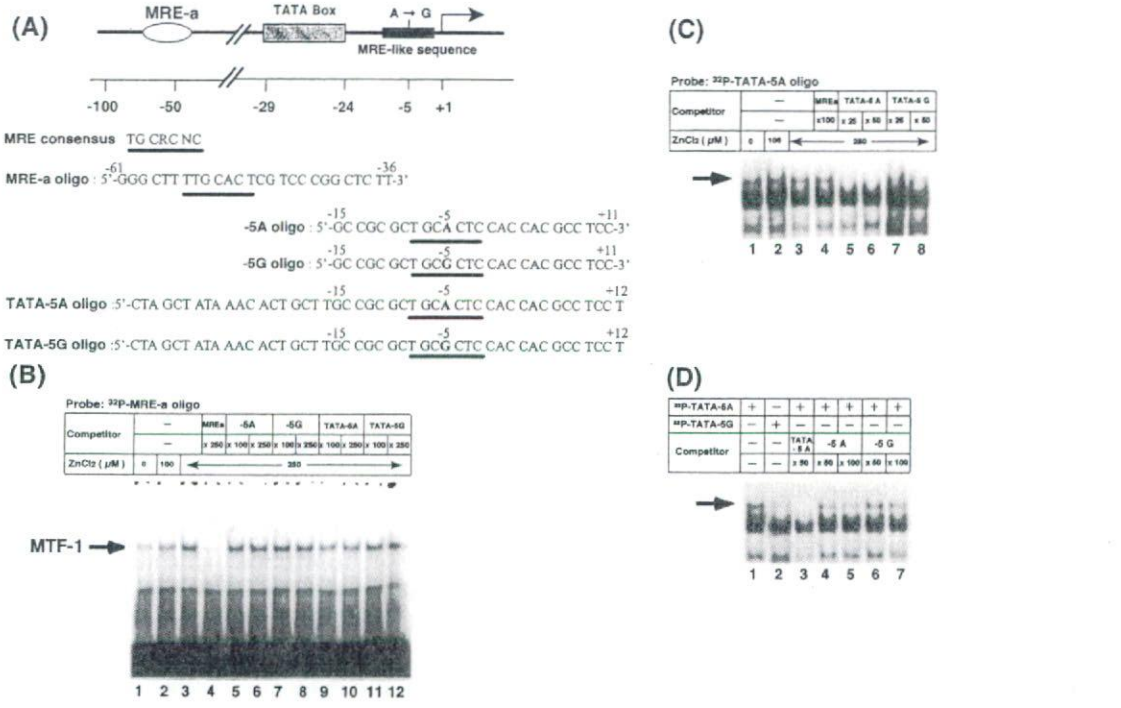

Fig. 3 Effects of the single-nucleotide A → G polymorphism on the binding of nuclear proteins to the core promoter region of the gene for MT-IIA. **a** Oligonucleotide probes for the electrophoretic mobility shift assay. The MRE core sequence is *underlined*. An *arrow* indicates the site of initiation of transcription. The numbers above sequences refer to positions relative to this site. **b** The ^{32}P-labeled MRE-a probe was incubated with a nuclear extract and the mixture was fractionated on non-denaturing polyacrylamide gel. For competition assays, unlabeled oligonucleotide probes were incubated with the nuclear extract prior to the addition of ^{32}P-labeled MRE-a. The *arrow* indicates the MTF-1/MRE complex. **c, d** Results of electrophoretic mobility shift assays with ^{32}P-labeled TATA−5A as probe. *Arrows* indicate complexes of transcriptional factor(s) and the TATA–5A probe

−5G oligonucleotides to react with the nuclear extract. However, no specific factors bound to either probe (data not shown).

A number of basic transcription factors are known to bind to the TATA box-containing core region of the promoter (Robert et al. 1996). Since the −5A and −5G oligonucleotides that we tested in the EMSA did not contain a TATA box, we extended them to include the TATA box and prepared TATA −5A and TATA −5G oligonucleotides (from position −15 to +34; Fig. 3a) and tested them in the EMSA. We found that proteins that bound to the ^{32}P-labeled TATA −5A oligonucleotide were present in the nuclear extract of HEK293 cells (Fig. 3c, lanes 1–3). In addition, a competition assay using unlabeled TATA −5A or TATA −5G oligonucleotides showed that the addition of a 25-fold molar excess of TATA −5A oligonucleotide inhibited binding, whereas a 50-fold molar excess of TATA −5G had a negligible inhibitory effect (Fig. 3C, lanes 5–8). The addition of 100-fold molar excess of MRE-a oligonucleotide, which bound to MTF-1 (as shown in Fig. 3b), did not inhibit the binding (Fig. 3c, lane 4). We also confirmed that neither the TATA −5A oligonucleotide nor the TATA −5G oligonucleotide bound MTF-1 (Fig. 3b, lanes 9–12).

The addition of Zn did not affect the binding of the ^{32}P-labeled TATA −5A oligonucleotide to nuclear proteins. This result suggests that the single-nucleotide substitution influenced the efficiency of basal transcription. Therefore, we labeled the TATA −5G oligonucleotide with ^{32}P and allowed it to react with a nuclear extract from Zn-untreated HEK293 cells. The extent of binding of the TATA −5G oligonucleotide to nuclear proteins (Fig. 3d, lane 2) was significantly lower than that of the TATA −5A oligonucleotide (Fig. 3d, lane 1). Furthermore, a competition assay in which a 100-fold molar excess of unlabeled −5A oligonucleotide or −5G oligonucleotide competed with the binding of the ^{32}P-labeled TATA -5A oligonucleotide for binding to nuclear proteins demonstrated an absence of competition (Fig. 3D, lanes 4–7). These results suggest that the single-nucleotide polymorphism in the promoter of the gene for MT-IIA might reduce the binding, to the core promoter region, of a nuclear protein that is involved in basic transcription, with a marked resultant

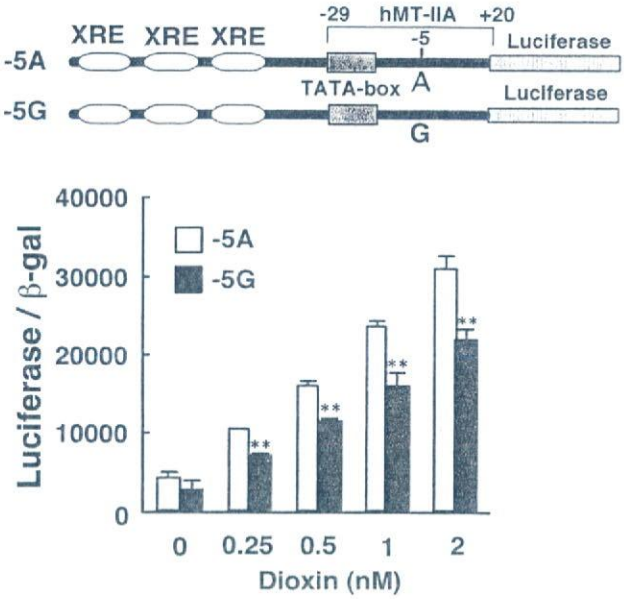

Fig. 4 Effects of the single-nucleotide A → G polymorphism on the promoter activity of dioxin-responsive elements. The region upstream of the TATA box of the gene for MT-IIA was replaced by a sequence that contained three dioxin-responsive elements (XREs) and performed a reporter-gene assay. After a 12-h incubation, HEK293 cells were treated with dioxin and incubated for a further 24 h. The activities of luciferase (an index of the activity of the promoter) and of β-galactosidase (an index of the efficiency of transcription) were measured. Results are mean ± SD ($n = 5$). A schematic representation of the relevant regions of the reporter plasmids is shown above the histograms. **Significantly different from the −5A type ($P < 0.0001$, Student's t test)

reduction in the efficiency of induction of transcription of the gene by heavy metals via the activation of MTF-1. If our hypothesis is correct, the A → G polymorphism should reduce the efficiency of induction of transcription even if the promoter region upstream of the TATA box is replaced by a region derived from the promoter of other genes. Therefore, we replaced the region upstream of the TATA box by a sequence that contained three dioxin-responsive elements (XREs) and performed a reporter-gene assay. We observed, as predicted, a marked reduction in the efficiency of induction by dioxin (Fig. 4). Point mutations in the core promoter region are known to inhibit the binding of nuclear proteins to this region (Chalkley and Verrijzer 1999; Yean and Gralla 1997). Yanai et al. (1997) reported that a polymorphism in the core promoter region of the gene for angiotensinogen reduced the binding of the nuclear factor AGCEI to this region. Similarly, in the case of the gene for a MT, the single-nucleotide polymorphism reduced basic transcriptional activity, thereby reducing the extent of induction of the synthesis of MT by heavy metals. The nuclear protein, whose binding to the core promoter region of MT-IIA gene is influenced by the A → G substitution, should be identified by future studies.

The MTs are characterized by free cysteine residues that account for approximately one-third of the total number of amino acid residues but do not form S–S bonds (Kägi 1991; Webb 1979). Exploiting its abundant intramolecular SH groups, MT reduces the toxicity of alkylating agents (Cagen and Klaassen 1979), γ-irradiation (Satoh et al. 1989), and pesticides (Satoh et al. 1992), as well as that of heavy metals such as Cd, and acts as a scavenger of free radicals to protect cells and animals against various types of oxidative stresses (Sato and Bremner 1993). In addition, MT is known to reduce the side effects of many anticancer drugs (Okazaki et al. 1998; Satoh et al. 1993b), such as cisplatin (Naganuma et al. 1987) and adriamycin (Naganuma et al. 1988), and MT-deficient mice have been shown to be particularly susceptible to toxicity of anticancer drugs (Zhang et al. 1998). Furthermore, MT has been shown to inhibit spontaneous or chemical carcinogenesis in mice (Satoh et al. 1993a). Thus, the A → G substitution in the core promoter region of the gene for MT-IIA might reduce the protective role of MT-IIA against such various stresses and carcinogenesis.

The single-nucleotide polymorphism found in this study raises a serious issue with respect to the effect of Cd on human health. In the population of middle-aged and elderly Japanese, the mean concentration of Cd in the kidney exceeds one-third of the JFCFA's estimated critical concentration (Yoshida et al. 1998). Olsson et al. (2002) reported that the level of ingested Cd in farmers in Sweden is not as high. However, a higher urinary concentration of Cd was associated with lower renal function (Olsson et al. 2002). It has also been reported that Cd, at the levels at which people routinely ingest it, has a potent estrogen-like activity and acts as an endocrine disruptor (Johnson et al. 2003). These reports suggest that the concentration of Cd that accumulates in the bodies of ordinary people is close to the toxic level. The A → G genetic polymorphism that influences the efficiency of synthesis of MT, which plays such an important role in reducing Cd toxicity, might be one of the major factors that determine individual differences in human sensitivity to Cd.

Acknowledgments This work was supported by a Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science and by Health and Labour Sciences Research Grants for Research on the Risk of Chemical Substances from the Ministry of Health, Labour and Welfare, Japan.

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Endocytic Ark/Prk Kinases Play a Critical Role in Adriamycin Resistance in Both Yeast and Mammalian Cells

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Abstract

To elucidate the mechanism of acquired resistance to Adriamycin, we searched for genes that, when overexpressed, render *Saccharomyces cerevisiae* resistant to Adriamycin. We identified *AKL1*, a gene of which the function is unknown but is considered, nonetheless, to be a member of the Ark/Prk kinase family, which is involved in the regulation of endocytosis, on the basis of its deduced amino acid sequence. Among tested members of the Ark/Prk kinase family (Ark1, Prk1, and Akl1), overexpressed Prk1 also conferred Adriamycin resistance on yeast cells. Prk1 is known to dissociate the Sla1/Pan1/End3 complex, which is involved in endocytosis, by phosphorylating Sla1 and Pan1 in the complex. We showed that Akl1 promotes phosphorylation of Pan1 in this complex and reduces the endocytic ability of the cell, as does Prk1. Sla1- and End3-defective yeast cells were also resistant to Adriamycin and overexpression of Akl1 in these defective cells did not increase the degree of Adriamycin resistance, suggesting that Akl1 might reduce Adriamycin toxicity by reducing the endocytic ability of cells via a mechanism that involves the Sla1/Pan1/End3 complex and the phosphorylation of Pan1. We also found that HEK293 cells that overexpressed AAK1, a member of the human Ark/Prk family, were Adriamycin resistant. Our findings suggest that endocytosis might be involved in the mechanism of Adriamycin toxicity in yeast and human cells. (Cancer Res 2006; 66(24): 11932-7)

Introduction

Adriamycin is an anticancer drug that is widely used in a clinical setting (1). The mechanism of action of Adriamycin, via inhibition of nucleic acid synthesis by intercalation into DNA and promotion of the cleavage of DNA by inhibition of DNA topoisomerase II, is well known, as is the mechanism of cytotoxicity, which involves the production of free radicals, but many other effects of the drug have also been reported, with many issues remaining to be clarified (2, 3). In cancer chemotherapy with Adriamycin, both the natural and the acquired resistance of cancer cells pose serious problems, as do the adverse effects of the drug (4). The acquisition of Adriamycin resistance involves promotion of the excretion of the drug from cells by overexpressed ATP-binding cassette transporters, such as P-glycoprotein and multidrug-resistance protein (5–7), and, in addition to the transporters involved in drug excretion, many other factors seem to be related to the mechanism of Adriamycin resistance. However, the properties of some Adriamycin-resistant cells cannot be explained by these phenomena, suggesting the presence of as yet unknown mechanisms of acquired resistance (8, 9). To shed further light on mechanisms of Adriamycin toxicity and the acquisition of Adriamycin resistance, we have identified genes involved in Adriamycin resistance in *Saccharomyces cerevisiae*. *S. cerevisiae* is widely used as a model eukaryote and its entire genome has been sequenced. Because many products of yeast genes have functions similar to those of human genes, information obtained from yeast cells might help to clarify the mechanism of acquisition by human cells of resistance to Adriamycin. We previously searched for genes that conferred Adriamycin resistance on yeast cells when such genes were overexpressed, using a yeast DNA genomic library, and we identified the *SSL2* (10, 11) and *BSD2* (12) genes. In the present study, we identified *AKL1* as a gene of which the overexpression rendered yeast cells resistant to Adriamycin. Akl1 is considered to be a member of the Ark/Prk kinase family and to be involved in the regulation of endocytosis on the basis of certain characteristics of its deduced amino acid sequence (13), but, to our knowledge, its function has not been fully analyzed and its relationship to drug resistance has not been investigated.

This study revealed the involvement of Akl1 in the regulation of endocytosis via phosphorylation of Pan1 in the Sla1/Pan1/End3 complex, as is Prk1 (14, 15). In addition, our results suggest that overexpressed Akl1 inhibits the internalization step of endocytosis, leading to Adriamycin resistance in yeast cells. Because human cells that overexpressed AAK1 (16), which is a member of the human Ark/Prk kinase family that is known to be involved in the regulation of endocytosis, were also Adriamycin resistant, it is possible that the Ark/Prk kinase family might be involved in Adriamycin toxicity via the regulation of endocytosis not only in yeast cells but also in human cells.

Materials and Methods

Yeast strains and media. The wild-type yeast strains used in this study were W303B (*MATα his3 can1-100 ade2 leu2 trp1 ura3*; ref. 17) and BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*). The deletion strains (*akl1Δ, ark1Δ, prk1Δ, end3Δ, sla1Δ, sla2Δ, vrp1Δ, rvs161Δ, vps23Δ*, and *vps27Δ*), derived from the parent BY4742 strain, were obtained from Euroscarf (Frankfurt, Germany). Yeast cells were grown in synthetic dextrose medium with or without leucine. For the characterization of cells that harbored the expression vector pYES2 (Invitrogen, Carlsbad, CA) or genes expressed under the control of the *GAL1* promoter, cells were grown in synthetic galactose medium that contained 2% galactose and 4% raffinose as the carbon source instead of dextrose.

Quantification of the toxicity of Adriamycin in yeast cells. Yeast cells were cultured (1×10^4/200 μL) in 96-well plates in synthetic dextrose medium that contained Adriamycin at various concentrations. After incubation for 48 hours, absorbance at 620 nm was determined spectrophotometrically. For the colony-formation assay, a suspension of yeast cells was incubated for 3 hours in liquid medium that contained

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doi:10.1158/0008-5472.CAN-06-3220

Adriamycin. After washing with synthetic dextrose liquid medium, the cells were resuspended in synthetic dextrose liquid medium and spotted onto a plate of agar-solidified medium.

Construction of a point mutant of *AKL1* (*AKL1* D181Y). Construction of a point mutant of *AKL1* (*AKL1*D181Y) was done as described by Hwang et al. (18) with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). For mutagenesis of *AKL1*, PCR was done using plasmid pRS315-*AKL1* as the template and the following oligonucleotides as the mutagenic primers: 5′-CCTTGATCCATCGCTATATCAAGATTG-3′ and 5′-CAATCTTGATATAGCGATGGATCAAGG-3′. The mutation in *AKL1* was verified with an automated DNA sequencer (LI-COR, Lincoln, NE).

Construction of an expression vector for various genes. The *ARK1* and *PRK1* genes were cloned separately by PCR with chromosomal DNA of *S. cerevisiae* as template. The following oligonucleotides were used as primers: 5′-GCGGCTCGCCAACTTGGAAG-3′ and 5′-CAGTTAAAGCCAC-TAGTTCCT-3′ for *ARK1*, and 5′-AGTGGTCTCTAGCGGGATCG-3′ and 5′-GCTGTTCAGAGAACCACAATG-3′ for *PRK1*. Each product of PCR was inserted into the pGEM-T easy vector (Promega, Madison, WI). Each insert was digested with the restriction endonuclease *Not*I and fragments were ligated into the single-copy plasmid pRS315. For the expression of each gene, we used the respective homologous promoter.

Construction of a *PAN1*-HA-expressing plasmid. For construction of the *PAN1*-hemagglutinin (HA) vector, the *PAN1* gene was amplified by PCR with chromosomal DNA of *S. cerevisiae* as template and primers 5′-GCTAATCTGTACAACTGAATATG-3′ and 5′-TCAAGCGTAATCTGGAA-CATCGTATGGGTATGGAAGGGGTGGGGGTGGAGGAATA-3′. The amplified DNA was inserted into the pGEM-T easy vector to produce plasmid pGEM-*PAN1*-HA. The insert was digested with the restriction endonuclease *Not*I and fragments were ligated into the pYES2 expression vector.

Phosphorylation of Pan1-HA. Yeast cells (3×10^7) expressing HA-tagged Pan1 (Pan1-HA) were cultured in 10 mL of synthetic galactose (−Ura, −Leu) medium for 4 hours and harvested. After washing with ice-cold water, cells were lysed by grinding with glass beads at 4°C. An aliquot of cell lysates (100 μg of protein) was immunoprecipitated with anti-HA agarose beads (Sigma, St. Louis, MO). Immunoprecipitates were fractionated by SDS-PAGE (7.5% polyacrylamide) and transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA). Immunoblotting was carried out using rabbit antibodies against phosphothreonine (Zymed, South San Francisco, CA) or rat antibodies against HA (Roche, Basel, Switzerland) and peroxidase-conjugated second antibodies (Dako A/S, Glostrup, Denmark). Immunoreactive proteins were detected with the enhanced chemiluminescence system from Amersham Pharmacia (Uppsala, Sweden).

Endocytic uptake of lucifer yellow. Fluid-phase endocytosis was assayed using lucifer yellow carbohydrazide (Molecular Probes, Eugene, OR). Yeast cells were incubated in synthetic dextrose medium in the presence of 4 mg/mL lucifer yellow carbohydrazide. After a 2-h incubation, cells were collected and washed thrice with PBS. Cells were resuspended in PBS and observed under a light microscope.

Construction of the AAK1-expression vector. The cDNA for human full-length AAK1 (clone KIAA1048) in pBluescript was provided by the Kazusa Research Institute (Kisarazu, Chiba, Japan). For construction of the AAK1-expression vector, the full-length AAK1 cDNA insert was amplified by PCR with primers 5′-CTCGAGACCATGTACCCATACGACGTGCCAGAC-TACGCTATGAAGAAGTTTTTCGACTCCCGG-3′ and 5′-GAATTCTTAAA-TAGCCTTGGCTTCTGGGGTGG-3′, and then the product of PCR was subcloned into the pcDNA3.1/Hygro expression vector (Invitrogen).

Measurement of the viability of AAK1-expressing HEK293 cells. Human embryonic kidney cells (HEK293 cells) were cultured at 37°C in DMEM supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine. Transfection of HEK293 cells with the plasmid that expressed AAK1 was done as described elsewhere (19) with TransIT-LT1 (Mirus, Madison, WI) according to the manufacturer's protocol. After incubation for 8 hours, cells (5×10^3) were plated in 96-well plates and cultured in 90-μL aliquots of medium. One day after plating, Adriamycin was added and cells were cultured for a further 3 days. Cell viability was assessed by the Alamar Blue assay (Biosource, Camarillo, CA). Each experiment was repeated at least thrice and representative results are presented.

Immunoblotting of HA-AAK1. Whole-cell extracts were prepared from individual transfectants by freezing and thawing. Immunoblotting with HA-specific antibody was done as described above.

Results

In a previous study, we searched for genes related to Adriamycin resistance using *S. cerevisiae* transfected with a chromosomal DNA library, and we obtained plasmids AR13 and AR17 that included genes that conferred resistance to Adriamycin on yeast cells when they were overexpressed (12). In the present study, we analyzed the DNA sequences of the genomic DNA fragments that had been

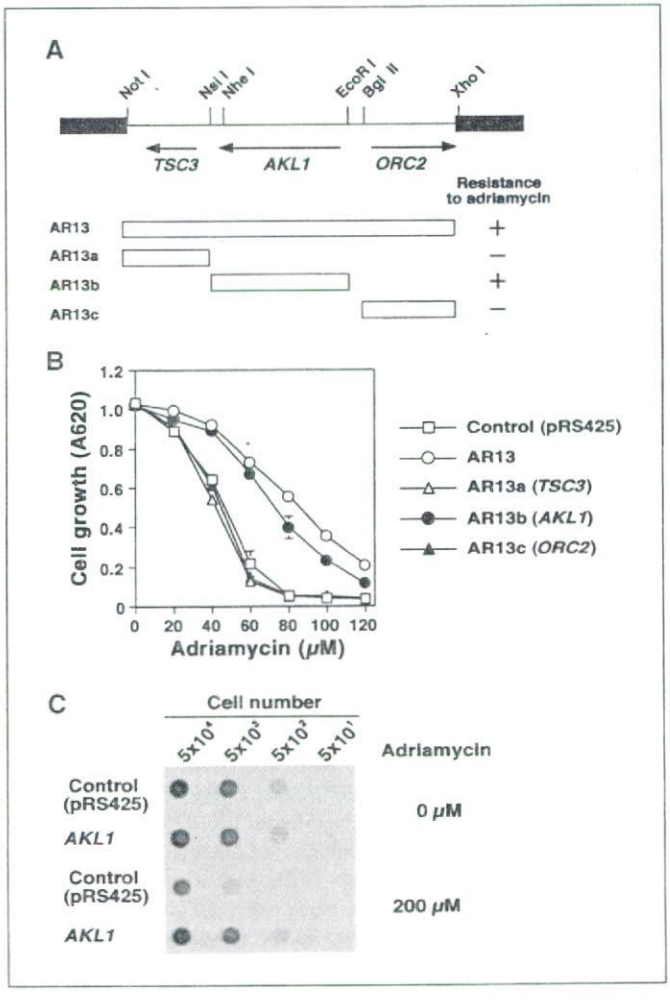

Figure 1. Identification of *AKL1* as an Adriamycin resistance gene. *A*, restriction map of the genomic DNA insert in plasmid AR13. *Thick black line*, vector pRS425; *thin line*, genomic DNA insert. Vertical lines above the genomic DNA insert indicate the restriction sites used to generate different subclones. The ability of three subclones (AR13a, AR13b, and AR13c) to confer resistance to Adriamycin is indicated (+, conferred resistance; −, did not confer resistance). ORFs are indicated by arrows that point in the direction of transcription, with the name of each ORF given below the respective arrow. *B*, sensitivity to Adriamycin of yeast cells that harbored plasmids with the indicated inserts. Yeast strains (W303B) carrying plasmids AR13a, AR13b, AR13c, or pRS425 were grown in synthetic dextrose (−Leu) medium that contained Adriamycin. After incubation for 48 hours at 30°C, absorbance was measured spectrophotometrically at 620 nm. *Points*, mean of results from three cultures; *bars*, SD. The absence of a bar indicates that the SD falls within the symbol. *C*, effects of overexpression of Akl1 on the sensitivity of yeast cells to Adriamycin. Yeast cells were incubated in the presence of Adriamycin for 3 hours and washed with synthetic dextrose (−Leu) medium. Then yeast cells were grown on a plate of agar-solidified synthetic dextrose (−Leu) for 24 hours.

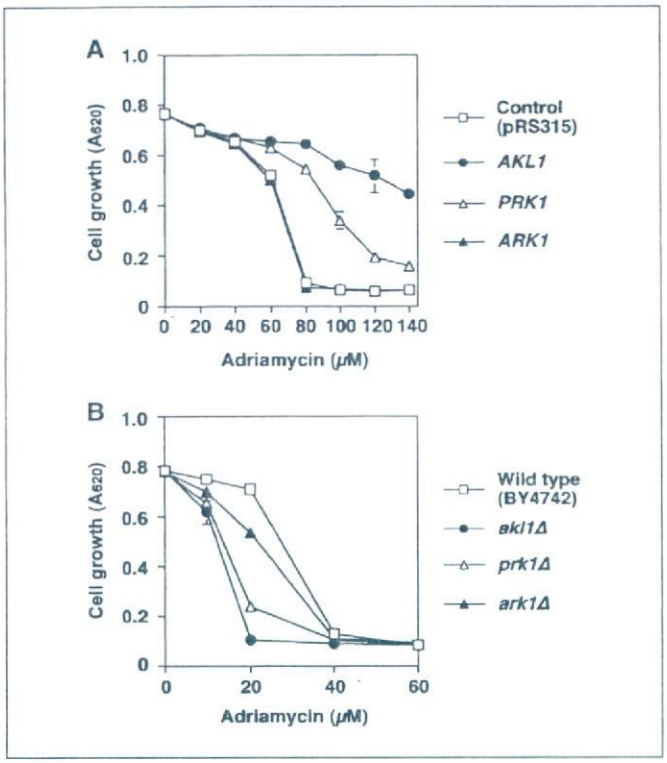

Figure 2. Involvement of members of the Ark/Prk kinase family in the sensitivity of yeast cells to Adriamycin. *A*, yeast cells (BY4742) harboring pRS315, pRS315-*AKL1*, pRS315-*ARK1*, or pRS315-*PRK1* were grown in synthetic dextrose (−Leu) medium that contained Adriamycin. *B*, yeast (BY4742) cells lacking Ark1, Prk1, or Akl1 (*ark1Δ*, *prk1Δ*, and *akl1Δ*, respectively) were grown in synthetic dextrose medium that contained Adriamycin. For further details, see Fig. 1*B* legend.

inserted into these plasmids, referring to the *Saccharomyces* Genome Database. A region of ~5 kbp derived from yeast chromosome no. 2 was present in both AR13 and AR17, and the region contained three open reading frames (ORF), designated *TSC3*, *AKL1*, and *ORC2* (Fig. 1*A*). To identify the gene involved in Adriamycin resistance, we cleaved AR13 with restriction enzymes and subcloned the resultant fragments (AR13a, AR13b, and AR13c) in pRS425. We introduced the three new plasmids into yeast W303B cells and examined the Adriamycin sensitivity. Only the yeast cells transfected with AR13b, which contained the *AKL1* ORF, exhibited Adriamycin resistance, showing that overexpression of *AKL1* conferred Adriamycin resistance on the yeast cells (Fig. 1*B* and *C*). Overexpression of the *AKL1* gene from a single-copy plasmid, pRS315, also led to Adriamycin resistance, suggesting that a severalfold increase in the intracellular level of Akl1 is sufficient to allow wild-type yeast cells to become Adriamycin resistant (Fig. 2).

Akl1 is classified as a member of Ark/Prk kinase family, which consists of serine/threonine kinases involved in the regulation of endocytosis, on the basis of its deduced amino acid sequence (13). However, to our knowledge, its function has not yet been analyzed. To investigate the involvement of the activity of Akl1 kinase in the Akl1 overexpression–induced reduction of Adriamycin toxicity, we prepared plasmids that expressed mutants of Akl1 with point mutations in the Akl1 kinase domain. All members of the Ark/Prk kinase family have a kinase domain in the NH_2-terminal half of the amino acid sequence, and the abolition of the kinase activity of Prk1 by substituting tyrosine (Y) for aspartic acid (D) at position 158 in this domain has been reported (14). Because the corresponding amino acid in Akl1 is aspartic acid at position 181 (Asp^{181}), we generated a mutant (Akl1D181Y) with tyrosine at this position. When Akl1D181Y was overexpressed, the sensitivity to Adriamycin of the yeast cells was similar to that of the control strain (Fig. 3). As described below, yeast cells had Akl1 kinase activity, and Asp^{181} was essential for this kinase activity (Fig. 4*A*). In view of these findings, we postulated that Akl1 kinase activity might be necessary for the Akl1 overexpression–induced acquisition of resistance to Adriamycin.

Akl1 is considered to function similarly to Ark1 and Prk1 because the kinase domain of Akl1 is strongly homologous to those of Ark1 and Prk1, two members of the Ark/Prk kinase family (13). Therefore, we investigated the sensitivity to Adriamycin of yeast cells that overexpressed Ark1 and Prk1, respectively. The sensitivity of Ark1-overexpressing yeast cells was similar to that of the control yeast cells, but Prk1-overexpressing yeast cells were apparently resistant to Adriamycin, although the level of resistance was lower than that of Akl1-overexpressing cells (Fig. 2*A*). When we prepared yeast cells with defective members of the Ark/Prk kinase family and investigated their Adriamycin sensitivity, we found that Ark1-defective yeast cells were slightly sensitive and Akl1- and Prk1-defective yeast cells were markedly sensitive to Adriamycin (Fig. 2*B*). These observations suggested that Akl1 and Prk1 might be intimately involved in the mechanism of Adriamycin toxicity, whereas the involvement of Ark1 might be limited.

Prk1 seems to promote the dissociation of the Sla1/Pan1/End3 complex via phosphorylation of both Sla1 and Pan1 (14, 15, 20). The Sla1/Pan1/End3 complex loses its function on dissociation, and Sla1- and End3-defective cells have low endocytic capacity (21, 22). When we investigated the sensitivity to Adriamycin of *SLA1*- and *END3*-defective yeast cells, we found that both defective lines of yeast cells were markedly resistant to Adriamycin (Fig. 5). Moreover, overexpression of Akl1 in these *SLA1*- and *END3*-defective cells did not increase the level of resistance (Fig. 5), suggesting that the presence of Sla1 and End3 is essential for the Akl1 overexpression–induced reduction in Adriamycin toxicity. In this series of experiments, we did not examine the effects of deletion of the *PAN1* gene because Pan1 is a protein that is essential for the proliferation of yeast cells.

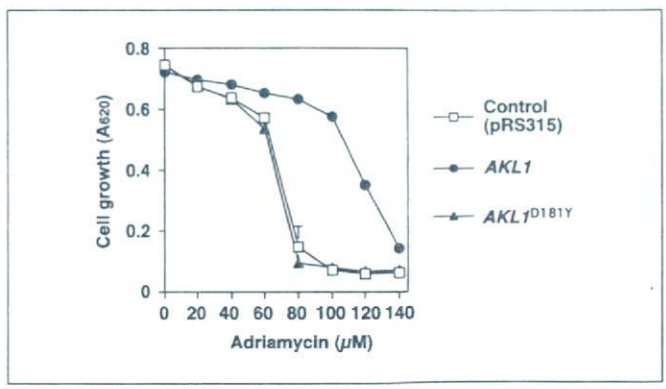

Figure 3. Akl1 kinase activity is required for the acquisition of Adriamycin resistance. Yeast cells (BY4742) harboring pRS315, pRS315-*AKL1*, or pRS315-*AKL1*D181Y were grown in synthetic dextrose (−Leu) medium that contained Adriamycin. For further details, see Fig. 1*B* legend.

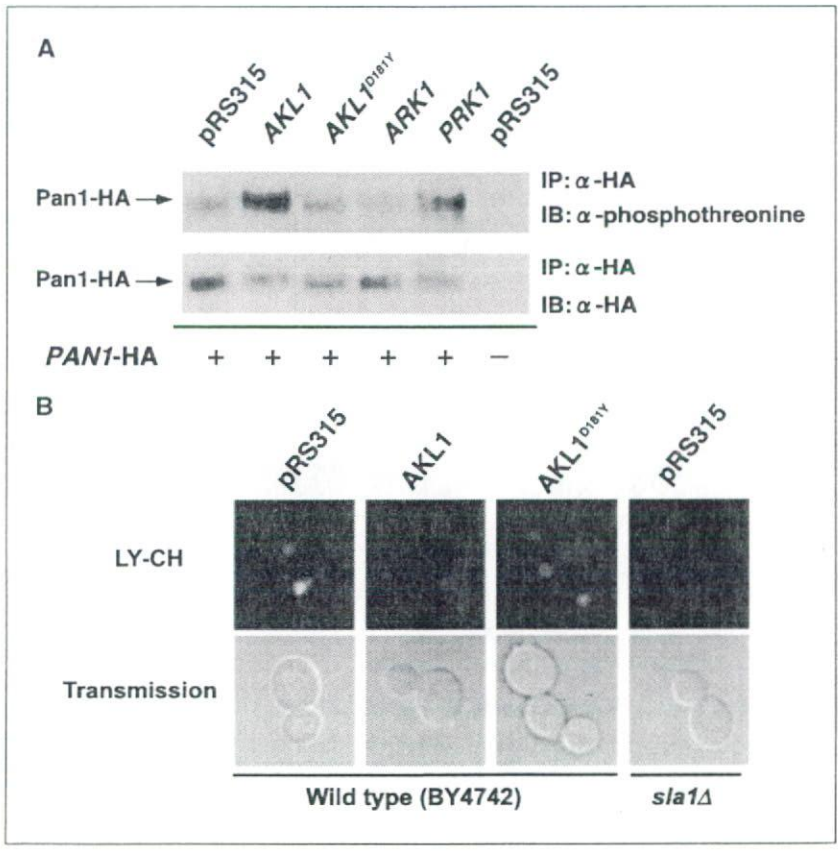

Figure 4. Effects of overexpression of Akl1 on the phosphorylation of Pan1-HA and the endocytic uptake of lucifer yellow. *A*, yeast (BY4742) cells expressing Pan1 tagged with HA at the COOH terminus (Pan1-HA) were transformed with pRS315, pRS315-*AKL1*, pRS315-*ARK1*, pRS315-*PRK1*, or pRS315-*AKL1*D181Y. Pan1-HA was immunoprecipitated (*IP*) with anti-HA agarose and immunoblotted (*IB*) with phosphothreonine-specific antibody (α-phosphothreonine; *top*) or HA-specific antibody (α-HA; *bottom*). See text for details. *B*, yeast cells at the logarithmic phase of growth were incubated with lucifer yellow for 2 hours. The localization of lucifer yellow was visualized with FITC fluorescence optics. The fluorescent images (*LY-CH*; *top*) and the transmission images (*bottom*) are shown.

Because our results suggested the involvement of the dissociation of the Sla1/Pan1/End3 complex via phosphorylation of Pan1 and Sla1 in resistance to Adriamycin, we investigated the effects of overexpression of Akl1 on the phosphorylation of Pan1 using yeast cells that expressed Pan1 fused to a HA tag at its COOH terminus (Pan1-HA). Phosphorylation of Pan1-HA was promoted in Adriamycin-resistant yeast cells that overexpressed Akl1 and Prk1, but it was unaffected in yeast cells that overexpressed Akl1 and point mutants of Akl1 (Akl1D181Y; Fig. 4*A*). These findings strongly suggested the involvement of the phosphorylation of Pan1 in the Akl1 overexpression–induced acquisition resistance to Adriamycin.

The overexpression of Prk1 is known to reduce endocytic ability, but, to our knowledge, no studies of the effects of overexpression of Akl1 on endocytosis have been done. We investigated the fluid-phase endocytotic activity of Akl1-overexpressing yeast cells using the uptake of a fluorescent dye, lucifer yellow, as an index. The overexpression of Akl1 decreased the uptake of lucifer yellow (Fig. 4*B*), showing that the capacity for endocytosis was reduced in Akl1-overexpressing yeast cells as it was in Sla1-defective yeast cells. By contrast, no endocytic abnormalities were noted in yeast cells that overexpressed the Akl1 point-mutant (Akl1D181Y), which might not have any kinase activity (Fig. 4*B*). These findings suggest that Akl1 acts negatively to control cellular endocytosis by promoting the dissociation of the Sla1/Pan1/End3 complex through phosphorylation of Pan1, as does Prk1.

In mammalian cells, adaptor-associated kinase 1 (AAK1; refs. 23, 24) and cyclin G–associated kinase (25, 26) are both members of the Ark/Prk kinase family, and overexpression of AAK1 has been reported to reduce the endocytosis of membrane proteins, such as transferrin receptors and low-density lipoprotein receptor–related protein (16). To investigate the possible involvement of the Ark/Prk kinase family in the acquisition of Adriamycin resistance by human cells, we fused AAK1 with a HA tag at its NH_2 terminus (HA-AAK1) and introduced it into HEK293 cells (Fig. 6*A*). The resultant AAK1-overexpressing HEK293 cells were found, as anticipated, to be resistant to Adriamycin (Fig. 6*B*). We confirmed that overexpression of AAK1 induced resistance to Adriamycin not

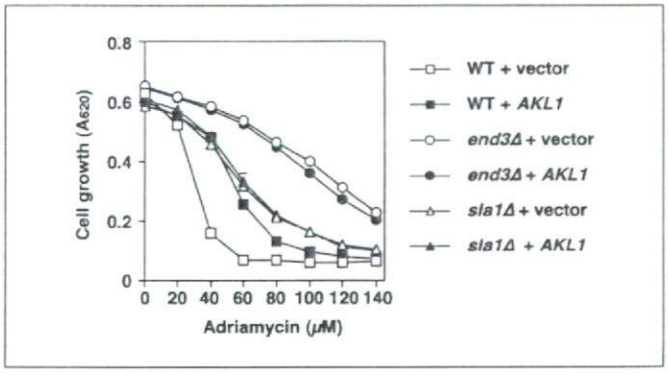

Figure 5. Relationship between Akl1 and the Sla1/End3/Pan1 complex in the acquisition of Adriamycin resistance. Yeast strains (BY4742, *sla1Δ*, and *end3Δ*) harboring pRS315 or pRS315-*AKL1* were grown in synthetic dextrose (−Leu) medium that contained Adriamycin. After incubation for 24 hours at 30°C, the absorbance was measured spectrophotometrically at 620 nm. *Points*, mean of results from three cultures; *bars*, SD. The absence of a bar indicates that the SD falls within the symbol.

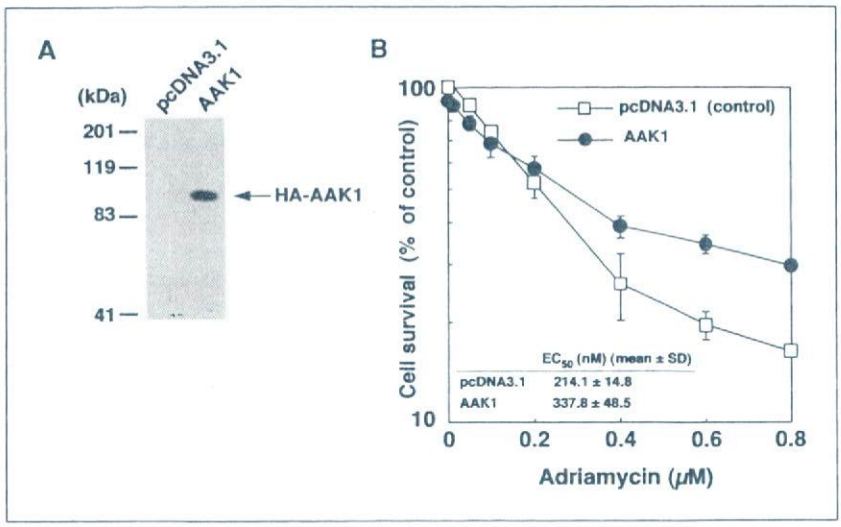

Figure 6. Effects of overexpression of AAK1, a member of the human Ark/Prk kinase family, on the sensitivity of HEK293 cells to Adriamycin. *A*, immunoblotting analysis of HA-tagged AAK1. HEK293 cells were transfected with pcDNA3.1-HA-AAK1 or the pcDNA3.1 empty vector. Each lane was loaded with whole-cell extract from the indicated transfectants. *B*, sensitivity of HA-AAK1-expressing HEK293 cells to Adriamycin. Transfectants expressing HA-AAK1 were cultured for 3 days in the presence of various concentrations of Adriamycin. *Points*, mean of results from three cultures; *bars*, SD. The absence of a bar indicates that the SD falls within the symbol. EC_{50} for cytotoxicity was defined as the effective concentration of Adriamycin that caused a 50% reduction in cell viability relative to non-Adriamycin-treated controls (which represent 100% viability).

only in HEK293 cells but also in HeLa cells (data not shown). Our findings suggest that the Ark/Prk kinase family might be involved in the acquisition of Adriamycin resistance not only in yeast cells but also in human cells.

Discussion

In the present study, we showed that overexpression of Akl1 renders *S. cerevisiae* resistant to Adriamycin. Akl1 has a kinase domain in its NH_2-terminal half and is considered to be a serine/threonine kinase that belongs to the Ark/Prk kinase family (13). Prk1 and Ark1 also belong to the Ark/Prk kinase family and these proteins phosphorylate different specific proteins (27, 28). In our study, overexpression of Prk1 rendered yeast cells Adriamycin resistant, but overexpression of Ark1 did not affect the sensitivity to Adriamycin (Fig. 2*A*). These findings suggest that similar mechanisms that are mediated by Akl1 and Prk1 might be involved in the resistance to Adriamycin.

This study is the first, to our knowledge, to reveal that Akl1, similarly to Prk1, phosphorylates Pan1 in the Sla1/Pan1/End3 complex, which is involved in endocytosis, and confirms that Asp^{181} is essential for the kinase activity (Fig. 4*A*). Cellular endocytic ability decreases when Pan1 is phosphorylated because the Sla1/Pan1/End3 complex dissociates (15). This dissociation might be responsible for the resistance of the yeast cells to Adriamycin.

The Sla1/Pan1/End3 complex regulates the internalization step of the endocytic pathway (15). Yeast cells with defects in genes for other factors that are involved in this internalization step of endocytosis, such as Sla2 (29, 30), Vrp1 (31), and Rvs161 (32), also exhibited marked resistance to Adriamycin,[3] whereas the Adriamycin sensitivity of yeast cells with defects in genes for Vps23 (33) and Vps27 (34), which are involved in the post-internalization step of endocytosis, was similar to that of control yeast cells (data not shown). The level of Adriamycin toxicity might be decreased when the internalization step of endocytosis is inhibited.

Because endocytosis is involved in the cellular uptake of various substances, reduced endocytosis might decrease the cellular uptake of Adriamycin. Reduced endocytosis has also been reported to increase the stability of Pdr5 (35), a transporter involved in the excretion of Adriamycin (36, 37). However, the overexpression of Akl1 did not decrease the intracellular accumulation of Adriamycin and, indeed, the level of accumulation increased slightly (data not shown). Therefore, the resistance to Adriamycin of Akl1-overexpressing yeast cells might not be due to a decrease in the intracellular accumulation of Adriamycin. Chen et al. identified a carcinoembryonic antigen–related membrane protein, p95, as an Adriamycin-resistance factor (38, 39). The mechanism of Adriamycin resistance that involves p95 has not yet been analyzed, but p95-overexpressing cells exhibit resistance to Adriamycin without any reduction in intracellular levels of Adriamycin (38). It is also possible that the Akl1 overexpression–induced reduction in the endocytic ability of the cell might have rendered yeast cells Adriamycin resistant by increasing the stability of membrane proteins, such as p95, that are not involved in drug excretion. Adriamycin is known to impair the functions of cell membranes, and some types of Adriamycin-resistant cell are resistant to such impairment, suggesting that some changes in cell membranes (such as changes in the lipid composition) might be involved in resistance to Adriamycin (40–42). Because the involvement of endocytosis in lipid homeostasis has been reported (43), endocytosis might be involved in the mechanism of Adriamycin toxicity via the regulation of the lipid composition of membranes.

Adriamycin also impairs formation of the cytoskeleton in human cells (44). The involvement of formation of the actin skeleton in the mechanism of cardiotoxicity of Adriamycin has also been reported (45). Ark1 and Prk1 are involved not only in the regulation of endocytosis but also in the formation of cytoskeleton (20). Therefore, we cannot rule out the possibility that the roles of members of the Ark/Prk kinase family in formation of the cytoskeleton might be involved in Adriamycin toxicity.

Overexpression of AAK1, a member of the human Ark/Prk kinase family, rendered both HEK293 and HeLa cells resistant to Adriamycin. AAK1 is considered to regulate the internalization step of endocytosis via the phosphorylation of an adaptor protein, AP2μ (24). Thus, the kinase-dependent regulation of endocytosis

[3] Unpublished data.

might be involved in the mechanism of Adriamycin toxicity not only in yeast cells but also in human cells. To our knowledge, no studies of the relationship between endocytosis and resistance to Adriamycin have been reported. Our results suggest novel mechanism for Adriamycin toxicity and the acquisition of resistance to Adriamycin in human cells.

Acknowledgments

Received 8/31/2006; accepted 10/10/2006.

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We thank Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan) for providing the Adriamycin used in this study.

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JOURNAL OF CELLULAR PHYSIOLOGY 210:378–384 (2007)

Cisplatin Upregulates *Saccharomyces cerevisiae* Genes Involved in Iron Homeostasis Through Activation of the Iron Insufficiency-Responsive Transcription Factor Aft1

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The response of *Saccharomyces cerevisiae* to cisplatin was investigated by examining variations in gene expression using cDNA microarrays and confirming the results by reverse transcription polymerase chain reaction (RT-PCR). The mRNA levels of 14 proteins involved in iron homeostasis were shown to be increased by cisplatin. Interestingly, the expression of all 14 genes is known to be regulated by Aft1, a transcription factor activated in response to iron insufficiency. The promoter of one of these genes, *FET3*, has been relatively well studied, so we performed a reporter assay using the *FET3* promoter and showed that an Aft1 binding site in the promoter region is indispensable for induction of transcription by cisplatin. The active domain of Aft1 necessary for activation of the *FET3* promoter by cisplatin is identical to the one required for activation by bathophenanthroline sulfonate, an inhibitor of cellular iron uptake. Furthermore, we found that cisplatin inhibits the uptake of ^{55}Fe(II) into yeast cells. These findings suggest that cisplatin activates Aft1 through the inhibition of iron uptake into the cells, after which the expression of Aft1 target genes involved in iron uptake might be induced. J. Cell. Physiol. 210: 378–384, 2007. © 2006 Wiley-Liss, Inc.

Cisplatin is an anticancer drug that is effective against various malignant tumors, including those of the testis, ovary, prostate gland, and uterus (Einhorn and Williams, 1979). However, acquisition of resistance to cisplatin by tumor cells and the development of adverse effects are disadvantageous in its clinical usage. A number of mechanisms have been implicated in the acquisition of cisplatin resistance, including decreases in the amount of cisplatin taken up by cells (Kikuchi et al., 1990; Gately and Howell, 1993), augmented cellular excretion of cisplatin (Ishikawa and Ali-Osman, 1993; Mistry et al., 1993; Fujii et al., 1994; Aebi et al., 1996; Fink et al., 1997), enhancement of detoxification mechanisms due to concentration increases in glutathione and metallothioneins (Naganuma et al., 1987; Kelley et al., 1988; Godwin et al., 1992; Ikeda et al., 2001), and inhibition of apoptotic signals (Siddik, 2003). However, in many types of tumor cells, the development of resistance cannot be explained only by these mechanisms, indicating the possible presence of other unknown processes.

Recently, comprehensive genetic analyses have been carried out of various organisms to elucidate the mechanisms behind the acquisition of resistance. Studies of the cells of budding yeasts (Brown et al., 1993; Fox et al., 1994; Furuchi et al., 2001; Niedner et al., 2001; Schenk et al., 2001, 2003; Huang et al., 2005), *Dictyostelium discoideum* (Niedner et al., 2001), and mammals (Niedner et al., 2001; Ishida et al., 2002) identified several genes involved in cisplatin-resistance. However, few studies have examined the cellular response to cisplatin. Genetic examination of budding yeasts is easily conducted and there is a wealth of information regarding the function of each gene. Furthermore, the functions of many yeast genes are common to those of mammalian genes including those of humans. Thus, the findings obtained from yeast studies provide an insight into the functions of human proteins.

Using budding yeast as a model organism, we used cDNA microarray technology to investigate variations in gene expression following cisplatin treatment. We found that the mRNA levels of many proteins involved in the maintenance of iron homeostasis were increased by cisplatin. Furthermore, we demonstrated for the first time that cisplatin inhibits iron uptake into eukaryotic cells, leading to iron deficiency and the consequent activation of the transcription factor Aft1. Our findings suggest that target genes of Aft1 involved in the maintenance of iron homeostasis are augmented in response to cisplatin.

Abbreviations: YPAD, yeast extract–peptone–adenine–dextrose; SD, synthetic dextrose; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; BPS, bathophenanthroline sulfonate, MUG, methyl umbelliferyl β-D galactopyranoside; WT, wild type.

Contract grant sponsor: Health and Labour Sciences Research Grants for Research on Risk of Chemical Substances from Ministry of Health, Labour and Welfare Japan; Contract grant number: H17-Risk of Chemical Substances-010.

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Received 5 June 2006; Accepted 25 July 2006

Published online in Wiley InterScience (www.interscience.wiley.com.), 9 November 2006.
DOI: 10.1002/jcp.20845

MATERIALS AND METHODS

Yeast strains and media

BY4742 (*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*) and *aft1Δ* (*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YGL071W:kanMX4*) were obtained from Euroscarf (Frankfurt, Germany). Yeast cells were grown on yeast extract–peptone–adenine–dextrose (YPAD) medium (1% yeast extract, 2% peptone, 0.004% adenine, and 2% glucose) or on synthetic dextrose (SD) medium supplemented with amino acids. Transformation of yeast cells was performed by the lithium acetate method (Naganuma et al., 2000).

Construction of pMELb2-FET3-lacZ, pMELb2-FIT3-lacZ, pMELb2- LM2-lacZ, and pMELb2-Aft1 binding site–lacZ

The *FET3* and *FIT3* promoters were amplified by polymerase chain reaction (PCR) using yeast chromosomal DNA as a template and the following primers: *FET3* promoter-F and *FET3* promoter-R for *FET3*, and *FIT3* promoter-F and *FIT3* promoter-R for *FIT3* (Table 1). The PCR product was digested with *Hind*III and *Bam*HI, and the fragment was cloned into the *Bam*HI–*Hind*III sites of pMELb2, a *LacZ* expression vector. The mutant *FET3* promoter (LM2) (Yamaguchi-Iwai et al., 1996) was amplified by PCR using pMELb2 *FET3-lacZ* as a template and the primer pair LM2-F and LM2-R (Table 1). The PCR product was then self-ligated.

The Aft1 binding site was created by annealing the oligonucleotides Aft1 binding site-F, 5′-TCGACGAGCACCTG-CAATGGGTGCACTTTTGAAG-3′, and Aft1 binding site-R, 5′-TCGACTTCAAAAGTGCACCCATTTGCAGGTGCTCG-3′. The annealed oligonucleotides were cloned into the *Xho*I sites of pMELb2.

Construction of pRS315-AFT1-GFP-GFP-HA. The *AFT1* gene containing 759-bp upstream from the transcriptional start site was amplified by PCR using yeast chromosomal DNA as a template and the primer pair AFT1-F and AFT1-R (Table 1). The PCR product was digested with *Sac*I and *Eco*RV, and the fragment was cloned into the *Sac*I–*Sma*I sites of pRS315-Met-GFP-GFP-HA (Isoyama et al., 2001).

Construction of yeast strains overexpressing mutant forms of Aft1p

The *AFT1* deletion mutants M1–M14 were constructed by creating pairs of *Kpn*I sites in the *AFT1* gene and self-ligating between the respective pairs of *Kpn*I sites. First, pRS315-*AFT1*-GFP-GFP-HA was digested with *Sac*I and *Apa*I, and the fragment cloned into the *Sac*I–*Apa*I site of the pGEM-T easy vector (Promega, Madison, WI). PCR mutagenesis was performed on pGEM-T easy-*AFT1*-GFP-GFP-HA using corresponding sets of primers (Table 1). The amplification added a *Kpn*I site to both ends of the PCR products so, after amplification, the PCR products were cleaved with *Kpn*I and self-ligated, resulting in the removal of the amino acids shown in Figure 5A. Each pGEM-T easy-*AFT1* deletion mutant (M1–M14) was digested with *Sac*I and *Apa*I and the fragment was ligated into pRS315. To create the deletion mutant M15, pRS315-*AFT1* was digested with *Hind*III and the fragment religated. Each deletion mutant (pRS315-*AFT1* mutants) was then introduced into *aft1Δ* cells.

Microarray

Yeast (5×10^6 cells/ml) was precultured in SD medium for 3 h at 30°C. Cells were then cultured in SD medium for 3 h with or without 100 μM cisplatin (Nippon Kayaku, Tokyo, Japan). The suspension was centrifuged at 2,200*g* for 5 min and the cells were collected. Total RNA was isolated using the hot acidic phenol method (Furuchi et al., 2002), after which poly $(A)^+$ mRNA was isolated from 250 μg total RNA using the Oligotex™ $-dt30^{mRNA}$ kit (JSR, Tokyo, Japan). Fluorescent-labeled cDNA was generated using an oligo dT primer with Fluorilink Cy3-dUTP or Fluorilink Cy5-dUTP and Super-Script™ II RT (Invitrogen, Carlsbad, CA). Cy3- and Cy5-labeled cDNA probes were combined and hybridized to Yeast Chip ver2.0. (Hitachi Software Engineering, Kanagawa, Japan) in 5× SSC at 65°C for 14–16 h. After washing, the microarray slides were simultaneously scanned by a GenePix 4000B array scanner (Axon Instruments, Union City, CA). Array-Pro Analyzer 4.5 analysis software (LI-COR Biosciences, Lincoln, NE) was used to analyze the results.

RT-PCR

Total RNA was extracted using the hot acidic phenol method (Furuchi et al., 2002). cDNA was synthesized from 3 mg total RNA using 0.5 mM oligo dT primer, 50 U Molony Murine Leukemia Virus reverse transcriptase, and 10 U RNA nuclease inhibitor. Reverse transcription polymerase chain reaction (RT-PCR) was performed using SYBR *premix Ex Taq*™ (TaKaRa, Shiga, Japan). Reverse transcribed mRNA was diluted 10-fold and 0.5 μl of the diluted solution was used as a template for PCR with specific primers (Table 1) to amplify the following genes: *ARN1, ARN2, ARN3, CCC2, CCP1, FIT1, FIT2, FIT3, FET3, FET4, FRE1, FRE2, FRE3, FTR1, HMX1, HSP30, TIS11, YHL035C*, and *YMR251W-R*. The amplified DNA was scanned by the iCycler iQ real-time PCR detection system (Bio-Rad, Philadelphia, PA). Each transcript was quantified relative to the Glyceraldehyde-3-phosphate dehydrogenase standard.

β-Galactosidase assay

β-Galactosidase reporter constructs were transformed into either BY4742 or *aft1Δ* cells. Yeast cells carrying a reporter plasmid at mid-logarithmic phase were cultured in SD medium with or without non-toxic concentration of cisplatin for 3 h. The culture was centrifuged at 2,200*g* for 5 min to remove the supernatant. Cells were resuspended in 25 μl Tris-Triton buffer (0.1 M Tris-HCl, pH 7.5; 0.05% Triton X-100). The suspensions were frozen, thawed, and incubated with 125 μl Z buffer (10 mM KCl, 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mM $MgSO_4$) containing 0.5 mg/ml methyl umbelliferyl β-D galactopyranoside (MUG) at 30°C for 5 min. The absorbance of MUG at $^{EX}360$ nm $^{EM}450$ nm was measured. After adjusting cell density (OD_{600}) and reaction time (min), the value was taken as an index of β-galactosidase activity. The assay was repeated at least three times.

Iron uptake assay

Ferrous uptake was assayed as described by Dancis et al. (1990) with a few modifications. Yeast cells ($OD_{600} = 0.5$) were collected by centrifugation and resuspended in ice-cold assay buffer (5% glucose, 50 mM sodium citrate, pH 6.5) to $OD_{600} = 5$. Then 1 μM $^{55}FeCl_3$ (Perkin Elmer, Wellesley, MA, 74 MBq/ml) and 1 μM ascorbate were added with or without non-toxic concentration of cisplatin. The suspensions were incubated at 30°C for 1, 2, or 3 h, and filtered through a glass filter. The radioactivity retained on the filters was measured using imaging plates (Fuji Photo Film, Kanagawa, Japan) and a BAS-5000 imaging analyzer (Fuji Photo Film).

RESULTS

To examine the effect of cisplatin treatment on budding yeasts, we used DNA microarray technology to study the variation in gene expression after incubation with 100 μM cisplatin for 3 h. A more than twofold increase in the expression of 18 genes and a more than twofold decrease in the expression of 2 genes was detected (Table 2).

Variation in expression following cisplatin treatment was confirmed by RT-PCR analysis for all genes, with the exception of *FIT1, YPR123C, HSP30*, and *CCP1* (Table 2). Interestingly, among the 16 genes whose expression was increased by cisplatin, 14 were shown to be involved in the maintenance of iron homeostasis. Fit2 and Fit3 hold iron within the cell wall (Protchenko et al., 2001); Arn 1–3 are components of a siderophore transporter complex produced by microorganisms and iron (III) (Lesuisse et al., 1998; Heymann et al., 1999, 2000); Fre 1–3 are iron reducing enzymes (Georgatsou and Alexandraki, 1994; Martins et al., 1998); Fet 4 is a low-affinity iron transporter (Dix et al., 1994); Fet3 is a constituent factor of a high affinity iron transport

TABLE 1. Primer Sequences used in plasmid construction and creation of deletion mutants

Primer pair	Sequence (5′-3′)[a]	Amplification
AFT1-F	GATCGAGCTCTCTAGATACACAGGGCAAGGTCATTA	*AFT1*
AFT1-R	GATCGATATCGCCATCTTCTGGCTTCACAT	
ARN1-F	ACCCTCTATAGAATTGGTTCT	*ARN1*
ARN1-R	CGACATATTCGCCATCCTC	
ARN2-F	GGCTAACGTTGTTTCTTGT	*ARN2*
ARN2-R	CCTGTAATTCTTTCCATTCCA	
ARN3-F	GTAACCTCATTATACCTTGC	*ARN3*
ARN3-R	CCTATCTTTACTGCTTGTAAA	
CCC2-F	GAGAGGACTGGCCAACG	*CCC2*
CCC2-R	CGGCCTGTGATTCTATGTC	
CCP1-F	CGCTGACTATGTCAGAACA	*CCP1*
CCP1-R	CCTTGTTCCTCTAAAGTC	
FET3-F	GCAAGGTTTGGGTCTTGTTC	*FET3*
FET3-R	GATGCTTTTCAGTGGAATGAC	
FET3 promoter-F	GCAAGCTTTTTCCGGGTGCGAATCAG	*FET3*
FET3 promoter-R	GCGGATCCAAACATCTAGTTCTAATTTTTTGC	
FET4-F	GGTGGTTGATTATCGGTACA	*FET4*
FET4-R	CCCAATTATGTGCTTGCAAC	
FIT1-F	TCTAAATCCACGTCTGCAGT	*FIT1*
FIT1-R	CGGTAGTGGTTTGAACTCTTG	
FIT2-F	ACAGTTATGACTGCCGTCTCG	*FIT2*
FIT2-R	ACACTTGCTCCTTGGAATGCA	
FIT3-F	TTTTGTCTGGACTGGTGAAGG	*FIT3*
FIT3-R	CAGCACCCATCAAACCAGTA	
FIT3 promoter-F	GCAAGCTTCTCCATAAACATTTCCTTTGTC	*FIT3*
FIT3 promoter-R	GCGGATCCAAACATTTTAGGGATTATTGTTATTAG	
FRE1-F	GCTATTTATCCGCACTTTGTC	*FRE1*
FRE1-R	CTTTCTTCTTCTAGTTCAACG	
FRE2-F	GGCTTATAAGCCGGAGTTG	*FRE2*
FRE2-R	GCATTGATACTCTTCAAAGTA	
FRE3-F	CGTACTCGAGGCTTACAAG	*FRE3*
FRE3-R	CTTCAAAGTATTCGATTGCC	
FTR1-F	GTGCTTCGAAATCCTCGCTG	*FTR1*
FTR1-R	CTCTTTTGCTCTTCCGTCAAC	
HMX1-F	CCCACTGAAGAAACACGCTT	*HMX1*
HMX1-R	GTGCTCTTCTAGTAGCAGAATCC	
HSP30-F	GGTGTGATATGCCAACGTC	*HSP30*
HSP30-R	CAGGTTCGGGTTCGTGG	
LM2-F	GGCCCATCTTCAAAAGTGCAGGGATTTGCAGGTGCTC	LM2
LM2-R	GAGCACCTGCAAATCCCTGCACTTTTGAAGATGGGCC	
TIS11-F	GCAACAGTTGTCTCAACA	*TIS11*
TIS11-R	GGTCATTCTCTGCAAAGC	
YHL035C-F	GAGGAACGCTTTAACAGCA	*YHL035C*
YHL035C-R	CATCTTACTTGATTGCTTGG	
YMR251W-F	CAGAGTTCAACTCTTGGGTG	*YMR251W*
YMR251W-R	GTGGGGTGATCCCAATC	
YPR123C-F	CCATATCCATCCCTGAAGA	*YPR123C*
YPR123C-R	CATTTGTTGGAAGAATCGAAT	
M1-F	GC**GGTACC**GAACATGCGTCACCGATTAATTCATCTGACAGC	M1
M1-R	CG**GGTACC**CATTGTCGTAGATTTTTCTGTTATTTTTTTGTT	
M2-F	GC**GGTACC**GAGGGTCGTGCAAGTGCAAGTGG	M2
M2-R	CG**GGTACC**TATGTCAGCCGGATTGAAGCCTTCC	
M3-F	GC**GGTACC**AACAATTTGATTCATCTGGATCCAGTACCCAAC	M3
M3-R	CG**GGTACC**ATTATGGTTGACTACATATTCACTAGCACTTTTGGG	
M4-F	GC**GGTACC**TTTGAAGATAAGTCCGATATTAAGCCTTGG	M4
M4-R	CG**GGTACC**TTGATTCAGCTTTTCCACATGATGAATATGC	
M5-F	GC**GGTACC**AAGCCCAAAGAAAAAAAGATGTGTATCGAGG	M5
M5-R	CG**GGTACC**TTTTCTTTTCCTTCTCGCGTTCCTTCCCC	
M6-F	GC**GGTACC**TGTGTATCGAGGTTTAATAACTGTCCG	M6
M6-R	CG**GGTACC**TATGGAGGATGTTTGATCGGGCG	
M7-F	GC**GGTACC**TCTAAAAGGCCATGCTTACCCTCTG	M7
M7-R	CG**GGTACC**TTTCTTGATTGCATCTACGTCATTATCC	
M8-F	GC**GGTACC**TGCTTACCCTCTGTAAATAACACCGG	M8
M8-R	CG**GGTACC**TGCATTCGATGAATCAAGGGATATGGACG	
M9-F	GC**GGTACC**AGGAAACCGAAAAGCCAGTGTAAGAATAAAG	M9
M9-R	CG**GGTACC**TGGCCTTTTAGATGCATTCGATGAATCAAG	
M10-F	GC**GGTACC**CAGTGTAAGAATAAAGACACACTCTTAAAAAG	M10
M10-R	CG**GGTACC**TACGTTATTGGTATTGATACTACCGGTG	
M11-F	GC**GGTACC**CCGGCATCCGATTTCAAGCTAAAC	M11
M11-R	CG**GGTACC**GCTTTTCGGTTTCCTTACGTTATTGG	
M12-F	GC**GGTACC**CAAGAAGCTTTAGTTGGCAGCTCTTC	M12
M12-R	CG**GGTACC**CAGTGGCAAGATTTCATTCAAATTGAAAGG	

[a]Bold letters indicate *Kpn*I sites.

system and an oxidizing enzyme of iron (Askwith et al., 1994); Ftr1 is an iron permease (Stearman et al., 1996); Hmx 1 is a factor resembling heme-oxygenase (Auclair et al., 2003), a copper transporter that transfers iron to Fet3 (Fu et al., 1995); and Tis11 is a factor that breaks down the mRNA of an iron-containing enzyme when the cell is deficient in iron (Puig et al., 2005) (Table 2).

TABLE 2. Yeast genes whose expression levels were changed by treatment with cisplatin

Gene	ID	Fold-increase		Function
		Microarray	RT-PCR	
FIT2[a]	*YOR382W*	49.3	19.1	Cell wall protein involved in iron uptake
FIT3[a]	*YOR383C*	30.6	10.9	Cell wall protein involved in iron uptake
ARN2[a]	*YHL047C*	9.2	7.9	Siderophore transporter
FIT1[a]	*YDR534C*	8.6	1.1	Cell wall protein involved in iron uptake
ARN3[a]	*YEL065W*	5.8	3.8	Siderophore transporter
FRE2[a]	*YKL220C*	4.8	4.5	Ferric and cupric reductase
HMX1[a]	*YLR205C*	4.7	3.0	Heme-binding protein
ARN1[a]	*YHL040C*	4.3	2.9	Siderophore transporter
FRE1[a]	*YLR214W*	4.2	3.1	Ferric and cupric reductase
FRE3[a]	*YOR381W*	3.2	1.9	Ferric reductase
FET3[a]	*YMR058W*	3.0	2.5	High-affinity Fe(II) transporter
FTR1[a]	*YER145C*	2.9	2.2	Iron permease
FET4[a]	*YMR319C*	2.2	2.5	Low-affinity Fe(II) transporter
CCC2[a]	*YDR270W*	2.5	2.3	Copper transporter
TIS11[a]	*YLR136C*	6.8	19.5	Protein of the CCCH zinc finger family
VMR1	*YHL035C*	2.0	2.0	Unknown
	YMR251W	4.5	3.9	Unknown
	YPR123C	2.3	1.0	Unknown
HSP30	*YCR021C*	0.3	0.8	Heat shock protein
CCP1	*YKR066C*	0.5	ND	Cytochrome-c peroxidase

ND, not detected.
[a]Genes whose expression is known to be regulated by Aft1.

It is known that the expression of many genes involved in cellular uptake of iron are regulated by the transcription factor Aft1 (Yamaguchi-Iwai et al., 1996). In the present study, we found that all 14 genes involved in maintaining iron homeostasis whose expression was augmented by cisplatin were regulated by Aft1 (Table 2). It is therefore possible that the augmentation of expression by cisplatin is also regulated by Aft1. To investigate this, we conducted a β-galactosidase reporter assay using plasmids expressing the *LacZ* gene under the control of *FIT3* and *FET3* promoters with Aft1 binding sites. The results showed that *FIT3* and *FET3* promoter activities increased in a dose-dependent manner with increasing concentrations of cisplatin (Fig. 1).

Next, we examined the effects of Aft1 deficiency on cisplatin activation of the *FET3* promoter (Yamaguchi-Iwai et al., 1996). Cisplatin had a negligible effect on *FET3* promoter activation in an Aft1-deficient, compared with a wild-type, yeast strain (Fig. 2A). Similarly, when the Aft1 binding site (TGCACCCA) was used in place of the *FET3* promoter (245–252 bp upstream from the *FET3* transcription start site), cisplatin effected an increase in β-galactosidase activity of the wild-type strain, but had no effect on reporter gene activity in the Aft1-deficient yeast (Fig. 2B). A previous study by Yamaguchi-Iwai et al. (1996) showed that Aft1 was unable to bind a mutant *FET3* promoter, LM2, with a CCC → GGG mutation in its Aft1 binding site. We therefore examined the effect of cisplatin by carrying out a reporter gene assay in yeast expressing *LacZ* under the control of the LM2 promoter and showed that cisplatin did not affect promoter activity (Fig. 2C). From the above findings, we conclude that cisplatin augments the expression of genes involved in maintaining iron homeostasis, such as *FET3*, by promoting Aft1 binding to a site present within the promoter region of Aft1 target genes.

Aft1 is usually present within the cytoplasm, but is translocated to the nucleus during periods of cellular iron deficiency, where it binds to the promoter of its target genes and accelerates their transcription (Yamaguchi-Iwai et al., 1995, 2002). Bathophenanthroline sulfonate (BPS) is a chelating agent of bivalent iron that is unable to penetrate the cell membrane and is capable of chelating iron in cell growth medium. As such, it is used as an inhibitor of iron uptake into cells (Alcain et al., 1994). We applied BPS to the reporter gene assay in wild-type and Aft1-deficient yeasts examined in Figure 2, and showed it to have similar, negligible effects on promoter activity in the Aft1-deficient strain (Fig. 3) as seen following cisplatin treatment.

It is possible that cisplatin activation of Aft1 utilizes similar mechanisms to those of iron chelating agents

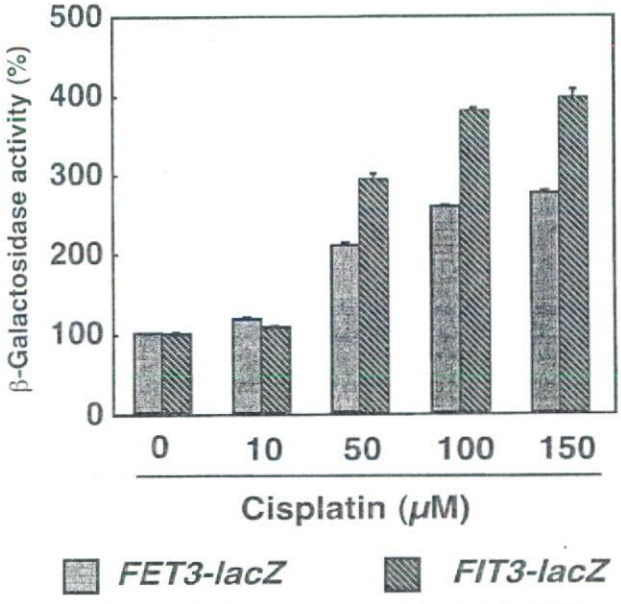

Fig. 1. *FET3* and *FIT3* promoter activation by cisplatin. Wild-type (WT) cells (BY4742) were transformed with either the *FET3-LacZ* or *FIT3-LacZ* construct. The transformed cells were incubated with 0, 10, 50, 100, or 150 μM cisplatin for 3 h and the specific activity of β-galactosidase was measured. Data are mean ± standard deviation (SD) from three independent experiments. β-galactosidase activity of a *FET3-lacZ* or *FIT3-lacZ* control is relatively indicated as 100%.

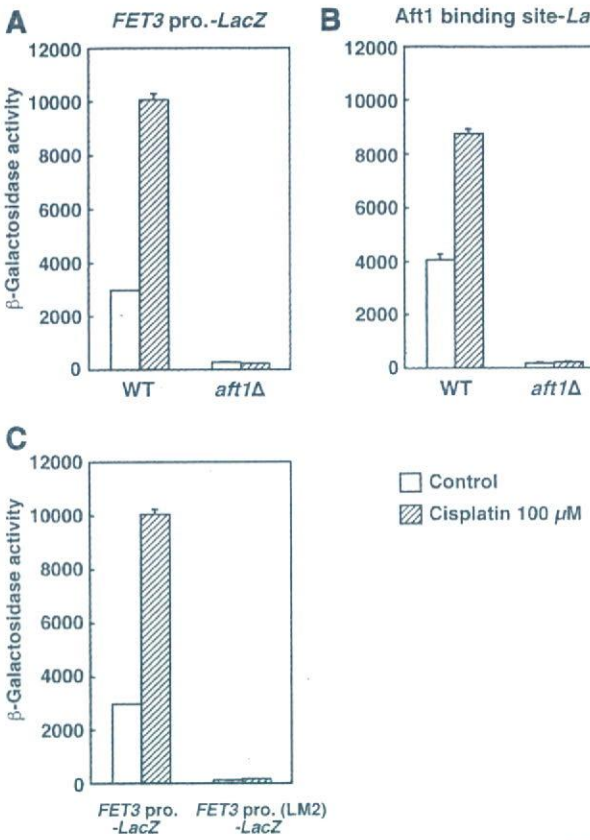

Fig. 2. Aft1 is necessary for cisplatin activation of the *FET3* promoter. WT cells (BY4742) were transformed with the *FET3-LacZ* (**A**), mutant *FET3-LacZ* (LM2) (**B**), or AFT1 binding site-*LacZ* (**C**) construct. The transformed cells were incubated with 0 or 100 μM cisplatin for 3 h and the specific activity of β-galactosidase was measured. Data are mean ± SD from three independent experiments.

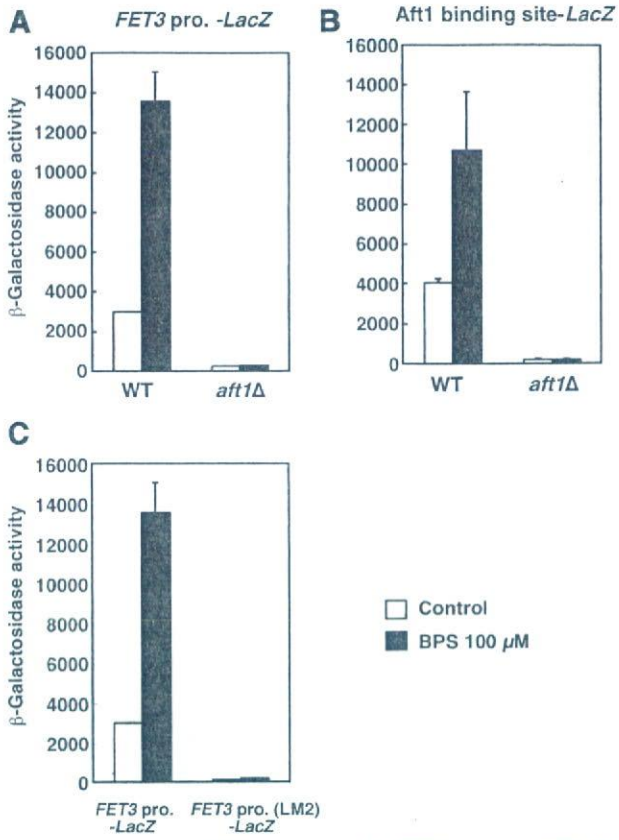

Fig. 3. Aft1 mediates BPS activation of the *FET3* promoter. WT cells (BY4742) were transformed with the *FET3-LacZ* (**A**), mutant *FET3-LacZ* (LM2) (**B**), or AFT1 binding site-*LacZ* (**C**) construct. The transformed cells were incubated with 0 or 100 μM BPS for 3 h and the specific activity of β-galactosidase was measured. Data are mean ± SD from three independent experiments.

that activate Aft1. We therefore examined Aft1 domains involved in activation by cisplatin and BPS and compared the effects of these compounds on Aft1 deletion mutants (Fig. 4A) expressed in Aft1-deleted yeast. The response of the Aft1 deletion mutants M1–M13 to both cisplatin and BPS was found to conform to a similar pattern. The *FET3* promoter could not be activated in yeasts expressing M4 (Δ97–107 a.a.), M7 (Δ271–331 a.a.), or M12 (Δ413–571 a.a.), but activation was observed following treatment with both compounds in yeasts expressing other mutants (Fig. 4B). M4 is a deletion mutant lacking the nuclear export signal (NES) (Yamaguchi-Iwai et al., 2002), while the cysteine (Cys 290) responsible for extra-nuclear transport of Aft1 is located within the domain deleted in M7 (Yamaguchi-Iwai et al., 1995). Both mutants are therefore constitutively present within the nucleus, irrespective of cisplatin or BPS treatment and, as such, are thought to be in a state of permanent transcription activation (Yamaguchi-Iwai et al., 2002). It is likely that activation of the *FET3* promoter in the M12 mutant was not observed following cisplatin and BPS treatment because of the deletion of the transcription domain (Yamaguchi-Iwai et al., 2002). These results indicate that one or more of the three Aft1 domains mutated in M4, M7, and M12 are responsible for activation of the *FET3* promoter by both cisplatin and BPS. Aft1 deletion mutants lacking the amino acids 108–157 and 208–270 were also constructed, but expression of mutated Aft1 was not observed in these yeasts under the conditions of the present study (data not shown). It is important to note that the above findings do not exclude the possibility that cisplatin augments *AFT1* transcription using mechanisms similar to those utilized by BPS.

As we believe that BPS activates Aft1 by inhibiting cellular uptake of iron, the effect of cisplatin on the uptake of ^{55}Fe(II) into yeast cells was examined. As shown in Figure 5, cisplatin inhibited iron uptake in a concentration-dependent manner. It is therefore reasonable to assume that cisplatin inhibition of iron uptake leads to activation of Aft1 and the subsequent induction of target genes involved in the maintenance of iron homeostasis.

DISCUSSION

The present study determined that expression of 16 yeast genes is accelerated by cisplatin. Among these are 14 genes that encode proteins involved in the maintenance of iron homeostasis (Table 2) and which are regulated by the transcription factor Aft1 (Yamaguchi-Iwai et al., 1995, 1996) that is activated by a decrease in intracellular iron concentrations (Yamaguchi-Iwai et al., 1995, 2002).

This study is the first to demonstrate the inhibition of cellular iron uptake by cisplatin. When cells are cultured

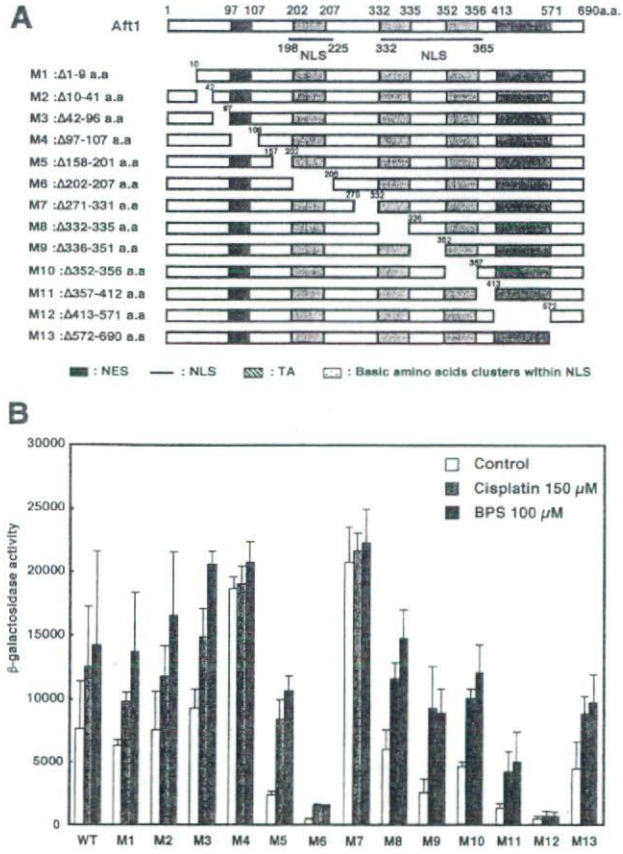

Fig. 4. Analysis of Aft1 domain requirements for cisplatin activation of the *FET3* promoter. **A**: Schematic representation of Aft1 structural domains and deletion mutants. **B**: Aft1 deletion mutants (M1–13) were introduced into WT cells carrying *FET3-LacZ*. Cells were then incubated with 150 μM cisplatin, 100 μM BPS or with no addition (control) and the specific activity of β-galactosidase was measured. Data are mean ± SD from three independent experiments.

in the presence of cisplatin, iron uptake is decreased and the expression of Aft1 target genes is activated. The toxic effects of cisplatin might be induced through its inhibition of uptake of iron, which is an essential element with an important role in oxidation–reduction reactions. Aft1 might function as a protective mechanism against cisplatin through its upregulation of genes involved in maintaining iron homeostasis such as *FET3, FET 4*, and *FTR 1* (Askwith et al., 1994; Dix et al., 1994). Indeed, yeast deficient in *AFT1* shows an extremely high sensitivity to the toxic effects of cisplatin (data not shown). Moreover, we have found that treatment of yeast with Fe(II) provided protection against toxicity of cisplatin (unpublished data). These facts suggest that activity of Aft1 and expression of its target genes might be involved in acquisition of resistance for cisplatin by acceleration of iron uptake.

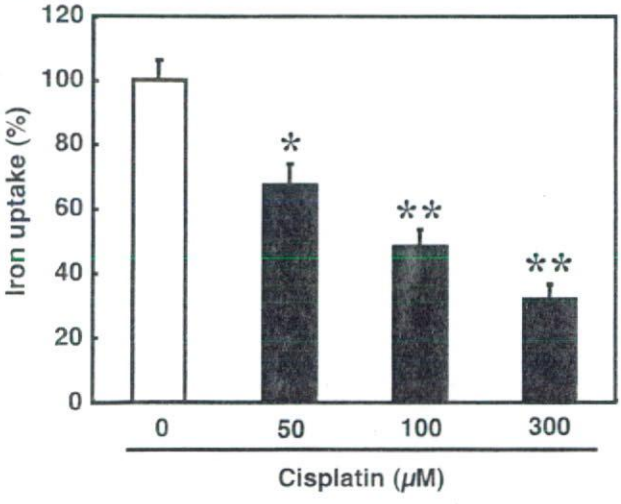

Fig. 5. Effect of cisplatin treatment on iron uptake. WT cells (BY4742) were incubated in assay buffer containing 1 mM $^{55}FeCl_3$ and ascorbate with 0, 50, 100, or 300 μM cisplatin for 2 h. Iron uptake by the cells was measured as described in the Materials and Method. *,**Significantly different from the control ($^{*}P < 0.005$, $^{**}P < 0.001$, Student's *t*-test).

The human transcription factors Irp1 and Irp2 exert concentration-dependent regulatory effects on the expression of factors involved in the maintenance of iron homeostasis, in a similar way to yeast Aft1 (Pantopoulos, 2004). As such, it would be of interest to examine whether human cells exhibit comparable responses to cisplatin to those observed in yeast in the present study.

Although the mechanisms by which cisplatin inhibits cellular uptake of iron are unclear, there are several possible theories. The first is the direct inhibition of proteins involved in iron uptake, as cisplatin is known to bind to various intracellular factors including DNA (Bose, 2002). A second possibility involves the indirect inhibition of copper uptake. It has recently been reported that cisplatin inhibits the activity of Ctrl, a transporter of copper in budding yeasts and human cells (Ishida et al., 2002; Holzer et al., 2004; Safaei and Howell, 2005). The copper oxidase Fet3, which forms a complex with Ftr 1 and is required for iron uptake in yeast, cannot maintain its activity in the absence of copper (De Freitas et al., 2003). It is conceivable that cisplatin inhibition of copper uptake leads to Fet3 inhibition and a subsequent decrease in the amount of iron taken up via the Fet3–Ftr l complex. However, as an alternative study reports that cisplatin does not influence intracellular copper levels in yeasts (Ohashi et al., 2003), further investigation is required into the effects of cisplatin on cellular copper uptake.

Previous microarray studies have been carried out into the effects of cisplatin on the expression of genes in budding yeasts (Birrell et al., 2002; Gatti et al., 2004). Interestingly, these studies did not find any effect on genes involved in the maintenance of iron homeostasis. Earlier investigators cultured yeast on YPAD medium, which is rich in nutrients, while in the present study, we used the synthetic minimum medium, SD medium. The iron concentration of the YPAD medium is higher than the SD medium, so it is possible that cellular concentration of iron did not decrease sufficiently to activate Aft1 in yeasts cultured in YPAD medium, even when iron uptake was inhibited by cisplatin. As an extension of this hypothesis, we speculate that the iron concentration of serum might influence the anticancer effects of cisplatin when used in chemotherapy for the treatment of cancers.

ACKNOWLEDGMENTS

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

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—Reviews—

メチル水銀に対する感受性決定因子としてのユビキチン・プロテアソームシステム

黄　基旭

A Ubiquitin-proteasome System as a Factor that Determine the Sensitivity to Methylmercury

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(Received November 27, 2006)

To elucidate the mechanism of toxicity of methylmercury (MeHg), we searched for factors that determine the sensitivity of yeast cells to MeHg and found that overexpression of Cdc34 or Rad23, both proteins related to the ubiquitin-proteasome (UP) system, induces resistance to MeHg toxicity. The acquisition of resistance to MeHg in Cdc34-overexpressing yeast cells requires the ubiquitin-conjugating activity of Cdc34 and the proteolytic activity of proteasomes. Therefore, it seems likely that certain as-yet-unidentified proteins that increase MeHg toxicity might exist in cells and that the toxicity of MeHg might be reduced by the enhanced degradation of such proteins through the UP system when Cdc34 is overexpressed. Unlike Cdc34, Rad23 suppresses the degradation of ubiquitinated proteins by proteasomes. This activity of Rad23 might be involved in the acquisition of resistance to MeHg toxicity when Rad23 is overexpressed. Overexpression of Rad23 might induce resistance to MeHg by suppressing the degradation of proteins that reduce the MeHg toxicity. Moreover, when we overexpressed Cdc34 in normal and Rad23-defective yeasts, resistance to MeHg was enhanced to almost the same extent in both lines of yeast cells. Thus it is possible that the binding of Rad23 to ubiquitinated proteins might be regulated by a mechanism that involves the recognition of substrate proteins and that the functions of Rad23 might not affect the protein-degradation system in which Cdc34 is involved. Many proteins that reduce or enhance MeHg toxicity and are ubiquitinated might exist in cells. The UP system and related proteins might determine the extent of MeHg toxicity by regulating the cellular concentrations of these various proteins.

Key words——methylmercury; toxicity; ubiquitin; proteasome; Cdc34; Rad23

1. はじめに

メチル水銀は主に中枢神経毒性を示す環境汚染物質であり，水俣病の原因物質としてもよく知られている．ヒトでのメチル水銀中毒はこれまでに水俣以外にも多くの国・地域で認められている．メチル水銀は食物連鎖によって魚類中に濃縮されるが，近年，妊娠中の女性が魚類を多く摂取することによって，未発達な胎児の脳にメチル水銀が影響を与える可能性が指摘され，世界的な社会問題ともなっている．しかし，メチル水銀による毒性発現機構及びそれに対する生体の防御機構はいまだほとんど解明されていない．

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本総説は，日本薬学会第 126 年会シンポジウム S16 で発表したものを中心に記述したものである．

これまでにわれわれは，メチル水銀毒性に対する防御機構を明らかにするために，真核単細胞生物であり，遺伝子産物の多くがヒトなどの哺乳動物と機能的に共通している出芽酵母（*Saccharomyces cerevisiae*）を用いてメチル水銀耐性獲得に係わる遺伝子の検索を行い，Bop3,[1] Cdc34,[2] GFAT[3] 及び Rad23[4] などを同定することに成功している．その中で，Cdc34 はユビキチン・プロテアソーム（UP）システムに係わるユビキチン転移酵素の一種であり，Rad23 も UP システム関連因子として知られている．UP システムは真核生物に広く保存されている蛋白質分解経路で，ユビキチン活性化酵素（E1），ユビキチン転移酵素（E2）及びユビキチンリガーゼ（E3）という 3 つの酵素の連続した働きによって細胞内で蛋白質にユビキチンを連結する．そして，ここでユビキチン化された蛋白質は，最終的にプロテアソームによって認識されて分解される

(Fig. 1).[5] 本稿では，選択的な蛋白質の分解経路であるUPシステムがメチル水銀毒性の発現機構において果たす役割について，筆者らの研究成果を中心に概説する．

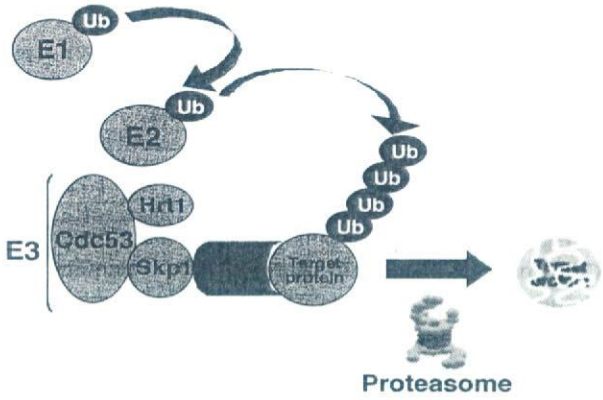

Fig. 1. Model for the SCF Complex-mediated Proteasomal Degradation

Proteins are targeted for degradation by the proteasome through the covalent attachment of ubiquitin (Ub) moieties. Ub activated by the ubiquitin-activating enzyme (E1) is transferred from E1 *via* ubiquitin-conjugating enzyme (E2) to the target protein. This final step is catalysed by ubiquitin ligase (E3). Multi-ubiquitinated proteins are degraded by the proteasome. Specific target recognition is the function of SCF complex, an E3. The complex is composed of a common core (Cdc53, Hrt1 and Skp1) and an F-box protein.

2. UPシステムを介した蛋白質分解の亢進によるメチル水銀の毒性軽減

Cdc34の構造中でメチル水銀耐性獲得に必要な機能ドメインを検索するために，E2活性に関与することが報告されている数ヵ所のドメインに変異を有するCdc34[6,7]をそれぞれ高発現する酵母を作製したところ，これらの酵母においてはメチル水銀に対する耐性は認められなかった（Fig. 2b）.[8] また，正常なCdc34を高発現させた酵母では総ユビキチン化蛋白質量の増加が認められたが，変異Cdc34を高発現させた酵母ではこのような現象も認められなかった（Fig. 2c）.[8] これらの結果は，Cdc34高発現によるメチル水銀耐性にCdc34が示すユビキチン転移活性が必須であることを示している．

Cdc34を介する蛋白質のユビキチン化には，E1であるUba1及びE3複合体の1つであるSCF（Skp1, Cdc53/cullin, F-box protein）が関与し，このE3複合体は4つのsubunit（Cdc53, Skp1, Hrt1, F-box protein）からなることが知られている（Fig. 1）.[9] しかし，この酵素群の各構成因子を高発現させてもE1高発現酵母でわずかなメチル水銀耐性が認められたものの，Cdc34高発現時に認められたよ

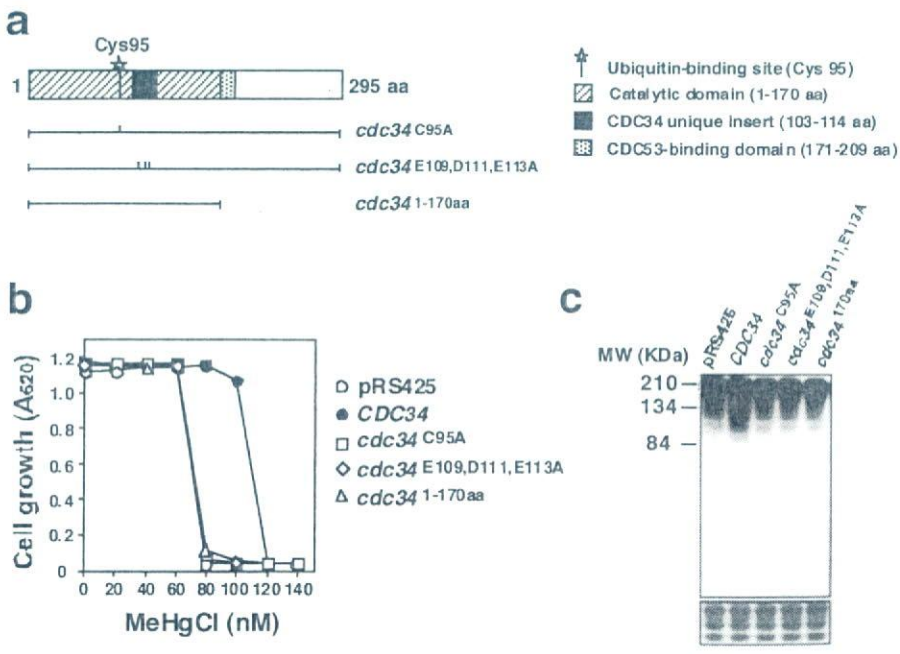

Fig. 2. Effects of the Overexpression of Cdc34 on the Sensitivity of Yeast Cells to Methylmercury

a: Structural domains of Cdc34 and construction of mutant proteins. b: Yeast strains carrying pRS425 (control), pRS425-*CDC34*, pRS425-*cdc34*^{C95A}, pRS425-*cdc34*$^{E109, D111, E113A}$ or pRS425-*cdc34*$^{1-170aa}$ were grown in SD (-leucine) medium in the presence of various concentrations of methylmercury. c: Lysates of each strain of yeast cells, cultured in control medium, were subjected to immunoblotting analysis with multiubiquitin-specific antibody. Staining with coomassie blue (lower panel) is shown as an indication of the amount of total protein loaded.

うな顕著な耐性は認められなかった．また，これら酵母の総ユビキチン化蛋白質量も正常酵母と同程度であった．[8]

一方，E2は遺伝子ファミリーを形成しており，出芽酵母ではメチル水銀耐性因子として見出したCdc34以外にも，12個のE2分子種が同定されている．[10] そこで，これらのうちのいくつかのE2の分子種を高発現する酵母を作製したところ，Cdc34を高発現させた酵母よりもその程度は劣るものの，Ubc4, 5又は7を高発現させた酵母もメチル水銀耐性が示し，これら酵母内のユビキチン化蛋白質量も正常酵母に比べて高値を示した．[8] ここで認められた耐性度の差は，恐らく各E2分子種の基質特異性の違いによるものと考えられる．以上の結果から，E2はユビキチン化反応の律速酵素であり，本酵素群の高発現は細胞内における蛋白質のユビキチン化を促進させることによってメチル水銀毒性に対して防御的に作用するものと考えられる．

ユビキチン化された標的蛋白質は最終的にプロテアソームに認識されて速やかに分解される．Cdc34高発現によるメチル水銀耐性はプロテアソーム阻害剤存在下では認められず（Fig. 3），また，遺伝子変異によって低プロテアソーム活性を示す酵母が対照酵母に比べて高いメチル水銀感受性を示すことも確認されたことから，Cdc34高発現によるメチル水銀耐性にはプロテアソームによるユビキチン化蛋白質の分解が必須であると考えられる．

以上の結果から，細胞内にはメチル水銀毒性の増強に関与し，かつ，UPシステムによって分解される蛋白質が存在し，Cdc34はこの蛋白質のプロテアソームでの分解を亢進させることによってメチル水銀毒性に対して防御的に作用するものと考えられる．[9]

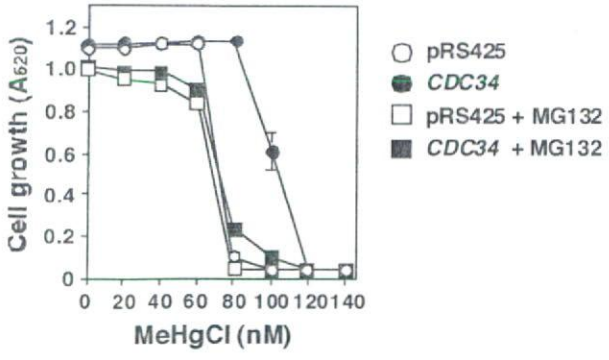

Fig. 3. Effects of a Proteasome Inhibitor on the Cdc34-mediated Resistance of Yeast Cells to Methylmercury

Yeast *erg6* cells that harbored pRS425 (control) or pRS425-*CDC34* were grown in SD (-uracil) liquid medium, with or without the proteasome inhibitor MG132 (50 μM), which had been dissolved in DMSO, and methylmercury at the indicated concentration.

一方，ヒトなどの高等動物においても多くのE2分子種が存在することが知られている．ヒトのCdc34を高発現させたHEK293細胞もメチル水銀に対して耐性を示すことから，ヒトにおいてもCdc34が関与するUPシステムがメチル水銀の毒性軽減機構として重要な役割を果たしている可能性が考えられる．したがって，メチル水銀毒性を増強させる蛋白質をヒト細胞中で同定することによって，ヒトにおけるメチル水銀の細胞内標的分子が明らかになるものと期待される．

3. メチル水銀の毒性軽減に係わるF-box蛋白質の検索

SCF複合体（E3）を構成する因子の中には，分解される基質蛋白質と直接結合するF-box蛋白質が存在し，酵母では17種類が知られている．[11] このF-box蛋白質を酵母に高発現させると，ユビキチン化される標的蛋白質とF-box蛋白質を介したSCF複合体との結合割合が増加し，標的蛋白質のユビキチン化とそれに続くプロテアソームでの分解が促進されると考えられる．したがって，メチル水銀毒性を増強させる蛋白質の分解に関与するF-box蛋白質が高発現すると，その酵母はメチル水銀に対して耐性を示すと予想される．そこで，17種のF-box蛋白質をそれぞれ高発現する酵母を作製したところ，Hrt3又はYlr224wの高発現酵母が対照酵母に比べて強いメチル水銀耐性を示した．[12] F-boxドメインを欠失させた両F-box蛋白質の変異体を高発現させた酵母はメチル水銀耐性を示さず，また，プロテアソーム阻害剤の存在下では，Hrt3又はYlr224wの高発現酵母が示すメチル水銀耐性が認められないことから（Figs. 4(a), (b)），[12] Hrt3及びYlr224w高発現による酵母のメチル水銀耐性獲得にはF-boxドメインを介したSCF複合体の形成とユビキチン化された蛋白質のプロテアソームでの分解が必要であると考えられる．

以上のことから，Cdc34又はF-box蛋白質が高発現すると，F-box蛋白質（Hrt3又はYlr224w）が認識する蛋白質のユビキチン化の亢進によってそれ

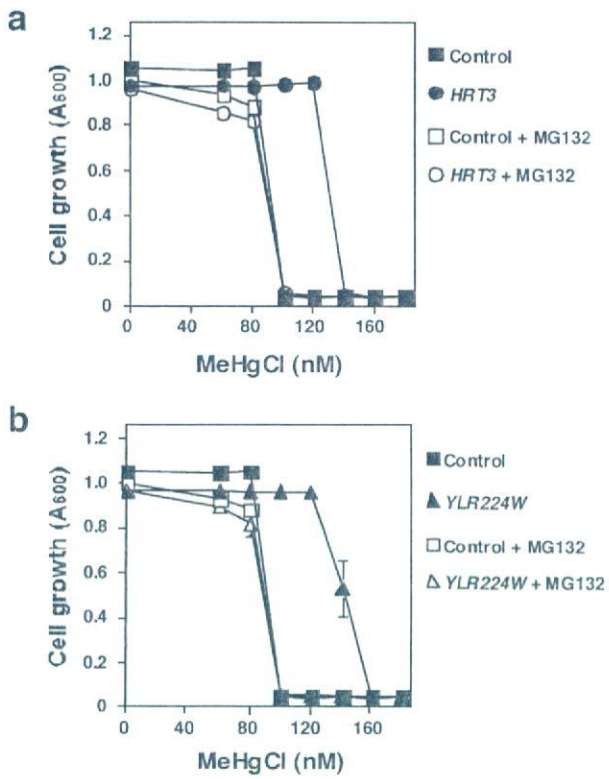

Fig. 4. Effects of a Proteasome Inhibitor on the Hrt3- and Ylr224w-mediated Resistance of Yeast Cells to Methylmercury

Yeast *erg6Δ* cells that harbored pKT10 or pKT10-*HRT3* (a) or pKT10 or pKT10-*YLR224W* (b) were grown in SD (-uracil) liquid medium, with or without the proteasome inhibitor MG132 (50 μM), which had been dissolved in DMSO, and methylmercury at the indicated concentration.

ら蛋白質のプロテアソームでの分解が促進され，その結果としてメチル水銀毒性が軽減されると考えられる．両 F-box 蛋白質の基質となる蛋白質は同定されていないが，両 F-box 蛋白質の基質蛋白質の中にメチル水銀毒性の増強に係わる蛋白質が含まれる可能性が高い．最近，われわれは両 F-box 蛋白質と特異的に結合し，かつ，メチル水銀の毒性増強に係わる蛋白質の同定に成功している．今後，これらの蛋白質がメチル水銀毒性の発現に果たす役割を検討することで，まだ不明な点が多いメチル水銀毒性発現機構の解明に重要な手掛かりが得られるものと期待される．

4. UP システムを介した蛋白質分解の抑制によるメチル水銀の毒性軽減

われわれは，高発現によって酵母にメチル水銀耐性を与える Cdc34 以外の UP システム関連因子として Rad23 を見出した．[4] Rad23 は UP システムによる蛋白質分解の促進及び抑制という相反する 2 つの機能によって UP システムによる蛋白質分解を調節していると考えられている．[13] Rad23 が示す蛋白質分解抑制作用は，Rad23 が有する 2 つの ubiquitin-associated（UBA）ドメインによるものであり，このドメインを介して Rad23 はユビキチン化蛋白質のユビキチン部分と結合し，それ以上ユビキチン鎖が伸長するのを阻害することによってその蛋白質の分解を抑制する．[14] 一方，Rad23 はユビキチン化蛋白質をプロテアソームに運搬する機能を有しており，これによって蛋白質の分解を促進させる．[15] この機能に必要な Rad23 中の領域は N 末端に存在する ubiquitin-like（UbL）ドメインであり，このドメインはユビキチンと相同性が高く，Rad23 はこのドメインを介してプロテアソームと結合することによってユビキチン化蛋白質の分解を亢進する役割を果たすと考えられている．[16]

Rad23 が有する 2 つの機能とメチル水銀毒性との関係を明らかにするために，Rad23 の UbL 及び 2 つの UBA ドメインをそれぞれ欠失させた truncation mutants を作製したところ，UbL ドメインを欠失した Rad23 を高発現させた酵母（ΔUbL）は，正常な Rad23 を高発現させた酵母よりも強いメチル水銀耐性を示し，両 UBA ドメインを欠失させた Rad23 を高発現させた酵母（ΔUBA1＋ΔUBA2）はメチル水銀耐性をほとんど示さなかった（Fig. 5(b)）．[4] すなわち，Rad23 の UbL ドメインはメチル水銀毒性を増強させる作用を有し，逆に UBA ドメインはメチル水銀耐性に係わっていると考えられる．正常な Rad23 を高発現させた酵母はメチル水銀耐性を示すことから，これらの結果は，少なくとも Rad23 高発現時には UbL ドメインに由来する機能よりも UBA ドメインに由来する機能の方が優位に作用していることを示している．また，正常な Rad23 を高発現させた酵母では総ユビキチン化蛋白質量の顕著な増加が認められたが，UbL ドメインを欠失した Rad23 を高発現させた際には総ユビキチン化蛋白質量がさらに著しく増加した．[4] 一方，両 UBA ドメインを欠失した Rad23 を高発現させた際には，総ユビキチン化蛋白質量の顕著な減少が認められた．[4] この結果は，Rad23 が有する UbL ドメインはユビキチン化蛋白質の細胞内濃度を減少させ，UBA ドメインは逆に増加させる機能を担っていることを示している．