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Overexpression of the Ubiquitin-Conjugating Enzyme Cdc34 Confers Resistance to Methylmercury in *Saccharomyces cerevisiae*

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ABSTRACT

A search was made for genes that confer resistance to methylmercury in yeast using a genomic DNA library derived from *Saccharomyces cerevisiae*. The genomic library was introduced into yeast and transformants that grew in the presence of a normally toxic concentration of methylmercury were selected. We sequenced the genomic DNA fragment in the plasmid from the clone with the highest resistance to methylmercury and analyzed the sequence for presence of an open reading frame that might confer resistance to methylmercury. We identified a gene, *CDC34* (also known as *UBC3*), that increased resistance to methylmercury when overexpressed in yeast. *CDC34* encodes a ubiquitin-conjugating enzyme; such proteins play important roles in the selective targeting of proteins for degradation. Overexpression of *UBC4* and of *UBC7*, two other genes for ubiquitin-conjugating enzymes, also conferred resistance to methylmercury. Yeast strains transformed with the *CDC34* gene were resistant not only to methylmercury but also to mercuric chloride and *p*-chloromercuribenzoate. To our knowledge, this is the first demonstration that overexpression of genes for ubiquitin-conjugating enzymes confers resistance to xenobiotics. Our results suggest that ubiquitination system might be involved in protection against the toxicity of mercury compounds, such as methylmercury, in eukaryotic cells.

Methylmercury, an environmental contaminant (Akagi and Naganuma, 2000), causes severe neurological disorders in humans (Takeuchi, 1982). Considerable individual variation in the sensitivity of humans to methylmercury has been reported (Tsubaki, 1968; Clarkson, 1972). Species- and strain-specific differences in toxic doses of methylmercury have also been observed in animals (Nordberg and Skerfving, 1972; Iverson et al., 1973; Soares et al., 1973). However, the reasons for differences in sensitivity to methylmercury remain to be clarified. In cultured cells, the toxicity of methylmercury also depends on the type of cells (Miura et al., 1994; Miura, 2000). It seems likely that levels of expression of certain gene(s) that protect cells against methylmercury toxicity might be involved in the differences in the sensitivity of different lines of cells to this mercury compound. Therefore, in the present study, we searched for novel genes that confer resistance to methylmercury in yeast (*Saccharomyces cerevisiae)* because yeast has been established as a model organism in which powerful genetic approaches can be used to elucidate fundamental but complex eukaryotic processes.

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Materials and Methods

Transformation. Yeast cells were transformed with a genomic DNA library as described previously (Miura et al., 1999; Naganuma et al., 2000). The yeast genomic DNA library (Furuchi et al., 2001) was prepared by cloning size-fractionated *Sau*3A I fragments (5 to 10-kbp in length) into the *Bam*HI cloning site of the *LEU2*-based multicopy plasmid YEp13. This library was introduced into *Saccharomyces cerevisiae* W303B (*MATa* his3 can1–100 ade2 leu2 trp1 ura3) by the lithium acetate procedure (Gietz et al., 1992; Miura et al., 1999). Transformants were grown in synthetic dextrose (SD) medium without leucine (−Leu).

Selection of Genes That Confer Resistance to Methylmercury. The above-described Leu^+ transformants were cultured (1×10^5 cells/200 μl/well) in SD (−Leu) medium for 24 h at 30°C in the presence of methylmercury (0.4 μM) in 96-well plates for 24 h. Transformed cells that rapidly grew and formed aggregates in the presence of methylmercury were isolated from individual wells and plated on agar-solidified SD (−Leu) medium at a cell density of 5×10^3 cells per 10-cm plate. After incubation for 24 h at 30°C, colonies were collected and their sensitivity to methylmercury was examined. The sensitivity of yeast cells from each colony was determined by culturing cells (1×10^5 cells/200 μl) in SD (−Leu) medium that contained various concentrations of methylmercury for 48 h. We chose eight colonies that were strongly resistant to methylmercury and isolated plasmids from them as described by Hoffman (1993). Then the plasmids were amplified in *Escherichia coli* (Miura et al.,

ABBREVIATIONS: SD, synthetic dextrose; SG, synthetic galactose; PCR, polymerase chain reaction; ORF, open reading frame.

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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

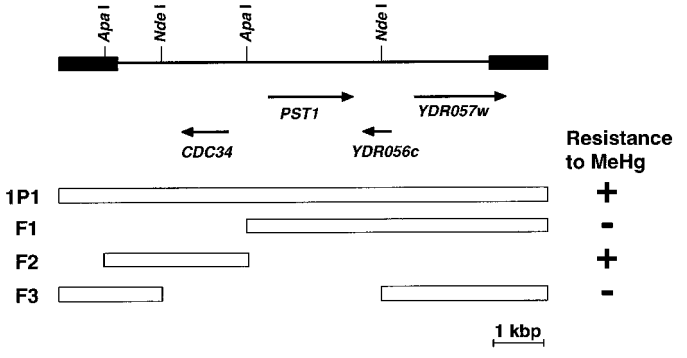

Fig. 1. Restriction map of the genomic DNA insert 1P1 that conferred resistance to methylmercury. The thick black line represents the vector YEp13; the thin line represents the genomic DNA insert. Vertical lines above the genomic DNA insert indicate the restriction sites used to generate different subclones. 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.

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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.

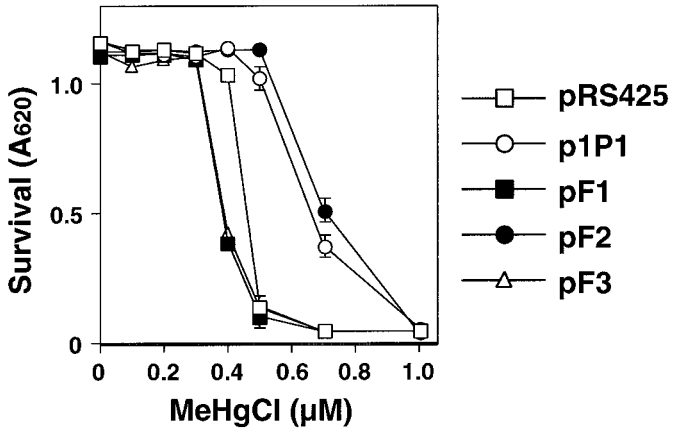

Fig. 2. Sensitivity to methylmercury of yeast cells that harbored plasmids with the indicated inserts. Yeast cells (1×10^5 cells/200 μl/well) carrying plasmids p1P1, pF1, pF2, pF3, or pRS425, as indicated, were grown in SD (−Leu) medium that contained methylmercuric chloride (MeHgCl). After a 48-h incubation, 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.

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-

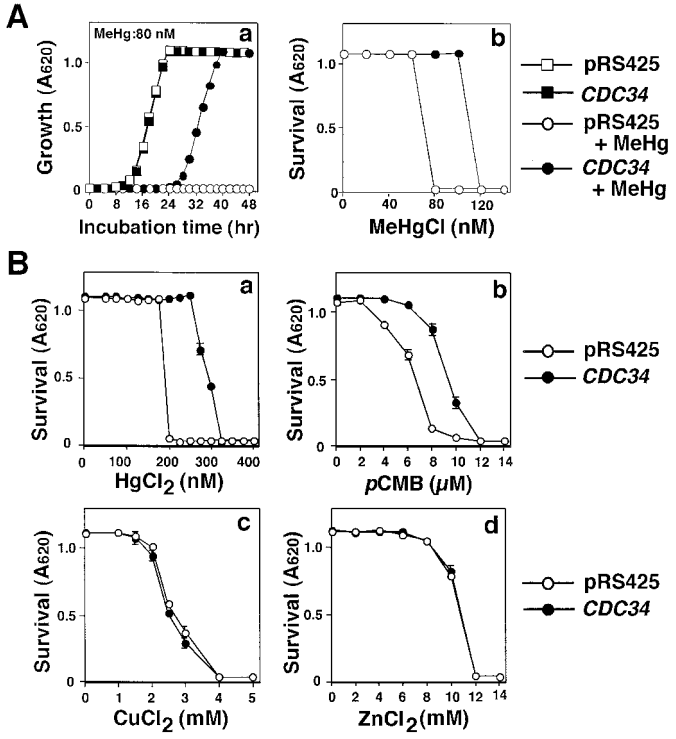

Fig. 3. Sensitivity of yeast cells that overexpressed Cdc34 to various metal compounds. Yeast cells (1×10^4 cells/200 μl/well) carrying pRS425 (control) or pRS425-CDC34 (pF2) were grown in SD (−Leu) medium that contained methylmercuric chloride (MeHgCl) (A, a and b), mercuric chloride ($HgCl_2$) (B, a), *p*-chloromercuribenzoate (*p*CMB) (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.

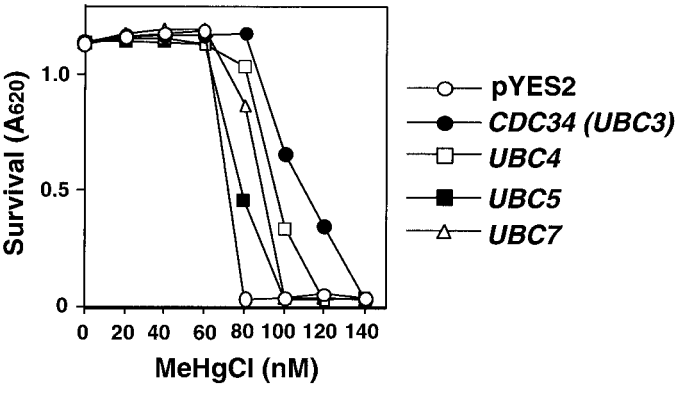

Fig. 4. Sensitivity of yeast cells that overexpressed Ubc4, Ubc5, or Ubc7 to methylmercury. Yeast cells (1×10^4 cells/200 μl/well) carrying pYES2, pYES-CDC34, pYES-UBC4, pYES-UBC5, or pYES-UBC7 were grown in SG medium (−Leu) that contained methylmercuric chloride (MeHgCl). After a 48-h incubation, absorbance 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.

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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.

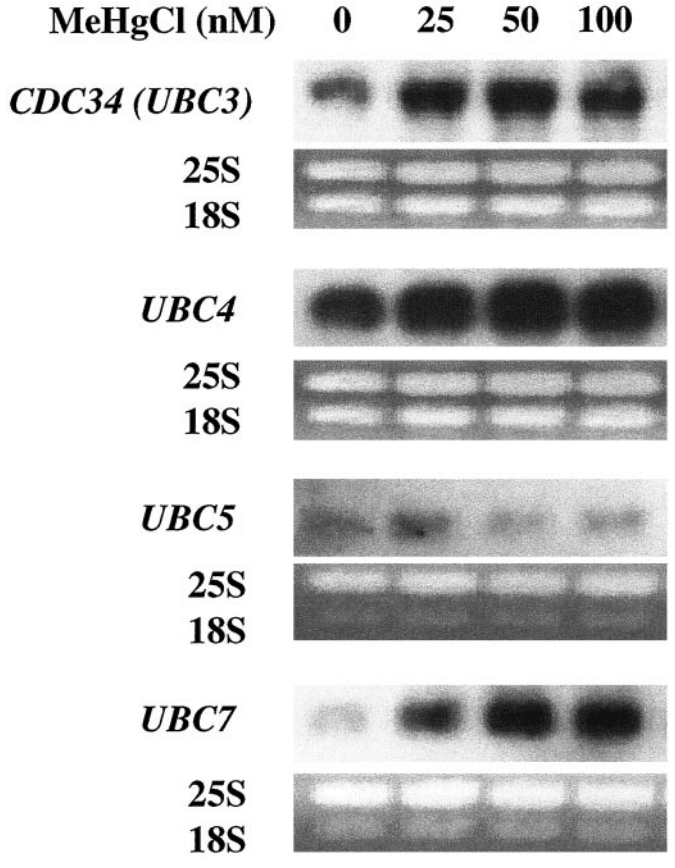

Fig. 5. Effects of methylmercury on the levels of transcripts of the ubiquitin-conjugating enzymes Cdc34, Ubc4, Ubc5, and Ubc7. Each lane was loaded with 20 μg of total RNA extracted from yeast cells (W303B) after treatment with methylmercuric chloride (MeHgCl) for 90 min. The bands of 25S and 18S RNA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

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