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Two Nuclear Proteins, Cin5 and Ydr259c, Confer Resistance to Cisplatin in *Saccharomyces cerevisiae*

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ABSTRACT

In an attempt to identify genes that can confer resistance to cisplatin, we introduced a yeast genomic library into *Saccharomyces cerevisiae* and selected for transformants that grew in the presence of a normally toxic concentration of cisplatin. Plasmids were rescued from the transformants and were analyzed for the presence of individual open reading frames that conferred resistance to cisplatin. We isolated two genes, *CIN5* and *YDR259c*, that increased resistance to cisplatin when overexpressed in *Saccharomyces cerevisiae*. These genes encoded two proteins, Cin5 and Ydr259c, that were homologous to yAP-1, a basic leucine zipper transcriptional factor that is known to mediate cellular resistance to various toxic agents. The two proteins exhibited stronger homology to each other (33.2% identity, 49.2% similarity) than to all other gene products in *S. cerevisiae*. Overexpression of each of these proteins also conferred resistance to two DNA-alkylating agents, methylmethanesulfonate and mitomycin C. An experiment with fusion proteins with green fluorescent protein revealed that Cin5 and Ydr259c were localized constitutively in the nuclei of yeast cells. Our results suggest that Cin5 and Ydr259c might be involved in pleiotropic drug-resistance and might protect yeast against the toxicity of cisplatin and other alkylating agents via a single mechanism. These two nuclear proteins might act as transcriptional factors, regulating the expression of certain genes that confer resistance to DNA-alkylating agents.

cis-Diamminedichloroplatinum(II) (cisplatin) is one of the most widely used chemotherapeutic agents for the treatment of human ovarian cancer and other tumors (Chu, 1994; Yoshida et al., 1994). In addition to its toxic side effects, a major limitation of cisplatin chemotherapy is acquired resistance. The increases in the dose of the drug that are necessary to overcome even a small increase in cellular resistance can cause severe toxicity (Von Hoff et al., 1979). An understanding of the molecular basis of both acquired and inherent resistance of cisplatin, therefore, might help us to improve clinical protocols significantly.

Laboratory studies with tumor tissues and cell lines suggest that resistance to cisplatin is nearly always multifactorial (Parker et al., 1991; Dabholkar and Reed, 1996; Gosland et al., 1996). The factors involved in resistance include impaired cellular uptake of cisplatin (Fujii et al., 1994; Shen et al., 1998), enhanced intracellular detoxification by systems that involve glutathione (Godwin et al., 1992; Ishikawa et al., 1994) or metallothionein (Naganuma et al., 1987; Kelley et al., 1988), altered patterns of DNA platination (Johnson et al., 1994), and enhanced repair of DNA damage (Masuda et al., 1988; Parker et al., 1991; Dabholkar and Reed, 1996; Gosland et al., 1996). However, the full details of the mechanism of cisplatin resistance are not yet clearly understood.

Genetic analyses of drug toxicity have been facilitated by the use of yeast model systems that have allowed cloning and analyses of genes that might contribute to drug resistance. In the present study, we isolated two genes that, when overexpressed from multicopy plasmids, conferred cisplatin resistance on *Saccharomyces cerevisiae*. These genes encode proteins in the yeast bZIP family, Cin5 and Ydr259c, the functions have not yet clearly been characterized. Cells that overexpressed each gene were also resistant to the DNA-alkylating agents methyl methanesulfonate and mitomycin C, but they remained sensitive to other compounds, such as doxorubicin and peplomycin. Our data suggest that both Cin5 and Ydr259c might be involved in pleiotropic drug-resistance and might protect yeast cells against the toxicity of DNA-alkylating agents such as cisplatin by the same mechanism.

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ABBREVIATIONS: cisplatin, *cis*-diamminedichloroplatinum(II); bZIP, basic leucine zipper; SD, synthetic dextrose; HA, influenza virus hemagglutinin; GFP, green fluorescent protein; ORF, open reading frame.

Materials and Methods

Yeast Strains and Media. Throughout this study, we used *Saccharomyces cerevisiae* W303B (*MATα his3 can1-100 ade2 leu2 trp1 ura3)*. The yeast genomic DNA library was made by cloning size-fractionated *Sau*3AI (5- to 10-kilobase) fragments into the *Bam*HI cloning site of the YEp13 vector. Cells were grown in yeast extract-peptone-dextrose medium or in synthetic dextrose (SD) medium without leucine (−Leu). Cells were transformed by the lithium acetate procedure (Gietz et al., 1992). Cultures were grown at 30°C. Plasmids were isolated from yeast cells as described by Hoffman (1993).

DNA Sequencing. The nucleotides sequences of genomic inserts in the isolated plasmids were determined with an automated sequencer (ABI, Natick, MA), with the YEp13-F primer (TGCTCGCTTCGCTACTTGA) or the YEp13-R primer (ATACCCACGCCGAAACAAGC).

Manipulation of DNA. Genes that supported growth in the presence of cisplatin (Nippon Kayaku Co. Ltd., Tokyo, Japan) were excised from clones and subcloned as DNA fragments in pRS425 vector. Sequencing identified genomic inserts as *CIN5* and *YDR259c*.

Construction of Genes for Green Fluorescent Protein (GFP)-Tagged Cin5 and Ydr259c. HA-*CIN5* and HA-*YDR259c* genes were amplified by the polymerase chain reaction with the following oligonucleotides as primers: 5′-GTCACAGCTGCCATGTACCCATACGATGTTCCAGATTACGCTTTAATGCAAATAAAAATGGAC-3′ and 5′-AGCACTTCTTCAGGGGGATG-3′ for *CIN5* and 5′-GTCACAGCTGCCATGTACCCATACGATGTTCCAGATTACGCTCAAAACCCTCCGTTGATTCGT-3′ and 5′-TACAATAAATAGGGAGCAGA-3′ for *YDR259c*. The products of polymerase chain reaction were ligated into the pGEM-T easy vector (Promega, Madison, WI). To generate cp-GFP-HA-*CIN5* and cp-GFP-HA-*YDR259c*, the *Nco*I-*Sal*I fragment of pRS cp-GFP-HA-*YAP1*(Kuge et al., 1997) was replaced by the *Nco*I-*Pvu*II fragment of pGFP536 (Shiroki et al., 1999) and the *Pvu*II-*Sal*I fragment of HA-*CIN5* or of HA-*YDR259C*.

Measurement of the Inhibition of Growth by Various Compounds. The effects of various compounds on yeast strains that

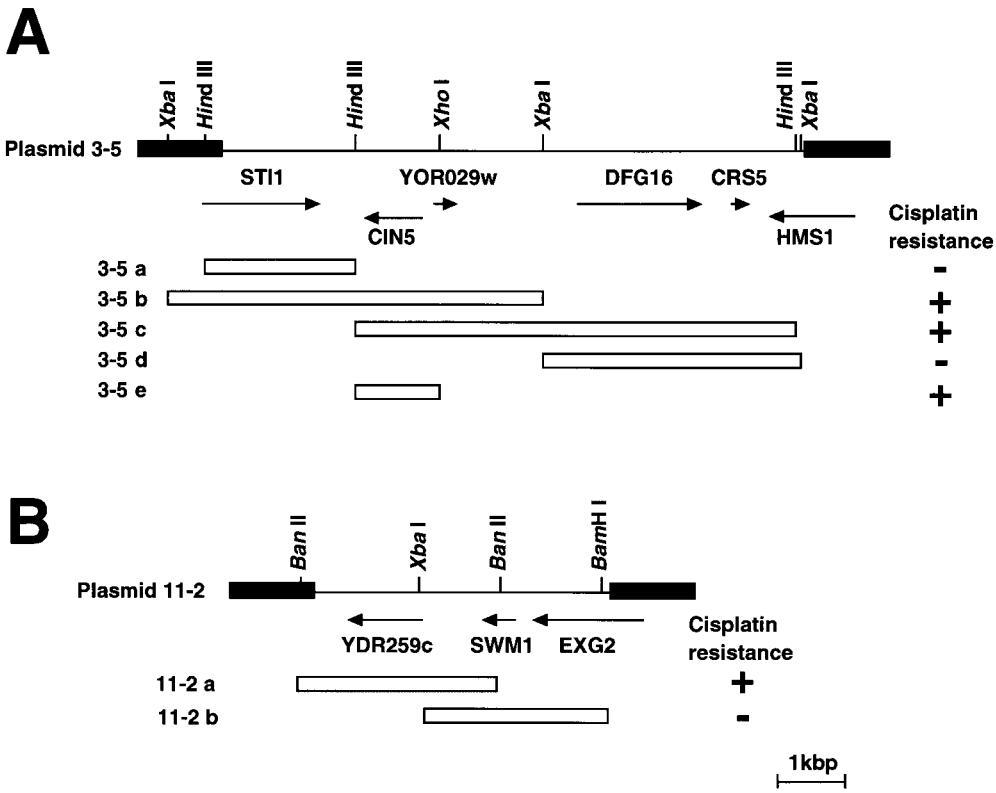


Fig. 1. Restriction map of the genomic DNA inserts in plasmids 3-5 (A) and 11-2 (B) and the ORFs that conferred resistance to cisplatin. The thick black lines represent the vector YEp13; the thin lines represent the genomic DNA inserts. The restriction sites used to generate different subclones are indicated by vertical lines above genomic DNA inserts 3-5 (A) and 11-2 (B). The ability of five subclones (p3-5a, -5b, -5c, -5d, and 5e) (A) and two subclones (p11-2a and -2b) (B) to confer cisplatin resistance is indicated (+, confers resistance; −, does not confer resistance). ORFs are indicated by black arrows pointing in the direction of transcription, with the name of the ORF given below each arrow.

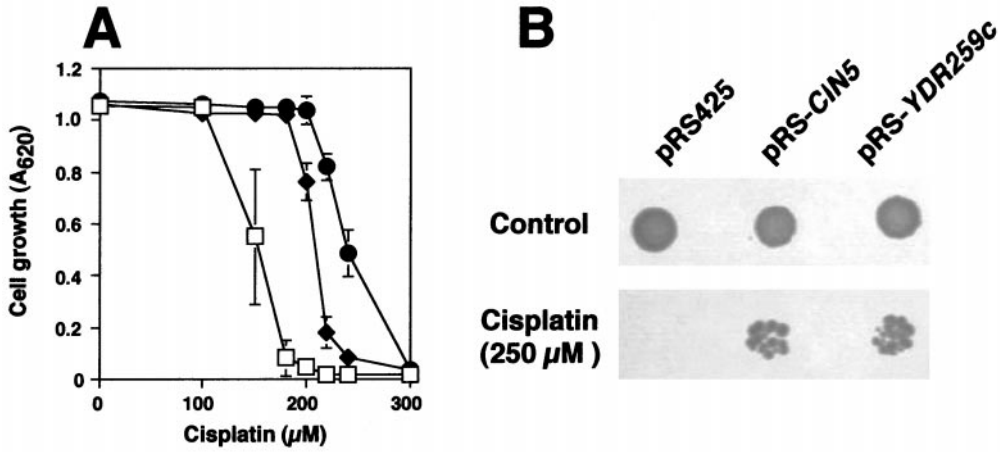


Fig. 2. Sensitivity of yeast cells that overexpressed Cin5 or Ydr259c to cisplatin. Yeast strains carrying pRS-*CIN5* (●), pRS-*YDR259c* (♦), or pRS425 (□) were grown in SD (−Leu) medium that contained cisplatin (A). After a 48-h incubation, absorbance was measured spectrophotometrically at 620 nm. Each point represents the mean value of results from three cultures with S.D. (bars). The absence of a bar indicates that the S.D. falls within the symbol. B, each strain was grown on a plate of agar-solidified SD (−Leu) medium for 3 days with or without cisplatin (250 μM). The results were confirmed by three separate experiments.

harbored the genes *CIN5* (pRS-*CIN5*) or *YDR259C* (pRS-*YDR259C*) or the empty pRS425 vector (control) were quantified by growing cells in SD (−Leu) medium. The levels of Cin5-mRNA or Ydr259C-mRNA in the yeast strains carrying pRS-*CIN5* or pRS-*YDR259C* were 3- or 13-fold higher than the control strain, respectively. Each suspension of cells (10^4 cells) was cultured in 200-μl aliquots of fresh medium that contained various concentrations of the respective compound. After 48 h, the absorbance at 620 nm (A_{620}) was determined spectrophotometrically to quantify the growth of each strain. For assay using agar-solidified medium, each overnight culture, diluted to 5×10^6 cells/ml, was spotted on plates of agar-solidified medium with or without cisplatin. Plates were monitored after incubation for 3 days at 30°C. Each experiment was repeated at least twice and representative results are shown.

Results and Discussion

In our search for novel genes that confer resistance of cisplatin, we introduced a yeast genomic DNA library in the vector YEp13 into W303B yeast cells. Transformants (5×10^3) were spread on plates of agar-solidified SD (−Leu) medium containing cisplatin (220 μM) and cultured for 3 days at 30°C. Under these conditions, yeast cells that had been transformed with the empty Yep13 vector did not form colonies. Single colonies of transformants that had grown in the presence of cisplatin were picked up and plasmids were rescued from these cells and amplified in *Escherichia coli.* Plasmids were reintroduced into W303B yeast cells to confirm the phenotype.

We obtained seven plasmids that conferred resistance to cisplatin and we sequenced and mapped the inserted genomic DNA in each plasmid using the *Saccharomyces* Genome Database. The inserted DNA of three of these plasmids (Nos. 1-2, 3-5, and 11-1) and that of the other four plasmids (Nos. 11-2, 11-3, 13-1, and 15-2) were found to have been derived almost identically from the same region of yeast chromosomes XV and IV, respectively. To identify the genes involved in cisplatin resistance, we subcloned the inserted

Fig. 3. Homology between Cin5 and Ydr259c. Alignment of the amino acid sequences of Cin5 and Ydr259c. Identical (*) and similar (●) residues are indicated. The bZIP motifs are underlined.

DNA in plasmids 3-5 and 11-2 in pRS425. The first gene responsible for the above-described phenotype was found in subclone 3-5e (Figs. 1A and 2) and the second gene was found in subclone 11-2a (Figs. 1B and 2). The insert of each subclone contained a single ORF: *CIN5* and *YDR259c*, respectively. The deduced products of these two ORFs exhibited the strongest homology to each other (33.2% identity, 49.2% similarity) among the deduced products of all yeast genes. Each contained a bZIP motif in the carboxyl-terminal domain. This motif is strongly conserved in the AP-1 transcription factors of yeast, such as Yap1 and Yap2 (Fernandes et al., 1997) (Fig. 3). A computer search of sequence databases for mammalian genes failed to identify sequences with high global similarity to both *CIN5* and *YDR259c*.

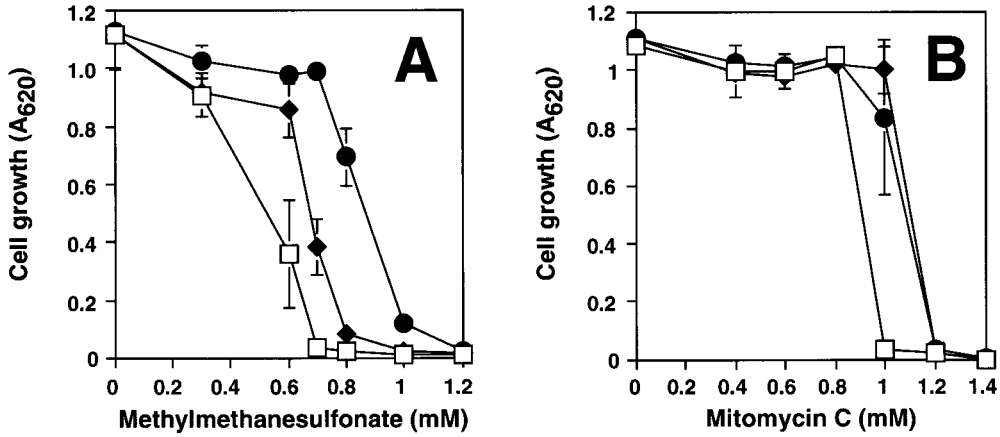
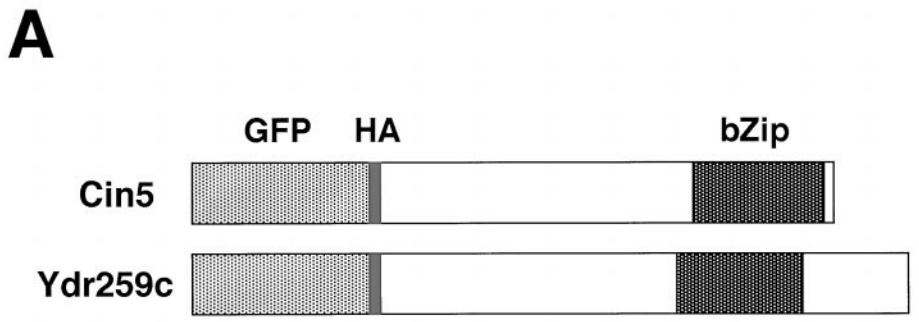


Fig. 4. Sensitivity of yeast cells that overexpressed Cin5 or Ydr259c to DNA-alkylating agents. Yeast cells carrying pRS-*CIN5* (●), pRS-*YDR259c* (♦), or pRS425 (□) were grown in the SD (−Leu) medium that contained methylmethanesulfonate (A) or mitomycin C (B). After a 48-h incubation, absorbance was measured spectrophotometrically at 620 nm. Each point represents the mean value of results from three cultures with S.D. (bars). The absence of a bar indicates that the S.D. falls within the symbol. The results were confirmed by three separate experiments.



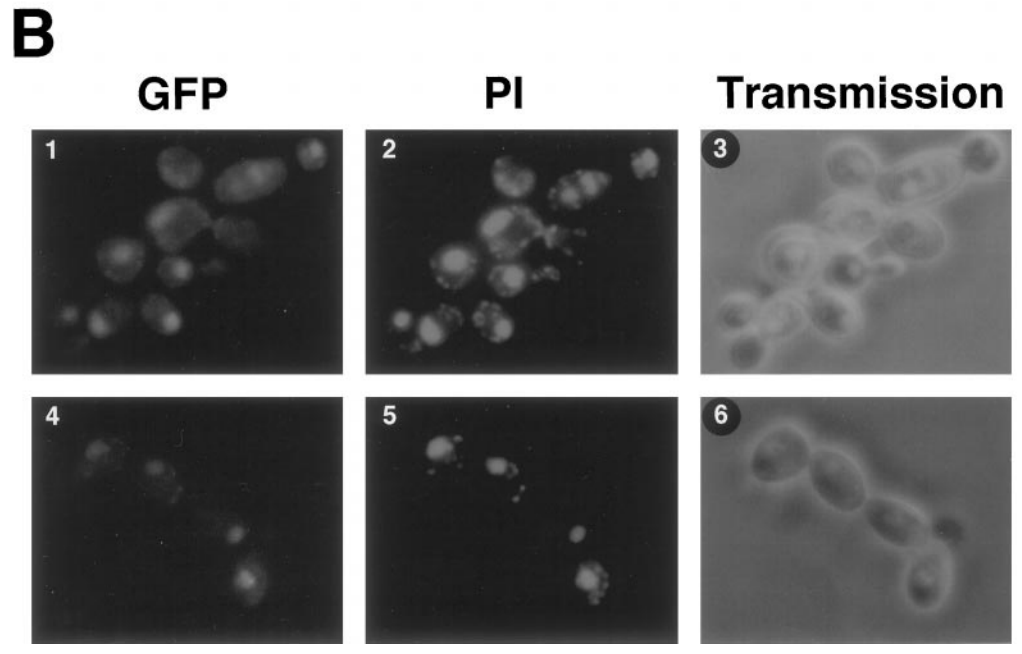


Fig. 5. Localization of GFP-Cin5 and GFP-Ydr259c in yeast cells. A, a schematic diagram of the GFP-*CIN5* and GFP-*YDR259c* fusion genes. B, localization of GFP-fusion proteins in yeast cells that harbored pRS cp-GFP-*CIN5* (panels 1–3) or pRS cp-GFP-*YDR259c* (panels 4–6), as analyzed by fluorescence microscopy. Images due to GFP fluorescence (panels 1 and 4), propidium iodide fluorescence (panels 2 and 5), and transmitted light (panels 3 and 6) are shown.

Yeast cells transformed with *CIN5* and *YDR259c* (pRS-*CIN5* and pRS-*YDR259c*) were resistant not only to cisplatin but also to methylmethansulfonate and mitomycin C (Fig. 4). By contrast, these strains were not significantly resistant to doxorubicin and 5-fluorouracil (data not shown). Recent observations indicate that overexpression of Cin5 or of Ydr259c also increases tolerance to sodium and lithium (Mendizabal et al., 1998), and overexpression of Cin5 confers resistance to quinidine, mefloquine, and chloroquine (Delling et al., 1998). These observations suggest that the proteins encoded by *CIN5* and *YDR259c* might be involved in pleiotropic drug resistance and might protect yeast cells against the toxicity of cisplatin and other chemicals through a single mechanism. We found that deletion of the *CIN5* or *YDR259c* gene in wild-type yeast (W303B) did not sensitize the cells to cisplatin (data not shown), an indication that the functions of endogenous Cin5 and Ydr259c can substitute for one another at the basal cellular levels of both proteins.

We examined the cellular localization of Cin5 and Ydr259c by inducing the expression of fusion proteins of Cin5 or Ydr259c with GFP in W303B cells (Fig. 5A). Both GFP-Cin5 and GFP-Ydr259c were detected as single spots in individual yeast cells (Fig. 5B, 1 and 4). A comparison with the sites of DNA staining clearly revealed that the proteins were localized in the nuclei (Fig. 5B, 2 and 5). The fusion proteins were constitutively localized in nuclei and their localization was unchanged by treatment of cells with cisplatin (data not shown).

As mentioned above, Cin5 and Ydr259c exhibited significant similarity to each other and each contained a highly conserved bZIP motif in the carboxyl-terminal domain, a characteristic of yeast AP-1 transcription factors, such as Yap-1 and related proteins (Fernandes et al., 1997). Overexpression of Yap-1 confers multidrug resistance that is mediated by the *trans*-activation of various genes (Toone and Jones, 1999). Yap-1 is involved in the expression of several ATP-binding cassette-type transporters (Wemmie et al., 1994; Bauer et al., 1999). In humans, these transporters are clinically relevant because they are responsible for resistance of tumors to chemotherapeutic drugs. In the present study, yeast cells overexpressing Cin5 or Ydr259c showed no significant differences from control cells in terms of the accumulation of platinum after treatment with cisplatin (data not shown), a result that suggests that neither Cin5 nor Ydr259c influences the expression of transporter proteins. Moreover, overexpression of Yap-1 in W303B cells failed to increase the resistance to cisplatin (data not shown). Our results suggest that Cin5 and Ydr259c might have a different function from Yap-1, even though all three proteins include a highly conserved bZIP motif. However, it is possible that Cin5 and Ydr259c, which are constitutively localized in the nuclei of yeast cells, might act as transcriptional factors and might regulate expression of genes involved in resistance to cisplatin and other DNA-alkylating agents. Further analysis is necessary to elucidate the mechanisms of pleiotropic resistance that results from the overexpression of Cin5 and Ydr259c.

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