiquitination of target proteins that include Sic1, whose elimination is necessary for progression of the cell cycle (Verma et al., 1997).

Figure 3 shows the effects of the overexpression of Cdc34 on the cytotoxicity of several metal compounds. Yeast cells (W303B/pCDC34) that had been transformed with pRS425-CDC34 were resistant not only to methylmercury (Fig. 3A) but also to mercuric chloride (Fig. 3B, a) and *p*-chloromercuribenzoate (Fig. 3B, b). By contrast, the cells were not significantly resistant to copper chloride (Fig. 3B, c) and zinc

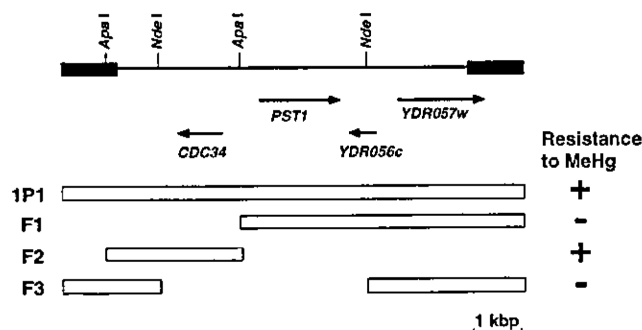

Fig. 1. Restriction map of the genomic DNA insert 1P1 that conferred resistance to methylmercury. The thick black line represents the vector YEp13; the thin line represents the genomic DNA insert. Vertical lines above the genomic DNA insert indicate the restriction sites used to generate different subclones. The ability of three subclones (pF1, pF2 and pF3) to confer resistance to methylmercury (MeHg) is indicated (+, conferred resistance; −, did not confer resistance). ORFs are indicated by black arrows that point in the direction of transcription, with the name of each ORF given below the respective arrow.

chloride (Fig. 3B, d). The toxic effects of mercury compounds in animals depend on the chemical form of the metal but most mercury compounds, including methylmercury, mercuric chloride, and *p*-chloromercuribenzoate, have strong affinity for the thiol groups in the cysteine residues of proteins (Naganuma et al., 2000). The affinities of copper and zinc compounds for thiol groups are severalfold lower than those of mercury compounds (Simpson, 1961; Lenz and Martell, 1964). Cdc34 includes only one cysteine residue that is essential for its function (Liu et al., 1995). It is possible that methylmercury and other mercury compounds might bind to the cysteine thiol of Cdc34 and inhibit its activity. Verma et al. (1997) indicated that Cdc34 is an essential protein in *S. cerevisiae* and that destruction of the *CDC34* gene is lethal for the yeast, as described above. The cells of yeast strain W303B/pCDC34 contained a higher concentration of Cdc34 protein than that in the control yeast cell. Thus, it is possible that Cdc34 might be one of the targets of methylmercury and that complete inhibition of Cdc34 in W303B/pCDC34 cells might require higher concentrations of methylmercury than in the control cells.

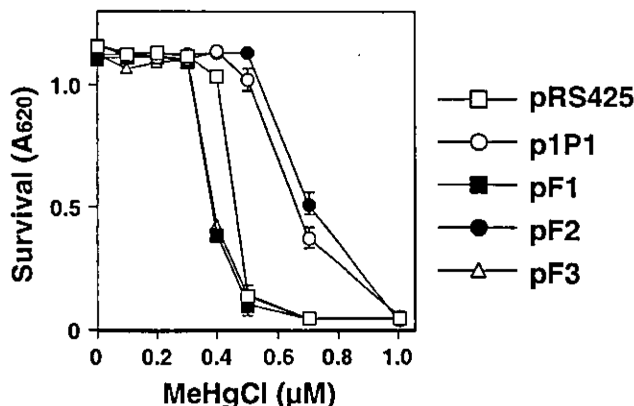

Fig. 2. Sensitivity to methylmercury of yeast cells that harbored plasmids with the indicated inserts. Yeast cells (1×10^5 cells/200 μl/well) carrying plasmids p1P1, pF1, pF2, pF3, or pRS425, as indicated, were grown in SD (−Leu) medium that contained methylmercuric chloride (MeHgCl). After a 48-h incubation, absorbance at 620 nm was measured spectrophotometrically. Each point and bar represent the mean value and S.D. results from four cultures. The absence of a bar indicates that the S.D. falls within the symbol.

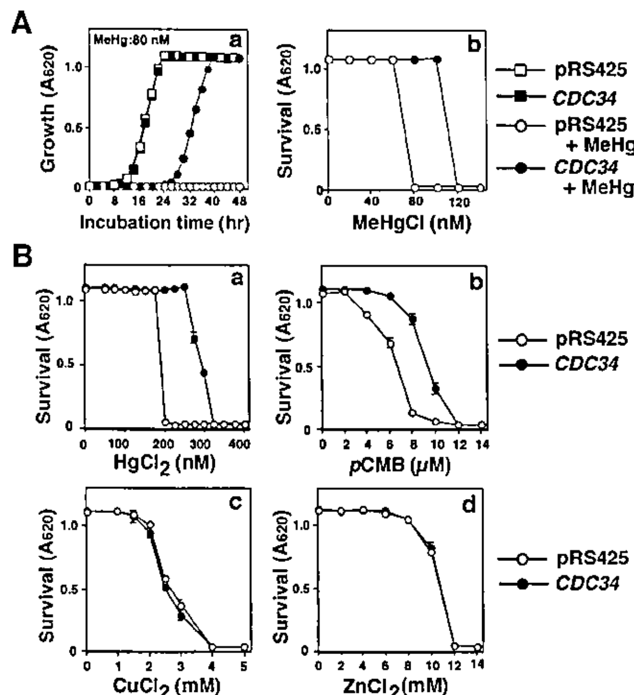

Fig. 3. Sensitivity of yeast cells that overexpressed Cdc34 to various metal compounds. Yeast cells (1×10^4 cells/200 μl/well) carrying pRS425 (control) or pRS425-CDC34 (pF2) were grown in SD (−Leu) medium that contained methylmercuric chloride (MeHgCl) (A, a and b), mercuric chloride ($HgCl_2$) (B, a), *p*-chloromercuribenzoate (pCMB) (B, b), copper chloride ($CuCl_2$) (B, c) or zinc chloride ($ZnCl_2$) (B, d). After the addition of methylmercury (A, a) or a 48-h incubation with each compound (A, b; B, a-d), absorbance at 620 nm was measured spectrophotometrically. Each point and bar represent the mean value and S.D. of results from four cultures. The absence of a bar indicates that the S.D. falls within the symbol.

To examine this hypothesis, we examined the effects of the overexpression of ubiquitin-conjugating enzymes other than Cdc34, namely Ubc4, Ubc5, and Ubc7, which are known to be inessential for cell growth. If methylmercury were to exert its toxic effects by inhibiting the function of Cdc34 that is essential for cell growth, yeast cells should not grow even when one of these nonessential ubiquitin-conjugating enzymes is overexpressed. However, we found that overexpression of Ubc4 and of Ubc7 also conferred resistance to methylmercury to a greater or lesser extent (Fig. 4), a result that suggests that Cdc34 might not be the target of methylmercury toxicity. Figure 5 shows the effects of methylmercury on levels of transcripts of these ubiquitin-conjugating enzymes in control yeast cells. The levels of Cdc34, Ubc4, and Ubc7 transcripts were increased upon treatment of cells with methylmercury. These ubiquitin-conjugating enzymes might be involved in the defense against methylmercury toxicity. Treatment of cells with methylmercury might accelerate the accumulation of a certain toxic proteins that induce the suppression of cell growth. Some ubiquitin-conjugating enzymes, such as Cdc34, Ubc4, and Ubc7, might be able to recognize the toxic protein(s) as a common substrate for ubiquitination; thus, overexpression of these enzymes might enhance the ubiquitin-

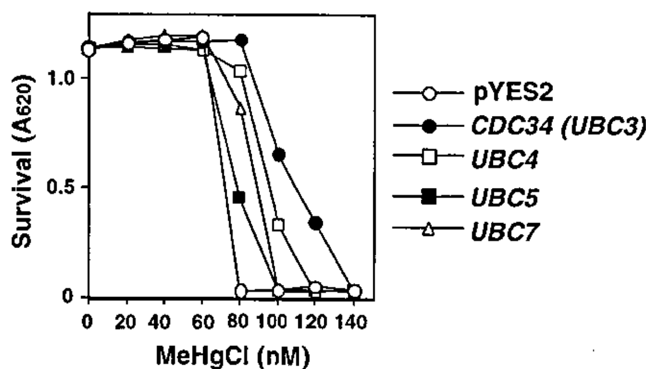

Fig. 4. Sensitivity of yeast cells that overexpressed Ubc4, Ubc5, or Ubc7 to methylmercury. Yeast cells (1×10^4 cells/200 μl/well) carrying pYES2, pYES-CDC34, pYES-UBC4, pYES-UBC5, or pYES-UBC7 were grown in SG medium (−Leu) that contained methylmercuric chloride (MeHgCl). After a 48-h incubation, absorbance at 620 nm was measured spectrophotometrically. Each point and bar represent the mean value and S.D. of results from four cultures. The absence of a bar indicates that the S.D. falls within the symbol.

dependent proteolysis of this toxic protein(s). Further studies are required if we are to understand fully the mechanism of action of Cdc34. However, the present study provides the first evidence, to our knowledge, that overexpression of genes for ubiquitin-conjugating enzymes confers resistance to xenobiotic, such as methylmercury. The ubiquitination system might provide a novel mechanism for protection against the toxicity of mercury compounds.

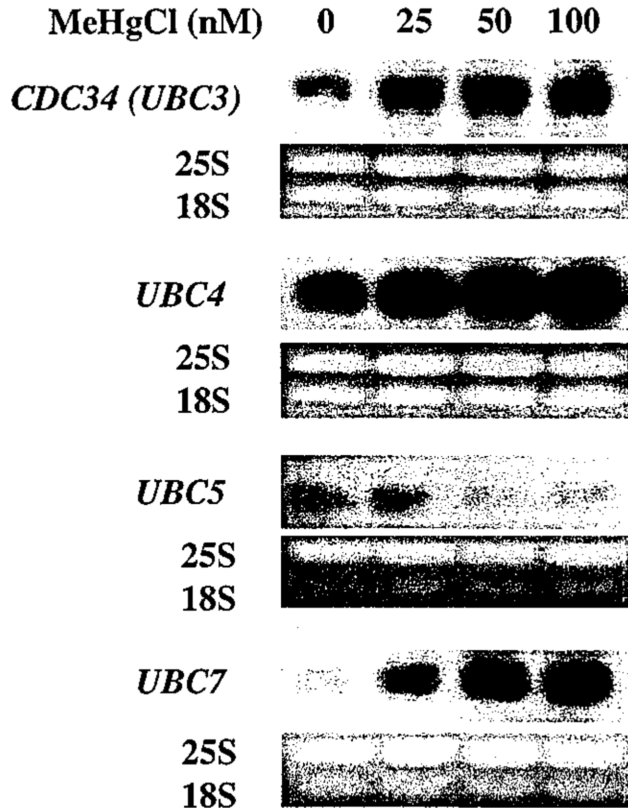

Fig. 5. Effects of methylmercury on the levels of transcripts of the ubiquitin-conjugating enzymes Cdc34, Ubc4, Ubc5, and Ubc7. Each lane was loaded with 20 μg of total RNA extracted from yeast cells (W303B) after treatment with methylmercuric chloride (MeHgCl) for 90 min. The bands of 25S and 18S RNA (lower) provide an indication of the amount of total RNA loaded in each lane.

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A ubiquitin-proteasome system is responsible for the protection of yeast and human cells against methylmercury[1]

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SPECIFIC AIMS

The aim of our study was to elucidate the mechanism responsible for the toxic effects of methylmercury (MeHg). In this report, a ubiquitin-proteasome system is shown for the first time to be involved in the protection against MeHg toxicity.

PRINCIPAL FINDINGS

1. Effect of overexpression of Cdc34 on toxicity of MeHg

To characterize the mechanisms of toxicity and the defense against MeHg, we searched for factors that determine the sensitivity of yeast cells to MeHg. We screened yeast cells that had been transformed with a yeast genomic DNA library for resistance to MeHg and isolated clones that grew in the presence of a normally toxic concentration of MeHg. Analysis of the clone with the highest resistance to MeHg revealed that the *CDC34* gene conferred resistance to MeHg. As shown in **Fig. 1*A***, yeast cells that overexpressed the product of *CDC34* exhibited significant resistance to MeHg. *CDC34* encodes a ubiquitin-conjugating enzyme, Cdc34, that is involved in ubiquitin-dependent proteolysis.

The ubiquitin-dependent pathway to protein degradation involves the covalent attachment of ubiquitin to substrate proteins to yield ubiquitin-protein conjugates. Ubiquitin is activated initially by ubiquitin-activating enzyme (E1) via formation of a thiolester bond with this enzyme. The activated ubiquitin is then transferred to one of many distinct ubiquitin-conjugating enzymes (E2s) by transthiolation. The E2 enzymes catalyze the ubiquitination of substrate proteins either directly or in conjunction with a distinct ubiquitin ligase (E3s) composed of multiple proteins. The ubiquitination of a substrate protein is followed by degradation of the protein by the proteasome.

2. Role of activity of Cdc34 to form a complex with ubiquitin

To investigate the way Cdc34 confers resistance to MeHg, we examined the ability of Cdc34 to form a complex with ubiquitin. Cdc34 has only one cysteine moiety, located at position 95, known to be the binding site for ubiquitin (Fig. 1*B*). As shown in Fig. 1*C*, the yeast cells that overexpressed Cdc34^{C95A}, in which cysteine 95 was replaced by alanine, failed to exhibit resistance to MeHg. This suggested that the activity of Cdc34 to form a complex with ubiquitin might be required for such resistance. Alternatively, MeHg might bind strongly to cysteine 95 of Cdc34 overexpressed in MeHg-resistant yeast cells, since MeHg has high affinity for the thiol groups on cysteine residues in proteins. Such strong binding might reduce the intracellular concentration of free MeHg and suppress the toxicity of MeHg in yeast. Therefore, we next examined the effects of overexpression of mutant Cdc34 proteins (Cdc34$^{E109, D111, E113A}$ and Cdc34$^{1-170aa}$) on the sensitivity of yeast cells to MeHg.

The mutant Cdc34$^{E109, D111, E113A}$ protein in which glutamate 109, aspartate 111, and glutamate 113 are replaced by alanine is unable to perform the function of Cdc34 essential for cell growth. The altered amino acids are located in a 12-residue segment (Fig. 1*B*) of Cdc34 not found in most other E2s and might be involved in the unique activity of Cdc34. The second mutant, Cdc34$^{1-170aa}$, has no E3 binding domain, which is also essential for the ubiquitination of substrate proteins by Cdc34. The sensitivity of yeast cells that overexpressed Cdc34$^{E109, D111, E113A}$ or Cdc34$^{1-170aa}$ to MeHg was not significantly different from that of control yeast cells (Fig. 1*C*). These results indicated that the binding of MeHg to cysteine 95 of Cdc34 is not involved in the resistance to MeHg since the mutant proteins both include cysteine 95. Binding of Cdc34 not only to ubiquitin, but also to E3, might be required for protection against the toxicity of MeHg. Our results suggest that the function of Cdc34 as a ubiquitin-conjugating enzyme is essential for resistance to MeHg. The level of total ubiquitinated proteins in yeast cells

[1] To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.01-0899fje; to cite this article, use *FASEB J.* (March 12, 2002) 10.1096/fj.01-0899fje

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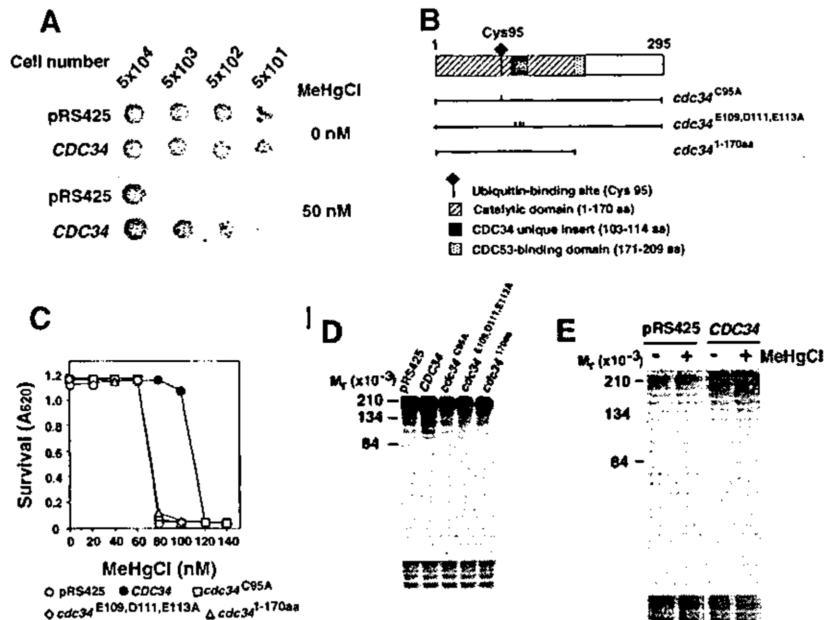
Figure 1. Effects of the overexpression of Cdc34 on the sensitivity of yeast cells to MeHg. *A)* Yeast strains carrying pRS425 (control) or pRS425-CDC34 were grown on a plate of agar-solidified synthetic dextrose (SD) medium (-leu) for 3 days with or without MeHgCl. *B)* Structural domains of Cdc34 and construction of mutant proteins. *C)* Yeast strains carrying pRS425 (control), pRS425-CDC34, pRS425-cdc34^{C95A}, pRS425-cdc34$^{E109, D111, E113A}$, or pRS425-cdc34$^{1-170aa}$ were grown in SD (-leu) medium in the presence of various concentrations of MeHgCl. *D)* Lysates of each strain of yeast cells cultured in control medium were subjected to immunoblotting analysis with multiubiquitin-specific antibody. Staining with Coomassie blue (lower panel) indicates the amount of total protein loaded. *E)* Yeast cells carrying pRS425 (control) or pRS425-CDC34 were cultured for 3 h in the presence or absence of MeHgCl (100 nM). Lysates of these cells were subjected to immunoblotting analysis with multiubiquitin-specific antibody.



that overexpressed Cdc34 was higher than that in control and yeast cells that expressed Cdc34$^{E109, D111, E113A}$ or Cdc34$^{1-170aa}$ (Fig. 1*D*). Although multiple enzymes (E1, E2, and E3) are involved in the ubiquitination of target proteins, this result indicates that overexpression of Cdc34 alone is sufficient to increase the ubiquitination of proteins.

3. Effect of overexpression of ubiquitination-related enzymes other than Cdc34

SCF (*S*kp1, *C*dc53/cullin, *F*-box protein) is one of several E3 complexes and is involved in the Cdc34-mediated ubiquitination of proteins. Four subunits of SCF—Cdc53, Hrt1, Skp1, and F-box protein—have been identified. Overexpression of individual subunits of SCF (particularly Cdc53, Hrt1, and Skp1) did not significantly affect the sensitivity of yeast cells to MeHg or the level of total ubiquitin-protein conjugates. By contrast, overexpression of E1(Uba1) conferred limited resistance to MeHg but the level of total ubiquitinated proteins did not change significantly. Increased levels of total ubiquitinated proteins were observed only in yeast cells that overexpressed Cdc34. Thus, Cdc34 seems to be a rate-limiting enzyme in the protein-ubiquitination system that requires Cdc34 as E2. The basal cellular concentration of Cdc34 might be lower than that of the other components of the ubiquitin system.

4. Effect of MeHg on ubiquitin-conjugating activity of Cdc34

Treatment with MeHg did not decrease levels of total ubiquitinated proteins in control yeast cells nor reduce the elevated levels of ubiquitinated proteins that were induced by overexpression of Cdc34(Fig. 1*E*). Slight increases in the levels of ubiquitinated proteins were observed in control cells and Cdc34-overexpressing yeast cells after treatment with MeHg. These data suggest that MeHg has no inhibitory effects on the ubiquitin-conjugating activities of Cdc34 and most other E2s.

5. Involvement of proteasome activity

The conjugation of ubiquitin to target proteins serves as a signal for degradation of these proteins in proteasomes. We postulated that MeHg might induce the accumulation of some protein(s) with an undesirable effect on cell growth and an inherent signal for ubiquitination. Degradation of the protein(s) by proteasomes after ubiquitination might act as a cellular defense against MeHg toxicity. To examine this possibility, we investigated the effects of a proteasome inhibitor, carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132), on the resistance to MeHg conferred by overexpression of Cdc34 (**Fig. 2*A***). We constructed yeast strain *erg6 (ise1)*, which is very permeable to MG132, because proteasome inhibitors such as MG132 are unable to penetrate wild-type yeast cells. The *erg6* mutant cells that overexpressed Cdc34 (*erg6*/CDC34) were significantly more resistant to MeHg than *erg6* cells that harbored the control vector (*erg6*/pRS425). Treatment with MG132 almost completely eliminated the protective effect of the overexpression of Cdc34 against MeHg toxicity, which suggested that proteasome activity is essential for the Cdc34-mediated resistance to MeHg. We also observed the enhancement of MeHg toxicity by MG132 (Fig. 2*A*). MG132 alone had only a slightly inhibitory effect on the growth of *erg6* mutant cells under our experimental conditions. To confirm the role of proteasome activity in the protection against the toxicity of MeHg, we performed an experiment with the proteasome-defective strain WCG4-11a. In WCG4-11a cells, the mutant *PRE1* gene encodes a

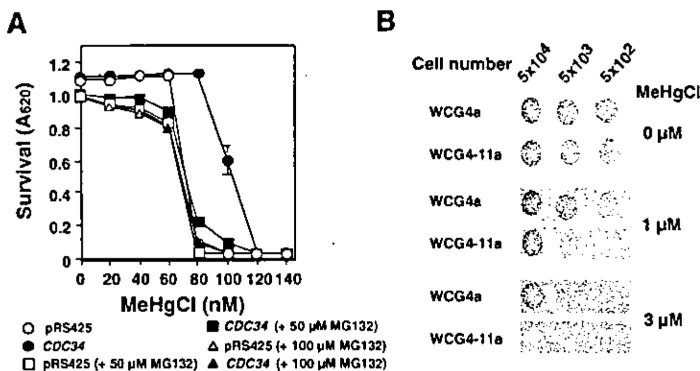
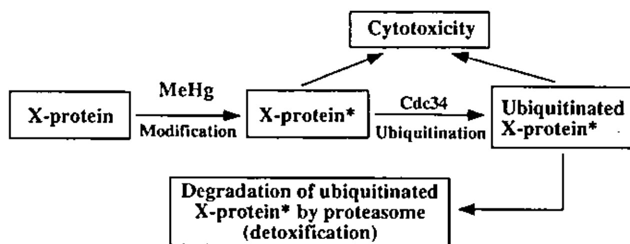


Figure 2. Involvement of proteasome activity in resistance to MeHg. *A)* Yeast *erg6* cells that harbored pRS425 (control) or pRS425-CDC34 were grown in SD (-leu) medium with or without the proteasome inhibitor MG132 in the presence of various concentrations of MeHgCl. *B)* Yeast WCG4a cells (control) and WCG4–11a cells (proteasome defective cells) were grown on a plate of agar-solidified YPD (yeast extract-peptone-dextrose) medium for 3 days with or without MeHgCl.

missense version of the 22.6 kDa subunit of the yeast proteasome and the cells cannot degrade proteins that undergo ubiquitin-dependent proteolysis in wild-type yeast cells. As shown in Fig. 2*B*, proteasome-defective WCG4–11a cells were hypersensitive to MeHg compared with wild-type cells (WCG4a). These results clearly indicated that proteasomes play a protective role in the defense against MeHg toxicity.

CONCLUSIONS AND SIGNIFICANCE

The results presented here suggest a novel hypothetical mechanism for MeHg toxicity and the defense against such toxicity. MeHg might accelerate the synthesis or modification of a certain protein(s). The protein (designated X-protein) modified by MeHg suppresses cell growth and includes a signal for ubiquitination by Cdc34 and related enzymes. Ubiquitination of X-protein and its subsequent degradation by the proteasome protect the cell against MeHg toxicity. In normal cells, MeHg might be cytotoxic when the cellular concentration of X-protein exceeds the cell's capacity for ubiquitination. X-Protein might still have growth-suppressive activity after ubiquitination since the sensitivity to MeHg observed in the presence of MG132 was almost identical in *erg6* cells that overexpressed Cdc34 (*erg6*/CDC34) and in cells that harbored the empty vector (*erg6*/pRS425) (Fig. 2*A*) even though levels of total ubiquitinated proteins after treatment with MG132 were significantly higher in *erg6*/CDC34 cells than in *erg6*/pRS425 cells (data not shown). Thus, not only the ubiquitination of X-protein, but also proteasome activity for degradation of ubiquitinated X-protein, seem to be crucial for protection against the cytotoxicity of MeHg. The identification and characterization of X-protein or the protein converted to X-protein by MeHg-initiated modification should help us to explain the mechanism of toxicity of MeHg.



Schematic diagram.

The ubiquitin-proteasome system is strongly conserved from yeast to human cells. To determine the effects of overexpression of human Cdc34 (hCdc34) in human cells (HEK293 cells), we established three lines of transfectants that stably expressed hCdc34. These clones all exhibited significant resistance to MeHg compared with two control clones that had been transfected with the empty vector (data not shown). This result indicates that the ubiquitin-proteasome system plays an important role in the protection against MeHg toxicity not only in yeast cells, but also in human cells. FJ

Tohoku J. Exp. Med., 2002, **196**, 65-70

Review

Investigation of Intracellular Factors Involved in Methylmercury Toxicity

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Naganuma, A., Furuchi, T., Miura, N., Hwang, G.-W. and Kuge, S. *Investigation of Intracellular Factors Involved Methylmercury Toxicity.* Tohoku J. Exp. Med., 2002, **196** (2), 65-70 —— Methylmercury is a known pollutant that causes severe central nervous system disorders. It is capable of passing through the blood-brain barrier and accumulates in cerebral cells. However, little is known regarding the mechanism of its toxicity at the molecular level. Using yeast cells, we searched for the genes involved in the expression of methylmercury toxicity, and found that genes encoding L-glutamine•D-fructose-6-phosphate amidotransferase (GFAT) and ubiquitin transferase (Ubc3) confer methylmercury resistance on the cells. It has also been shown that GFAT is the target molecule of methylmercury in yeast cells. These findings provide important clues about the mechanism underlying methylmercury toxicity in mammals. ——— methylmercury; resistance; yeast; ubiquitin; glucosamine-6-phosphate
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The mechanism underlying mercury poisoning remains to be elucidated

Despite the efforts of many researchers, including ourselves, the mechanisms involved in methylmercury toxicity and the defense against this toxicity remain unknown (Akagi and Naganuma 2000; Miura 2000). Although more than 40 years have passed since the first outbreak of Minamata disease, we still only know that the causative agent is methylmercury which is highly cytotoxic, but how it exerts it toxicity at the molecular level remains to be determined. We have attempted to clarify the mechanism of methylmercury toxicity using experimental animals, because we consider it important to investigate the in vivo toxicity in detail, since studies using only in vitro experimental systems did not clarify the mechanism of the neurotoxicity of methylmercury. However, studies using rats and mice would be meaningless unless findings significant and rele-

Received December 12, 2001; revision accepted for publication February 28, 2002.
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This paper was presented at the Sendai Symposium: Paradigm Shift in Heavy Metal Toxicology held on November 21-22, 2001.

vant to humans are obtained. We have obtained several toxicologically important findings with respect to methylmercury using experimental animals (Naganuma and Imura 1979, 1980, 1984; Naganuma et al. 1980a; Tanaka et al. 1991, 1992a, b; Urano et al. 1997), but could not determine the mechanism of methylmercury toxicity at the molecular level.

Does methylmercury target specific molecules in cells ?

By what mechanism does methylmercury exert it toxic effects on cells? Methylmercury has a high affinity to the SH residues of cysteine, and inhibits most of the enzymes whose activities depend on the SH residues in the enzyme molecules. Therefore, some researchers consider that methylmercury does not exert its toxicity by attacking specific targets in cells but suppresses the growth of cells by the nonspecific inhibition of the activities of proteins with SH residues. In fact, increases in the concentration of intracellular free SH residues reduce methylmercury toxicity (Miura et al. 1994a, b). On the other hand, it was reported that methylmercury toxicity was suppressed by antioxidants such as vitamin E in animal experiments (Yonaha et al. 1983; Sarafian and Verity 1991), but this cannot be explained by the nonspecific inhibition of intracellular active substances by methylmercury.

Investigation of target molecules using yeast

If it is assumed that there are target molecules of methylmercury in cells, the problem of finding them remains. Many researchers have attempted to identify such target molecules, but most tested candidates among known intracellular factors. However, unknown factors cannot be identified by such a method. In this study, we randomly tested target molecules at the genetic level. We focused on the fact that drug resistance is sometimes involved in elevation of the concentration of the intracellular target of the drug. Screening for the genes conferring methylmercury resistance on cells transformed with individual genes would indicate the genes encoding the target molecules of methylmercury together with the genes for the defense against its toxicity. We investigated yeast genes obtained from a library, since yeast genes can be easily identified because the nucleotide sequence of the entire genome has been clarified. Plasmids carrying a chromosome fragment (usually containing 2-4 genes) were transfected into yeast cells, and genes contained in the chromosome fragments were expressed at high levels in the cells. Among such yeast cells, those that could grow on a medium containing methylmercury at a concentration that would not permit the growth of normal yeast cells were selected. Since these yeast cells acquired methylmercury resistance following the introduction of gene fragments, genes conferring methylmercury resistance must be contained in the introduced gene fragments. Plasmids were isolated from the yeast cells that

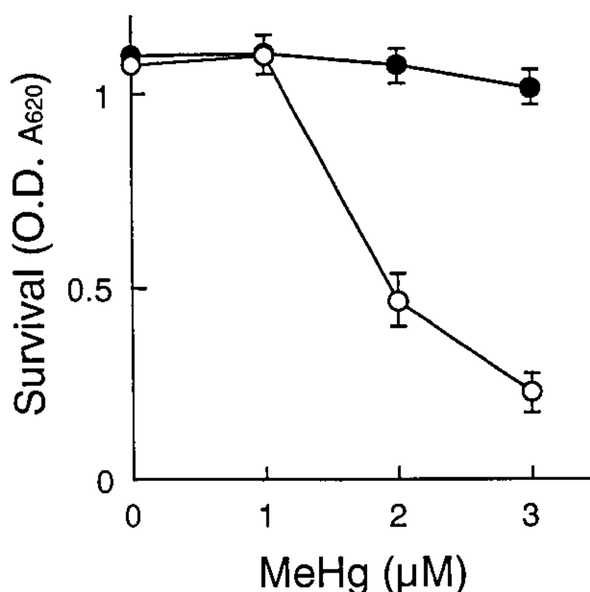

Fig. 1. Sensitivity of yeast transfected with the *GFAT* gene to toxicity of methylmercury (MeHg). W303B/pGFAT and W303B/pYES2 (control) were suspended in a medium in the presence of MeHg and cultured with shaking at 30°C for 24 hours.
○, pYES2 (Control); ●, GFAT.

had acquired methylmercury resistance, and the chromosome fragments carried in the plasmids were investigated. We identified *GFA1* (Fig. 1) and *CDC34* as the genes involved in methylmercury resistance (Miura et al. 1999; Naganuma et al. 2000; Furuchi et al. 2002; Hwang et al. 2002). *GFA1* is the gene encoding L-glutamine•D-fructose-6-phosphate amidotransferase (GFAT), which is a catalytic enzyme involved in the production of glucosamine-6-phosphate from glutamine and fructose. *CDC34* is the gene encoding ubiquitin transferase (Ubc3), which is involved in the ubiquitination of intracellular proteins.

GFAT is the target molecule of methylmercury in yeast

Glucosamine-6-phosphate generated by the reaction catalyzed by GFAT is the precursor of all amino sugars synthesized intracellularly. Yeast cells cannot survive without amino sugars, because glycoproteins cannot be produced in their absence. Therefore, GFAT is an the essential enzyme for the growth of yeast cells (Watzele and Tanner 1989). Since GFAT is an SH enzyme, methylmercury inhibits GFAT activity. The inhibitory effects of methylmercury on various SH enzymes were determined (Naganuma et al. 2000) (Fig. 2). The activity of GFAT was almost completely inhibited by 4 μM methylmercury, while those of other SH enzymes were hardly affected by methylmercury at this concentration. The inhibition constant (Ki) of methylmercury was 4 μM for GFAT, while the Ki values for other SH enzymes were higher than 10-fold this value. These results indicate that methylmercury has a high affinity for GFAT and specifically inhibits its activity, suggesting that GFAT is the target molecule of methylmercury.

Yeast cells transfected with the *GFA1* gene are highly resistant to methylmercury, and a

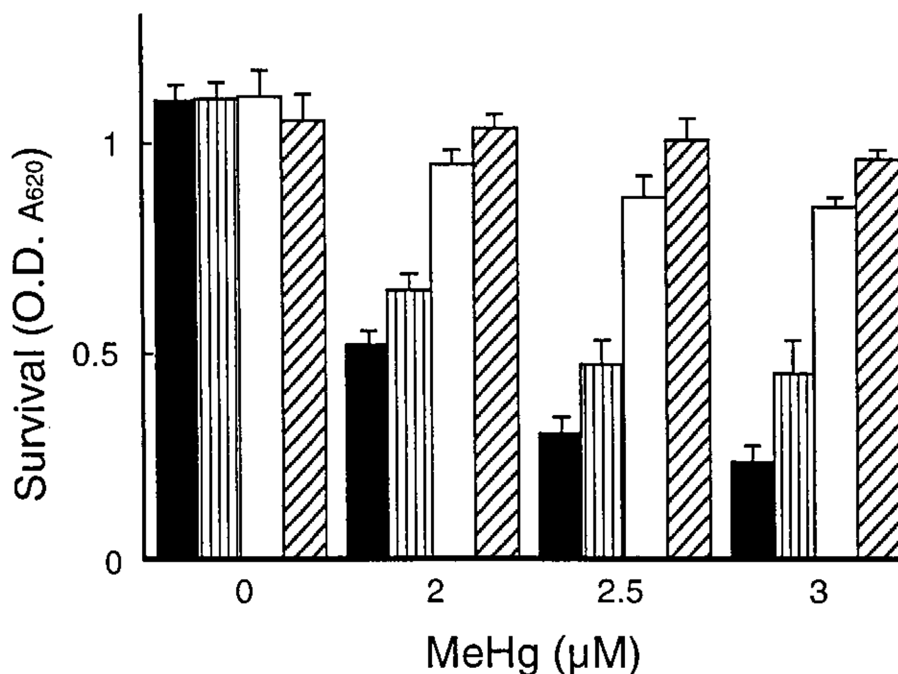

Fig. 2. Effect of MeHg on activities of GFAT and other SH-enzymes in extract of wild-type yeast (W303B). After a 3-minute incubation in yeast extract with MeHg at 37°C, the activities of GFAT, alcohol dehydrogenase (ADH), glutathione reductase (GR) and lactate dehydrogenase (LDH) were determined.
GlcN (mM) ■, 0; ▥, 6.25; □, 12.5; ▨, 25.

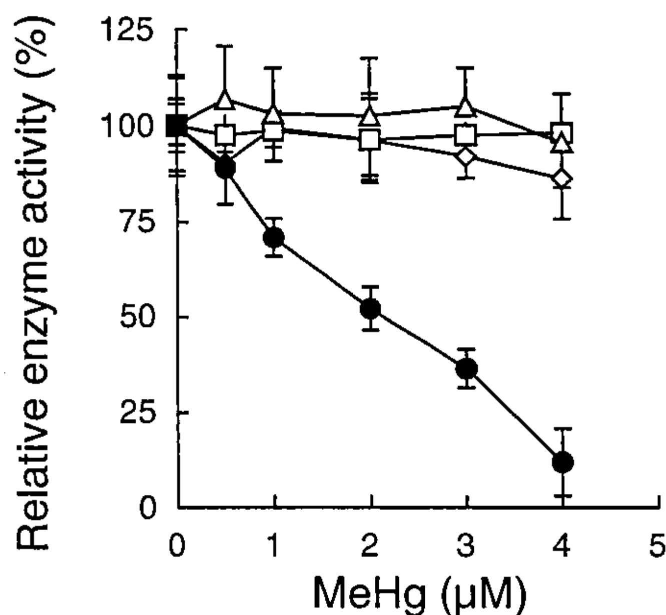

Fig. 3. Effect of an increase in intracellular levels of glucosamine-6-phosphate on toxicity of MeHg. Wild-type yeast (W303B) cells suspended in the medium were pretreated with glucosamine, which is converted to glucosamine-6-phosphate by hexokinase in yeast, for 1 hour followed by a 24-hour incubation with MeHg.
●, GFAT; △, ADH; □, GSHR; ◇, LDH.

relatively large amount of GFAT is synthesized by the cells. Therefore, it remains possible that strong binding of GFAT at a high concentration to methylmercury reduced the concentration of free methylmercury and suppressed methylmercury toxicity, inducing methylmercury resistance in the yeast cells. Therefore, we determined the effects of increasing the intracellular concentration of glucosamine-6-phosphate induced by the reaction catalyzed by GFAT on methylrercury toxicity. Since glucosamine-6-phosphoric acid added to media is not taken up by cells, glucosamine was added to media. Glucosamine is not synthesized by cells, but extracellularly added glucosamine is taken up by cells and transformed to glucosamine-6-phosphate by hexokinase. The toxicity of methylmercury towards the yeast cells was markedly reduced depending on the concentration of added glucosamine (Naganuma et al. 2000) (Fig. 3).

In conclusion, (1) yeast cells with high-level GFAT expression are resistant to methylmercury, (2) methylmercury specifically inhibits GFAT activity, (3) methylmercury toxicity is markedly reduced by the addition of glucosamine, which is transformed to glucosamine-6-phosphate, the product of the GFAT reaction, to cells, and (4) GFAT is an essential enzyme in yeast. These results suggest that GFAT is the main target molecule of methylmercury in yeast (Naganuma et al. 2000).

Ubiquitination as a defense mechanism against methylmercury toxicity

As described above, we showed that the gene encoding Ubc3, in addition to that encoding GFAT, confers methylmercury resistance on yeast. Ubc3 is an important enzyme in the ubiquitination of intracellular proteins. The ubiquitin system, which consists of a ubiquitin activation enzyme (E1), ubiquitin transferase (E2) and ubiquitin ligase (E3), is involved in the degradation of abnormal intracellular proteins. In this system, ubiquitin is activated by E1 and then binds to E2, while E3 recognizes

target proteins such as abnormal proteins. E2 bound to ubiquitin binds to E3, and transfers ubiquitin to the target protein. Finally, the target protein ubiquitinated by these reactions is recognized by proteasomes and rapidly degraded (Hochstrasser 1996; Hershko and Ciechanover 1998).

E2 proteins belong to the family of ubiquitin transferases, and the UBC domain is preserved as the catalytic domain in all E2 proteins. It is known that cysteine residues involved in the binding to ubiquitin, which is essential to the expression of E2 activity, are present in the UBC domain. To clarify the mechanism by which Ubc3 confers methylmercury resistance on yeast cells, we produced yeast cells overexpressing a mutant Ubc3 by substituting this cysteine residue with alanine, and found that these yeast cells were not methylmercury-resistant (Hwang et al. 2002). Therefore, the ubiquitin transfer activity of Ubc3 is considered essential to the acquisition of methylmercury resistance.

Thirteen enzymes of the E2 family of yeast have been identified, each considered to exhibit substrate specificity. Therefore, we produced yeast cells overexpressing Ubc2, Ubc4, Ubc5 or Ubc7 of the E2 family, and determined their methylmercury resistance. Methylmercury resistance was observed in the yeast cells overexpressing Ubc4, Ubc5 and Ubc7 (Furuchi et al. 2002). Our study was the first to show that high-level expression of E2 family enzymes confers resistance to toxic chemicals. Among the yeast cells overexpressing E1 (Uba1) or E3 (CDC53, SKP1, HRT1) proteins, only the yeast cells overexpressing Uba1 of the E1 family exhibited weak methylmercury resistance (Hwang et al. 2002). These results suggest that E2 is the rate-limiting enzyme in the ubiquitination reaction, and the amount of ubiquitinated protein within cells was markedly increased by the overexpression of Ubc3.

It is considered that protein denatured by active oxygen is ubiquitinated by the ubiquitin system and degraded. It is hypothesized that cytotoxicity results when abnormal proteins do not undergo normal degradation and accumulate in cells. Therefore, it is suggested that some modification of a specific protein within cells by methylmercury causes cytotoxicity, and enhancement of the degradation of the protein by ubiquitination reduces this toxicity. In this case, the ubiquitin system acts to protect cells against methylmercury toxicity, and the protein ubiquitinated following modification by methylmercury would be the target molecule of methylmercury toxicity.

There are genes in humans that are homologous to the genes encoding GFAT and Ubc3, which have been identified as proteins conferring methylmercury resistance on yeast cells. Therefore, both proteins are likely to be involved in methylmercury toxicity in human cells. To clarify the mechanisms of the toxicities of other toxic chemicals, investigation of resistance factors at the genetic level using yeast is considered useful.

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AYUMI

メチル水銀の毒性発現機構

Molecular mechanism of methylmercury toxicity

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◎水俣病の原因物質として知られるメチル水銀は，脳に蓄積して重篤な中枢神経障害を引き起こす重要な環境汚染物質である．水俣病の発見から50年近くが経過した現在，わが国では環境汚染物質としてのメチル水銀の危険性は一般的にはすっかり忘れ去られてしまったように思われる．しかしアメリカでは，胎児の脳に対するメチル水銀の影響を危惧して，2001年に，妊娠中または妊娠する可能性のある女性は大型魚類を摂取するべきではないとの勧告が出された．メチル水銀は胎児中に蓄積しやすく，発達途中の胎児脳はメチル水銀に対する感受性が高いので，比較的微量のメチル水銀によっても脳機能に障害が生じる可能性があるためである．水銀による環境汚染は現在も世界的に進行しているが，メチル水銀の毒性発現機構はほとんどわかっていない．著者らは酵母にメチル水銀耐性を与える細胞内因子を検索し，2つの酵素を同定することに成功した．本稿では，メチル水銀の毒性発現におけるこれら2つの酵素の役割について概説する．

メチル水銀，酵母，耐性遺伝子，ユビキチン

メチル水銀は水俣病の原因物質として知られており，重篤な中枢神経障害を引き起こす．メチル水銀の健康影響をより正確に理解し評価するためには，その毒性発現機構の解明が必須と考えられるが，水俣病の発症確認からすでに50年近くが経過したにもかかわらず，メチル水銀の毒性発現機構は不明のままであり，解明の糸口さえつかめていない．

著者らもメチル水銀の毒性発現機構の解明をめざし，長年にわたって実験動物などを使ってさまざまな検討を続けてきたが，ほとんど目的を達成することができなかった．そこで最近，メチル水銀毒性の発現機構に関する新知見を確実に集積することが重要と考え，より単純な実験系として酵母を選び，メチル水銀の毒性発現に影響を与える細胞内因子の遺伝子レベルでの検索を試みたところ，メチル水銀に対する感受性を左右する酵母遺伝子としてグルタミンとフルクトース6リン酸からグルコサミン6リン酸を生成する反応を触媒する酵素であるL-グルタミン：D-フルクトース6リン酸アミドトランスフェラーゼ(GFAT)[1]，および細胞内蛋白質分解系のひとつであるユビキチン/プロテアソームシステムを構成するユビキチン転移酵素Cdc34[2]を同定することに成功した．

GFATは酵母におけるメチル水銀の標的分子である[3]

グルコサミン6リン酸は細胞内で合成されるすべてのアミノ糖の原材料となる物質であり，アミノ糖がないと糖蛋白質などが合成されず酵母は生存することができない．したがって，GFATは酵母の増殖に必須の酵素ということになる．GFATはSH酵素であり，メチル水銀はこのGFATの活性を阻害する．他のSH酵素の活性に対するメチル水銀の阻害効果をGFATと比較すると，酵母抽出液中のGFAT活性は4 μMのメチル水銀添加に

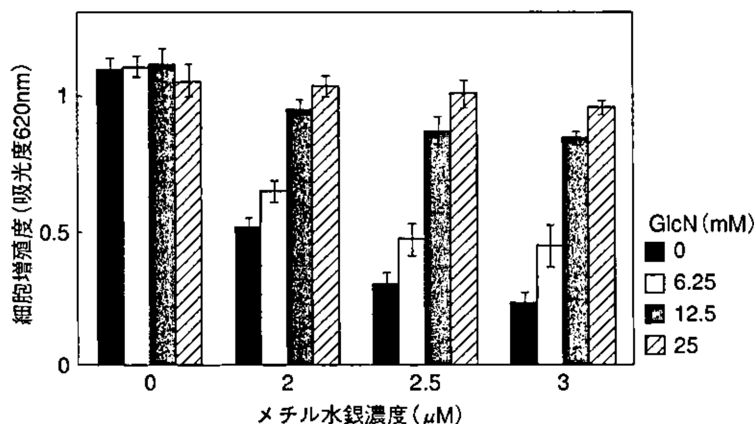

図1 細胞内グルコサミン6リン酸濃度の上昇がメチル水銀毒性に与える影響

グルコサミン(GlcN)は細胞内でリン酸化されてグルコサミン6リン酸に変換される.

よってほぼ完全に阻害されるが，他のSH酵素の活性の阻害には10倍以上の濃度のメチル水銀が必要である．したがって，メチル水銀はGFATに対して高い親和性を有し，特異的にその活性を阻害するものと考えられる.

GFAT遺伝子を導入した酵母はメチル水銀に対して高い耐性を示すが，この酵母内ではGFAT蛋白質が比較的多量に合成されている．したがって，高濃度に存在するGFAT蛋白質がメチル水銀と強固に結合すると，遊離のメチル水銀濃度が減少することによってメチル水銀の毒性発現が抑制され，その結果として本酵母がメチル水銀に対して耐性を示すという可能性も考えられる．そこで，GFATが触媒する反応の産物であるグルコサミン6リン酸の細胞内濃度を上昇させた際のメチル水銀感受性を検討したところ，メチル水銀の酵母に対する毒性はグルコサミンの添加濃度に依存して顕著に軽減された(図1).

以上を要約すると，①GFATを高発現する酵母はメチル水銀に対して耐性を示す，②メチル水銀は特異的にGFAT活性を阻害する，③GFAT反応の産物であるグルコサミン6リン酸を細胞内に供給することによってメチル水銀の毒性は顕著に軽減される，④GFATは酵母に必須の酵素である，ということになり，これらの事実からGFATは酵母におけるメチル水銀の主要な標的分子であると考えられる.

GFATは，酵母のみならずヒトをはじめとする哺乳動物においても必須の酵素として働いていることから，哺乳動物でもメチル水銀の標的となっている可能性が考えられる．しかし，この可能性に関しては，すくなくとも著者らの予備的な検討では否定的な結果が得られており，ヒト培養細胞にGFATを高発現させてもメチル水銀に対する感受性には変化が認められなかった．したがって，ヒト細胞中にはGFATよりもメチル水銀に対して高い親和性を有する因子が存在する可能性が考えられる.

いずれにしろ，本研究は真核生物でのメチル水銀の細胞毒性発現機構をはじめて明らかにしたものであり，このような方法で標的分子を同定できる可能性を示せたことは，今後のメチル水銀研究の発展を考えるうえで重要な意味をもつ．また，これまでメチル水銀は細胞内の生理活性物質を不特定に阻害することによって細胞毒性を発揮するとの考え方もあったが，本知見は酵母中に特定の標的分子が存在するということを明確に示しており，標的分子を明らかにすることがヒトにおけるメチル水銀毒性発現機構解明の最短距離と考えられる.

メチル水銀毒性に対する防御機構としてのユビキチン/プロテアソームシステム[4]

メチル水銀耐性にかかわる遺伝子として上述のように，GFTA以外にユビキチン転移酵素(Cdc34)をコードする遺伝子CDC34が同定された．ユビ

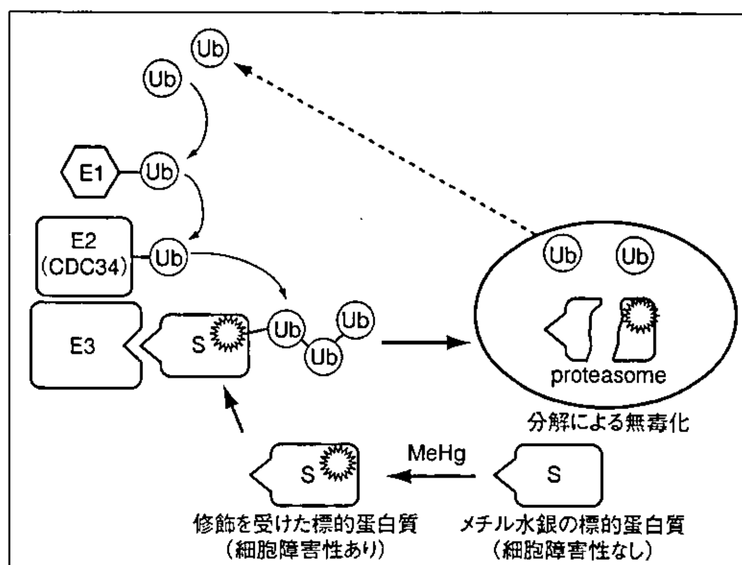

図 2 メチル水銀毒性発現機構とそれに対する防御機構としてのユビキチン/プロテアソームシステム
Ub：ユビキチン，E1：ユビキチン活性化酵素，E2：ユビキチン転移酵素，E3：ユビキチン結合酵素，S：基質(標的蛋白質).

キチン/プロテアソームシステムは異常蛋白質の分解に関与する重要な細胞内機能である．このうちユビキチンシステムはユビキチン活性化酵素(E1)，ユビキチン転移酵素(E2)，およびユビキチン結合酵素(E3，複数の蛋白質の複合体)から構成され，このシステムにおいてユビキチンはまずE1によって活性化された後にE2と結合する．一方，標的となる蛋白質(異常蛋白質など)はE3によって認識される．ユビキチンと結合したE2は，E3を構成する蛋白質に結合した後に，ユビキチンを標的蛋白質に付加する．これら一連の反応によってユビキチン化された標的蛋白質は，最終的にプロテアソームに認識されて速やかに分解される．

Cdc34の構造中で，メチル水銀耐性に必要なドメインを検索するため，Cdc34のユビキチン結合部位またはE3結合部位に変異与えたミュータントを作製したところ，いずれのミュータントを高発現させた酵母もメチル水銀に対して耐性を示さなかった．一方，これらの酵母内でユビキチン化された蛋白質の総量を測定したところ，Cdc34高発現酵母ではユビキチン化蛋白質量がコントロール酵母に比べて著しく高かったが，メチル水銀に対して耐性を示さなかったCdc34のミュータントを高発現させた酵母では，ユビキチン化蛋白質量がコントロール酵母とほとんど同程度であった．以上の結果は，Cdc34高発現によるメチル水銀耐性にはCdc34がユビキチンおよびE3と結合することが必要であることを示しており，Cdc34のE2としての機能であるユビキチン転移反応がメチル水銀耐性に必須であると考えられる．

一方，ユビキチンシステムに関与するE2以外の蛋白質，すなわちE1(Uba1)およびE3複合体(Cdc53，Skp1，Hrt1)を高発現させても，E1高発現酵母でわずかなメチル水銀耐性が認められたものの，Cdc34高発現酵母で認められるような顕著な耐性は観察されなかった．また，これら酵母のユビキチン化蛋白質量も正常酵母と同程度であった．以上の結果はE2がユビキチン化反応の律速蛋白質である可能性を示唆するものであり，E2の高発現は細胞内における蛋白質のユビキチン化を促進させることによってメチル水銀毒性に対して防御的に作用するものと考えられる．

ユビキチン化された標的蛋白質は最終的にプロテアソームに認識されて速やかに分解される．プロテアソーム阻害薬存在下ではCdc34高発現によるメチル水銀耐性が認められず，また，遺伝子変異によって低プロテアソーム活性を示す酵母がコ

ントロール酵母に比べて高いメチル水銀感受性を示すことから，Cdc34高発現によるメチル水銀耐性にはプロテアソームによるユビキチン化蛋白質の分解が必須であると考えられる．

以上の結果から，ユビキチン/プロテアソームシステムが関与するメチル水銀の毒性発現機構およびそれに対する防御機構として，以下のような可能性が考えられる．すなわち，ある種の特定な蛋白質がメチル水銀によって何らかの修飾を受け，この修飾蛋白質が細胞内に蓄積することによって細胞障害が引き起こされる．一方，メチル水銀によって修飾を受けた蛋白質のユビキチン化を介したプロテアソームでの分解の促進がメチル水銀毒性に対して防御的に作用する(図2)．したがって，メチル水銀により分解が促進される蛋白質を同定することによってメチル水銀毒性の発現機構が解明できる可能性がある．

一方，ヒトなどの高等動物においても多くのE2遺伝子が存在することが知られている．そこで，ヒトの*CDC34*(*hCDC34*)をHEK293細胞に導入し，hCdc34高発現細胞を作製してメチル水銀に対する感受性を検討したところ，hCdc34を高発現させたすべてのクローンがメチル水銀に対して耐性を示した．このことから，ヒトにおいてもCdc34が関与するユビキチン/プロテアソームシステムがメチル水銀の毒性軽減機構として働いている可能性が考えられる．したがって，メチル水銀によって修飾を受けてユビキチン化される標的蛋白質をヒト細胞中で同定することによって，ヒトにおけるメチル水銀の細胞内標的分子が明らかになるものと期待される．

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●お知らせ●

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　　　　9月29日(日)午前9時00分～午後4時10分
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Biochemical and Biophysical Research Communications 310 (2003) 148–152

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Copper(II) protects yeast against the toxicity of cisplatin independently of the induction of metallothionein and the inhibition of platinum uptake

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Received 19 August 2003

Abstract

We have made the unexpected discovery that copper sulfate protects *Saccharomyces cerevisiae* from the toxic effects of cisplatin. Addition of copper to the culture medium of yeast cells at concentrations above 0.1 μM significantly reduced the toxicity of cisplatin. Since a high-affinity copper transporter, Ctr1, has been reported to play a major role in the uptake of cisplatin, we examined the effects of copper on the cellular uptake of cisplatin. We found that the cellular concentration of platinum was not significantly affected by treatment of cells with 1 μM copper. It is known that mammalian metallothionein is induced by copper and is involved in acquired resistance to cisplatin. Copper significantly increased the level of mRNA for yeast metallothionein at a concentration that has effectively reduced the toxicity of cisplatin. However, the toxicity of cisplatin in cells with a disrupted gene for ACE1, a factor that regulates transcription of the yeast gene for metallothionein, was also significantly reduced by treatment with copper. These results suggest that copper protects yeast cells from cisplatin toxicity independently of induction of the synthesis of metallothionein and of the inhibition of platinum uptake. Since copper is one of the trace elements that are essential for cell function and since a relatively low concentration of copper (0.1 μM) significantly reduced cisplatin toxicity, it is possible that copper might play an important role in the expression of cisplatin toxicity.
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Keywords: Cisplatin; Resistance; Copper; Metallothionein; Yeast

Cisplatin [*cis*-diamminedichloroplatinum(II)] is an effective antitumor agent for several types of cancer [1,2], but the effectiveness of chemotherapy with cisplatin is often limited by the development of resistance to this drug [3]. Various mechanisms of cisplatin resistance have been described and they include decreased accumulation of cisplatin [4–7], enhanced intracellular detoxification by a system that involves glutathione [8–12] or metallothionein [13–16], altered patterns of DNA platination [17], and increased repair of damaged DNA [18–22]. However, the details of the mechanism of cisplatin resistance remain to be clarified.

We have been searching for cellular factors that confer resistance to cisplatin in yeast because yeast has been established as a model organism in which powerful genetic techniques can be used to elucidate fundamental but complex eukaryotic processes. Previously, we identified *CIN5* and *YDR259c* as genes that confer resistance to cisplatin in yeast [23]. We described here the unexpected finding that copper has a powerful protective effect against cisplatin toxicity. Copper is one of the trace elements that is essential for cell function and a relatively low concentration of copper (0.1 μM) significantly reduced cisplatin toxicity. Thus, copper might play an important role in the expression of cisplatin toxicity.

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doi:10.1016/j.bbrc.2003.09.008

Materials and methods

Yeast and media. *Saccharomyces cerevisiae* W303B (*MATα his3 can1-100 ade2 leu2 trp1 ura3*) was grown in yeast extract–peptone–adenine–dextrose (YPAD) medium (1% yeast extract, 2% peptone, 0.004% adenine, and 2% glucose) or in synthetic dextrose (SD) medium supplemented with amino acids [24].

Quantitation of the toxicity of cisplatin in yeast. Yeast cells were cultured (1×10^4 cells/200 μl) in SD medium that contained cisplatin (Nippon Kayaku, Tokyo, Japan) at various concentrations. After a 48-h incubation, we measured the absorbance of the culture at 620 nm to quantify cell growth. We treated yeast cells with metal compounds by incubating them with copper sulfate, silver nitrate, cadmium chloride or zinc chloride for 12 h. After the 12-h incubation, we washed the yeast cells once with SD medium and then resuspended them in fresh SD medium that contained cisplatin, as indicated.

Disruption of the ACE1 gene in yeast. The *ACE1* gene of *S. cerevisiae* W303B was disrupted as described previously [25]. For construction of the *ace1::HIS3* vector, the *HIS3* gene was amplified by PCR with the following oligonucleotides as primers: 5′-ATG GTCGTAATTAACGGGGTCAAATATGCCTGGAAACGTGTA TC-3′and 5′-TTATTGTGAATGTGAGTTATGCGAAGATACTTG TTTGTATAGCTC-3′. The product of PCR was introduced into W303B cells for construction of the *ACE1* disruptant (*ace1Δ*), and disruption of the gene was verified by PCR and subsequent analysis of the products.

Northern blotting analysis. Cells (5×10^6 cells/ml) were cultured in 30 ml of SD medium that contained a metal compound for 12 h. Total RNA was prepared as described elsewhere [26]. The probe for the CUP1 gene was obtained by PCR with the yeast genome as template- and gene-specific oligonucleotides. Northern blotting was performed using the digoxigenin system from Roche Applied Science (Indianapolis, IN) in accordance with the manufacturer's instructions.

Quantitation of the cellular accumulation of platinum. W303B cells (1×10^6 cells/ml) were cultured in SD medium (30 ml) that contained 150 μM cisplatin for 4 h at 30 °C. The cellular platinum content was determined by ICP-MS (HP4500; Yokokawa Analytical Systems, Tokyo, Japan).

Results and discussion

As shown in Fig. 1A, copper significantly protected yeast cells against cisplatin toxicity at concentrations of copper above 0.1 μM and the effect of copper was dose-dependent. The effective concentration of copper that reduced cisplatin toxicity was considerably lower than the concentration at which copper is cytotoxic (more than 1 mM; Fig. 1B).

The involvement of copper in cisplatin toxicity has been explained in two ways. One way involves the regulation of the high-affinity copper transporter Ctr1. Ctr1 plays a major role in the uptake of cisplatin by yeast and mammalian cells [27,28]. The level of Ctr1 falls in the presence of copper [29,30], and thus, copper has an inhibitory effect on the uptake of cisplatin [27,28]. We monitored the cellular uptake of cisplatin after treatment of yeast cells with copper at a concentration (1 μM) that effectively prevented the cytotoxic effects of cisplatin under our conditions. However, the cellular concentration of platinum was not significantly affected by treatment of cells with 1 μM copper as shown in Fig. 1C. Ishida et al. [28] reported that 100 μM copper (II) significantly decreased the accumulation of platinum in yeast cells treated with cisplatin, but copper at 10 μM had only a minimal effect. In the present study, 1 μM copper significantly reduced the toxicity of cisplatin without affecting the uptake of cisplatin (Figs. 1A and C). These results suggest that a relatively low

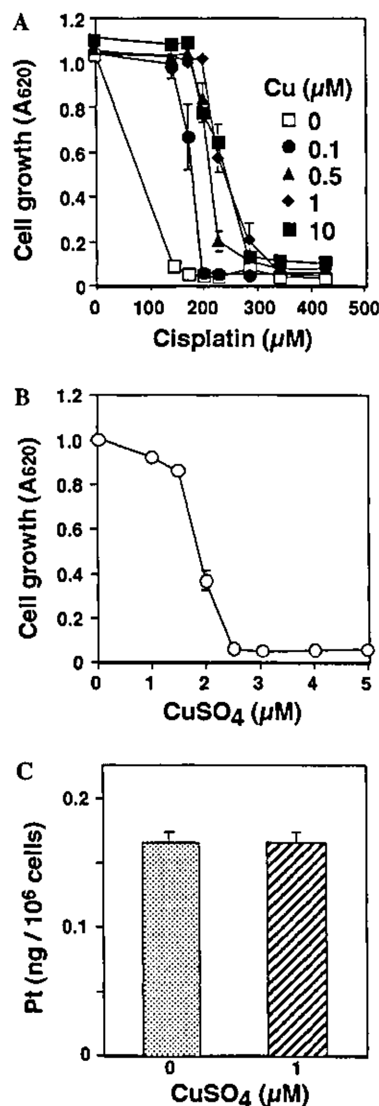

Fig. 1. (A) Effects of copper on the sensitivity of yeast cells to cisplatin. Yeast cells (W303B; 1×10^4 cells/200 μl/well) were grown in SD medium that contained cisplatin and copper sulfate as indicated. After a 48-h incubation, absorbance at 620 nm was measured spectrophotometrically. (B) Effects of copper sulfate on the growth of yeast cells. Yeast cells (W303B; 1×10^4 cells/200 μl/well) were grown in SD medium that contained copper sulfate as indicated. After a 48-h incubation, absorbance at 620 nm was measured spectrophotometrically. (C) Effects of copper sulfate on the uptake of platinum. Yeast cells (W303B; 1×10^6 cells/ml) were cultured in SD medium (30 ml) that contained 150 μM cisplatin for 4 h and then cellular platinum was quantitated. Each result is shown as the mean value with SD of results from three cultures. The absence of a bar indicates that the SD falls within the symbol.

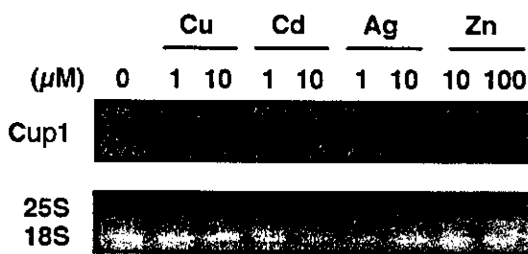

Fig. 2. Effects of metal compounds on the expression of Cup1 mRNA in yeast cells. Each lane was loaded with 25 μg of total RNA that had been extracted from cells after treatment with copper sulfate (Cu), cadmium chloride (Cd), silver nitrate (Ag), or zinc chloride (Zn), as indicated. The bands of 25S and 18S rRNA (lower panel) provide an indication of the amount of total RNA loaded in each lane.

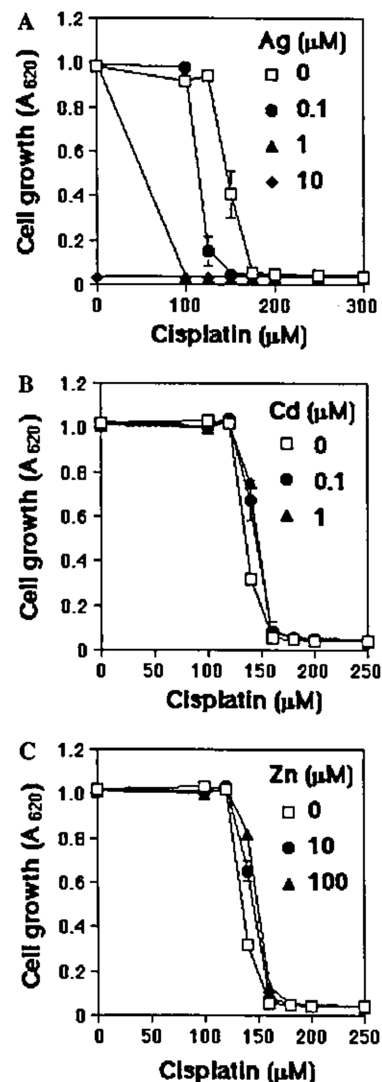

Fig. 3. The effects of metal compounds on the sensitivity of yeast cells to cisplatin. Cells (W303B; 1×10^4 cells /200 μl/well) were grown in SD medium that contained cisplatin plus silver nitrate (A), cadmium chloride (B), and zinc chloride (C) at indicated concentrations. After a 48-h incubation, absorbance at 620 nm was measured spectrophotometrically. Each point and bar represent the mean value and SD of results from three cultures. The absence of a bar indicates that the SD falls within the symbol.

concentration of copper can prevent cisplatin toxicity independently of suppression of the expression of Ctr1.

Synthesis of mammalian metallothionein, a protein that protect cells against the toxicity of certain drugs, metals, and oxidative stresses, is induced by heavy metals, such as copper, zinc, cadmium, and silver [31]. Copper protects mice against the lethal toxicity of cisplatin as effectively as other metallothionein-inducing metals [15]. Metallothionein is one of the major factors involved in the resistance to cisplatin in mammalian cells [13,32–34]. Transcription of the yeast gene for metallothionein, *CUP1*, is also induced by copper and silver, but not by zinc and cadmium [35–37]. The protective effect of yeast metallothionein (Cup1) against cisplatin toxicity has not been examined but the possibility exists that copper might reduce the toxicity of cisplatin via induction of the synthesis of Cup1 in yeast. To examine this possibility, we investigated the relationship between the effects of copper on the level of Cup1 mRNA and on cisplatin toxicity. As shown in Fig. 2, the level of Cup1 mRNA was significantly elevated by treatment of cells with copper sulfate at concentrations at which this salt efficiently reduced the toxicity of cisplatin (see Fig. 1A). We also examined the effects of other metals, namely, silver, cadmium, and zinc, on the toxicity of cisplatin and the level of Cup1 mRNA. Cadmium and zinc slightly reduced the toxicity of cisplatin (Figs. 3B and C). However, the effective concentrations of these metals for depression of the toxicity of cisplatin (1 μM for cadmium and 10 μM for zinc) did not significantly affect the level of Cup1 mRNA (Fig. 2). Silver induced the synthesis of Cup1 mRNA at concentrations above 1 μM (Fig. 2), but treatment with silver at concentrations of 0.1 and 1 μM significantly enhanced the toxicity of cisplatin (Fig. 3A). This result suggests that not all metal compounds protect the yeast cells from the toxicity of cisplatin and that the induction of Cup1 mRNA does not always reduce the toxicity of cisplatin.

Transcription of the *CUP1* gene is regulated by ACE1, a copper-dependent transcription factor [38]. To investigate the role of Cup1 in the effects of copper on cisplatin toxicity, we generated a yeast strain with a disrupted *ACE1* gene (*ace1Δ*). As shown in Fig. 4, the extent of the copper-dependent induction of *CUP1* mRNA in *ace1Δ* yeast cells was very low compared with that in the wild-type yeast W303B cells. The mutant yeast cells exhibited slight resistance to cisplatin, but treatment with copper significantly reduced the toxicity of cisplatin even in these *ace1Δ* cells (Fig. 5). Our results suggest that induction of the synthesis of Cup1 does not play a major role in the protection by copper against cisplatin toxicity. Although mammalian metallothionein protects cells against cisplatin toxicity [13,32,34], the

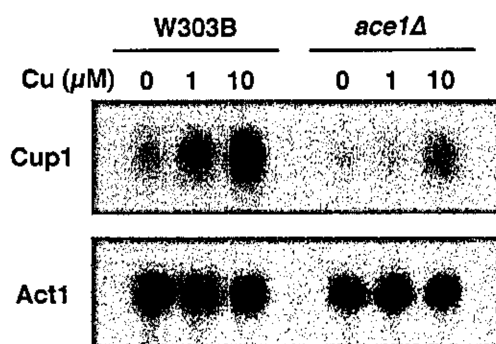

Fig. 4. Effects of copper on the expression of Cup1 mRNA in wild-type yeast (W303B) and yeast with disrupted *ACE1* gene (*ace1Δ*). Each lane was loaded with 25 μg of total RNA that had been extracted from cells after treatment with copper sulfate for 12 h. The bands of actin (Act1) RNA (lower panel) provide an indication of the amount of total RNA loaded in each lane.

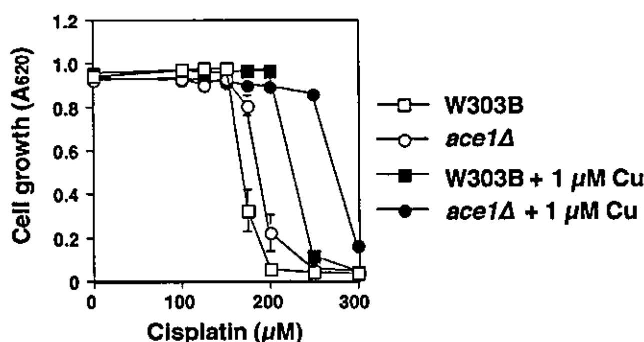

Fig. 5. Effects of copper on the sensitivity of wild-type (W303B) and *ace1Δ* yeast cells to cisplatin. Yeast cells (1×10^4 cells/ 200 μl/well) were grown in SD medium that contained cisplatin with or without 1 μM copper sulfate. After a 48-h incubation, absorbance at 620 nm was measured spectrophotometrically. Each point and bar represent the mean value and SD of results from three cultures. The absence of a bar indicates that the SD falls within the symbol.

yeast metallothionein Cup1 might have only limited ability to depress the toxicity of cisplatin.

In the present study, we found that a relatively low concentration of copper provided protection against cisplatin toxicity independently of the induction of the synthesis of metallothionein and of the inhibition of platinum uptake. Although the mechanism of such protection is unknown, this study provides new insight into the biological function of copper, an essential trace element, in the protection of eukaryotic cells against an anticancer drug, cisplatin.

Acknowledgment

The authors thank Nippon Kayaku (Tokyo) for providing the cisplatin used in this study.

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