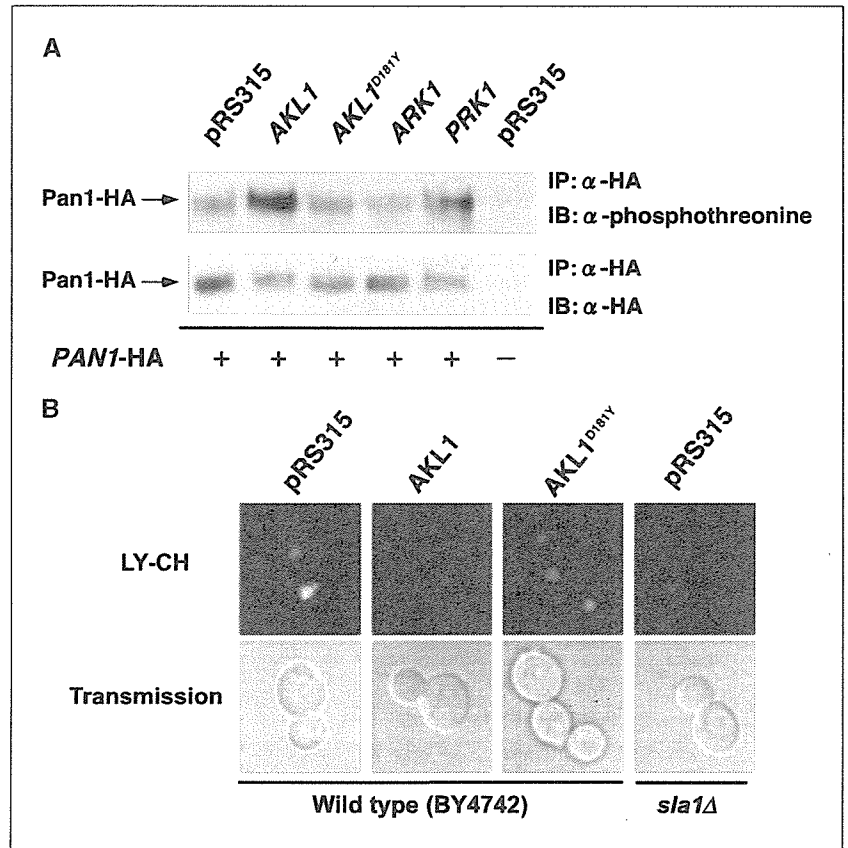


Figure 4. Effects of overexpression of Akl1 on the phosphorylation of Pan1-HA and the endocytic uptake of lucifer yellow. *A*, yeast (BY4742) cells expressing Pan1 tagged with HA at the COOH terminus (Pan1-HA) were transformed with pRS315, pRS315-*AKL1*, pRS315-*ARK1*, pRS315-*PRK1*, or pRS315-*AKL1D181Y*. 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. 4A). 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. 4B), 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. 4B). 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 NH₂ terminus (HA-AAK1) and introduced it into HEK293 cells (Fig. 6A). The resultant AAK1-overexpressing HEK293 cells were found, as anticipated, to be resistant to Adriamycin (Fig. 6B). We confirmed that overexpression of AAK1 induced resistance to Adriamycin not

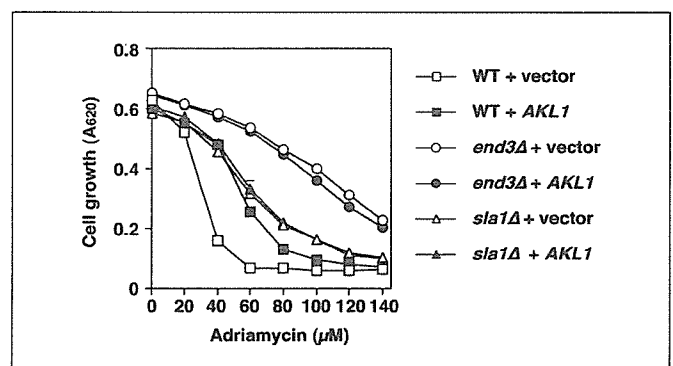


Figure 5. Relationship between Akl1 and the Sla1/End3/Pan1 complex in the acquisition of Adriamycin resistance. Yeast strains (BY4742, *sla1 Δ* , and *end3 Δ*) harboring pRS315 or pRS315-*AKL1* were grown in synthetic dextrose (-Leu) medium that contained Adriamycin. After incubation for 24 hours at 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.

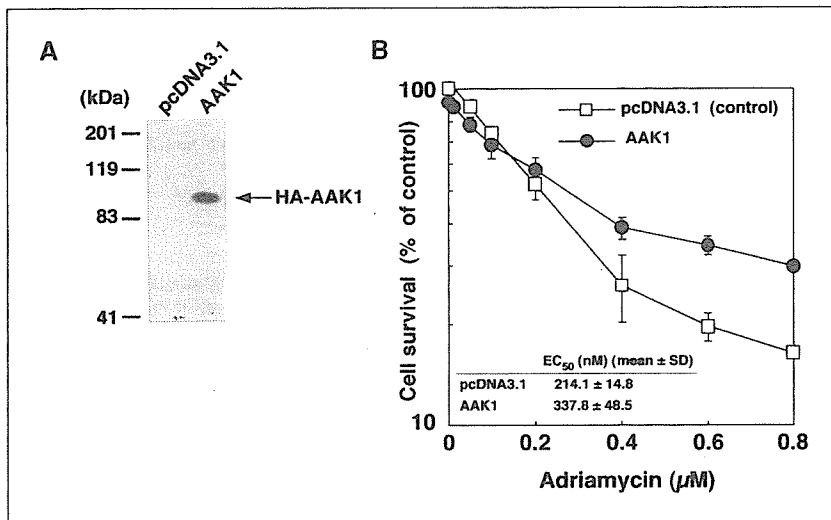


Figure 6. Effects of overexpression of AAK1, a member of the human Ark/Prk kinase family, on the sensitivity of HEK293 cells to Adriamycin. *A*, immunoblotting analysis of HA-tagged AAK1. HEK293 cells were transfected with pcDNA3.1-HA-AAK1 or the pcDNA3.1 empty vector. Each lane was loaded with whole-cell 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. EC₅₀ 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 NH₂-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. 2A). 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 Asp¹⁸¹ is essential for the kinase activity (Fig. 4A). 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, AP2 μ (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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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 ⁵⁵Fe(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 (*MAT α* ; *his3 Δ 1*; *leu2 Δ 0*; *lys2 Δ 0*; *ura3 Δ 0*) and *aft1 Δ* (*MAT α* ; *his3 Δ 1*; *leu2 Δ 0*; *lys2 Δ 0*; *ura3 Δ 0*; *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'-TCGACGAGCACCTGCAATGGGTGCACCTTTGAAG-3', and Aft1 binding site-R, 5'-TCGACTTCAAAAAGTGCACCCATTTGCAGGTGCTCG-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 *aft1 Δ* 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 μ M cisplatin (Nippon Kayaku, Tokyo, Japan). The suspension was centrifuged at 2,200g 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 μ g total RNA using the OligotexTM-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 SuperScriptTM 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 \times 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*TM (TaKaRa, Shiga, Japan). Reverse transcribed mRNA was diluted 10-fold and 0.5 μ l 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 *aft1 Δ* 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 2,200g for 5 min to remove the supernatant. Cells were resuspended in 25 μ l 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 μ l Z buffer (10 mM KCl, 60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 1 mM MgSO₄) containing 0.5 mg/ml methyl umbelliferyl β -D galactopyranoside (MUG) at 30°C for 5 min. The absorbance of MUG at λ_{ex} 360 nm λ_{em} 450 nm was measured. After adjusting cell density (OD₆₀₀) 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 (OD₆₀₀ = 0.5) were collected by centrifugation and resuspended in ice-cold assay buffer (5% glucose, 50 mM sodium citrate, pH 6.5) to OD₆₀₀ = 5. Then 1 μ M ⁵⁵FeCl₃ (Perkin Elmer, Wellesley, MA, 74 MBq/ml) and 1 μ M 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 μ M 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	<i>AFT1</i>
AFT1-R	GATCGATATCGCCATCTTCTGGCTTCACAT	
ARN1-F	ACCCTCTATAGAATTGGTTCT	<i>ARN1</i>
ARN1-R	CGACATATTGCCATCCTC	
ARN2-F	GGCTAACGTTGTTCTTGT	<i>ARN2</i>
ARN2-R	CCTGTAATTCCTTCCATTCCA	
ARN3-F	GTAACCTCATTATACCTTGC	<i>ARN3</i>
ARN3-R	CCTATCTTACTGCTTGTA	
CCC2-F	GAGAGGACTGGCCAACG	<i>CCC2</i>
CCC2-R	CGGCCTGTGATCTATGTC	
CCP1-F	CGCTGACTATGTCAGAACA	<i>CCP1</i>
CCP1-R	CCTGTTCCTCTAAAGTC	
FET3-F	GCAAGGTTTGGGTCCTTGTTC	<i>FET3</i>
FET3-R	GATGCTTTTTCAGTGGAAATGAC	
FET3 promoter-F	GCAAGCTTTTCCGGGTGCGAATCAG	<i>FET3</i>
FET3 promoter-R	CGGGATCCAAACATCTAGTTCTAATTTTTTGC	
FET4-F	GGTGGTTGATATCGGTACA	<i>FET4</i>
FET4-R	CCCAATTATGTCTTGCAAC	
FIT1-F	TCTAAATCCACGTCTGCACT	<i>FIT1</i>
FIT1-R	CGGTAGTGGTTGAACTCTTG	
FIT2-F	ACAGTTATGACTGCCGCTCG	<i>FIT2</i>
FIT2-R	ACACTTGCTCCTTGGAATGCA	
FIT3-F	TTTTGTCTGGACTGGTGAAGG	<i>FIT3</i>
FIT3-R	CAGCACCCATCAAACCGTA	
FIT3 promoter-F	GCAAGCTTCTCCATAAACATTTCTTTTGC	<i>FIT3</i>
FIT3 promoter-R	CGGGATCCAAACATTTTAGGGATTATTGTTATTAG	
FRE1-F	GCTATTTATCCGCACCTTGTTC	<i>FRE1</i>
FRE1-R	CTTCTCTCTTAGTTCAACG	
FRE2-F	GGCTTATAAGCCGGAGTTG	<i>FRE2</i>
FRE2-R	GCATTGATACTCTTCAAAGTA	
FRE3-F	CGTACTCGAGGCTTACAAG	<i>FRE3</i>
FRE3-R	CTTCAAAGTATTCGATTGCC	
FTR1-F	GTGCTTCGAAATCCTCGCTG	<i>FTR1</i>
FTR1-R	CTCTTTTGTCTTCCGTCAAC	
HMX1-F	CCCCTGAAGAAACACGCTT	<i>HMX1</i>
HMX1-R	GTGCTCTTCTAGTAGCAGAATCC	
HSP30-F	GGTGTGATATGCCAACGTC	<i>HSP30</i>
HSP30-R	CAGGTTCCGGGTTCTGTGG	
LM2-F	GGCCCATCTTCAAAGTGCAGGGATTGCAGGTGCTC	<i>LM2</i>
LM2-R	GAGCACCTGCAAAATCCCTGCACCTTTGAAGATGGGCC	
TIS11-F	GCAACAGTTGTCTCAACA	<i>TIS11</i>
TIS11-R	GGTCATTCTCTGCAAAGC	
YHL035C-F	GAGGAACGCTTTAACAGCA	<i>YHL035C</i>
YHL035C-R	CATCTTACTTGATTGCTTGG	
YMR251W-F	CAGAGTTCAACTCTTGGGTG	<i>YMR251W</i>
YMR251W-R	GTGGGGTGATCCCAATC	
YPR123C-F	CCATATCCATCCCTGAAGA	<i>YPR123C</i>
YPR123C-R	CAITTTGTTGGAAGAATCGAAT	
M1-F	GCGGTACCGAACATGCGTACCGGATTAATTCATCTGACAGC	<i>M1</i>
M1-R	CGGGTACCCATTGTCGTAGATTTTTCTGTTATTTTTTTGTT	
M2-F	GCGGTACCGAGGGTCTGCAAGTCAAGTGG	<i>M2</i>
M2-R	CGGGTACCTATGTCAGCCGGATTGAAGCCTTCC	
M3-F	GCGGTACCAACAATTTGATTTCATCTGGATCCAGTACCCAAC	<i>M3</i>
M3-R	CGGGTACCATTAATGTTGACTACATATTCAGTACACTTTTGGG	
M4-F	GCGGTACCTTTGAAGATAAGTCCGATATTAAGCCTTGG	<i>M4</i>
M4-R	CGGGTACCTTGATTTCAGCTTTTCCACATGATGAATATGC	
M5-F	GCGGTACCAAGCCCAAGAAAAAAGATGTGTATCGAGG	<i>M5</i>
M5-R	CGGGTACCTTTTCTTTTCTCTCGGTTCTTCCCTCC	
M6-F	GCGGTACCTGTGTATCGAGGTTAATAACTGTCCG	<i>M6</i>
M6-R	CGGGTACCTATGGAGGATGTTTGTATCGGGCG	
M7-F	GCGGTACCTCTAAAGGCCATGCTTACCTCTG	<i>M7</i>
M7-R	CGGGTACCTTTCTTGTATGTCATCTACGTCAATATCC	
M8-F	GCGGTACCTGCTTACCCTCTGTAATAACACCGG	<i>M8</i>
M8-R	CGGGTACCTGCAATCGATGAATCAAGGGATATGGACG	
M9-F	GCGGTACCAAGAAACCGAAAAGCCAGTGTAAAGATAAAG	<i>M9</i>
M9-R	CGGGTACCTGGCCTTTAGATGCATTTCGATGAATCAAG	
M10-F	GCGGTACCCAGTGTAAAGATAAAGACACACTCTTAAAAAG	<i>M10</i>
M10-R	CGGGTACCTACGTTATTGGTATTGATACTACCGGTG	
M11-F	GCGGTACCCCGGCATCCGATTTCAAGCTAAAC	<i>M11</i>
M11-R	CGGGTACCGCTTTTCCGTTTCTTACGTTATTGG	
M12-F	GCGGTACCAAGAAGCTTTAGTTGGCAGCTCTTC	<i>M12</i>
M12-R	CGGGTACCCAGTGGCAAGATTTCAATTCAAATTGAAAGG	

^aBold letters indicate *KpnI* 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	
<i>FIT2</i> ^a	<i>YOR382W</i>	49.3	19.1	Cell wall protein involved in iron uptake
<i>FIT3</i> ^a	<i>YOR383C</i>	30.6	10.9	Cell wall protein involved in iron uptake
<i>ARN2</i> ^a	<i>YHL047C</i>	9.2	7.9	Siderophore transporter
<i>FIT1</i> ^a	<i>YDR534C</i>	8.6	1.1	Cell wall protein involved in iron uptake
<i>ARN3</i> ^a	<i>YEL065W</i>	5.8	3.8	Siderophore transporter
<i>FRE2</i> ^a	<i>YKL220C</i>	4.8	4.5	Ferric and cupric reductase
<i>HMX1</i> ^a	<i>YLR205C</i>	4.7	3.0	Heme-binding protein
<i>ARN1</i> ^a	<i>YHL040C</i>	4.3	2.9	Siderophore transporter
<i>FRE1</i> ^a	<i>YLR214W</i>	4.2	3.1	Ferric and cupric reductase
<i>FRE3</i> ^a	<i>YOR381W</i>	3.2	1.9	Ferric reductase
<i>FET3</i> ^a	<i>YMR053W</i>	3.0	2.5	High-affinity Fe(II) transporter
<i>FTR1</i> ^a	<i>YER145C</i>	2.9	2.2	Iron permease
<i>FET4</i> ^a	<i>YMR319C</i>	2.2	2.5	Low-affinity Fe(II) transporter
<i>CCC2</i> ^a	<i>YDR270W</i>	2.5	2.3	Copper transporter
<i>TIS11</i> ^a	<i>YLR136C</i>	6.8	19.5	Protein of the CCCH zinc finger family
<i>VMR1</i>	<i>YHL035C</i>	2.0	2.0	Unknown
	<i>YMR251W</i>	4.5	3.9	Unknown
	<i>YPR123C</i>	2.3	1.0	Unknown
<i>HSP30</i>	<i>YCR021C</i>	0.3	0.8	Heat shock protein
<i>CCP1</i>	<i>YKR066C</i>	0.5	ND	Cytochrome-c peroxidase

ND, not detected.

^aGenes 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 \rightarrow 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

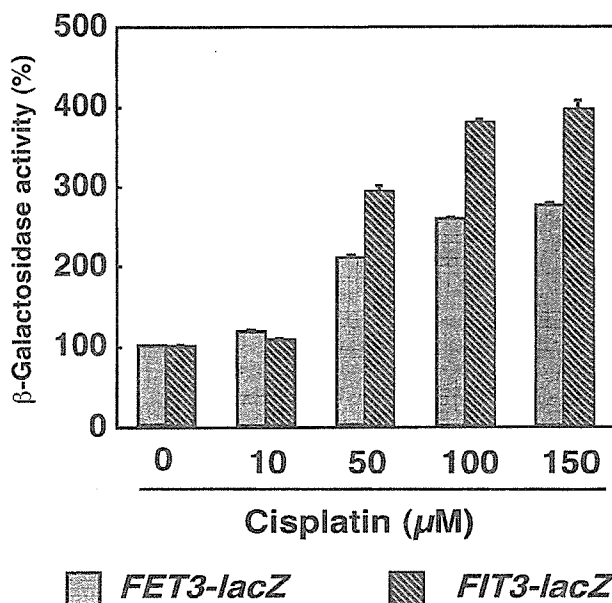


Fig. 1. *FET3* and *FIT3* promoter activation by cisplatin. Wild-type (WT) cells (BY4742) were transformed with either the *FET3-LacZ* or *FIT3-LacZ* construct. The transformed cells were incubated with 0, 10, 50, 100, or 150 μ M cisplatin for 3 h and the specific activity of β -galactosidase was measured.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

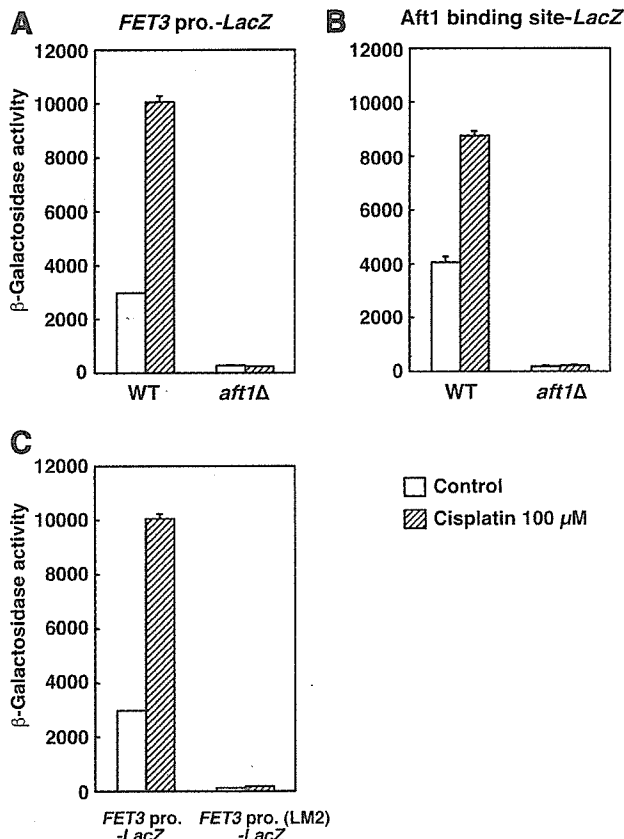


Fig. 2. Aft1 is necessary for cisplatin activation of the *FET3* promoter. WT cells (BY4742) were transformed with the *FET3*-LacZ (A), mutant *FET3*-LacZ (LM2) (B), or AFT1 binding site-LacZ (C) construct. The transformed cells were incubated with 0 or 100 μ M cisplatin for 3 h and the specific activity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

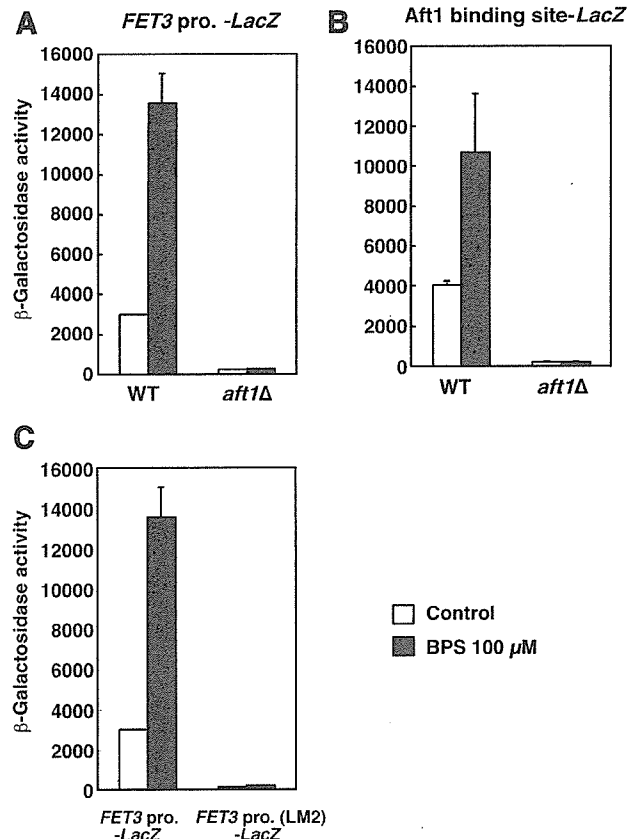


Fig. 3. Aft1 mediates BPS activation of the *FET3* promoter. WT cells (BY4742) were transformed with the *FET3*-LacZ (A), mutant *FET3*-LacZ (LM2) (B), or AFT1 binding site-LacZ (C) construct. The transformed cells were incubated with 0 or 100 μ M BPS for 3 h and the specific activity of β -galactosidase was measured. Data are mean \pm 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 (Δ 97–107 a.a.), M7 (Δ 271–331 a.a.), or M12 (Δ 413–571 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 $^{55}\text{Fe(II)}$ 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

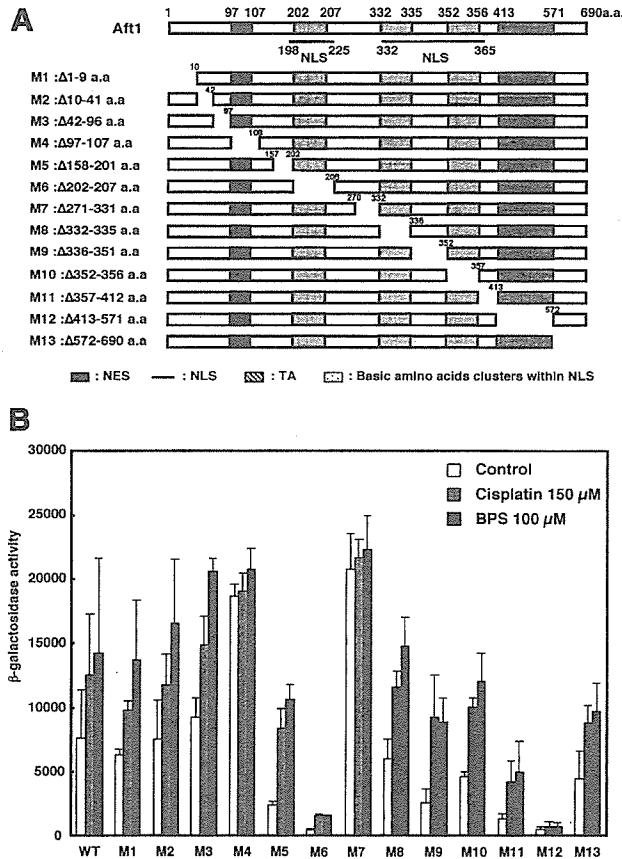


Fig. 4. Analysis of Aft1 domain requirements for cisplatin activation of the *FET3* promoter. **A**: Schematic representation of Aft1 structural domains and deletion mutants. **B**: Aft1 deletion mutants (M1–13) were introduced into WT cells carrying *FET3-LacZ*. Cells were then incubated with 150 μM 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

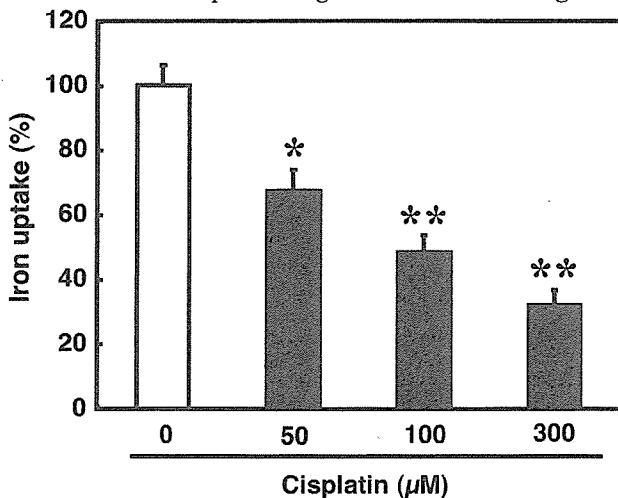


Fig. 5. Effect of cisplatin treatment on iron uptake. WT cells (BY4742) were incubated in assay buffer containing 1 mM $^{55}\text{FeCl}_3$ and ascorbate with 0, 50, 100, or 300 μM cisplatin for 2 h. Iron uptake by the cells was measured as described in the Materials and Method. *, ** Significantly different from the control ($P < 0.005$, $P < 0.001$, Student's *t*-test).

Journal of Cellular Physiology DOI 10.1002/jcp

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*, *FET4*, and *FTR1* (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.

The human transcription factors Irf1 and Irf2 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 1 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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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

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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。

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⁾ GFAT³⁾ 及び Rad23⁴⁾ 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

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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。

(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。

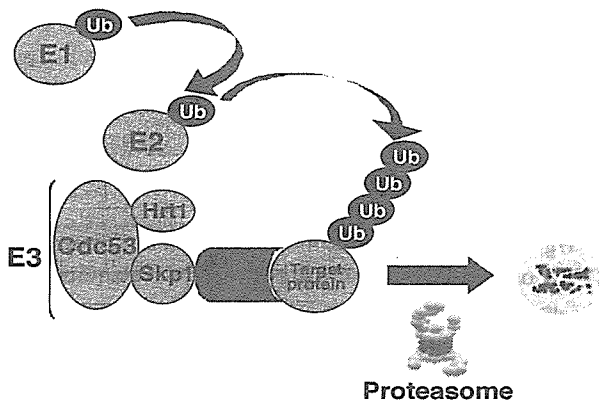


Fig. 1. Model for the SCF Complex-mediated Proteasomal Degradation

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カ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

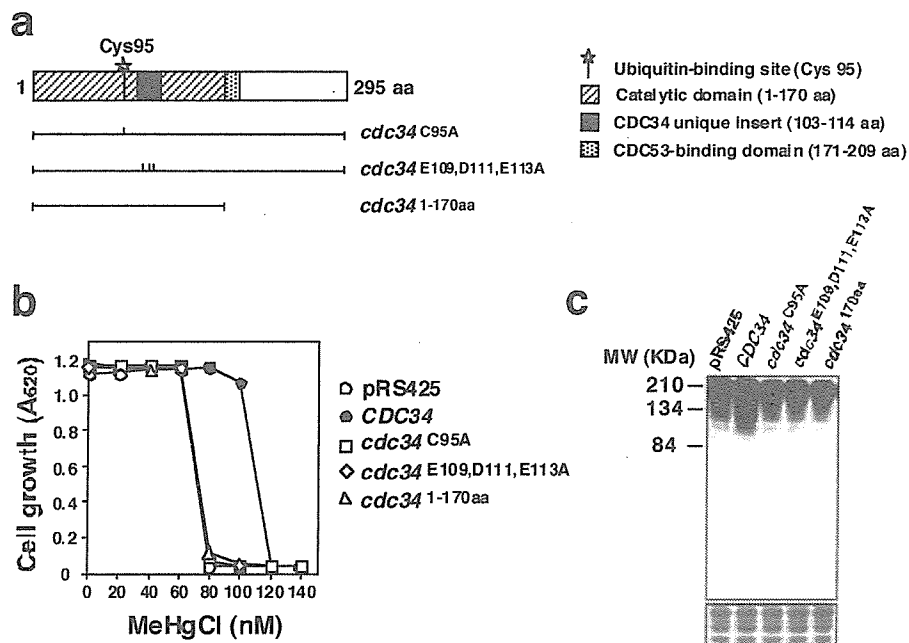


Fig. 2. Effects of the Overexpression of Cdc34 on the Sensitivity of Yeast Cells to Methylmercury

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

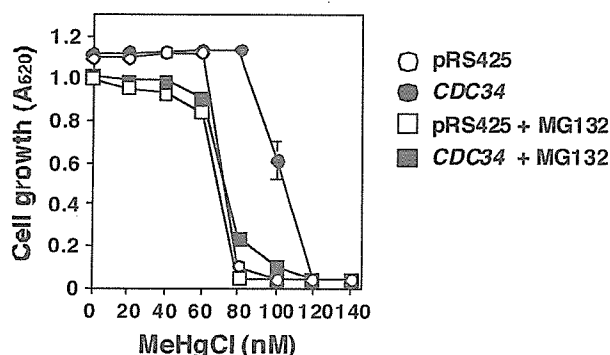


Fig. 3. Effects of a Proteasome Inhibitor on the Cdc34-mediated Resistance of Yeast Cells to Methylmercury

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 μ M), which had been dissolved in DMSO, and methylmercury at the indicated concentration.

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⁾

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

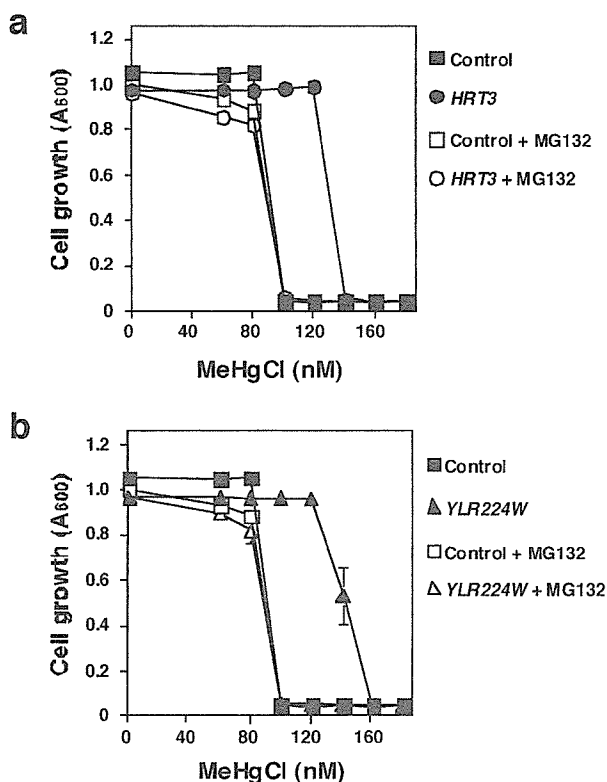


Fig. 4. Effects of a Proteasome Inhibitor on the Hrt3- and Ylr224w-mediated Resistance of Yeast Cells to Methylmercury

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溶性が高く、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 (Δ UbL) 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 (Δ UBA1+ Δ UBA2) 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。

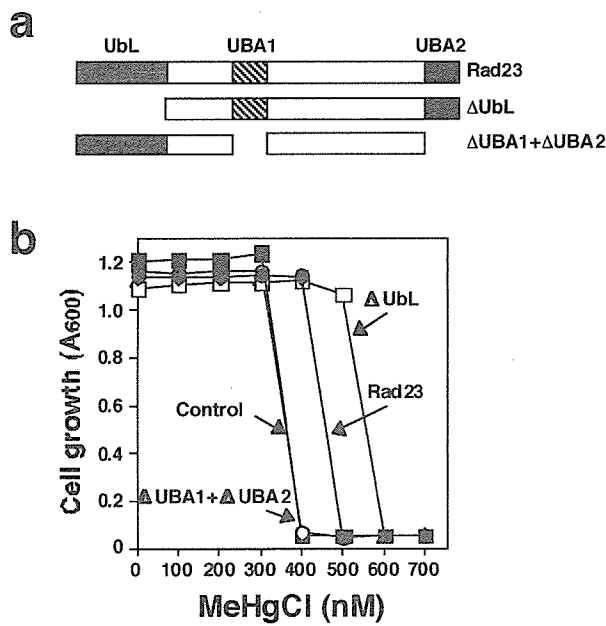


Fig. 5. Effects of Overexpression of Mutant Forms of Rad23 on the Sensitivity of Yeast Cells to Methylmercury

a: Schematic representation of the structural domains of Rad23 and the mutant proteins generated in this study. Rad23 contains a ubiquitin-like (UbL) domain and two ubiquitin-associated (UBA) domains. b: Yeast cells that overexpressed FLAG-Rad23 or mutant derivatives were cultured in SD (-uracil) liquid medium that contained methylmercury at the indicated concentrations.

以上の結果から、Rad23 が有する 2 つの機能のうち、UBA ドメインを介したユビキチン化蛋白質の分解抑制作用はメチル水銀毒性を軽減し、UbL ドメインを介したユビキチン化蛋白質の分解促進作用はメチル水銀毒性を増強するが、Rad23 高発現酵母中では、ユビキチン化蛋白質の分解抑制作用の方が分解促進作用を凌いでいるために、Rad23 高発現酵母はメチル水銀に対して耐性を示すと考えられる。したがって、細胞内にはメチル水銀毒性の軽減に関与し、かつ、UP システムによって分解される蛋白質が存在し、Rad23 はこの蛋白質の分解を抑制することによってメチル水銀毒性に対して防御的に作用していると考えられる。

5. メチル水銀毒性発現における Cdc34 と Rad23 との係わり

Rad23 は、Cdc34 とは逆に、メチル水銀毒性を軽減する蛋白質の分解を抑制することによってメチル水銀毒性を軽減する可能性が示された。しかし、Cdc34 は蛋白質のユビキチン化に関与し、Rad23 はユビキチン化蛋白質のユビキチン鎖と結合することから、Cdc34 と Rad23 がメチル水銀毒性に間接的に係わる同一の蛋白質を基質として認識する可能性も否定できない。そこで、正常酵母と Rad23 欠損

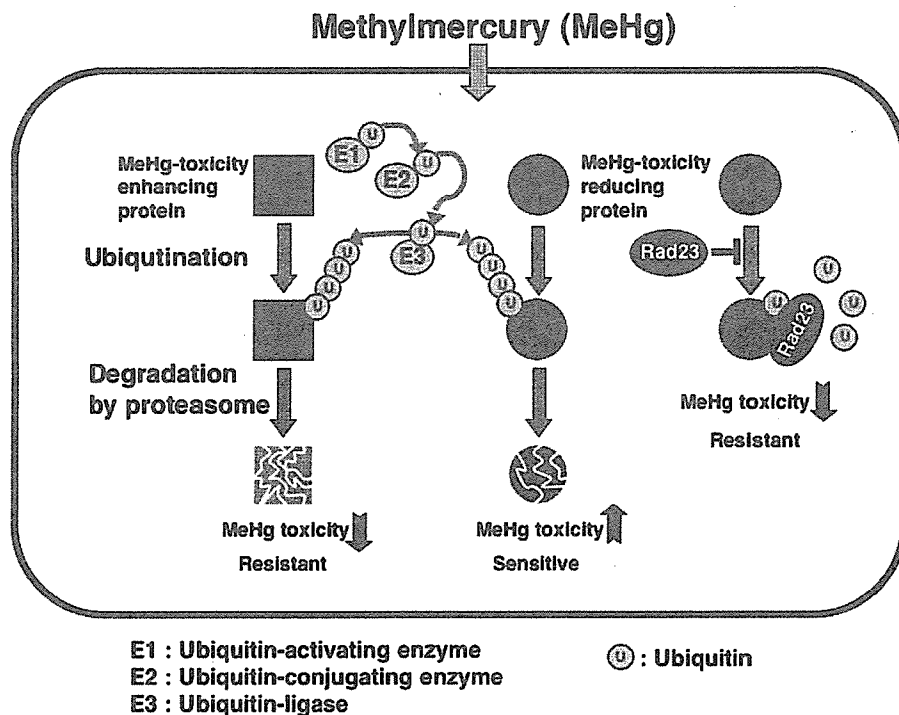


Fig. 6. Regulation of Methylmercury Toxicity by Ubiquitin-proteasome System

酵母にそれぞれ Cdc34 を高発現させたところ、両酵母のメチル水銀に対する耐性度はほぼ同程度に上昇した。このことから Rad23 とユビキチン化蛋白質のユビキチン鎖との結合は何らかの厳密な基質蛋白質認識機構によって調節されており、Cdc34 が E2 として関与する蛋白質分解システムに Rad23 の機能はほとんど影響を与えないと考えられる。細胞内にはメチル水銀毒性を軽減又は増強し、かつ、ユビキチン化を受ける複数の蛋白質が存在し、UP システム及び関連蛋白質はそれら蛋白質の細胞内濃度を複雑に調節することによってメチル水銀毒性の発現程度を規定していると考えられる (Fig. 6)。

6. おわりに

近年、様々な神経変性疾患の発症に UP システムが関わっていることが多く報告されており、UP システムの異常は脳機能に大きな障害をもたらすものと考えられる。一方、メチル水銀も主に中枢神経障害を引き起こすことから、UP システムとメチル水銀毒性との関係も注目すべき事実と考えられる。これまでに筆者らが得た知見は、メチル水銀に対する感受性決定機構として UP システムが重要な役割を果たしている可能性を強く示唆するものである。これらの知見はメチル水銀細胞毒性の発現機構及びそれに対する防御機構の解明に有用な手掛かりを与えるだけでなく、UP システムの新しい役割の解明にも大きく貢献し得るものと思われる。今後、Cdc34 及び Rad23 の基質となる蛋白質の中からメチル水銀毒性に係わる蛋白質を同定することによって、UP システムによるメチル水銀毒性調節機構が解明されるものと期待される。

謝辞 本総説に関するすべての研究は東北大学大学院薬学研究科において永沼 章教授の指導の下、多くの大学院生等の協力によって実施されたものであり、この場を借りてそれらの方々に心から感謝申し上げます。

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