Title
Mechanisms of cisplatin resistance in yeast and mammals

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Mechanisms of cisplatin resistance in yeast and mammals

by

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DISSERTATION

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UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

DEDICATION

To my parents, Tsuyoshi and Takako Ishida

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My years in graduate school have been a rewarding experience professionally and personally. I am grateful to Ira Herskowitz for his guidance in my developing as an independent scientist. I have enjoyed his intellectual challenges and his enthusiasm in my research, and have learned many invaluable lessons by working with him on a talk or a paper. He is a genuin teacher -- even before I joined his lab, he would go through the trouble of correcting all the grammatical errors in my genetics exams. He always tries to help a student develop in every possible aspect, not only in science. He would be happy to find out I took some time off to go see a Giants game or practice the piano. As I wrote my thesis, his absence and the lack of his input and challenges were painful. I am hoping for his quick return.

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I am most grateful to my parents, Tsuyoshi and Takako Ishida, for their continuous love, support, and encouragement. Without them, I would not have reached where I now stand.

MECHANISMS OF CISPLATIN RESISTANCE
IN YEAST AND MAMMALS

Seiko Ishida

ABSTRACT

Cisplatin is one of the most widely used anticancer drugs. The acquisition or presence of resistance significantly undermines the curative potential of the drug against many malignancies. Molecular mechanisms that underlie cisplatin resistance are poorly understood. We used the budding yeast *Saccharomyces cerevisiae* to identify genes that mediate cisplatin resistance. Selection of transposon-mutagenized yeast cells for growth in the presence of toxic concentrations of cisplatin allowed us to identify four genes, *YNR051c*, *GPA2*, *NMD2*, and *MAC1*, which govern cisplatin sensitivity. Mutants lacking each of these genes are more resistant to cisplatin than wild-type cells, and exhibit decreased levels of cisplatin inside the cells and on DNA. These results suggest that reduced cellular accumulation of the drug may be a common mechanism by which eukaryotic cells can become resistant to the drug. The *mac1Δ* mutant exhibited the highest level of resistance to cisplatin among the four mutants.

To understand the basis for increased resistance in cells deleted for the *MAC1* gene, which encodes a transcription factor, we deleted each of the Mac1p target genes and observed that cells deleted for the *CTR1* gene, which encodes a high-affinity copper transporter, were as resistant as the *mac1Δ* mutant and exhibited reduced intracellular accumulation of cisplatin.

Copper, which causes a reduction in the level of Ctr1 protein (Ctr1p), enhances survival of yeast cells to cisplatin and reduces cellular accumulation of the drug in wild-type cells but not in the *ctr1Δ* mutant. Similarly, cisplatin reduces copper uptake in wild-type yeast cells and causes internalization and downregulation of Ctr1p, indicating a further link between cisplatin uptake and copper transport mediated by Ctr1p. Mouse cell lines lacking one or both *CTR1* alleles exhibit increased cisplatin resistance and decreased cisplatin accumulation in parallel with *CTR1* gene dosage. We propose that cisplatin uptake is mediated by the copper transporter Ctr1p in yeast and mammals. The link between Ctr1p and cisplatin transport may explain some cases of cisplatin resistance and suggests ways of modulating sensitivity and toxicity to this important anticancer drug.

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CHAPTER ONE

INTRODUCTION

Cancer is one of the leading causes of death in developed countries. Its incidence is related to both genetic and environmental factors. Methods of treatment are determined by the stages of cancer. Local measures such as surgery and radiation are effective when the tumor has not metastasized. However, even when the tumor appears confined, micrometastasis is common, which requires a systemic approach in treatment. For this reason, chemotherapy is employed in treating most cases of cancer.

Anticancer drugs have been developed through empirical screening of compounds and rational design. Mechanisms of action for cytotoxicity involve DNA damage, inhibition of nucleotide synthesis, and interference with microtubule assembly. Due to heterogeneity of tumor cells, multiple anticancer drugs are used simultaneously in chemotherapy. Ideal anticancer drugs would eradicate cancer cells without harming normal cells. Unfortunately, currently available agents do not meet this criterion, and clinical use of these drugs involves a weighing of benefits against toxicity in search for a favorable therapeutic effect.

A major impediment in cancer chemotherapy is drug resistance. Some cancers such as non-small cell lung cancer and colon cancer exhibit intrinsic resistance, i.e., absence of response on the first exposure, to currently available agents. Acquired resistance develops in a number of cancers that were initially sensitive to the drug. Resistance can be highly specific to a single drug, accompanied by a change in the level of expression of one or more specific genes. A well-characterized example is resistance to a folic acid antagonist methotrexate, which is attributed to increased levels of dihydrofolate reductase (DHFR) due to amplification of the *DHFR* gene locus (Banerjee *et al.*, 1995). Methotrexate binds to the active site of DHFR and blocks its catalytic activity required for nucleotide synthesis; increasing the

level of DHFR, the target of methotrexate, titrates out the inhibitory effect of methotrexate on DHFR. In other instances, cancers exhibit resistance to multiple drugs of different structures. Such multidrug resistance is often associated with increased expression of the *MDR* gene, which encodes a cell surface glycoprotein involved in drug efflux (Litman *et al.*, 2001).

Cisplatin is one of the most widely used anticancer drugs, possessing a very broad spectrum of activity. Its cytotoxic effect was discovered serendipitously in 1965 by Rosenberg *et al.* who were studying the effects of electric fields on the growth of the bacterium *Escherichia coli* (Rosenberg *et al.*, 1965). The bacterial cells, which normally divide rapidly, grew to 300 times their usual length and did not divide. This effect was found to derive from the presence of platinum complexes, one being *cis*-diamminedichloroplatinum [II] (cisplatin) (Figure 1-1), formed by electrolysis at the platinum electrodes (Rosenberg *et al.*, 1967). Anticancer activity of cisplatin was demonstrated against mouse sarcoma and leukemia (Rosenberg *et al.*, 1969), followed by clinical trials. In 1979, cisplatin was approved by the FDA for the treatment of several human cancers.

Cisplatin-based chemotherapy is curative for the majority of patients with advanced testicular cancer, which was almost uniformly fatal in the pre-cisplatin era (Loehrer and Einhorn, 1984). Cisplatin is also effective for ovarian, bladder, cervical, head and neck, and small cell lung cancers. Unfortunately, many patients with these cancers eventually relapse and become refractory to cisplatin. In addition, cisplatin has minimal activity against some common cancers such as colorectal cancer. Increasing dosage to overcome resistance can cause serious side effects in the kidneys and the ears. Therefore, understanding the mechanism of intrinsic and acquired resistance to cisplatin is critical in developing a more effective cure for cancer.

Mode of action of cisplatin

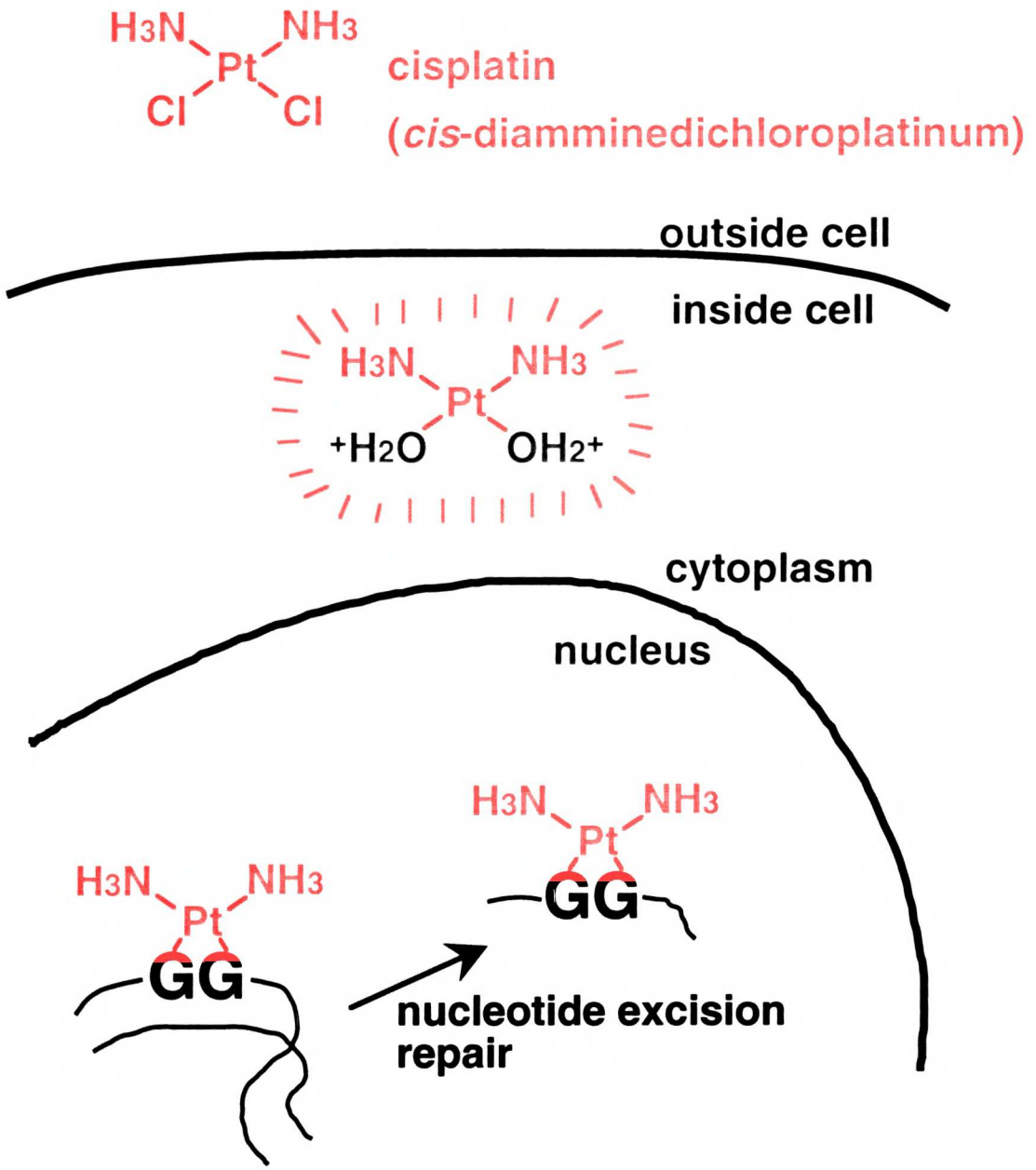
Cisplatin is commonly administered intravenously. Cisplatin retains its neutral structure in an environment of relatively-high chloride concentration such as plasma, where the chloride concentration is ~100 mM (Rosenberg, 1985). The mechanism by which cisplatin enters the cell remains unknown (Gately and Howell, 1993). The observations that accumulation of cisplatin is not saturable up to its solubility limit of 3.3 mM and that structural analogs fail to inhibit cisplatin accumulation support a passive diffusion model. However, the existence of agents and conditions that modulate cisplatin accumulation suggests that some component of cisplatin uptake is mediated by a form of transport mechanism. Factors that affect cisplatin accumulation include pH, osmolarity, sodium, potassium, and intracellular signalling mechanisms such as protein kinase C (PKC), protein kinase A (PKA), and the calcium/calmodulin pathway.

Once cisplatin passes through the plasma membrane into the cytoplasm of the cell, where the chloride concentration drops to ~3 mM, its chlorides are displaced by water molecules (Figure 1-1). This aquated product is the reactive form, which is a potent electrophile that can react with any nucleophile, including the sulfhydryl groups on proteins and nitrogen donor atoms on nucleic acids.

Work from many laboratories has implicated DNA as a critical target for cisplatin cytotoxicity, the most revealing evidence being the hypersensitivity to cisplatin of both prokaryotic and eukaryotic cells deficient in DNA repair (Beck and Brubaker, 1973; Fraval *et al.*, 1978; McA'Nulty and Lippard, 1996). The most prevalent cisplatin-induced DNA adduct is the intrastrand crosslink in which the platinum is covalently bound to the N^7

Figure 1-1. Cisplatin: its mode of action.

Cisplatin (*cis*-diamminedichloroplatinum [II]) remains intact in the circulating plasma, where the chloride concentration is ~100 mM. Once cisplatin enters the cell, where the chloride concentration drops to ~3 mM, the chlorides in cisplatin are replaced with water molecules. This aquated product is the reactive form and is a potent electrophile. Its cytotoxic target is believed to be DNA, where it forms a crosslink between two adjacent guanines on the same strand of DNA. This adduct can be removed by the nucleotide excision repair.


H3N
NH3
Pt
Cl
Cl
cisplatin
(*cis*-diamminedichloroplatinum)
outside cell
inside cell
H3N
NH3
Pt
+H2O
OH2+
cytoplasm
nucleus
H3N
NH3
Pt
GG
H3N
NH3
Pt
GG
nucleotide excision
repair

positions of the imidazole ring of two adjacent guanines (Fichtinger-Schepman *et al.*, 1985). The intrastrand crosslinks are repaired by the nucleotide excision repair pathway (Beck *et al.*, 1985).

Genes involved in cisplatin resistance

Postulated mechanisms of cisplatin resistance include decreased drug accumulation, a decreased level of DNA adduct formation, enhanced removal of the adducts, and altered response to DNA damage (Figure 1-2).

Decreased accumulation is common in cell lines selected for cisplatin resistance *in vitro*. The methods used in most of these investigations have generally not allowed discrimination between decreased uptake and increased efflux as determinants of decreased cisplatin accumulation. Overexpression of *ATP7B*, a copper-transporting ATPase in the post-Golgi, confers decreased cisplatin accumulation and increased cisplatin resistance, suggesting that ATP7B protein functions as a pump to assist cisplatin efflux (Komatsu *et al.*, 2000).

Increased cisplatin resistance has been associated with deficiency in mismatch repair in ovarian, colon, and endometrial carcinoma cell lines (Aebi *et al.*, 1996; Drummond *et al.*, 1996; Fink *et al.*, 1996). Mismatch repair is a post-replication repair system that corrects unpaired or mispaired nucleotides. Mismatch repair deficiency predisposes cells to genomic instability. It is thought to be important in hereditary nonpolyposis colon cancer and has also been identified in a variety of sporadic tumors. Knockout cell lines deleted for either the *MSH2* or *PMS2* mismatch repair gene demonstrate increased resistance to cisplatin (Fink *et al.*, 1997). The human mismatch repair protein hMSH2 binds a cisplatin G-G intrastrand adduct (Duckett *et al.*, 1996; Mello *et al.*, 1996). It is hypothesized that mismatch

Figure 1-2. Possible mechanisms of cisplatin resistance.

Cisplatin resistance could arise from decrease in the level of DNA adducts due to reduced formation of the adducts or enhanced repair, or changes in response to DNA damage. The mechanisms for a reduced level of adduct formation may involve decreased drug uptake, increased efflux, decreased reactivity of the drug, and decreased accessibility of DNA for adduct formation. Decreased levels of drug accumulation are frequently observed in cisplatin resistant cell lines. Enhanced repair has also been noted in some resistant cell lines.

Possible mechanisms of cisplatin resistance

- low levels of cisplatin-DNA adducts

  ↓ formation of cisplatin-DNA adduct

  drug accumulation ↓

  uptake ↓

  efflux ↑

  cisplatin reactivity ↓

  accessibility of DNA to cisplatin ↓

  ↑ removal of DNA adducts

- altered response to DNA damage

repair proteins sense the damage and initiate a series of events that result in cell cycle arrest and death. Two models have been proposed to explain the connection between the mismatch repair pathway and sensitivity to DNA damage caused by specific anticancer drugs. In one model, DNA lesions are recognized and processed by the mismatch repair pathway, but because mismatch repair excises a tract from the newly incorporated strand, damage in the parental strand is not removed. Repeated processing attempts create persistent gaps that may trigger cell cycle arrest or cell death. Alternatively, a more direct signaling pathway could be responsible. Assembly of mismatch repair proteins at the specific lesions, whose effect on DNA structure is different from that of unpaired or mispaired bases (Takahara *et al.*, 1995), could initiate a signaling cascade leading to cell death.

In the budding yeast *Saccharomyces cerevisiae*, deletion of the *IXR1* gene, which encodes an HMG box protein that binds to cisplatin-DNA adducts, results in increased cisplatin resistance (Brown *et al.*, 1993). It is thought that Ixr1p shields cisplatin-DNA adducts from repair (McA'Nulty and Lippard, 1996). Mammalian HMG proteins have also been shown to interact with cisplatin-DNA adducts and inhibit repair of the adduct *in vitro* (Pil and Lippard, 1992; Huang *et al.*, 1994). Overproduction in yeast of Pde2p, a phosphodiesterase that functions in the PKA pathway, confers increased cisplatin resistance to mutants defective for excision repair (Burger *et al.*, 2000). Studies in mammalian cells also suggest potential links between the PKA pathway and cisplatin sensitivity (Cvijic *et al.*, 1998). It has recently been reported that overexpression of two yeast genes, *CIN5* and *YDR259c* (Furuchi *et al.*, 2001), and deletion of the *SKY1* gene in the excision repair-deficient *rad4Δ* mutant (Schenk, P. W. *et al.*, 2001), increase cisplatin resistance. The

basis for increased cisplatin resistance in cells overexpressing *PDE2*, *CIN5*, or *YDR259c*, or in cells deleted for *SKY1*, is not known.

In our study, we took a genetic approach to identify genes that are involved in cisplatin resistance in yeast. From the screen used and in subsequent studies, we identified nine genes whose deletion results in increased resistance to cisplatin. We demonstrated that the mechanism of cisplatin resistance involving Ctr1 is conserved between yeast and mammals, and propose that Ctr1 is a transporter for cisplatin and thus will be a good target for drug development to increase efficacy of cisplatin.

CHAPTER TWO

IDENTIFICATION AND CHARACTERIZATION OF MUTANTS WITH INCREASED CISPLATIN RESISTANCE IN *SACCHAROMYCES CEREVISIAE*

ABSTRACT

The therapeutic potential of cisplatin, one of the most widely used anticancer drugs, is limited by the occurrence of resistance. We used the budding yeast *Saccharomyces cerevisiae* to identify genes that mediate cisplatin resistance. Selection of transposon-mutagenized yeast cells for growth in the presence of toxic concentrations of cisplatin allowed us to identify four genes, *YNR051c*, *GPA2*, *NMD2*, and *MAC1*, which govern cisplatin sensitivity. Mutants lacking each of these genes are more resistant to cisplatin than wildtype cells, and exhibit decreased levels of cisplatin inside the cells and on DNA. These results suggest that reduced cellular accumulation of the drug may be a common mechanism by which eukaryotic cells can become resistant to the drug.

INTRODUCTION

Cisplatin (*cis*-diamminedichloroplatinum) is among the most active and widely used anticancer drugs. However, acquisition or presence of resistance significantly undermines the curative potential of cisplatin against many cancers. The cytotoxic target for cisplatin is thought to be DNA (Beck and Brubaker, 1973; Fraval *et al.*, 1978; McA'Nulty and Lippard, 1996), where cisplatin forms intrastrand crosslinks between two adjacent guanines (Fichtinger-Schepman *et al.*, 1985). Nucleotide excision repair is the major mode of repair for cisplatin-induced DNA damage (Beck *et al.*, 1985). Resistance to cisplatin has been studied extensively using cell lines selected in the presence of cisplatin *in vitro*. Mechanisms of resistance observed in cisplatin-resistant cell lines involve decreased accumulation of the drug inside the cell, decreased level of cisplatin-DNA adducts, and enhanced removal of the adducts (Scanlon *et al.*, 1991). When I began working on this project, two groups of genes were implicated in cisplatin resistance: mismatch repair genes in human and the *IXR1* gene in the budding yeast *Saccharomyces cerevisiae*. Mismatch repair-deficient cell lines are more resistant to cisplatin than when complemented with a chromosome carrying either *hMSH2* or *hMLH1* (Aebi *et al.*, 1996). In human cells, mismatch repair proteins bind to cisplatin-DNA adducts and it is hypothesized that futile attempts at mismatch repair are the cause of cell death (Fink *et al.*, 1996). Deletion of the *IXR1* gene in yeast results in increased cisplatin resistance (Brown *et al.*, 1993). Ixr1p, which binds to cisplatin-DNA adducts, is thought to shield the lesions from nucleotide excision repair proteins (McA'Nulty and Lippard, 1996).

For a number of reasons, we chose to use yeast to understand the mechanism of cisplatin resistance. First, yeast cells are sensitive to cisplatin.

This allows us to look for mutants with increased resistance to cisplatin. Second, many of the genes involved in cell survival are conserved between mammals and yeast. Thus, the mechanisms of cisplatin resistance may be similar between the two species. Third, genetic manipulations are easier in yeast than mammalian cells. This facilitates rapid identification of genes involved in cisplatin resistance. Here we describe isolation of mutants with increased cisplatin resistance, identification of genes disrupted in the mutants, epistasis analysis of the mutants, and characterization of the mutants by measuring cisplatin accumulation inside the cell and on DNA. *YNR051c*, *GPA2*, *NMD2*, and *MAC1* were found to be involved in governing cisplatin sensitivity. Mutants deleted for each of these genes exhibit decreased levels of cellular drug accumulation, suggesting that this could be a major mechanism of cisplatin resistance in eukaryotes.

MATERIALS AND METHODS

Yeast growth conditions and media

Standard yeast growth conditions and media were used as described in Guthrie and Fink (1991).

Yeast strains

Isogenic strains carrying deletions in *YNR051c*, *GPA2*, *NMD2*, *MAC1*, *RAD2*, *GPR1*, and *PDE2* were generated in YSI1 (W303 background; *MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 GAL*$^+$ *psi*$^+$) by the protocol of Sakumoto *et al.* (1999). A polymerase chain reaction (PCR) fragment was used to replace the entire ORF with the *Candida glabrata HIS3* gene. Double mutants were obtained by crossing single mutants as described in Appendix.

Selection for cisplatin-resistant mutants

The *mTn-lacZ/LEU2*-mutagenized library was a generous gift from Michael Snyder (Burns *et al.*, 1994). Strain YSI1 was mutagenized with the transposon library. Transformants were selected on SC -Leu plates, pooled, and plated on SC -Leu plates containing 0.2 mM cisplatin (Bristol Laboratories) and 2.7 mg/ml NaCl at a density of ~100 cells per plate. Colonies that formed on cisplatin plates at 30°C were streaked out on SC -Leu plates for single colonies and subjected to further analyses.

Cisplatin sensitivity assay

Log-phase cells grown in rich medium (YPD) were exposed for two hours to different concentrations of cisplatin (Bristol Laboratories) in YPD liquid medium, washed once with SD to remove cisplatin, and incubated on YPD

plates at 30°C for two days to allow formation of colonies. Data are expressed as percentages of colonies formed compared to control cultures not exposed to cisplatin.

Yeast Transformations

Yeast transformations were performed by the lithium acetate method (Ito *et al.*, 1983).

Atomic Absorption spectrophotometry

Platinum was measured using a Perkin-Elmer Atomic Absorption Spectrophotometer 3300 with an HGA-400 Graphite Furnace System. A volume of 20 µl was introduced into the graphite furnace and the peak area was read during a five-second atomization step at 2500°C. The amount of platinum in the samples was determined from a calibration curve prepared using cisplatin solutions.

RESULTS

Isolation of mutants with increased cisplatin resistance

Mutants with increased resistance to cisplatin were identified based upon their ability to grow on solid minimal medium containing a toxic dose of cisplatin. To isolate such mutants, it was necessary to determine the minimal concentration of cisplatin that inhibits growth of wild-type cells. SC plates were prepared containing various concentrations of cisplatin, on which equal numbers of wild-type cells were plated. When wild-type cells were plated at a density of 100 to 200 cells per plate, no or one colony was able to form on a plate containing 0.2 mM cisplatin. We therefore decided to use this concentration to select for mutants with increased cisplatin resistance.

A Tn3-mutagenized library was used to mutagenize wild-type *MATa* cells (YSI1) in the W303 strain background (Burns *et al.*, 1994). Of the 27,000 mutants screened, 77 formed colonies on cisplatin plates. The levels of survival after two-hour exposure to 1 mM cisplatin were more than ten times that of wild-type cells in 17 of the mutants. They were backcrossed to wild-type *MATα* cells (YSI2), and cisplatin resistance of the tetrads was analyzed. Eight mutants showed a link between the presence of the transposon (Leu^+) and cisplatin resistance.

Deletion of *YNR051c*, *GPA2*, *NMD2*, or *MAC1* results in increased resistance to cisplatin

Five of the eight cisplatin-resistant mutants carried a transposon insertion at two different loci of the *MAC1* open reading frame (ORF). The remaining three mutants had a disruption in the ORF of *YNR051c*, *GPA2*, or *NMD2*. Gpa2p is a G protein α that regulates the intracellular cAMP level

Figure 2-1. Isolation of cisplatin-resistant mutants.

Of the 27,000 transposon-insertion mutants screened, 77 formed colonies on 0.2 mM cisplatin plates. The levels of survival after two-hour exposure to 1 mM cisplatin were more than ten times that of wild-type cells in 17 of the mutants. Eight mutants showed a link between the presence of the transposon (Leu^+) and cisplatin resistance. Five of them carried a transposon insertion at two different loci of the *MAC1* ORF; the rest of the mutants had an insertion in the ORF of *YNR051c*, *GPA2*, or *NMD2*.

Isolation of Cisplatin Resistant Mutants

27,000 transposon-insertion mutants screened

77 survivors on 0.2 mM cisplatin plates

17 mutants with more than ten times the percentage of wild-type cells surviving two hour treatment with 1 mM cisplatin

8 mutants with the resistance linked to transposon

4 genes identified to be disrupted in the mutants

(Kubler *et al.*, 1997); Nmd2p is involved in nonsense-mediated mRNA decay (Cui *et al.*, 1995; He and Jacobson, 1995); and Mac1p is a transciption factor that controls trascription of catalase genes and genes involved in uptake of copper and iron (Jungmann *et al.*, 1993; Yamaguchi-Iwai *et al.*, 1997; Labbe *et al.*, 1997; Martins *et al.*, 1998). To determine whether loss of function of these genes results in increased cisplatin resistance, the entire ORF of *YNR051c*, *GPA2*, *NMD2*, or *MAC1* was deleted in the strain YSI1. As shown in Figure 2-1, the percentage of cells that survive a two-hour treatment with 1 mM cisplatin was 300 times that of wild-type cells in *ynr051cΔ* (YSI3), 60 times in *gpa2Δ* (YSI4), 700 times in *nmd2Δ* (YSI5), and 1300 times in *mac1Δ* (YSI6). The degree of resistance as measured by IC_{10} was 1.9-fold in *ynr051cΔ* compared to that of wild-type cells, 1.6-fold in *gpa2Δ*, 2.0-fold in *nmd2Δ*, and 2.2-fold in *mac1Δ*. The original transposon-insertional mutants that exhibited increased cisplatin resistance were thus loss of function mutants, and their resistant phenotypes were due to defects in these genes.

YNR051c, _GPA2_, _NMD2_, and _MAC1_ belong to different epistasis groups in governing sensitivity to cisplatin

We asked whether *YNR051c*, *GPA2*, *NMD2*, and *MAC1* fall in the same genetic pathway to mediate cisplatin sensitivity. If, for example, *YNR051c* and *GPA2* act in the same pathway, the double mutant *ynr051cΔ gpa2Δ* should not be more resistant than either of the single mutants, *ynr051cΔ* or *gpa2Δ*. Our analyses of double mutants shown in Figure 2-2 demonstrate that each of the four mutants belongs to different epistasis groups, suggesting that the mechanisms of resistance are different in these mutants.

Figure 2-2. Cisplatin resistance of mutants deleted for *YNR051c*, *GPA2*, *NMD2*, or *MAC1*.

Log-phase cells grown in rich medium (YPD) were exposed for two hours to different concentrations of cisplatin in YPD and incubated on YPD plates at 30°C for two days to allow formation of colonies. Data are expressed as percentages of colonies formed compared to control cultures not exposed to cisplatin. Strains are wild-type (YSI1), *ynr051cΔ* (YSI3), *gpa2Δ* (YSI4). *nmd2Δ* (YSI5), and *mac1Δ* (YSI6).

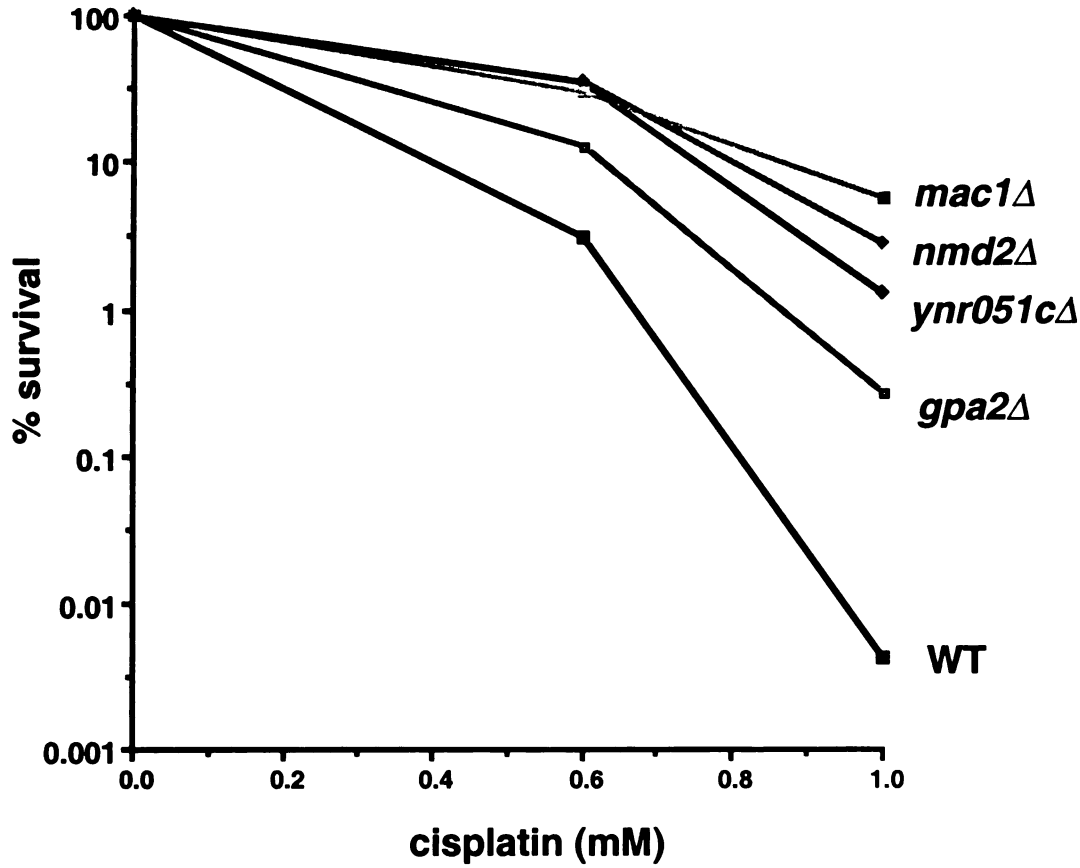
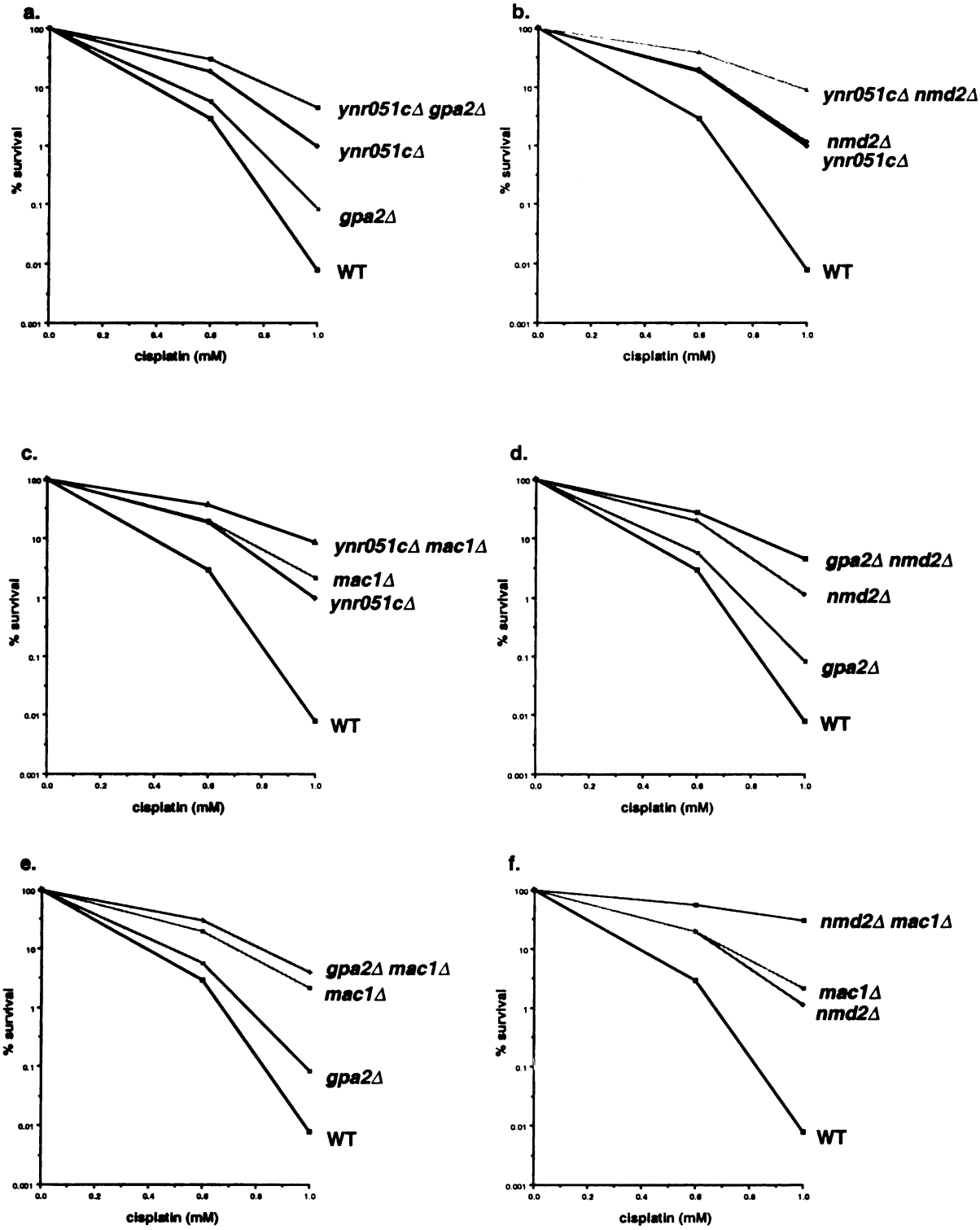

100
10
1
0.1
0.01
0.001
% survival
0.0
0.2
0.4
0.6
0.8
1.0
cisplatin (mM)
mac1Δ
nmd2Δ
ynr051cΔ
gpa2Δ
WT

Figure 2-3. Epistasis analyses of *ynr051cΔ*, *gpa2Δ*, *nmd2Δ*, and *mac1Δ*. Cisplatin resistance between single and double mutants were compared: **a.** *ynr051cΔ* (YSI3), *gpa2Δ* (YSI4), and *ynr051cΔ gpa2Δ* (YSI10); **b.** *ynr051cΔ*, *nmd2Δ* (YSI5), and *ynr051cΔ nmd2Δ* (YSI11); **c.** *ynr051cΔ*, *mac1Δ* (YSI6), and *ynr051cΔ mac1Δ* (YSI12); **d.** *gpa2Δ*. *nmd2Δ*, and *gpa2Δ nmd2Δ* (YSI13); **e.** *gpa2Δ*, *mac1Δ*, and *gpa2Δ mac1Δ* (YSI14); **f.** *nmd2Δ*, *mac1Δ*, and *nmd2Δ mac1Δ* (YSI15). Assays were performed as in Figure 2-2.


a.
ynr051cΔ gpa2Δ
ynr051cΔ
gpa2Δ
WT
% survival
cisplatin (mM)
b.
ynr051cΔ nmd2Δ
nmd2Δ
ynr051cΔ
WT
% survival
cisplatin (mM)
c.
ynr051cΔ mac1Δ
mac1Δ
ynr051cΔ
WT
% survival
cisplatin (mM)
d.
gpa2Δ nmd2Δ
nmd2Δ
gpa2Δ
WT
% survival
cisplatin (mM)
e.
gpa2Δ mac1Δ
mac1Δ
gpa2Δ
WT
% survival
cisplatin (mM)
f.
nmd2Δ mac1Δ
mac1Δ
nmd2Δ
WT
% survival
cisplatin (mM)

The levels of cisplatin-DNA adducts are reduced in *ynr051cΔ*, *gpa2Δ*, *nmd2Δ*, and *mac1Δ*, independently of the nucleotide excision repair gene *RAD2*.

Since the formation of cisplatin-DNA adducts is thought to be the major cause of cisplatin toxicity, we measured the amount of platinum bound to purified DNA in each mutant after a two-hour exposure to 1 mM cisplatin. All of the mutants exhibited a decreased amount of platinum on DNA compared to wild-type cells (Figure 2-3): the level of adducts was reduced to 62% in *ynr051cΔ*, 85% in *gpa2Δ*, 72% in *nmd2Δ*, and 58% in *mac1Δ*. The reduction in the adduct level in the mutants does not seem to be caused by enhanced excision repair, since deletion of one of the repair genes, *RAD2*, did not restore the adduct level to that of wild-type (Figure 2-4). The differences in adduct levels in the mutants reflected the degree of sensitivity to cisplatin in most cases: the level of adducts was wild-type > *gpa2Δ* > *nmd2Δ* > *ynr051cΔ* > *mac1Δ*, and cislpatin sensitivity was wild-type > *gpa2Δ* > *ynr051cΔ* > *nmd2Δ* > *mac1Δ*. It is interesting that *nmd2Δ* accumulates more adducts than *ynr051cΔ* but is more resistant than *ynr051cΔ*. *nmd2Δ* may have an altered response to DNA adducts that allows cells to survive more DNA damage.

The levels of cisplatin in cells are reduced in *ynr051cΔ*, *gpa2Δ*, *nmd2Δ*, and *mac1Δ*

One of the mechanisms that lead cells to reduce the level of cisplatin-DNA adducts is decreased drug accumulation inside the cells. We therefore measured the amount of platinum in whole cells after a two-hour treatment of cells with 1 mM cisplatin. The levels of cellular platinum were reduced in all of the mutants (Figure 2-4): wild-type (100%) > *ynr051cΔ* (79%) > *nmd2Δ* (76%) > *gpa2Δ* (74%) > *mac1Δ* (59%). In *nmd2Δ* and *mac1Δ*, the levels of accumulation of platinum in cells and on DNA were reduced to similar

Figure 2-4. The levels of cisplatin-DNA adducts in *ynr051cΔ*, *gpa2Δ*, *nmd2Δ*, and *mac1Δ* in the presence or absence of *RAD2*.

DNA was purified from cells treated with 1 mM cisplatin for two hours in YPD. The amount of platinum was measured using an atomic absorption spectrophotometer. Numbers represent absorption by atomized platinum divided by A_{260} of each DNA sample. Strains are wild-type (YSI1), *ynr051cΔ* (YSI3), *gpa2Δ* (YSI4), *nmd2Δ* (YSI5), *mac1Δ* (YSI6), *rad2Δ* (YSI16), *ynr051cΔ rad2Δ* (YSI18), *gpa2Δ rad2Δ* (YSI19), *nmd2Δ rad2Δ* (YSI20), and *mac1Δ rad2Δ* (YSI21).

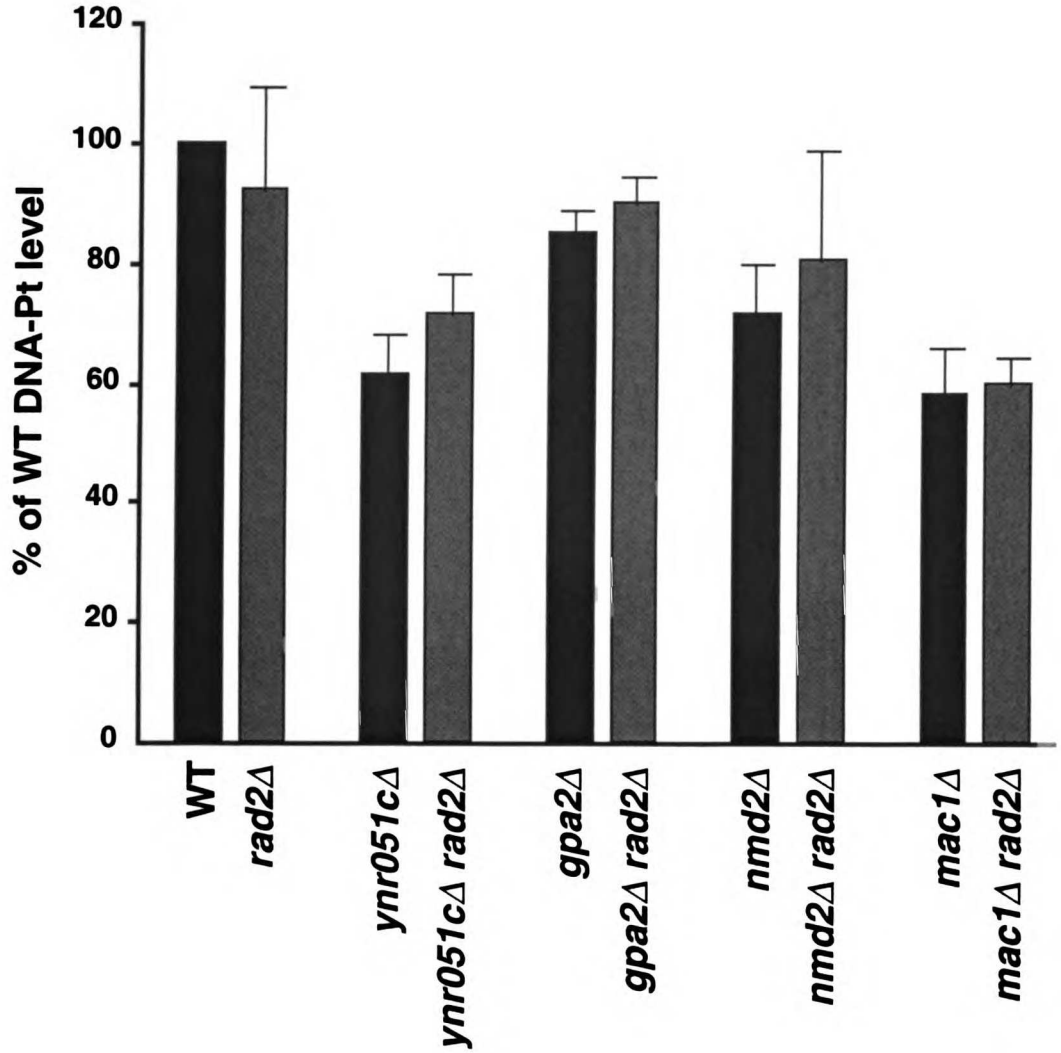
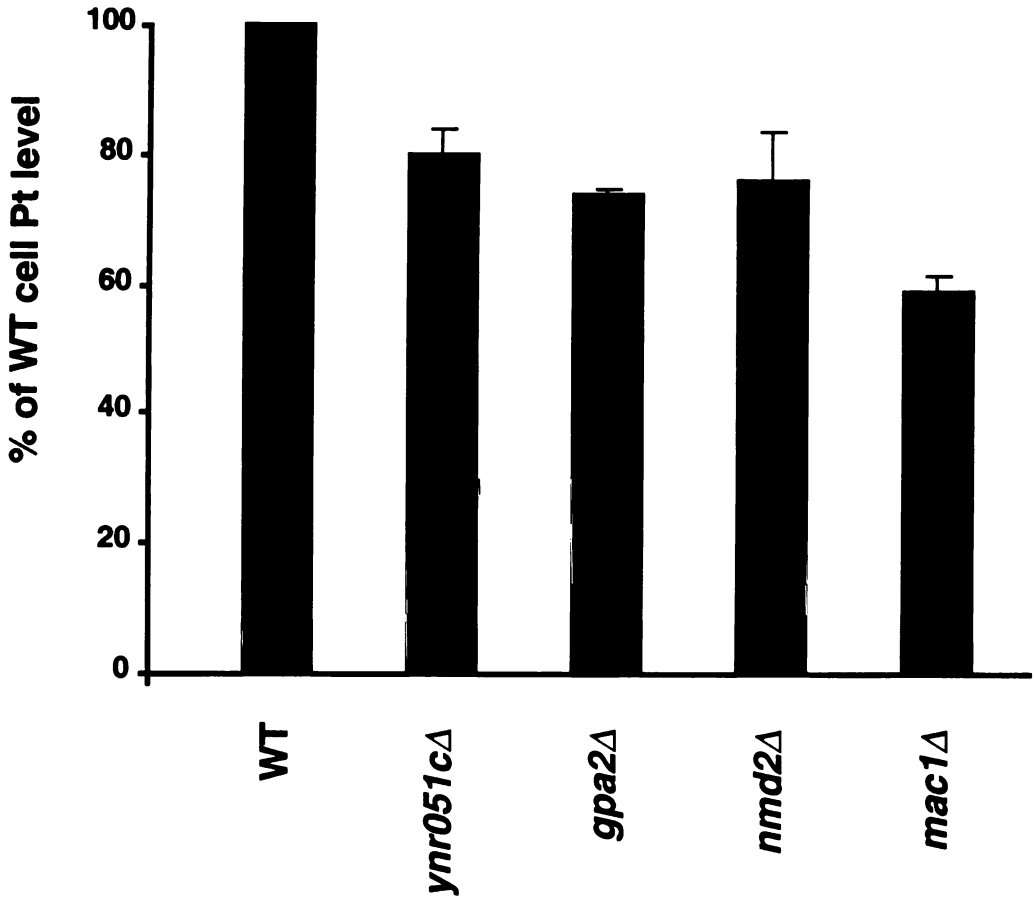

% of WT DNA-Pt level
120
100
80
60
40
20
0
WT
rad2Δ
ynr051cΔ
ynr051cΔ rad2Δ
gpa2Δ
gpa2Δ rad2Δ
nmd2Δ
nmd2Δ rad2Δ
mac1Δ
mac1Δ rad2Δ

Figure 2-5. The levels of cellular cisplatin accumulation in *ynr051cΔ*, *gpa2Δ*, *nmd2Δ*, and *mac1Δ*.

The amount of platinum in whole cells was measured using the atomic absorption spectrophotometer after incubating cells with 1 mM cisplatin for two hours. Cells were collected by centrifugation and washed twice with minimal medium (SD). Numbers were obtained by dividing the reading from the spectrophotometer by A_{600} of the cultures. Strains are wild-type (YSI1), *ynr051cΔ* (YSI3), *gpa2Δ* (YSI4), *nmd2Δ* (YSI5), and *mac1Δ* (YSI6).


% of WT cell Pt level
100
80
60
40
20
0
WT
ynr051cΔ
gpa2Δ
nmd2Δ
mac1Δ

degrees (cell platinum in *nmd2Δ* 76%, on DNA 72%; cell platinum in *mac1Δ* 59%, on DNA 58%), suggesting that decreased accumulation of cisplatin inside the cells is the major mechanism that accounts for increased resistance observed in these mutants. In *ynr051cΔ*, cellular platinum level was 79% of that of wild-type cells, and cisplatin-DNA adduct level was further reduced to 62%. *ynr051cΔ* may possess other mechanisms in addition to decreased drug accumulation that lead to decrease in the DNA adduct levels. *gpa2Δ* exhibited 74% of wild-type level of cellular drug accumulation, but the adduct level was 85%. This suggests that *gpa2Δ* is deficient in cellular drug accumulation, but either the ability to form DNA adducts is greater or repair is repressed in this mutant, resulting in the increased percentage of intracellular platinum bound to DNA. This observation suggests that decreased drug accumulation is the major cause of increased cisplatin resistance in *gpa2Δ*.

DISCUSSION

We have isolated mutants that are able to grow in the presence of a toxic dose of cisplatin. The use of transposon-insertional mutagenesis made it possible to identify genes disrupted in the cisplatin-resistant mutants. We have shown that deletion of *YNR051c*, *GPA2*, *NMD2*, or *MAC1* results in increased resistance to cisplatin. The degree of resistance in these mutants was 1.6 to 2.2 times that of wild-type cells. Low levels of resistance are generally believed to be sufficient to cause lack of clinical responsiveness. Changes of less than two-fold may account for treatment failure in human ovarian carcinoma xenografts (Andrews *et al.*, 1990). The four mutants, *ynr051cΔ*, *gpa2Δ*, *nmd2Δ*, and *mac1Δ*, were categorized into different epistasis groups based on double mutant analyses. To gain insight on the mechanisms of increased cisplatin resistance in these mutants, the levels of cisplatin in the cells and on DNA were measured. Postulated function of these genes in regulating cisplatin sensitivity is discussed below.

The role of Ynr051cp in cisplatin sensitivity

In *ynr051cΔ*, cellular cisplatin accumulation is reduced to 79% of that of wild-type cells, and the cisplatin-DNA adduct level was further reduced to 62%. *ynr051cΔ* may possess multiple mechanisms that lead to decreased drug accumulation inside the cells and on DNA. In a high-throughout two-hybrid assay, Ynr051c protein was shown to interact with Yap6p (Ito *et al.*, 2001). Interestingly, *YAP6* was identified in a high-copy screen for cisplatin resistance (Furuchi *et al.*, 2001). Overexpression of *YAP6* results in increased resistance to cisplatin. However, deletion of *YAP6* does not cause increased sensitivity to cisplatin. One model to explain the roles of Ynr051cp and Yap6p

in cisplatin resistance is that the level of free Yap6p inside the cells determines cisplatin resistance, and in wild-type cells, all of the Yap6p is bound by Ynr051cp and thus inhibited from confering resistance. In the absence of *YNR051c*, or when *YAP6* is overexpressed, there is more free Yap6p, which somehow results in increased cisplatin resistance. Overproduction of Yap6p does not affect cellular accumulation of the drug (Furuchi *et al.*, 2001). Yap6p is localized in the nucleus, and is postulated to function as a transcription factor. Its target genes may be directly involved in reducing the level of cisplatin-DNA adducts and/or cellular cisplatin level. Ynr051cp has also been shown to interact with a presumptive membrane protein Psl10p by two-hybrid assay (Ito *et al.*, 2001). Ynr051cp may regulate cisplatin uptake by acting as a transporter or activating other transporters, or by altering membrane fluidity or permeability. Determining the localization of Ynr051cp will be informative in understanding its role in cisplatin sensitivity.

The role of Gpa2p in cisplatin sensitivity

gpa2Δ exhibited 74% of wild-type level of cellular drug accumulation, and the adduct level was 85% of that of wild-type. This suggests that decreased cellular accumulation of the drug is the major mechanism for increased resistance in *gpa2Δ*.

GPA2 encodes a G protein α that regulates the cAMP level in the cell (Kubler *et al.*, 1997). It acts with Gpr1p, a plasma membrane G protein-coupled receptor (Xue *et al.*, 1998). The cellular cAMP level is also controlled by Pde2p, a phosphodiesterase that destroys cAMP (Sass *et al.*, 1986). In order to assess involvement of *GPR1* and *PDE2* in cisplatin sensitivity, I deleted each of these genes and measured cisplatin sensitivity. *gpr1Δ* exhibited

increased resistance and *pde2Δ* was more sensitive to cisplatin than wild-type cells (Ishida, unpublished observation). Thus, the level of intracellular cAMP may influence cisplatin sensitivity: high cellular cAMP level results in sensitivity and low cAMP level causes resistance to cisplatin.

The mechanism by which cells manifest resistance to cisplatin when the cellular cAMP level is low is not clear. Overexpression of *PDE2* has been shown to confer increased resistance to cisplatin in the *rad4Δ* mutant defective in nucleotide excision repair (Burger *et al.*, 2000). Therefore, it is unlikely that the increased resistance is caused by enhanced excision repair. We have seen a reduction in drug accumulation inside the cell in *gpa2Δ*. In the *gpa2Δ* mutant, the amount of *FLO11* mRNA is reduced to less than 10 % of that of wild-type cells (Lorenz *et al.*, 2000). Flo11p is a cell surface protein required for cell flocculation and invasiveness. Decreased level of Flo11p in *gpa2Δ* may lead to reduction in permeability of the cell wall and/or plasma membrane to cisplatin. *FLO11* mRNA level is also controlled by ploidy and mating type (Galitski *et al.*, 1999). Cells with increased ploidy have less *FLO11* transcripts, and '**a**/α' cells have less *FLO11* mRNA than '**a**' or 'α' cells. I have tested the effects of cell type and ploidy on cisplatin resistance, and found that diploid cells are more resistant to cisplatin than haploid cells of the same mating type, and that the '**a**/α' cell type confers resistance (Ishida, unpublished observation). This observation is consistent with a hypothesis that a decreased level of Flo11p contributes to increased cisplatin resistance. Our hypothesis predicts that *flo11Δ* cells are more resistant to cisplatin. One may then ask whether the effects of ploidy and cell type are dependent on *FLO11*.

Gpa2p interacts with Ynl124p by two-hybrid assay (Ito *et al.*, 2001). The human homolog of Ynl124p is ALF, which is a transcription factor abundant

in the testis (De Vries *et al.*, 2000). High ALF level in the testis could explain the high responsiveness of testicular cancer to cisplatin. It will be interesting to test whether cisplatin sensitivity is altered in *ynl124Δ*.

In mammals, the cAMP-dependent protein kinase A (PKA) pathway has been suggested to play a role in regulating cisplatin sensitivity (Cvijic *et al.*, 1998). The PKA tetrameric holoenzyme contains two regulatory (R) subunits and two catalytic (C) subunits, and is activated when two cAMP molecules cooperatively bind each R subunit, resulting in the release of the active C subunits. Cells harboring defective R subunits, but not defective C subunits, show increased resistance to cisplatin. Nuclear extracts from these cells demonstrated an increase in recognition and binding of nuclear factors to a cisplatin-damaged DNA probe (Liu *et al.*, 1996). This suggests that cisplatin resistance observed in the R subunit mutants may be controlled at the DNA level after the formation of adducts. R subunits may negatively regulate DNA repair pathways or cell survival after DNA damage.

The role of Nmd2p in cisplatin sensitivity

Nmd2p promotes degradation of mRNAs containing nonsense mutations in concert with Upf1p and Upf3p (Culbertson, 1999). Nmd2p is proposed to pass information from Upf3p on mRNA to the Upf1p helicase that unwinds the mRNA, promoting its decapping and decay. Deletion of *UPF1* or *UPF3* results in increased cisplatin resistance similar to that of *nmd2Δ* in the same assay and strain background, suggesting that the nonsense-mediated mRNA decay pathway regulates cisplatin sensitivity (Roby Bhachatarrya, unpublished observation). The Upf1p/Nmd2p/Upf3p complex is involved in global expression of the yeast transcriptome (Lelivelt and Culbertson, 1999). Loss of *UPF1*, *NMD2*, or *UPF3* changes the abundance

of 8% of 6220 cellular mRNAs, 90% of which exhibit an increase and 10% of which exhibit a decrease in abundance (Culbertson, 1999). Increased cisplatin resistance observed in the *nmd2Δ* mutant could result from increased abundance of total cellular mRNA which can react with cisplatin, thereby leaving less cisplatin available for reacting with DNA. However, our findings indicate that a decrease in cellular drug accumulation is the major mechanism of cisplatin resistance in *nmd2Δ*: cellular platinum in *nmd2Δ* was 76% of the wild-type level, and 72% on DNA. Transcript levels of genes whose products are involved in cisplatin uptake or efflux may be regulated by the nonsense-mediated mRNA decay pathway. However, the mRNA levels of genes governing cisplatin sensitivity identified in our screen and in the subsequent work, namely *YNR051c*, *GPA2*, *MAC1*, *CTR1*, *CTR3* and *FRE1*, were not significantly altered in *upf1Δ*, *nmd2Δ*, *upf3Δ*, or the triple *upf1Δ nmd2Δ upf3Δ* mutant (http://144.92.19.47/default.htm).

The role of Mac1p in cisplatin sensitivity

Mac1p is a transcription factor that regulates transcription of genes involved in copper and iron uptake and catalase genes (Jungmann *et al.*, 1993; Yamaguchi-Iwai *et al.*, 1997; Labbe *et al.*, 1997; Martins *et al.*, 1998). The level of cisplatin accumulation in *mac1Δ* cells was 59% of that in wild-type cells, and the DNA adduct level was reduced to a similar degree (58%), suggesting that decreased drug accumulation in the cells is the mechanism of increased resistance in *mac1Δ*. Analysis of mutants deleted for the Mac1p target genes revealed that a copper transporter Ctr1p and a copper/iron reductase Fre1p are involved in cisplatin sensitivity (see chapter three, Fig. 3-1). The cellular cisplatin level in *ctr1Δ* (YSI22) was reduced to 67% of that of wild-type, and to 86% in *fre1Δ*, (YSI23) while *mac1Δ* exhibited 59% of the wild-type cell

platinum level (Ishida, unpublished observation). Reduced level of Ctr1p and Fre1p in the *mac1Δ* mutant is likely to be responsible for defective drug accumulation observed in *mac1Δ*. Further characterization of the role of Ctr1p in cisplatin uptake in yeast and mammalian cells is described in the next chapter.

Acknowledgements

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CHAPTER THREE

RESISTANCE TO THE ANTICANCER DRUG CISPLATIN DUE TO DEFECTS IN THE COPPER TRANSPORTER CTR1 IN YEAST AND MAMMALS

SUMMARY

Cisplatin is a chemotherapeutic drug used to treat a variety of cancers (Loehrer and Einhorn, 1984). However, both intrinsic and acquired resistance to cisplatin, as well as toxicity, limit its effectiveness (Giaccone, 2000). Molecular mechanisms that underlie cisplatin resistance are poorly understood. Here we demonstrate that deletion of the yeast *CTR1* gene, which encodes a high-affinity copper transporter (Dancis *et al.*, 1994), results in increased cisplatin resistance and reduced intracellular accumulation of cisplatin. Copper, which causes a reduction in the level of Ctr1 protein (Ctr1p) (Ooi *et al.*, 1996), enhances survival of wild-type yeast cells to cisplatin and reduces cellular accumulation of the drug. Mouse cell lines lacking one or both *CTR1* alleles exhibit increased cisplatin resistance and decreased cisplatin accumulation in parallel with *CTR1* gene dosage. We propose that cisplatin uptake is mediated by a copper transporter, Ctr1p, in yeast and mammals. The link between Ctr1p and cisplatin transport may explain some cases of cisplatin resistance and suggests ways of modulating sensitivity and toxicity to this important anticancer drug.

INTRODUCTION

The therapeutic effect of cisplatin is due to formation of intrastrand crosslinks in DNA (Zamble *et al.*, 1995). A number of cisplatin-resistant cancer cell lines have been identified that exhibit decreased intracellular drug accumulation, decreased formation of cisplatin-DNA adducts, and/or increased repair (Scanlon *et al.*, 1991). Even small differences in cisplatin accumulation lead to large increases in resistance to the drug in cell lines (Scanlon *et al.*, 1991). The genetic basis for cisplatin resistance, however, remains unclear. In the budding yeast, *Saccharomyces cerevisiae*, inactivation of the *IXR1* gene, which encodes an HMG box protein that binds to cisplatin-DNA adducts, leads to increased cisplatin resistance (Brown *et al.*, 1993). It is thought that Ixr1p shields cisplatin-DNA adducts from repair (McA'Nulty and Lippard, 1996). Mammalian HMG proteins have also been shown to interact with cisplatin-DNA adducts and inhibit repair of the adduct *in vitro* (Pil and Lippard, 1992; Huang *et al.*, 1994). Overproduction in yeast of Pde2p, a phosphodiesterase that functions in the cAMP-dependent protein kinase (PKA) pathway, confers increased cisplatin resistance to mutants defective for excision repair (Burger *et al.*, 2000). Studies in mammalian cells also suggest potential links between the PKA pathway and cisplatin sensitivity (Cvijic *et al.*, 1998). It has recently been reported that overexpression of two yeast genes, *CIN5* and *YDR259c* (Furuchi *et al.*, 2001), and deletion of the *SKY1* gene in the excision repair-deficient *rad4Δ* mutant (Schenk *et al.*, 2001), result in increased cisplatin resistance. The basis for the observed increase in cisplatin resistance in cells overexpressing *PDE2*, *CIN5*, or *YDR259c*, or in cells deleted for *SKY1*, is not known.

RESULTS AND DISCUSSION

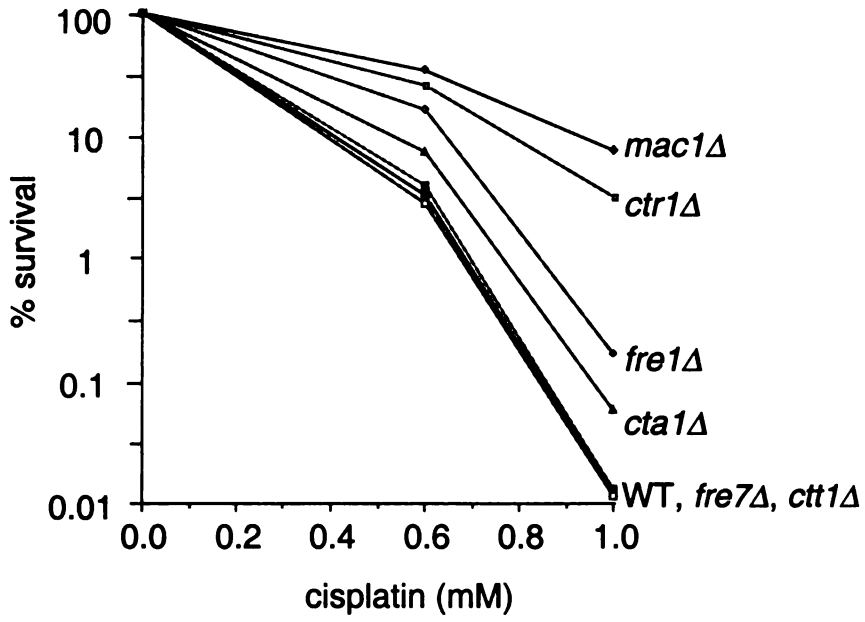
Yeast cells deleted for *CTR1*, one of the target genes of Mac1p, are as resistant to cisplatin as *mac1Δ*

In order to identify genes involved in cisplatin resistance, we mutagenized wild-type yeast cells with a transposon library and selected for mutants able to grow in the presence of a toxic dose of cisplatin (chapter two). The mutant that showed the highest degree of resistance was defective in the *MAC1* gene: deletion of the complete *MAC1* open reading frame (ORF) resulted in a 2.5-fold increase in survival relative to wild-type cells (Figure 3-1). *MAC1* encodes a transcription factor that activates transcription of catalase genes and genes involved in uptake of copper and iron (Jungmann *et al.*, 1993; Yamaguchi-Iwai *et al.*, 1997; Labbe *et al.*, 1997; Martins *et al.*, 1998). To address whether increased cisplatin resistance of the *mac1Δ* mutant is due to inability to transcribe a Mac1p target gene, we deleted each of its target genes and measured the sensitivity of these mutants to cisplatin. Of the five mutants, only the *ctr1Δ* strain showed a level of resistance similar to that of the *mac1Δ* mutant (Figure 3-1). These observations suggest that *CTR1* is the major target for Mac1p with respect to cisplatin resistance.

Mutants lacking genes involved in intracellular copper trafficking and utilization are not as resistant to cisplatin as *ctr1Δ*

CTR1 encodes a high-affinity copper transporter (Dancis *et al.*, 1994a). Cells deleted for *CTR1* are resistant to growth inhibition caused by high levels of copper in the medium, fail to grow on low-copper medium, and are defective in enzymes such as superoxide dismutase or in cellular activities such as iron uptake and respiration that require copper (Dancis *et al.*, 1994a, b).

Figure 3-1. Cisplatin resistance of mutants defective in Mac1p target genes. Log-phase cells were exposed for two hours to different concentrations of cisplatin (Bristol Laboratories) in rich medium (YPD) and incubated on YPD plates at 30°C for two days to allow formation of colonies. Data are expressed as percentages of colonies formed compared to control cultures not exposed to cisplatin. Strains are wild-type (YSI1), *mac1Δ* (YSI6), *ctr1Δ* (YSI22), *fre1Δ* (YSI23), *fre7Δ* (YSI24), *cta1Δ* (YSI25), and *ctt1Δ* (YSI26).


100
10
1
0.1
0.01
% survival
0.0
0.2
0.4
0.6
0.8
1.0
cisplatin (mM)
mac1Δ
ctr1Δ
fre1Δ
cta1Δ
WT, fre7Δ, ctt1Δ

We measured cisplatin sensitivity in strains defective in intracellular copper trafficking and utilization, namely, in *atx1Δ*, *ccc2Δ*, *fet3Δ*, *lys7Δ*, *sod1Δ*, *cox17Δ*, and *sco1Δ* mutants (Valentine and Gralla, 1997) (Figure 3-2), but did not observe significant changes (Figure 3-3). It thus appears that defects in proteins involved in intracellular copper distribution or utilization do not play a major role in cisplatin resistance in yeast.

Deletion of a low-affinity copper transporter, *CTR2* or *FET3*, does not cause increased resistance to cisplatin; cells deficient in another high-affinity copper transporter *CTR3* exhibit increased resistance

We also tested for involvement of other yeast copper transporters in cisplatin sensitivity -- another high-affinity transporter, Ctr3p, and two low-affinity transporters, Ctr2p and Fet4p (Knight *et al.*, 1996; Kampfenkel *et al.*, 1995; Hassett *et al.*, 2000). The *CTR3* gene is not transcribed in the strain background used for our studies due to the presence of a transposon in its upstream regulatory sequence (Knight *et al.*, 1996): thus our Ctr1p-deficient strain and its isogenic *CTR1* parent strain are also defective for Ctr3p. Deletion of a low-affinity copper transporter, *CTR2* or *FET4*, in this *ctr3* background did not affect cisplatin resistance (Figure 3-4). To investigate the role of Ctr3p in cisplatin uptake, we measured cisplatin resistance in a set of isogenic strains constructed in a *CTR3* background (Knight *et al.*, 1996). We observed the level of cisplatin resistance in these strains to be *ctr1 ctr3* > *ctr1 CTR3* >> *CTR1 ctr3* > *CTR1 CTR3* (Figure 3-5). Although the yeast Ctr1 and Ctr3 proteins have redundant roles in copper uptake, they have distinct structural features that could be responsible for differential function in cisplatin uptake and resistance.

Figure 3-2. Copper trafficking and utilization in yeast.

Once copper enters the cell via Ctr1p, it is bound by copper chaperones, Atx1p, Lys7p, or Cox17p. Atx1p directs copper to a post-Golgi compartment, by way of Ccc2p, a P-type ATPase transmembrane copper transporter, for final insertion into Fet3p, a multicopper oxidase essential for high-affinity iron uptake; Lys7p targets copper to copper-zinc superoxide dismutase Sod1p, a primary antioxidant enzyme in the cytosol; and Cox17p guides copper to the mitochondria for insertion into cytochrome c oxidase (CCO), the terminal oxidase of the respiratory chain. Sco1p is one of the components of CCO.

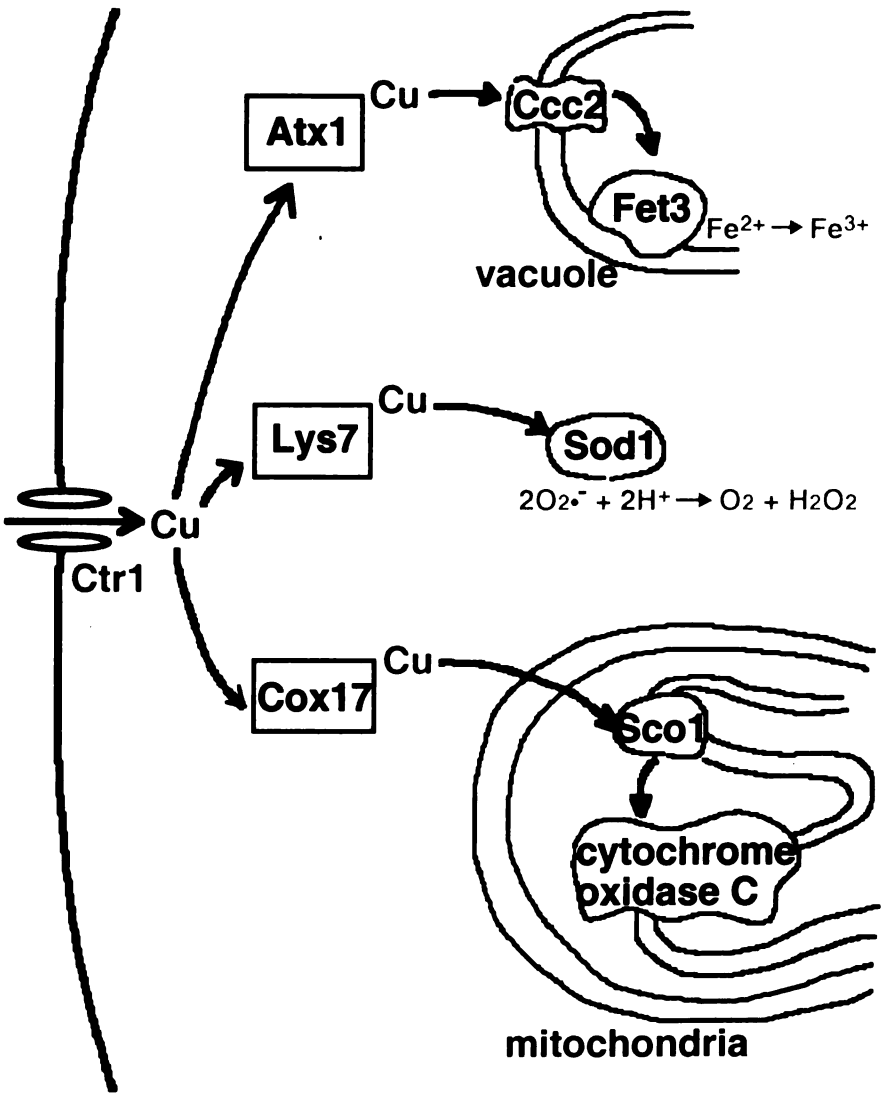

Atx1
Cu
Ccc2
Fet3
Fe2+ → Fe3+
vacuole
Lys7
Cu
Sod1
2O2•- + 2H+ → O2 + H2O2
Cu
Ctr1
Cox17
Cu
Sco1
cytochrome
oxidase C
mitochondria

Figure 3-3. Cisplatin sensitivity of mutants defective in intracellular copper trafficking and utilization.

Sensitivity to cisplatin was assayed as described in Figure 3-1. Strains are wild-type (YSI1), *atx1Δ* (YSI28), *ccc1Δ* (YSI29), *fet3Δ* (YSI30), *lys7Δ* (YSI31), *sod1Δ* (YSI32), *cox17Δ* (YSI33), and *sco1Δ* (YSI34).

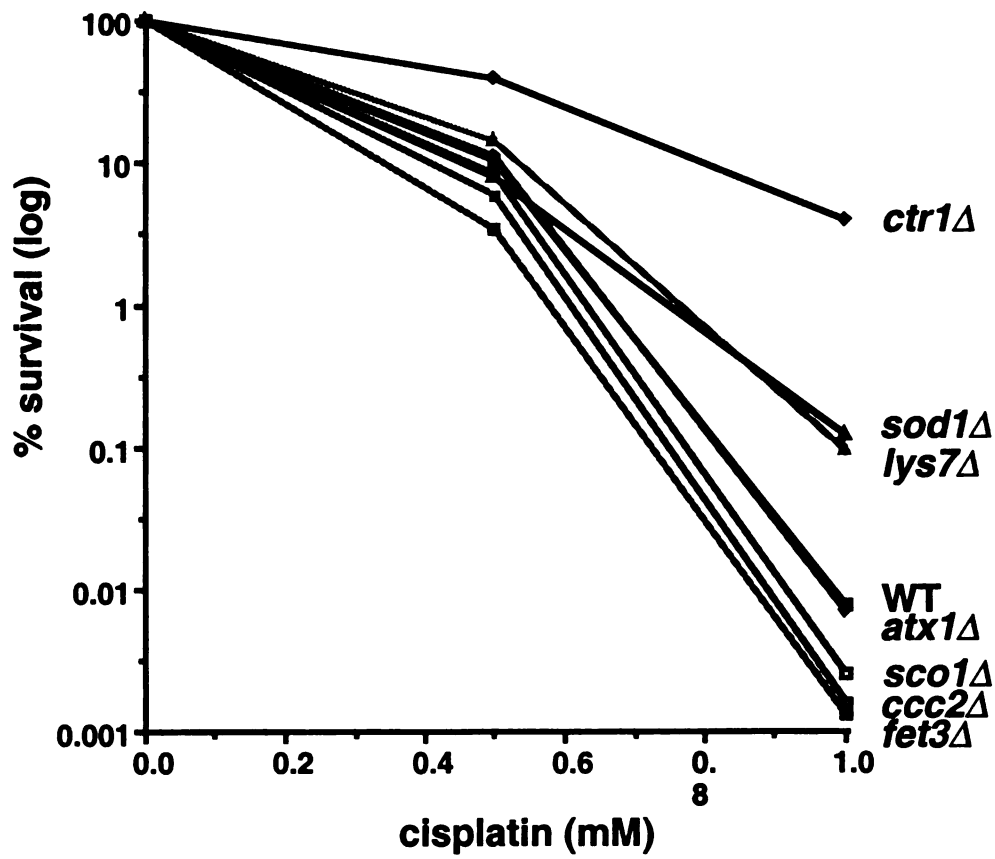

100
10
1
0.1
0.01
0.001
% survival (log)
0.0
0.2
0.4
0.6
0.8
1.0
cisplatin (mM)
ctr1Δ
sod1Δ
lys7Δ
WT
atx1Δ
sco1Δ
ccc2Δ
fet3Δ

Figure 3-4. Cisplatin sensitivity of mutants defective in low-affinity copper transporters.

Sensitivity to cisplatin was assayed as described in Figure 3-1. Strains are wild-type (YSI1), *ctr1Δ* (YSI22), *ctr2Δ* (YSI35), and *fet4Δ* (YSI36).

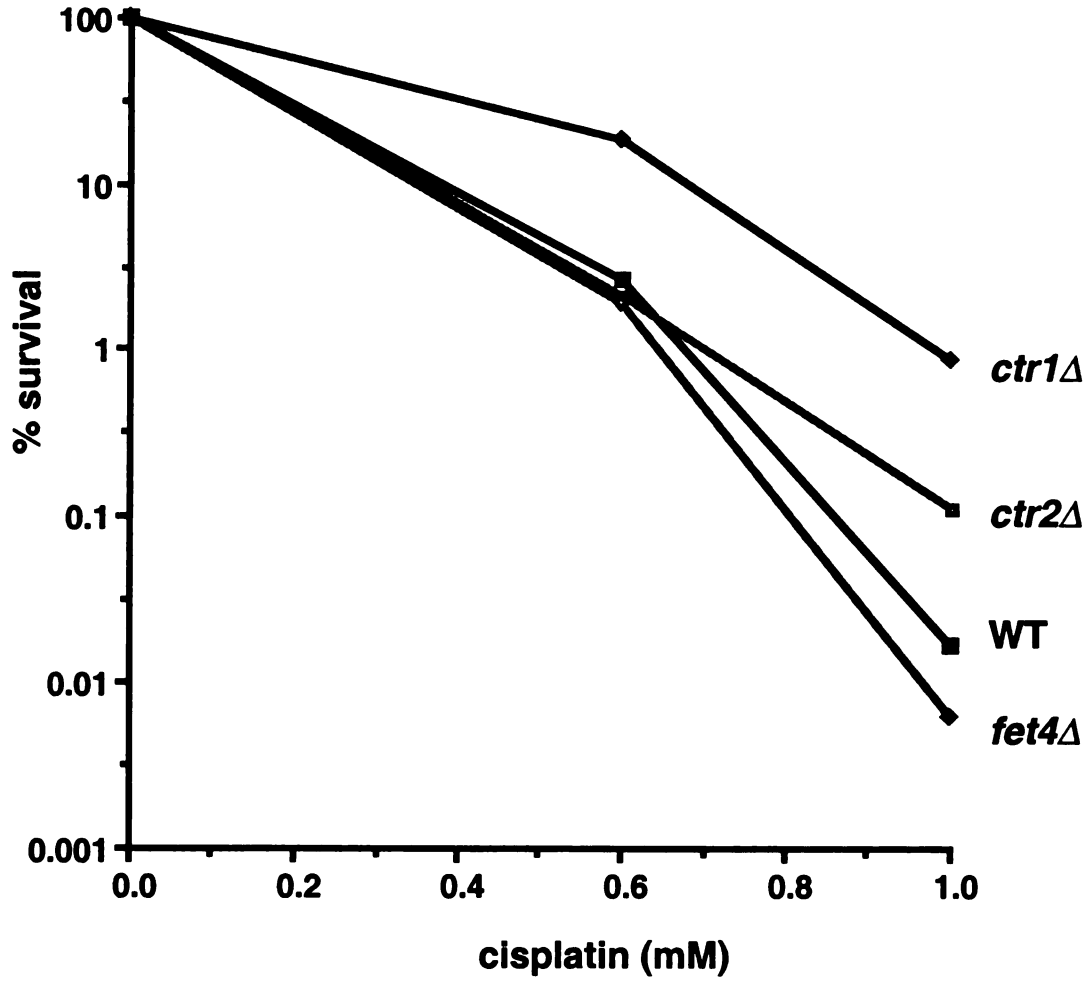
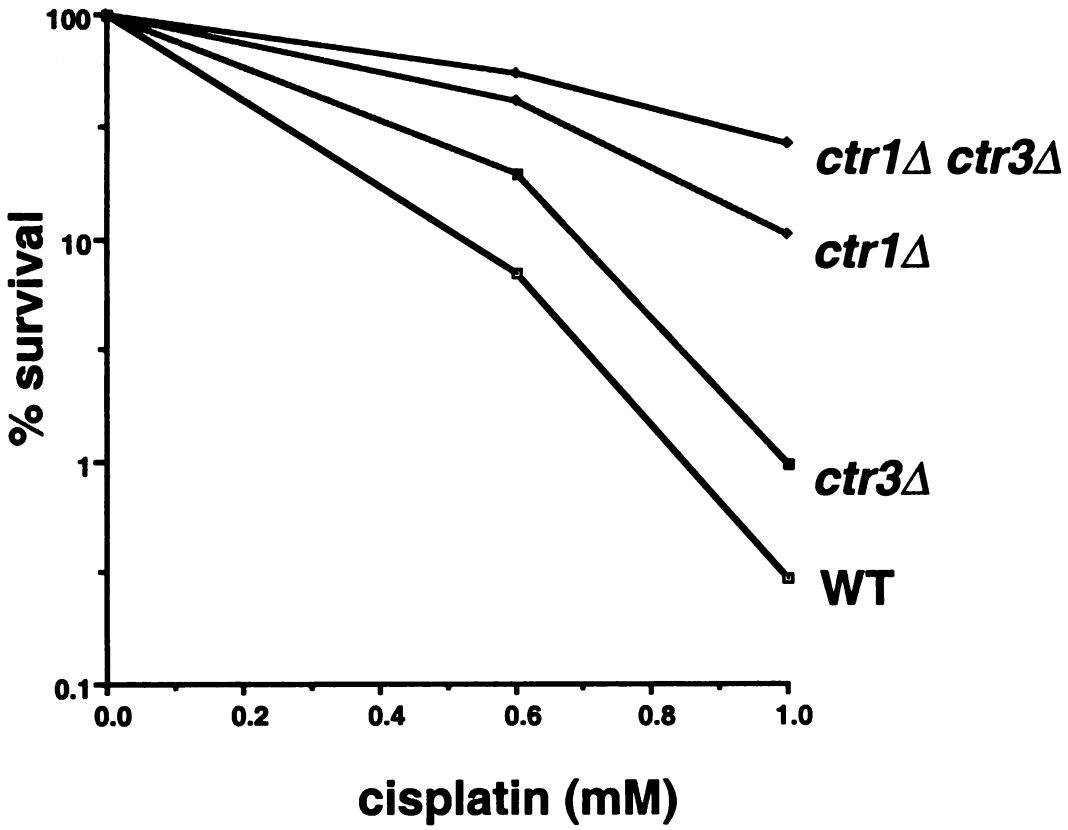

100
10
1
0.1
0.01
0.001
% survival
0.0
0.2
0.4
0.6
0.8
1.0
cisplatin (mM)
ctr1Δ
ctr2Δ
WT
fet4Δ

Figure 3-5. Cisplatin sensitivity of mutants defective in high-affinity copper transporters in the BR10 strain background.

Log-phase cells were exposed for three hours to different concentrations of cisplatin (Bristol Laboratories) in rich medium (YPD) and incubated on YPD plates at 30°C for two days to allow formation of colonies. Data are expressed as percentages of colonies formed compared to control cultures not exposed to cisplatin. Strains are wild-type (DTY1), *ctr1Δ* (SKY52), *ctr3Δ* (SKY44), and *ctr1Δ ctr3Δ* (SKY46).


100
10
1
0.1
% survival
0.0
0.2
0.4
0.6
0.8
1.0
cisplatin (mM)
ctr1Δ ctr3Δ
ctr1Δ
ctr3Δ
WT

The level of DNA adducts is decreased in the *ctr1Δ* mutant indepently of the nucleotide excision repair

To understand the basis for cisplatin resistance in *ctr1Δ* strains, we first compared the level of cisplatin-DNA adducts in wild-type and *ctr1Δ* mutant cells, since the formation of DNA adducts is the major cause of cisplatin toxicity (Zamble and Lippard, 1995). As shown in Figure 3-6, the amount of cisplatin bound to DNA during a two-hour incubation with 1 mM cisplatin in the mutant cells was approximately 59% of that in wild-type cells, in which platinum was calculated to be bound to DNA at a ratio of one molecule per 14 base pairs. To determine whether the reduced level of cisplatin-DNA adducts in the mutant is due to enhanced excision repair, we examined the level of adduct formed in a *ctr1Δ* strain incapable of excision repair due to deletion of *RAD2* (Wang *et al.*, 1993). We observed that the level of adduct was also reduced in the *ctr1Δ rad2Δ* strain, to 35% of the level formed by the isogenic *CTR1 rad2Δ* strain (Figure 3-6), indicating that enhanced excision repair is not the mechanism by which *ctr1Δ* mutants exhibit increased cisplatin resistance.

The level of cellular cisplatin accumulation is reduced in the *ctr1Δ* mutant

To determine whether cellular accumulation of cisplatin is affected in the *ctr1Δ* mutant, we measured the level of platinum in wild-type and mutant cells treated with 1 mM cisplatin for two hours. Figure 3-7 shows that the level of cisplatin accumulation in the *ctr1Δ* mutant was only 56% of that exhibited by wild-type cells. This decrease in cellular accumulation of cisplatin is likely to reflect decrease in drug uptake and not increase in efflux, since we did not see a significant increase in drug clearance in the *ctr1Δ* mutant relative to wild-type after cisplatin was removed from the medium and further incubated for six hours (Ishida, unpublished). Therefore,

Figure 3-6. Accumulation of cisplatin-DNA adducts in wild-type and isogenic *ctr1Δ* mutant strains.

DNA was purified from cells treated with 1 mM cisplatin for two hours in YPD. The amount of platinum was measured using an atomic absorption spectrophotometer. Numbers represent absorption by atomized platinum divided by A_{260} of each DNA sample. Strains are wild-type (YSI1), *ctr1Δ* (YSI22), *rad2Δ* (YSI16), and *ctr1Δ rad2Δ* (YSI27).

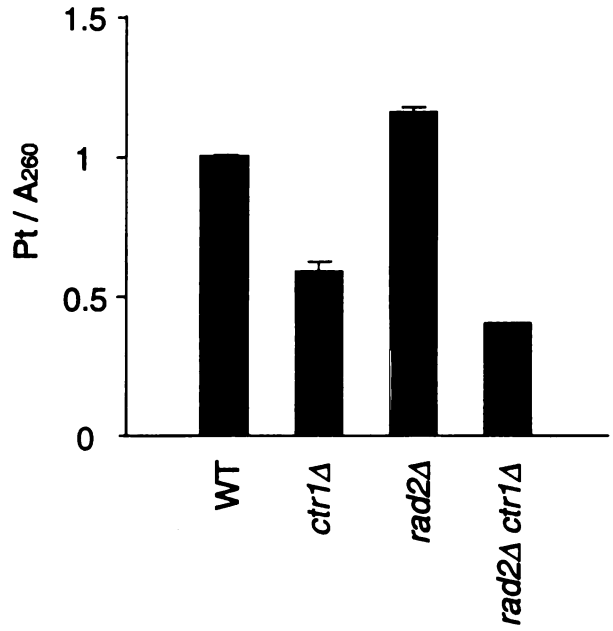
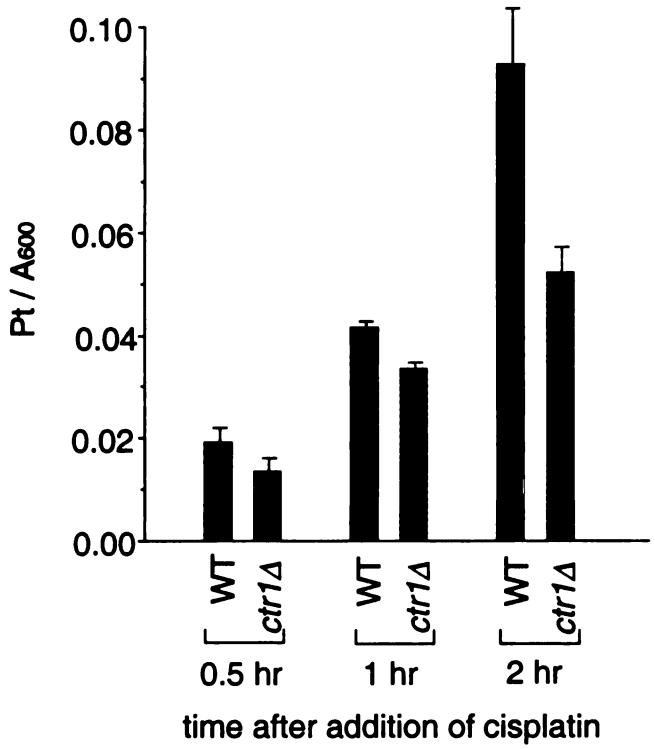

1.5
1
0.5
0
Pt / A260
WT
ctr1Δ
rad2Δ
rad2Δ ctr1Δ

Figure 3-7. Intracellular platinum accumulation in wild-type and *ctr1Δ* mutant strains.

The amount of platinum in whole cells was measured using the atomic absorption spectrophotometer after incubating cells with 1 mM cisplatin for the indicated times. Cells were collected by centrifugation and washed twice with minimal medium (SD). Numbers were obtained by dividing the reading from the spectrophotometer by A_{600} of culture at each time point. Strains are wild-type (YSI1) and *ctr1Δ* (YSI22).


0.10
0.08
0.06
0.04
0.02
0.00
Pt / A600
WT
ctr1Δ
WT
ctr1Δ
WT
ctr1Δ
0.5 hr
1 hr
2 hr
time after addition of cisplatin

decreased drug uptake is likely to be responsible for the lower cisplatin adduct level observed in the *ctr1Δ* mutant and consequent increased survival.

Copper enhances cell survival to cisplatin and decreases cisplatin accumulation inside the cell in a *CTR1*-dependent manner

Decreased cisplatin accumulation observed in *ctr1Δ* led us to propose that the copper transporter Ctr1p also functions as a cisplatin transporter. If so, copper might compete with cisplatin for transport into cells. We observed that addition of exogenous copper to the medium resulted in enhanced survival of wild-type but not of *ctr1Δ* mutant cells exposed to cisplatin (Figure 3-8). When 0.1 mM copper was added to the medium, the survival of wild-type cells was increased two-fold (Figure 3-8); cellular accumulation of cisplatin was decreased two-fold (Figure 3-9). In contrast, copper had no effect on the survival and cisplatin accumulation in *ctr1Δ* mutant cells (Figures 3-8, 9). These observations indicate that the ability of copper to reduce cisplatin uptake and thereby increase resistance to cisplatin is dependent on *CTR1*. We can imagine at least two mechanisms by which copper might interfere with cisplatin uptake. One is a direct competition between copper and cisplatin for a site on a transporter whose abundance is limiting. The other is a copper-induced inactivation of the transporter. Although our experiments do not allow us to exclude direct competition for a site on Ctr1p, there is ample evidence for the second explanation. It is known from prior work that the level of Ctr1p is regulated by copper concentration: high copper triggers internalization and degradation of Ctr1p as well as inactivation of the Mac1 protein (Ooi *et al.*, 1996; Labbe *et al.*, 1997; Dancis *et al.*, 1994b). In our experiments, the level of Ctr1p was reduced four-fold after two hours of incubation with 0.1 mM copper (Figure 3-10). Copper did not have to be

Figure 3-8. Effect of copper on survival of wild-type and the *ctr1Δ* mutant exposed to cisplatin.

Wild-type (YSI1) and *ctr1Δ* (YSI22)were treated for two hours with various concentrations of cisplatin in the presence of 0.01 mM, 0.1 mM, or no $CuSO_4$. Data were analyzed as in Figure 3-1.

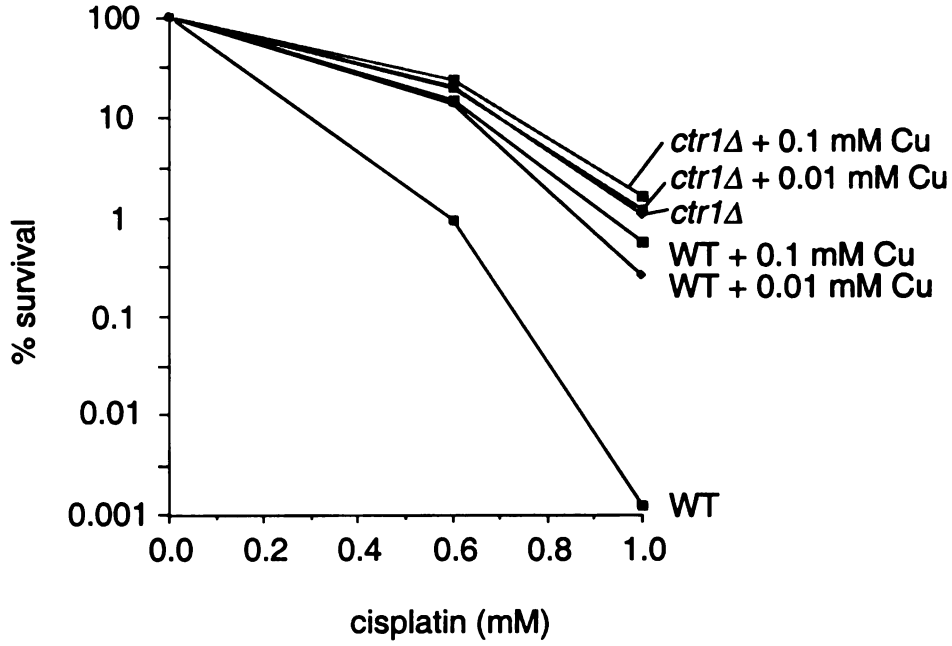

100
10
1
0.1
0.01
0.001
% survival
0.0
0.2
0.4
0.6
0.8
1.0
cisplatin (mM)
ctr1Δ + 0.1 mM Cu
ctr1Δ + 0.01 mM Cu
ctr1Δ
WT + 0.1 mM Cu
WT + 0.01 mM Cu
WT

Figure 3-9. Effect of copper on cellular accumulation of platinum in wild-type and *ctr1Δ* mutants.

Cells of wild-type (YSI1) and *ctr1Δ* (YSI22) were exposed to 1 mM cisplatin in the presence of 0.01 mM, 0.1 mM, or no $CuSO_4$. Data were analyzed as in Figure 3-7.

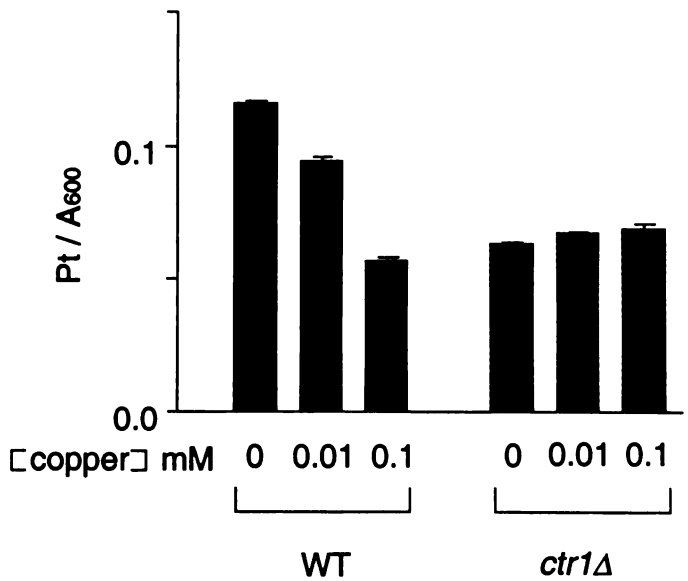
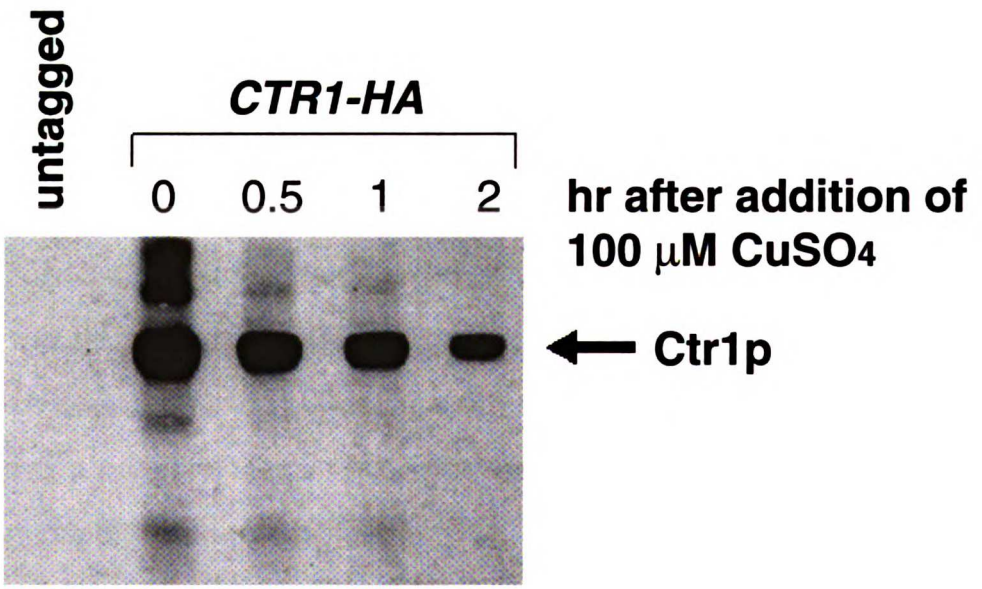

0.1
0.0
Pt / A600
[copper] mM
0
0.01
0.1
0
0.01
0.1
WT
ctr1Δ

Figure 3-10. Effect of copper on Ctr1 protein level.

The Ctr1 protein was tagged at its C-terminus with an HA epitope by modification of the genomic *CTR1* locus (YSI37). The level of Ctr1 protein was determined by Western blot using anti-HA antibodies. 100 μM $CuSO_4$ was added to the culture, and samples were taken at the indicated times.


untagged
CTR1-HA
0
0.5
1
2
hr after addition of
100 μM CuSO4
Ctr1p

present in the medium during cisplatin treatment to inhibit cisplatin accumulation: cells preincubated in high copper medium, washed to remove copper, and then returned to a normal medium (YPD) for cisplatin treatment also exhibited increased cisplatin resistance (Ishida, unpublished observation). These observations indicate that copper creates a phenocopy of a *ctr1Δ* mutant by causing down-regulation of Ctr1p and thus decreasing uptake of cisplatin.

Cisplatin causes internalization and downregulation of Ctr1 protein, and inhibits copper uptake

A further indication of a link between cisplatin uptake and the Ctr1 protein is the observation that cisplatin caused the level of Ctr1p to be decreased: incubation of wild-type cells with 0.1 mM cisplatin for two hours in the presence of a protein synthesis inhibitor led to a three-fold decrease in Ctr1p (Figure 3-11). Furthermore, when cells that produce a Ctr1p-GFP fusion protein were treated with 1 mM cisplatin, a reduced level of the fusion protein was detected at the plasma membrane, and clustering of Ctr1p-GFP appearing as a punctate signal was observed (Figure 3-12). We have also found that cisplatin reduces uptake of ^{64}Cu into wild-type cells: when treated for two hours with 1 mM cisplatin, the level of ^{64}Cu uptake was 16% of that of mock-treated cells (Figure 3-13). Such a decrease was not observed in the *ctr1Δ* mutant. These observations provide further support for a link between Ctr1p and cisplatin transport.

Deletion of mouse *CTR1* results in increased cisplatin resistance and decreased cisplatin accumulation in mouse cells

Mammals contain homologues of the yeast *CTR1* gene, *hCTR1* in humans and *mCTR1* in mouse, which complement a yeast *ctr1* mutant for

Figure 3-11. Degradation of Ctr1p upon cisplatin treatment.

100 µg/ml cycloheximide was added to the *CTR1-HA* cells (YSI37) 30 minutes before cisplatin treatment to block new protein synthesis. Cells were then exposed to 0.1 mM or no cisplatin, and samples were taken at the indicated times. Ctr1p level was analyzed as in Figure 3-10.

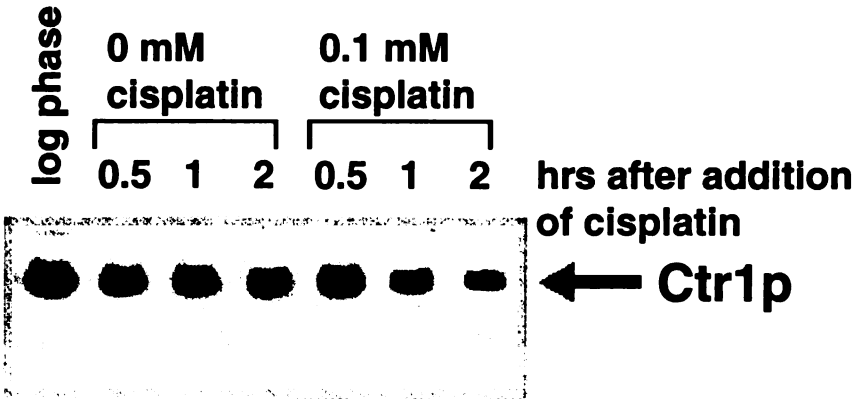

log phase
0 mM cisplatin
0.1 mM cisplatin
0.5 1 2 0.5 1 2
hrs after addition of cisplatin
Ctr1p

Figure 3-12. Localization of Ctr1p after cisplatin or copper treatment.

The Ctr1 protein was tagged at its C-terminus with green fluorescent protein (GFP) by modification of the genomic *CTR1* locus (YSI38). Cells were incubated with 100 μg/ml cycloheximide for 30 minutes to block new protein synthesis, treated with 1 mM cisplatin or 0.1 mM $CuSO_4$ for four hours, and analyzed by fluorescence microscopy.

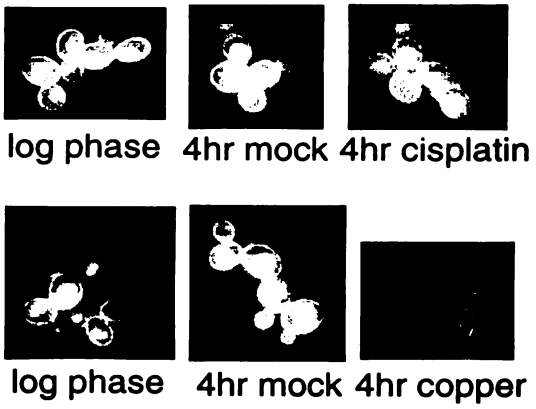
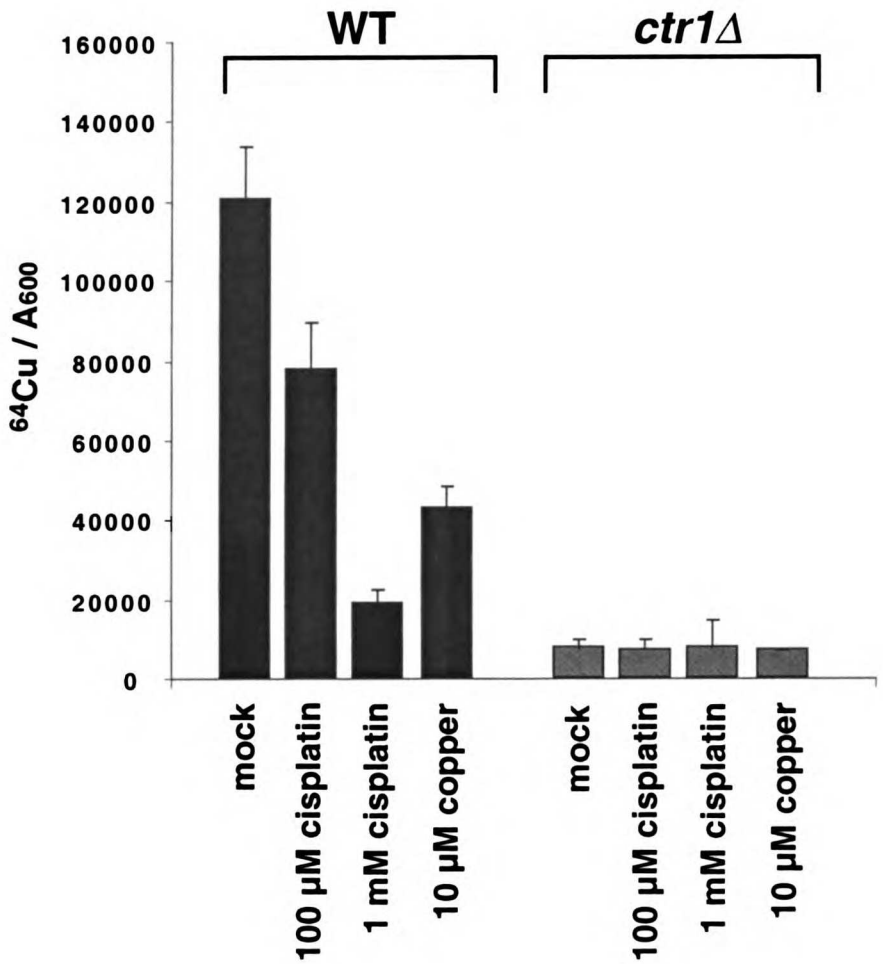

log phase
4hr mock
4hr cisplatin
log phase
4hr mock
4hr copper

Figure 3-13. Effect of cisplatin on ^{64}Cu uptake in wild-type and *ctr1Δ* mutant cells.

Log-phase wild-type (YSI1) or *ctr1Δ* (YSI22) cells were treated with ^{64}Cu (5 μM as $CuSO_4$) in the presence of 100 μM or 1 mM cisplatin, or 10 μM cold $CuSO_4$ for two hours. Cellular ^{64}Cu level was counted with a -counter, and the values were divided by cell OD at A_{600}.


WT
ctr1Δ
160000
140000
120000
100000
80000
60000
40000
20000
0
64Cu / A600
mock
100 µM cisplatin
1 mM cisplatin
10 µM copper
mock
100 µM cisplatin
1 mM cisplatin
10 µM copper

intracellular copper deficiency (Zhou and Gitschier, 1997; Lee *et al.*, 2000). In order to test for a potential role of mammalian *CTR1* in cisplatin resistance, we prepared mouse cell lines from two independent embryos that are wild-type, heterozygous, or homozygous for a knock-out allele of *mCTR126* (Lee and Thiele, manuscript in preparation) and tested their cisplatin sensitivity. As shown in Figure 3-14, the resistance observed in homozygous mutant cells was increased to eight times that of wild-type cells, and in heterozygous cells it was four times that of wild-type cells. Similar results for survival were obtained using an independent pair of wild-type and homozygous mutant cell lines (data not shown). The levels of cisplatin accumulation were also reduced accordingly (Figure 3-15): heterozygous cells exhibited 35% reduction in cisplatin accumulation compared to wild-type cells; homozygous mutant cells exhibited a 70% decrease. A similar graded reduction in copper uptake was also observed with the wild-type, heterozygous, and homozygous mutant cell lines (Lee and Thiele, manuscript in preparation). There was no significant difference between the two cell lines derived from two independent embryos of the same genetic background with respect to copper uptake. These observations suggest that mCtr1 protein functions as a cisplatin transporter in mouse cells and that its human homologue, hCtr1, which is 92% identical to mCtr1, may mediate cisplatin uptake as well. We did not see any effect of exogenous copper on cell survival after cisplatin treatment or cellular accumulation of cisplatin in the wild-type mouse cell lines (data not shown). Thus the effect of copper on uptake of cisplatin observed in yeast may be due to differences between mouse and yeast Ctr1 proteins or to differences in assay conditions. We do not know whether copper causes inactivation of mouse Ctr1p or whether copper and cisplatin compete for binding on mouse Ctr1p.

Figure 3-14. Cisplatin resistance of mouse embryonic cell lines lacking *mCTR1*.

Cisplatin was added 24 hours after plating of cells (80% confluence) and incubated for two hours. Cells were detached with 10 mM EDTA in PBS. For determining survival, cells were stained with trypan blue to detect dead cells; unstained cells were counted using a hemocytometer. Data are expressed as percentages of unstained cells compared to control cultures not exposed to cisplatin.

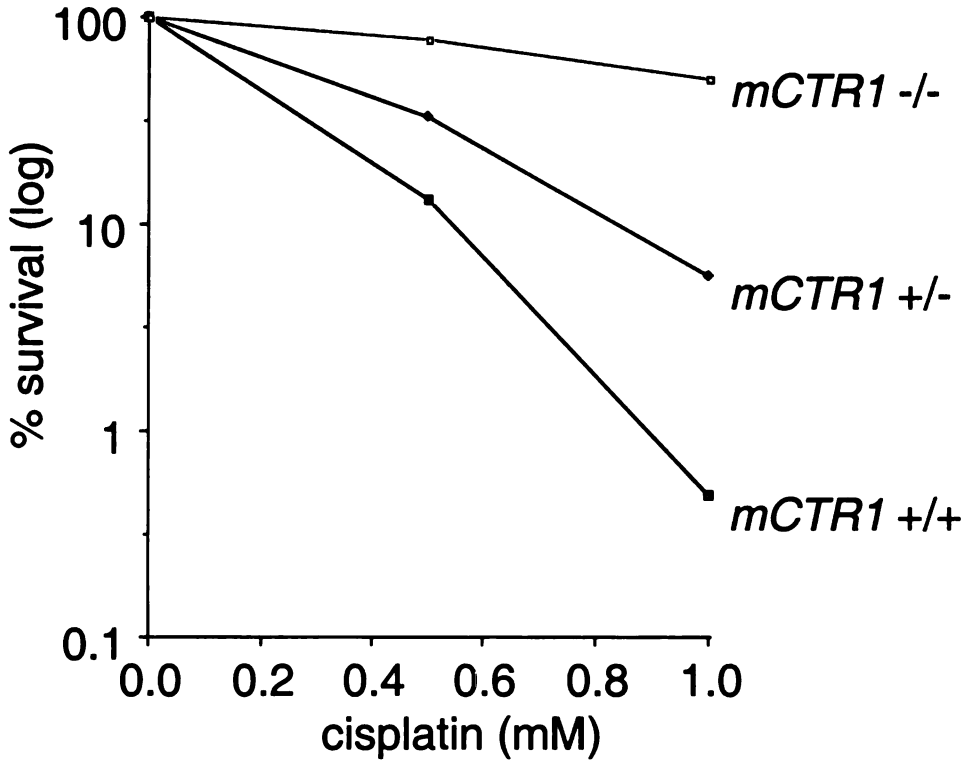
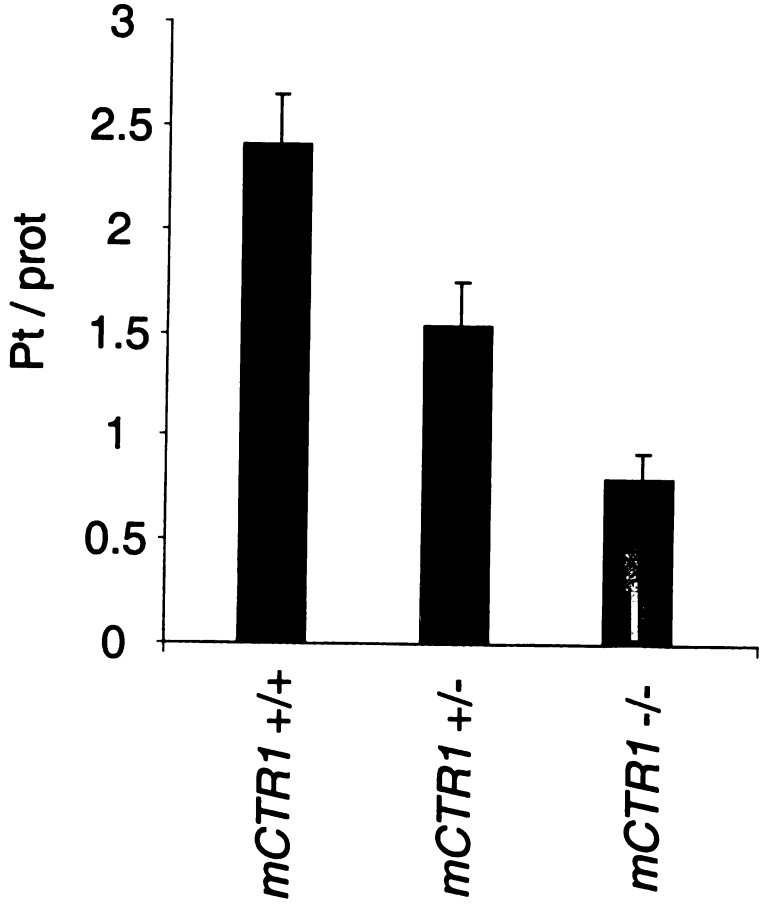

100
10
1
0.1
% survival (log)
0.0
0.2
0.4
0.6
0.8
1.0
cisplatin (mM)
mCTR1 -/-
mCTR1 +/-
mCTR1 +/+

Figure 3-15. Cisplatin accumulation by mouse embryonic cell lines lacking *mCTR1*.

Cisplatin was added 24 hours after plating of cells (80% confluence) and incubated for two hours. Cells were detached with 10 mM EDTA in PBS. Cells were washed twice with PBS and lysed with 1% Triton X-100/ 0.1% SDS. After a three-minute centrifugation at 15000 rpm at 4°C, the supernatant was used to determine protein concentration by the BCA protein assay (Pierce) and platinum content by atomic absorption spectrophotometry. The platinum reading was normalized to protein concentration.


3
2.5
2
1.5
1
0.5
0
Pt / prot
mCTR1 +/+
mCTR1 +/-
mCTR1 -/-

Copper-transporting P-type ATPase in the post-Golgi does not affect cisplatin accumulation in yeast

Recent studies with the human *ATP7B* gene provide another link between copper-binding proteins and cisplatin resistance. *ATP7B* encodes a copper-transporting P-type ATPase which is localized to the post-Golgi compartment (Suzuki and Gitlin, 1999) and facilitates copper secretion from the liver to the bile. Human epidermoid carcinoma cells that overexpress *ATP7B* display increased cisplatin resistance and decreased cisplatin accumulation, leading to the proposal that ATP7B protein functions as a pump to assist efflux of cisplatin, presumably by routing it to the Golgi and subsequently into secretory vesicles (Komatsu *et al.*, 2000). These observations suggest that ATP7B protein may recognize both copper and cisplatin. Although we have not been able to overexpress the yeast *ATP7B* homologue *CCC2* in yeast and test cisplatin sensitivity, we have observed that deletion of *CCC2*, *ATX1* (which encodes a copper chaperone for Ccc2p), or *FET3* (which encodes an iron oxidase that receives copper from Ccc2p), did not significantly alter cisplatin sensitivity (Figure 3-3). Thus in yeast, the post-Golgi copper transporter Ccc2p does not appear to play a major role in cisplatin resistance.

Mechanisms of cisplatin uptake

Studies with cisplatin-resistant human cell lines have indicated that a 25-75% decrease in cisplatin uptake contributes to a three- to 30-fold increase in cisplatin resistance (Scanlon *et al.*, 1991). Our observations on the yeast and mouse *ctr1Δ* mutants fall into a similar range, with a 40-50% decrease in uptake and a two- to eight-fold increase in resistance. The mechanism of cisplatin uptake has been unclear. Inability to saturate the rate of cisplatin

uptake supports a simple diffusion model, whereas the presence of a variety of agents that do not alter plasma membrane permeability but affect cisplatin uptake has suggested that uptake may be mediated by plasma membrane proteins (Gately and Howell, 1993). It has been proposed that about 50% of the intial rate of uptake is due to passive diffusion and that the remaining 50% is due to facilitated diffusion through an as yet unidentified gated channel (Gately and Howell, 1993). Ctr1p could be responsible for the facilitated uptake of cisplatin. We do not yet understand the mechanism of cisplatin uptake by Ctr1p, nor even the uptake of copper. Ctr1p has three putative transmembrane domains and forms at least a dimer (Dancis *et al.*, 1994b). Ctr1p might serve as a channel, or cisplatin uptake may be coupled to endocytosis and/or degradation of Ctr1p.

Clinical perspectives

Our work offers new perspectives on the molecular mechanism of intrinsic and acquired resistance to cisplatin, which represent a major impediment to successful treatment of cancer, and may facilitate development of more effective therapy. Differences in the effectiveness of cisplatin treatment among different cancers or individuals may reflect changes in the activity of Ctr1p. It may be possible, for example, to selectively enhance the activity of Ctr1p in tumors that are intrinsically resistant or in tumors that have acquired resistance and thus restore killing by cisplatin. Similarly, it may be possible to reduce cisplatin-induced nephrotoxicity and ototoxicity by downregulating Ctr1p. We suggest that Ctr1p activity could exert an important influence on cisplatin efficacy and could be a determining factor in designing chemotherapy for cancer patients.

METHODS

Strains

All the strains used in this study are listed in Appendix. Isogenic strains carrying deletions in *MAC1*, *CTR1*, *FRE1*, *FRE7*, *CTA1*, *CTT1*, *RAD2*, *ATX1*, *CCC2*, *FET3*, *LYS7*, *SOD1*, *COX17*, *SCO1*, *CTR2*, and *FET4* were generated in YSI1 (W303 background; *MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 GAL*$^+$ *psi*$^+$) by the protocol of Sakumoto *et al.* (*Yeast* **15**, 1669 [1999]). A polymerase chain reaction (PCR) fragment was used to replace the entire ORF with the *Candida glabrata HIS3* gene. A *ctr1Δ rad2Δ* strain (YSI27) was a product of a cross between YSI22 (*MATa ctr1Δ*) and YSI17 (*MATα rad2Δ*; obtained from a cross of wild-type *MATα* [YSI2] and *MATa rad2Δ* [YSI16]). Strains *CTR1-HA* (YSI37) and *CTR1-GFP* (YSI38) were generated in YSI1 by the protocol of Longtine *et al.* (1998). A PCR fragment was used to add an HA epitope sequence or the GFP gene, followed by the *HIS3* gene, at the C-terminus of the genomic *CTR1*. The Ctr1-GFP fusion is thus read from the native *CTR1* promoter. Both *CTR1-HA* and *CTR1-GFP* complemented the cisplatin resistant phenotype of the *ctr1Δ* mutant. *ctr3Δ* strains were generous gifts from Dennis Thiele (see Appendix).

Atomic absorption spectrophotometry

Platinum was measured using a Perkin Elmer Atomic Absorption Spectrometer (AAnalyst 100 and 3300) with an HGA-800 and -400 Graphite Furnace System. A volume of 20 µl was introduced into the graphite furnace and the peak area was read during a five-second atomization step at 2500°C. The amount of platinum in the samples was determined from a calibration curve prepared using cisplatin solutions.

Cell lines and tissue culture

Mouse cell lines were kindly provided by Jaekwon Lee and Dennis Thiele (University of Michigan). The cell lines were cultured in DMEM (Gibco) with 20% FBS, 110 mg/L pyruvate, 50 mg/L uridine, 1 mM nonessential amino acids, 1x antibiotic/antimycotic solution (Gibco), and 55 μM β-mercaptoethanol.

^{64}Cu uptake assay

Log-phase cells were treated with ^{64}Cu (5 μM as $CuSO_4$) in the presence of 100 μM or 1 mM cisplatin, or 10 μM cold $CuSO_4$ for two hours. ^{64}Cu uptake was quenched by adding ice-cold 50 mM EDTA/ 0.1 M Tris-succinate pH 6.0. Cells were then washed on filters twice with ice-cold 10 mM EDTA/ 0.1 M Tris-succinate pH 6.0 solution. The filters were counted with a γ-counter, and the values were divided by cell OD at A_{600}.

Acknowledgements

We thank Marc Shuman for cisplatin and for leading our initial foray into cisplatin resistance in yeast, Dennis Thiele, Yien Kuo and Jane Gitschier for use of the atomic absorption spectrophotometer, Jaekwon Lee and Dennis Thiele for the mouse *CTR1* knockout cell lines, Sergi Puig for showing me the ^{64}Cu uptake assay, and members of Herskowitz and Thiele laboratories for helpful discussions. Dennis Thiele kindly provided me with the space and materials to perform the work on mouse cell lines and the ^{64}Cu uptake assay in his laboratory at University of Michigan, and also allowed us to use their atomic absorption spectrophotometer. This study was supported by a Stewart Trust Cancer Research Award and a Sandler Basic Science Award to Ira Herskowitz, and by National Institutes of Health grant GM62555 to Dennis J. Thiele and GM61390 to Ira Herskowitz. Seiko Ishida was supported by Howard Hughes Medical Institute Predoctoral Fellowship. Jaekwon Lee was supported by an American Heart Association Postdoctoral Fellowship.

CHAPTER FOUR

CONCLUSIONS AND PERSPECTIVE

Cisplatin is one of the most widely used anticancer drugs effective in the treatment of a variety of cancers. However, intrinsic or acquired resistance to cisplatin reduces its efficacy, which undermines its curative potential. The goal of my project was to identify genes that modulate cisplatin sensitivity and to understand how the gene products function in the process. I identified several yeast genes whose deletion leads to increased cisplatin resistance, and demonstrated that in yeast and mammals, the copper transporter Ctr1 mediates cisplatin uptake and thus governs cisplatin sensitivity. Here I will discuss the use of yeast in understanding mechanisms of cisplatin resistance and postulate mechanisms of cisplatin uptake via Ctr1. Finally, I will speculate on the clinical relevance of our findings.

Yeast as an experimental system to understand mechanisms of cisplatin resistance

Although considerable effort has been made to define the cellular and molecular mechanisms responsible for cisplatin resistance, there has been no demonstration of a mammalian gene being directly involved in cisplatin resistance. Our work has proven yeast to be useful in identifying genes responsible for cisplatin resistance in yeast and mammals. For one of the yeast genes I obtained in my studies, *CTR1*, I was able to demonstrate that the gene product is required for cisplatin uptake in yeast, and subsequently showed that its mammalian homolog functions in the same manner in mammals.

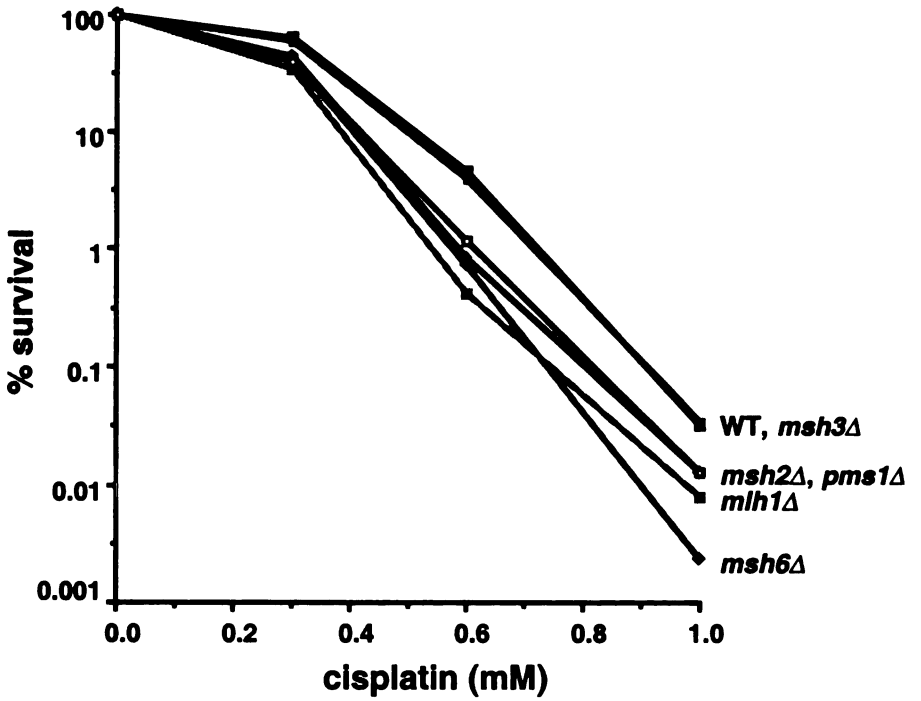
So far there are five yeast genes implicated by others to play a role in cisplatin resistance: *IXR1*, *PDE2*, *ZDS2*, *CIN5*, *YAP6*, and *SKY1* (Brown *et al.*, 1993; Burger *et al.*, 2000; Furuchi *et al.*, 2001; Schenk *et al.*, 2001). Our studies add nine more genes to this list: *YNR051c*, *GPA2*, *NMD2*, *MAC1*, *CTR1*, *CTR3*,

FRE1, *UPF1*, and *UPF3*. All of the 14 genes either have mammalian homologs or are involved in functions that are also present in mammals. For six of them, *IXR1*, *PDE2*, *SKY1*, *GPA2*, *CTR1*, and *CTR3*, there is evidence consistent with potential roles in cisplatin resistance of their mammalian counterparts or the pathways which these genes regulate in mammalian cells (Perez, 1998; Cvijic *et al.*, 1998; Schenk *et al.*, 2001; this study).

Genes that are involved in mammalian-specific functions may not be uncovered by using yeast in the study of cisplatin resistance. Mismatch repair deficiency has been associated with cisplatin resistance in mammalian cells (Fink *et al.*, 1997). In yeast, however, deletion of genes that have been demonstrated biochemically and genetically to be involved in mismatch repair, namely, *MSH2*, *MSH3*, *MSH6*, *MLH1*, or *PMS1*, did not result in increased cisplatin resistance (Figure 4-1). In mammals, mismatch repair deficiency is also associated with resistance to other DNA-damaging agents (Lage and Dietel, 1999). It has been postulated that detection of DNA damage by the mismatch repair system triggers a pathway that leads to apoptosis. Cisplatin-induced cell death shares certain features with apoptosis, such as chromatin condensation and the activation of a DNA endonuclease (Eastman, 1990), and key regulators of apoptosis such as p53, c-Abl, p73 and Bcl-2 have been associated with cisplatin resistance (Jordan and Carmo-Fonseca, 2000). However, to date there has been no clear evidence of the existence of an active cell death pathway such as apoptosis in yeast. Lack of involvement of mismatch repair genes in cisplatin resistance in yeast may reflect differences in the way mammalian cells and yeast cells eventually die after exposure to toxic doses of cispatin.

Figure 4-1. Cisplatin sensitivity of yeast mutants defective in mismatch repair genes.

Log-phase cells were exposed for two hours to different concentrations of cisplatin (Bristol Laboratories) in rich medium (YPD) and incubated on YPD plates at 30°C for two days to allow formation of colonies. Data are expressed as percentages of colonies formed compared to control cultures not exposed to cisplatin. Strains are wild-type (YSI1), *msh2Δ* (YSI39), *msh3Δ* (YSI40), *msh6Δ* (YSI41), *mlh1Δ* (YSI42), and *pms1Δ* (YSI43).


100
10
1
0.1
0.01
0.001
% survival
0.0
0.2
0.4
0.6
0.8
1.0
cisplatin (mM)
WT, msh3Δ
msh2Δ, pms1Δ
mlh1Δ
msh6Δ

Decreased cellular accumulation of cisplatin is a common mechanism for cisplatin resistance *in vitro*

Studies of cell lines resistant to cisplatin suggest that mechanisms of resistance may be multifactorial. In the majority of cases, resistant cell lines generated by exposure to a high concentration of cisplatin accumulate less cisplatin than the parental cell lines (Gately and Howell, 1993). All of the yeast mutants with increased cisplatin resistance that I obtained in my studies are defective in drug accumulation to various degrees. We proposed in chapter three that Ctr1p is a transporter for cisplatin based on its localization, deletion phenotype, and its behavior upon cisplatin treatment. Ctr1p may be defective in other resistant mutants of yeast and mammals. It would be interesting to study a potential role of Ctr1p in other cisplatin-resistant mutants of yeast that are defective in drug uptake, by measuring the level of Ctr1p and its localization. Studying the effects of *ctr1* deletion in these resistant mutants may allow us to further understand the mechanism of cisplatin uptake and how it is regulated. In mammals, raising antibodies against mouse or human Ctr1 has been a challenge. One could measure *CTR1* transcript levels by Northern blot analyses and show that resistant cell lines with decreased drug accumulation have less *CTR1* mRNA than parental cell lines. The function of Ctr1 could also be downregulated by other mechanisms besides decreased protein levels, such as mutations in *CTR1*, which can be revealed by sequencing the *CTR1* locus, or defects in other proteins that are required for the cisplatin uptake function of Ctr1.

Mechanism of cisplatin uptake by Ctr1

Although we have shown that Ctr1 is necessary in both yeast and mouse cells for cisplatin uptake, we do not know whether it is sufficient. The

level of cisplatin accumulation in yeast *ctr1Δ* mutants transformed with a high-copy plasmid carrying the yeast *CTR1* under the transcriptional control of the strong *ADH* promoter was two times compared to that of *ctr1Δ* transformed with an empty vector, and yeast transformed with human *CTR1* (*hCTR1*) showed a 50% increase in drug accumulation (Ishida, unpublished observations). However, in both cases, there was no significant change in the level of cisplatin sensitivity relative to vector-transfected cells. We know from comparison between wild-type and *ctr1Δ* that a two-fold difference in cisplatin accumulation could result in a several-order of magnitude difference in cisplatin sensitivity. The results with high-copy plasmids may imply the presence of a factor that assists Ctr1p in cisplatin uptake. The abundance of this factor may be limited; therefore, overexpression of *CTR1* alone is not sufficient to confer sensitivity to cisplatin. The increase in cisplatin accumulation observed in yeast cells transformed with either yeast *CTR1* or *hCTR1* may only reflect cisplatin bound to the Ctr1p on the cell surface and not the actual amount of drug taken inside the cell. There may exist a protein that is required to bring cisplatin inside the cell once it is bound to Ctr1p.

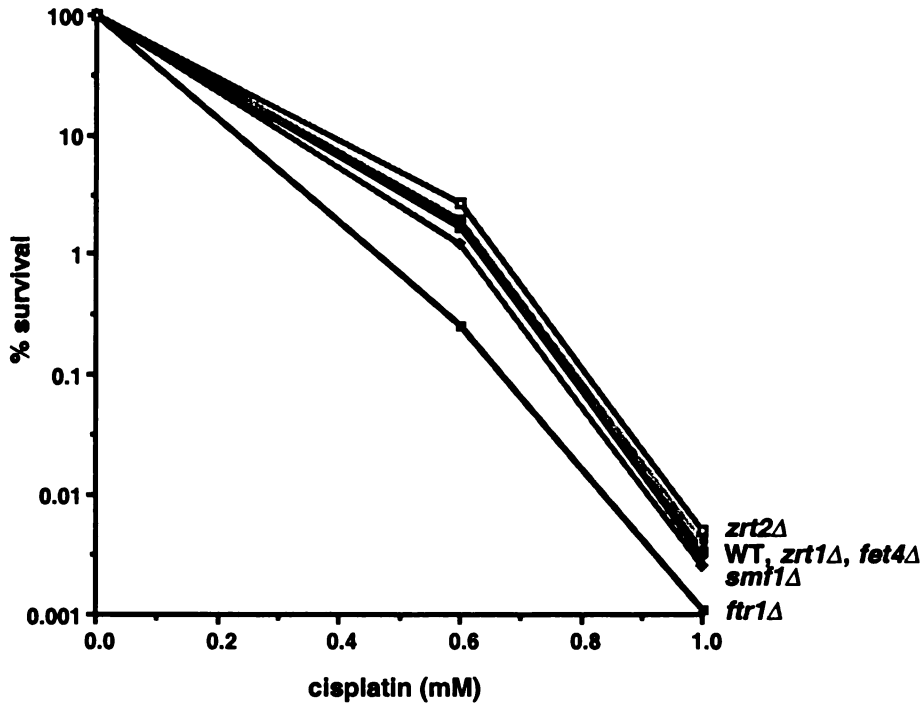
The mechanisms for copper uptake and cisplatin uptake via Ctr1p may be different. Regions and amino acids required for efficient copper uptake in yeast Ctr1p have been mapped (Sergi Puig and Dennis Thiele, manuscript in preparation). We are now testing if these mutations affect cisplatin uptake. We have observed degradation and disappearance of Ctr1p from the plasma membrane upon cisplatin treatment (chapter three). Therefore, cisplatin may not enter the cell through Ctr1p but may enter by binding to Ctr1p and its subsequent internalization and degradation. Ligand-stimulated endocytosis and degradation of plasma membrane protein have been extensively studied

in yeast, its prototype being Ste2p, the α-factor receptor (Hicke, 1997). Upon binding of α-factor, Ste2p is phosphorylated. This serves as a signal for its ubiquitination, followed by its endocytosis and degradation in the vacuole. A similar mechanism may account for cisplatin uptake via Ctr1p, which can be tested using various mutants affected in these processes and analyzing Ctr1p biochemically.

Why would a cell have a transporter for cisplatin, which is not a natural compound? This is likely to be due to mistaken identity. In solution, copper ion is bound by water molecules, and its structure may resemble the neutral planar structure of cisplatin. We have tested the possible involvement of other metal ion transporters in yeast: Fet4 (Fe^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}), Ftr1p (Fe^{2+}), Smf1p (Mn^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+}, Ni^{2+}, Li^{+}, Rb^{+}, K^{+}, Ca^{2+}, Sr^{2+}, Na^{+}), Zrt1p (Zn^{2+}, Fe^{2+}), and Zrt2p (Zn^{2+}). Deletion of each of these metal ion transporters did not result in increased cisplatin resistance (Figure 4-2).

Uptake of cisplatin has been postulated to occur by both passive diffusion and protein-mediated transport (Gately and Howell, 1993). The inability to saturate cisplatin uptake and the inability of structural analogs to inhibit uptake support a passive diffusion model, whereas the existence of pharmacologic agents that are able to modulate uptake but not cause general permeabilization of the membrane are suggestive of protein-mediated uptake. Cisplatin uptake is not completely abolished in cells lacking *CTR1*: in yeast *ctr1Δ* the level of cisplatin accumulation is decreased to 56%, and in mouse cell lines homozygous for the *mCTR1* deletion it is reduced to 30% (chapter three). Our observations are consistent with both the diffusion and facilitated uptake models. The remaining uptake activity seen in the *ctr1Δ* mutants could reflect facilitated transport by yet unidentified transporters, or

Figure 4-2. Cisplatin sensitivity of mutants defective in metal transporters. Sensitivity to cisplatin was assayed as described in Figure 4-1. Strains are wild-type (YSI1), *fet4Δ* (YSI36), *ftr1Δ* (YSI44), *smf1Δ* (YSI45), *zrt1Δ* (YSI46), and *zrt2Δ* (YSI47).


100
10
1
0.1
0.01
0.001
% survival
0.0
0.2
0.4
0.6
0.8
1.0
cisplatin (mM)
zrt2Δ
WT, zrt1Δ, fet4Δ
smf1Δ
ftr1Δ

passive diffusion across the plasma membrane. Although it is clear from our work that Ctr1p is the major factor that governs cisplatin uptake in yeast and mammals, further studies of other yeast mutants with decreased drug accumulation and/or performing the mutant screen on a larger scale may uncover additional factors that control uptake of cisplatin.

Clinical relevance

Our results with mouse *CTR1* knockout cell lines clearly demonstrate the role of Ctr1p in mediating cisplatin uptake in mammalian cells. Whether Ctr1p is involved in cisplatin resistance of tumors in patients is a separate issue. Most of the cisplatin-resistant cell lines were established by exposing parental cell lines to cisplatin at high concentrations -- up to 25 times that in a patient's plasma -- for a long period of time, usually days to months, whereas cisplatin has a rapid plasma clearance after intravenous administration, with most of the drug excreted into the urine within the first few days (Loehrer and Einhorn, 1984). In addition to differences in drug concentrations, we do not know how active the drug remains in plasma versus tissue culture medium. Studies of cell lines are useful in investigating resistance mechanisms which occur in cultured cells, and which may potentially occur in tumors. To understand clinical mechanisms of resistance, analysis of specimens from patients is unavoidable. Once aberrations in the resistant tumors are found, one may introduce the same aberrations in an *in vitro* system to test whether the aberrations cause resistance.

Our findings with *CTR1* make it a good candidate gene that needs to be closely monitored in clinical settings. Mutations in the gene or changes in its expression level may influence the efficacy of cisplatin, and thus could be a crucial factor that may determine prognosis of the patient. Since Ctr1 is a cell

surface protein, it may serve as a new target for drugs that would either antagonize cisplatin uptake to protect normal tissues from damage or facilitate cisplatin uptake to sensitize tumor cells to cisplatin. Localized administration of such Ctr1 modifiers would also greatly enhance the efficacy of cisplatin against tumors and minimize its side effects.

APPENDIX

YEAST STRAINS

All the deletions were constructed in YSI1 (W303 background: *MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 GAL*$^+$ *psi*$^+$) by the protocol of Sakumoto *et al.* (1999). Double mutants were generated from crosses as indicated below.

		generated from
YSI1	*MAT**a***	
YSI2	*MATα*	
YSI3	*MAT**a** ynr051cΔ::HIS3*	
YSI4	*MAT**a** gpa2Δ::HIS3*	
YSI5	*MAT**a** nmd2Δ::HIS3*	
YSI6	*MAT**a** mac1Δ::HIS3*	
YSI7	*MATα gpa2Δ::HIS3*	YSI2 x YSI4
YSI8	*MATα nmd2Δ::HIS3*	YSI2 x YSI5
YSI9	*MATα mac1Δ::HIS3*	YSI2 x YSI6
YSI10	*MAT**a** ynr051cΔ::HIS3 gpa2Δ::HIS3*	YSI3 x YSI7
YSI11	*MAT**a** ynr051cΔ::HIS3 nmd2Δ::HIS3*	YSI3 x YSI8
YSI12	*MAT**a** ynr051cΔ::HIS3 mac1Δ::HIS3*	YSI3 x YSI9
YSI13	*MAT**a** gpa2Δ::HIS3 nmd2Δ::HIS3*	YSI4 x YSI8
YSI14	*MAT**a** gpa2Δ::HIS3 mac1Δ::HIS3*	YSI4 x YSI9
YSI15	*MAT**a** nmd2Δ::HIS3 mac1Δ::HIS3*	YSI5 x YSI9
YSI16	*MAT**a** rad2Δ::HIS3*	
YSI17	*MATα rad2Δ::HIS3*	YSI16 x YSI2

YSI18	*MATa ynr051cΔ::HIS3 rad2Δ::HIS3*	YSI3 x YSI17
YSI19	*MATa gpa2Δ::HIS3 rad2Δ::HIS3*	YSI4 x YSI17
YSI20	*MATa nmd2Δ::HIS3 rad2Δ::HIS3*	YSI5 x YSI17
YSI21	*MATa mac1Δ::HIS3 rad2Δ::HIS3*	YSI6 x YSI17

YSI22 *MATa ctr1Δ::HIS3*

YSI23 *MATa fre1Δ::HIS3*

YSI24 *MATa fre7Δ::HIS3*

YSI25 *MATa cta1Δ::HIS3*

YSI26 *MATa ctt1Δ::HIS3*

YSI27	*MATa ctr1Δ::HIS3 rad2Δ::HIS3*	YSI22 x YSI17

YSI28 *MATa atx1Δ::HIS3*

YSI29 *MATa ccc2::HIS3*

YSI30 *MATa fet3Δ::HIS3*

YSI31 *MATa lys7Δ::HIS3*

YSI32 *MATa sod1Δ::HIS3*

YSI33 *MATa cox17Δ::HIS3*

YSI34 *MATa sco1Δ::HIS3*

YSI35 *MATa ctr2Δ::HIS3*

YSI36 *MATa fet4Δ::HIS3*

YSI37 *MATa CTR1-HA HIS3*

YSI38 *MATa CTR1-GFP HIS3*

YSI39 *MATa msh2Δ::HIS3*

YSI40 *MATa msh3Δ::HIS3*

YSI41 *MATa msh6Δ::HIS3*

YSI42 *MATa mlh1Δ::HIS3*

YSI43 *MATa pms1Δ::HIS3*

YSI44 *MATa ftr1Δ::HIS3*

YSI45 *MATa smf1Δ::HIS3*

YSI46 *MATa zrt1Δ::HIS3*

YSI47 *MATa zrt2Δ::HIS3*

The follwing strains were gifts from Dennis Thiele. DTY1, SKY52, and SKY44 are isogenic in the BR10 background (*MATa gal1 trp1-1 his3 ade*) that does not carry a transposon in the upstream regulatory sequence of *CTR3*. SKY46 was obtained from a cross between SKY44 and SKY1 (Knight *et al.*, 1996).

DTY1	*MATa*
SKY52	*MATa ctr1Δ::TRP1*
SKY44	*MATa ctr3Δ::TRP1*
SKY46	*MATa ctr1Δ::URA3 ctr3Δ::TRP1*

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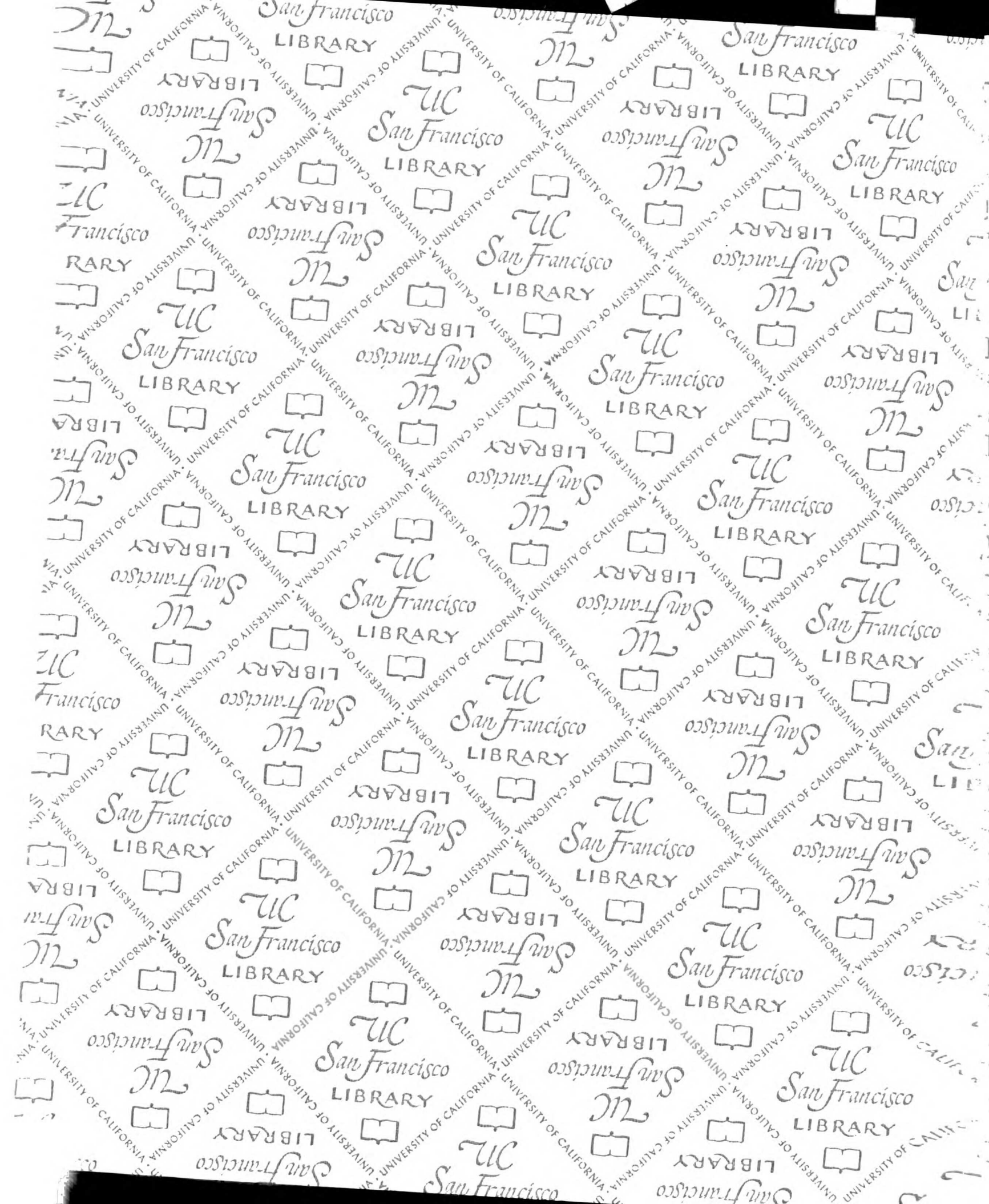
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