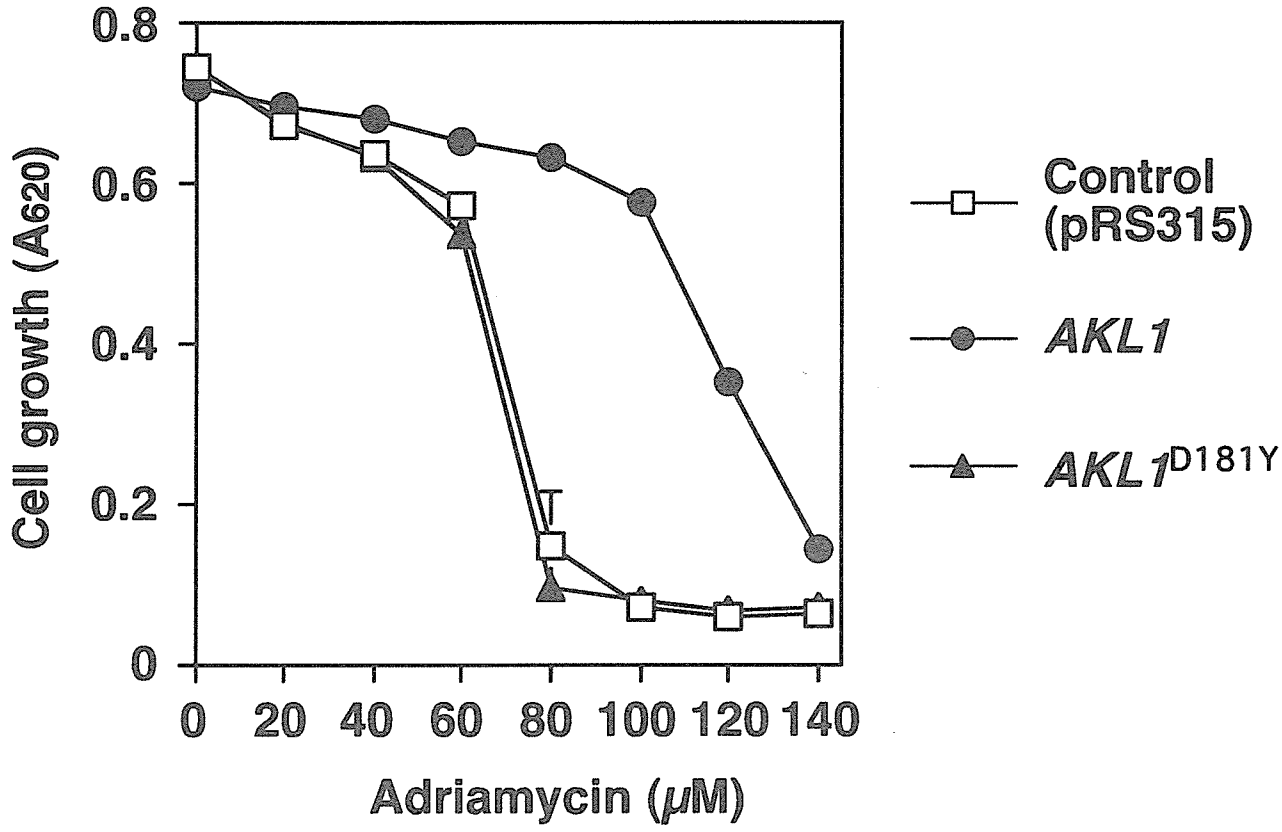

Figure 2 Akl1 kinase activity is required for the acquisition of adriamycin resistance. Yeast cells (BY4742) harboring pRS315, pRS315-*AKL1* or pRS315-*AKL1*^{D181Y} were grown in SD (-Leu) medium that contained adriamycin. For further details, see the legend to Fig. 1 (B).

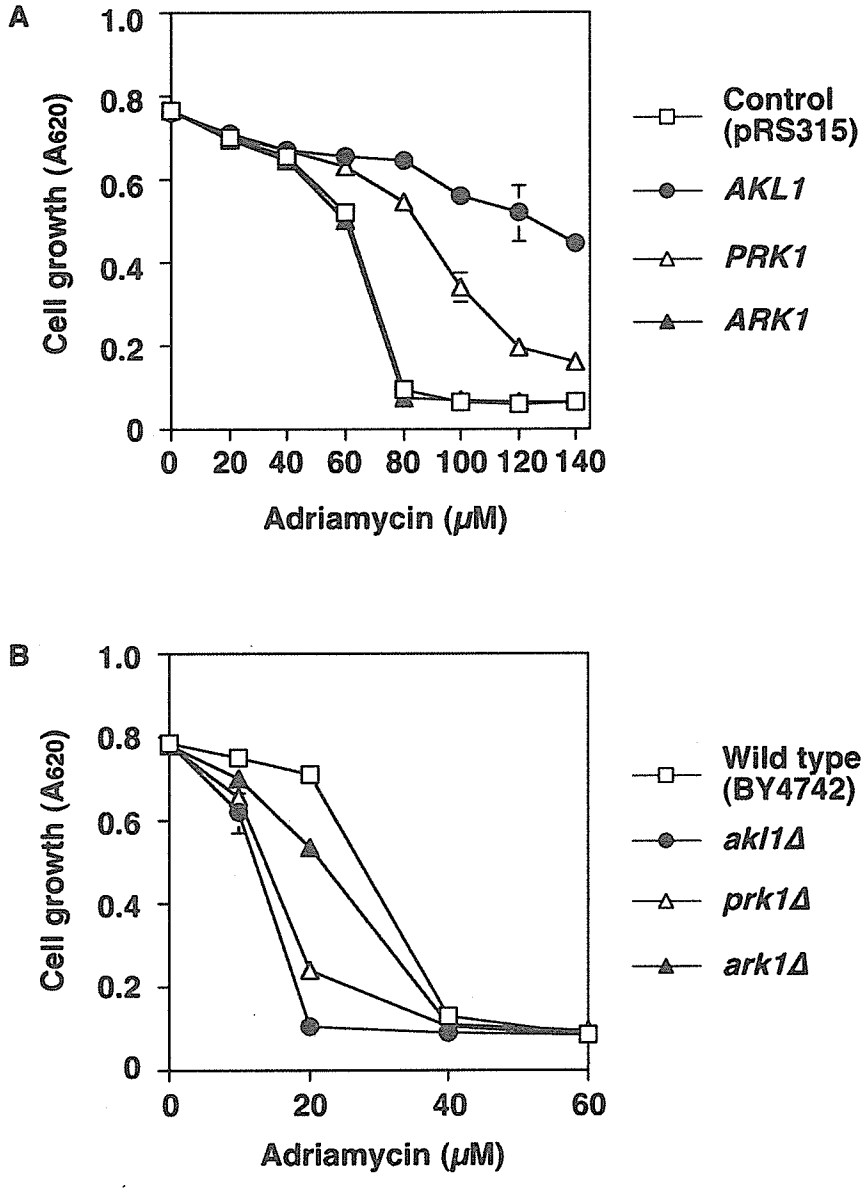

Figure 3 Involvement of members of the Ark/Prk kinase family in the sensitivity of yeast cells to adriamycin. (A) Yeast cells (BY4742) harboring pRS315, pRS315-*AKL1*, pRS315-*ARK1* or pRS315-*PRK1* were grown in SD (-Leu) medium that contained adriamycin. (B) Yeast (BY4742) cells lacking Ark1, Prk1, or Akl1 (*ark1*Δ, *prk1*Δ, and *akl1*Δ, respectively) were grown in SD medium that contained adriamycin. For further details, see the legend to Fig. 1 (B).

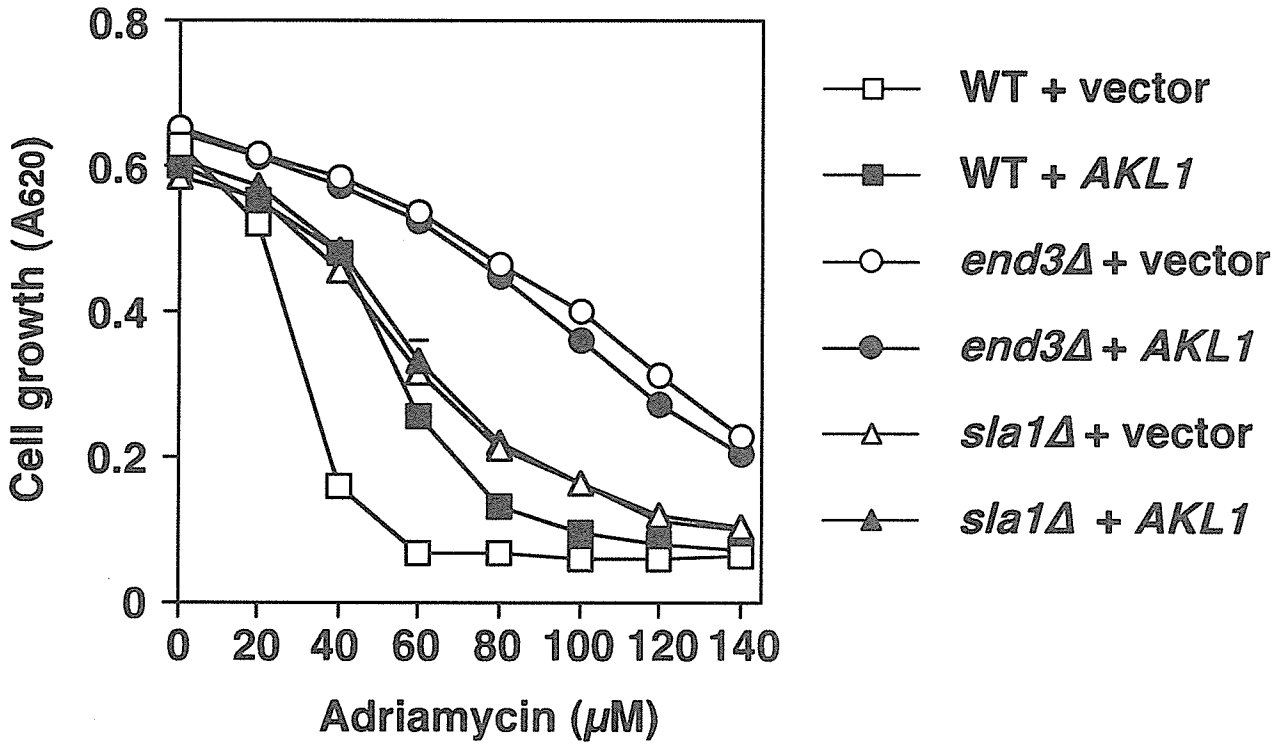

Figure 4 Relationship between Akl1 and the Sla1/End3/Pan1 complex in the acquisition of adriamycin resistance. Yeast strains (BY4742, *sla1*Δ, and *end3*Δ) harboring pRS315 or pRS315-*AKL1* were grown in SD (-Leu) medium that contained adriamycin. After incubation for 24 hr at 30 , the absorbance was measured spectrophotometrically at 620 nm. Each point represents the mean value of results from three cultures with S.D. (bar). The absence of a bar indicates that the S.D. falls within the symbol.

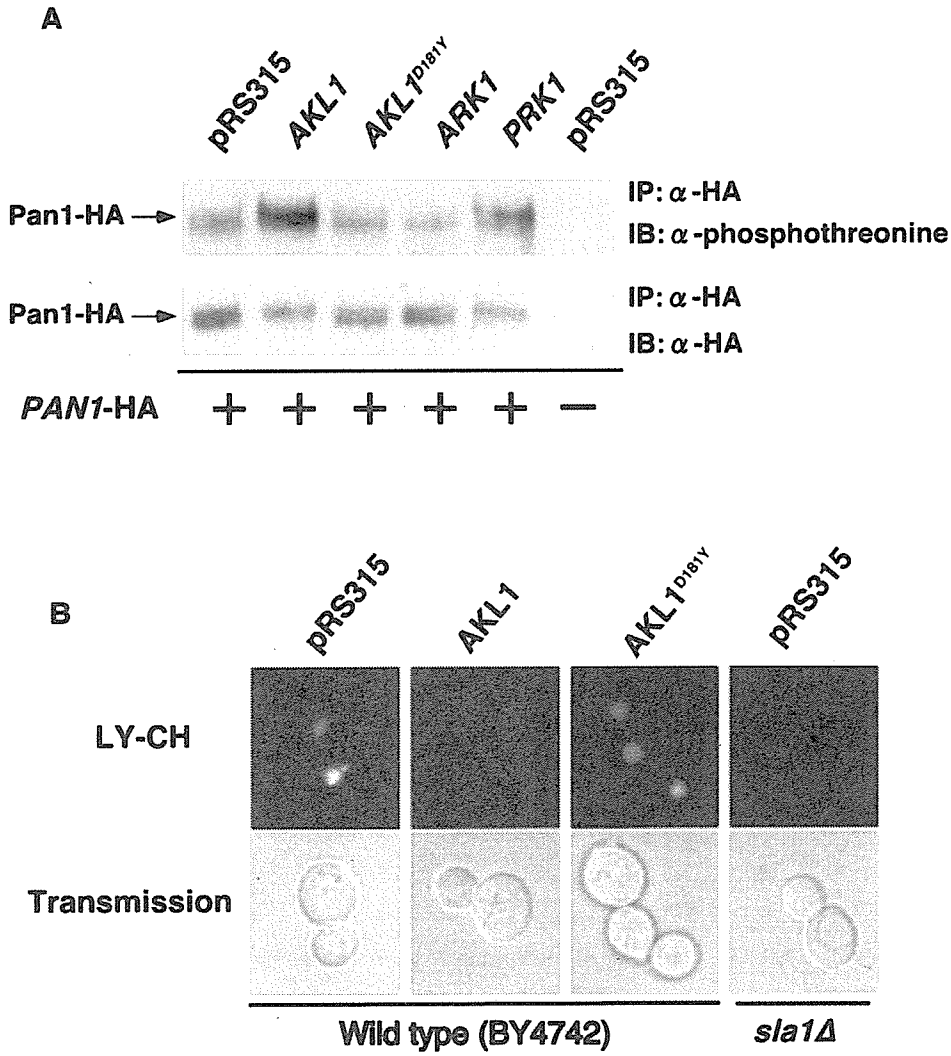

Figure 5 Effects of overexpression of Akl1 on the phosphorylation of Pan1-HA and the endocytic uptake of lucifer yellow. (A) Yeast (BY4742) cells expressing Pan1 tagged with HA at the carboxyl terminus (Pan1-HA) were transformed with pRS315, pRS315-*AKL1*, pRS315-*ARK1*, pRS315-*PRK1* or pRS315-*AKL1*^{D181Y}. Pan1-HA was immunoprecipitated (IP) with anti-HA agarose and immunoblotted with phosphothreonine-specific antibody (α-phosphothreonine; upper panel) or HA-specific antibody (α-HA; lower panel). See text for details. (B) Yeast cells at the logarithmic phase of growth were incubated with lucifer yellow for 2 hr. The localization of lucifer yellow was visualized with fluorescein isothiocyanate (FITC) fluorescence optics. The fluorescent images (LY-CH; upper panel) and the transmission images (lower panel) are shown.

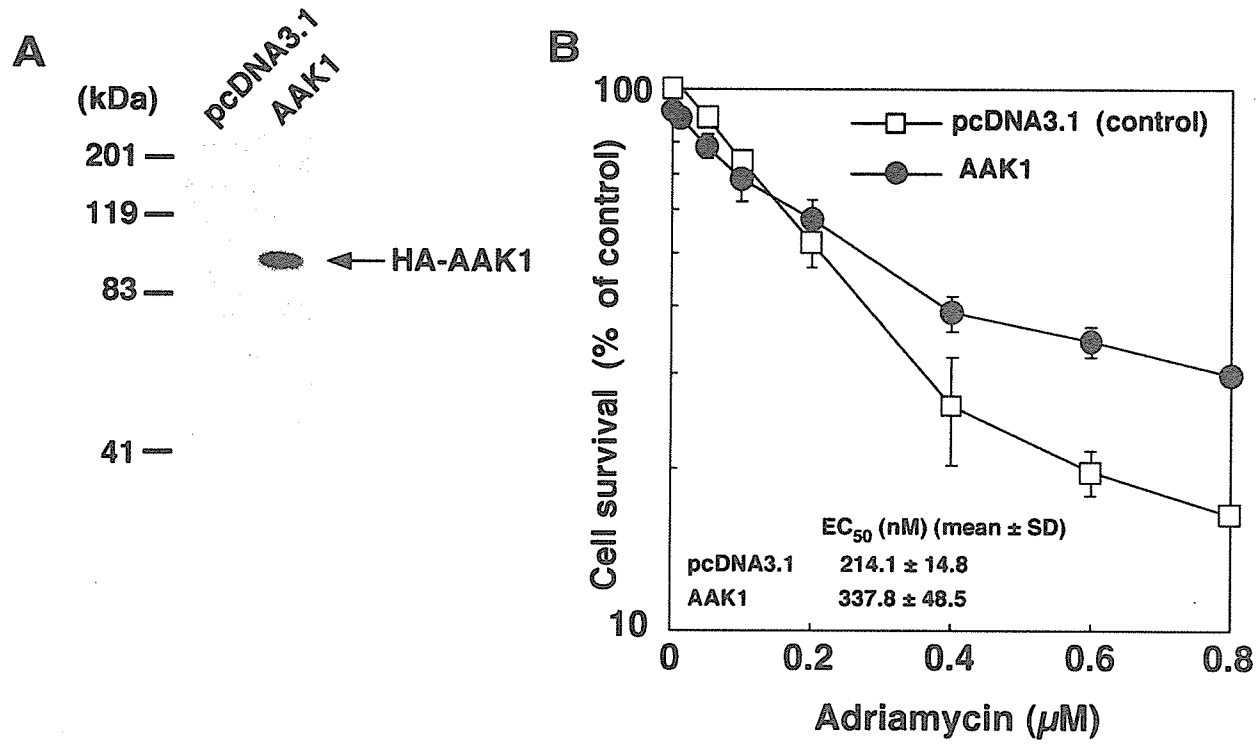

Figure 6 Effects of overexpression of AAK1, a member of the human Ark/Prk kinase family, on the sensitivity of HEK293 cells to adriamycin. (A) Immunoblotting analysis of HA-tagged AAK1. HEK293 cells were transfected with pcDNA3.1-HA-AAK1 or the pcDNA3.1 empty vector. Each lane was loaded with whole-cell 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. Each point and bar represents the mean value and SD of results from three cultures. 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).

別添５　　研究成果の刊行に関する一覧表

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書　籍

なし。

雑　誌

発表者氏名	論文タイトル	発表雑誌名	巻号	ページ	出版年
Hwang GW, Ishida Y, Naganuma A	Identification of F-box proteins that are involved in resistance to methylmercury in *Saccharomyces cerevisiae*	FEBS Lett	580	6813-6818	2006
Hwang GW, Naganuma A	DNA microarray analysis of transcriptional responses of human neuroblastoma IMR32 cells to methylmercury	J Toxicol Sci	31	537-538	2006
Kimura A, Ohashi K, Naganuma A, et al.	Cisplatin-induced expression of iron-retaining genes FIT2 and FIT3 in Saccharomyces cerevisiae	J Toxicol Sci	31	287-290	2006
Miura N, Kanayama Y, Naganuma A, et al.	Characterization of an immortalized hepatic stellate cell line established from metallothionein-null mice	J Toxicol Sci	31	391-398	2006

発表者氏名	論文タイトル	発表雑誌名	巻号	ページ	出版年
Kita K, Miura N, Naganuma A, et al.	Potential effect on cellular response to cadmium of a single-nucleotide A→ G polymorphism in the promoter of the human gene for metallothionein IIA	Hum Genet	120	553-560	2006
Takahashi T, Furuchi T, Naganuma A	Endocytic Ark/Prk kinases play a critical role in adriamycin resistance in both yeast and mammalian cells	Cancer Res	66	11932-11937	2007
Kimura A, Ohashi K, Naganuma A	Cisplatin upregulates Saccharomyces cerevisiae genes involved in iron homeostasis through activation of the iron insufficiency-responsive transcription factor Aft1	J Cell Physiol	210	378-384	2007
Hwang GW	A ubiquitin-proteasome system as a factor that determine the sensitivity to methylmercury	Yakugaku Zasshi	127	463-468	2007

Ⅳ. 研究成果の刊行物・別刷

FEBS Letters 580 (2006) 6813–6818

Identification of F-box proteins that are involved in resistance to methylmercury in *Saccharomyces cerevisiae*

Gi-Wook Hwang[a,b], Yosuke Ishida[a], Akira Naganuma[a,b,*]

[a] *Laboratory of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai 980-8578, Japan*
[b] *Tohoku University 21st Century COE Program "Comprehensive Research and Education Center for Planning of Drug Development and Clinical Evaluation", Sendai 980-8578, Japan*

Received 11 September 2006; revised 30 October 2006; accepted 15 November 2006

Available online 27 November 2006

Edited by Horst Feldmann

Abstract **We searched for F-box proteins that might be related to the mechanism that protects *Saccharomyces cerevisiae* against the toxic effects of methylmercury. We found that overexpression of Hrt3 and of Ylr224w rendered yeast cells resistant to methylmercury. Yeast cells that overexpressed Hrt3 and Ylr224w were barely resistant to methylmercury in the presence of a proteasome inhibitor. Our results suggest the existence of some protein(s) that enhances the toxicity of methylmercury in yeast cells and, also, that overexpression of Hrt3 or Ylr224w can confer resistance to methylmercury by enhancing the polyubiquitination of this protein(s) and its degradation in proteasomes.**

Keywords: Methylmercury; Resistance; Ubiquitin; F-box protein; Yeast

1. Introduction

Methylmercury is concentrated in fish via the food chain and, thus, most of the methylmercury that humans ingest comes from fish. Ingestion of methylmercury can cause severe damage to the human central nervous system [1,2]. However, mechanisms of methylmercury toxicity and cellular protective mechanisms against such toxicity remain poorly understood.

In order to characterize cellular mechanisms that protect against methylmercury toxicity, we have been searching for genes involved in the resistance to methylmercury in the budding yeast *Saccharomyces cerevisiae*, a unicellular eukaryote, many of whose gene products have functions similar to those of mammalians, including *Homo sapiens*. As a result, we have identified genes designated *BOP3* [3], *CDC34* [4] and *GFAT* [5], among others. The *CDC34* gene encodes Cdc34, a ubiquitin-conjugating enzyme [6] that is involved in the ubiquitin–proteasome system (UP system). The UP system is a proteolytic pathway that is strongly conserved in eukaryotes and operates as follows. A protein becomes bound to ubiquitin in the cell as a consequence of the actions of three enzymes, namely, a ubiquitin-activating enzyme (designated, generically, E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3). The resultant polyubiquitinated protein is then recognized and degraded by the proteasome [7–9]. We have analyzed the mechanism of acquisition of resistance to methylmercury that involves the overexpression of Cdc34 and we have shown that enhancement of cellular proteolysis by the UP system helps to protect cells against the toxic effects of methylmercury [10]. Thus, we postulated that some protein(s) involved in methylmercury toxicity might be included among the proteins whose degradation is enhanced by the enhanced activity of the UP system. Identification of such a protein(s) would provide an important clue to the mechanism of expression of the toxicity of methylmercury.

The SCF (*S*kp1/*C*dc53/*F*-box protein) complex is known as an E3 that is involved in the polyubiquitination of proteins in cooperation with Cdc34 (E2) [11,12]. Among the factors that make up this SCF complex in budding yeast, 17 different F-box proteins are known to bind directly to substrate proteins that are then degraded by the UP system [13–16]. F-box proteins have their own respective substrate-specificities, playing important roles in the selection of proteins that are degraded by the UP system. In order to identify the protein(s) involved in methylmercury toxicity that is degraded by the UP system, we need to identify the F-box protein(s) that is involved in the recognition of this protein(s). In the present study, therefore, we searched for F-box proteins that might be involved in the protection of yeast cells against the methylmercury toxicity.

2. Materials and methods

2.1. Culture and transformation of yeast cells

Saccharomyces cerevisiae W303B (*MAT*α *his3 can1-100 ade2 leu2 trp1 ura3*) was grown at 30 °C in yeast extract–peptone–dextrose (YPD) medium or synthetic dextrose (SD) medium. Plasmid DNA was introduced into W303B cells by the high-efficiency lithium acetate transformation method [17].

2.2. Construction of plasmids

The genes for F-box proteins were amplified by the polymerase chain reaction (PCR) with yeast genomic DNA as template and the following oligonucleotides as primers: CDC4-F and CDC4-R for the *CDC4* gene; COS3-F and COS3-R for the *COS3* gene; CTF13-F and CTF13-R for the *CTF13* gene; DIA2-F and DIA2-R for the *DIA2* gene; ELA1-F and ELA1-R for the *ELA1* gene; FLM13-F and FLM13-R for the *FLM13* gene; GRR1-F and GRR1-R for the *GRR1* gene; HRT3-F and HRT3-R for the *HRT3* gene; MET30-F and MET30-R for the *MET30* gene; RCY1-F and RCY1-R for the

*Corresponding author. Address: Laboratory of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai 980-8578, Japan. Fax: +81 22 795 6869.
E-mail address: naganuma@mail.pharm.tohoku.ac.jp (A. Naganuma).

0014-5793/$32.00
doi:10.1016/j.febslet.2006.11.045

RCY1 gene; UFO1-F and UFO1-R for the *UFO1* gene; YDR131C-F and YDR131C-R for the *YDR131C* gene; YDR219C-F and YDR219C-R for the *YDR219C* gene; YDR306C-F and YDR306C-R for the *YDR306C* gene; YJL149W-F and YJL149W-R for the *YJL149W* gene; YLR224W-F and YLR224W-R for the *YLR224W* gene; YNL311C-F and YNL311C-R for the *YNL311C* gene; FLAG-HRT3-F and HRT3-R for the FLAG-*HRT3* gene; FLAG-YLR224W-F and YLR224W-R for the FLAG-*YLR224W* gene; and SKP1-F and HA-SKP1-R for the *SKP1*-HA gene (see Table 1 for the sequences of all primers). The PCR-generated *CTF13*, *ELA1*, *UFO1*, *YDR219C* and *YJL149W* genes were ligated into the pGEM-T Easy vector (Promega, Madison, WI). Each insert was digested with the restriction endonuclease *Eco*RI and fragments were ligated into the pKT10-GAPDH (*URA3*) yeast expression vector. The PCR-generated *DIA2*, *FLM13*, *HRT3*, FLAG-*HRT3*, *RCY1*, *YDR131C*, *YDR306C* and *YNL311C* genes were ligated into the pTARGET vector (Promega). Each insert was digested with restriction endonucleases, as follows: *Sal*I and *Xho*I for the *DIA2* gene; and *Bam*HI and *Kpn*I for the *FLM13* and *RCY1* genes; and *Kpn*I and *Xho*I for the *HRT3*, *HRT3*-FLAG, *YDR131C*, *YDR306C* and *YNL311C* genes. The resultant fragments were ligated into the pKT10-GAPDH (*URA3*) yeast expression vector. The PCR-generated *CDC4*, *COS3*, *GRR1*, *MET30*, *YLR224W* and FLAG-*YLR224W* genes were ligated into the blunted *Puv*II cloning site of the pKT10-GAPDH (*URA3*) yeast expression vector. The PCR-generated *SKP1*-HA gene was ligated into the blunted *Puv*II cloning site of the pKT10-GAPDH (*TRP1*) yeast expression vector. Sequences of constructs were verified with an automated sequencer.

Table 1
Oligonucleotide primers used in this study

Primer	Oligonucleotide (5′ → 3′)
CDC4-F	GGCAAAAATTACGCTGTACG
CDC4-R	TGCTTATTCTCTCTGGGAAAGG
COS3-F	CTCGAAGCAAGAGGGGAAAAG
COS3-R	TGCTGTTAAAAGAGAGCAGGC
CTF13-F	TGACTGTGAGTCCCCAGAAGT
CTF13-R	TAAAATACCGCCGGTTTTCC
DIA2-F	GACATGCAAAATGATTAGCC
DIA2-R	AGGATACTGCATTATCATCAG
ELA1-F	AAATCGATTGATGTCGAGAT
ELA1-R	GCCTTCGGAGTTGGGTTACT
FLM13-F	TTAGTTACTAAAAGGCTCACA
FLM13-R	TGCTACTTTTGGAAACCTCC
GRR1-F	GTTTTGCGGTTTCCTTTATAC
GRR1-R	GGACAGTAAGTATTCAATGA
HRT3-F	CCATAAGCTAAACTCAAGG
HRT3-R	AACAACTGCAAAAAACATCG
MET30-F	GGGTGTGTGTTTGGTGATTTA
MET30-R	CAAGAAAAGACCACACACAGG
RCY1-F	AAACCAAAAGAAAACAAAAGC
RCY1-R	TCCGCACTTCATACCTAT
UFO1-F	CCGACACTAGGGAATAAGACA
UFO1-R	TGCTCTTCCAAATGTACATAC
YDR131C-F	TTTGAAAGGGCCCGAAAA
YDR131C-R	TAACCGCCATGTCTCACAGTA
YDR219C-F	ATAGTTCCTTCAACCACATAG
YDR219C-R	AAAGTCGGTTTGAGGCGTTT
YDR306C-F	CATATCAACCACAGTACTCAG
YDR306C-R	CACTGACTCTTATAAAACAAA
YJL149W-F	CACAGTGTTTACAACTCAGC
YJL149W-R	TATTTGAAGGGGAGTTGA
YLR224W-F	ATTGGCGCAAAGAAGACAGA
YLR224W-R	GCATAGACGTATATACACAT
YNL311C-F	ACGTTCAAACCAACCGAATC
YNL311C-R	AAAGTCCACTACAAAAAGTCA
FLAG-HRT3-F	AACTCAAGGAGCAAATGGACTACAAGGATGACG ATGACAAGATAGTAGATTATGAAAA
FLAG-YLR224W-F	AGAGATGGACTACAAGGATGACGATGACAAGAA TCAGAGCGATAGCAGCT
SKP1-F	CTAACAACGTAGCGCAGAT
HA-SKP1-R	TAGGCTAAGCGTAATCTGGAACATCGTATGGGTA ACGGTCTTCAGCCCATTC
HRT3ΔF-F	GTGCCATTTAAAGGTACCGCGAAGTACATATATTC
HRT3ΔF-R	CGTCAGGCAAGATGGTACCAATCCAGCAGGGTTG
YLR224WΔF-F	CCACAGCTATAAGGGTACCAGTTTGGCGTGG
YLR224WΔF-R	CCAGTGGTAAATCGGTACCGCTGCTATCGCTC

2.3. Quantitation of the toxicity of methylmercury in yeast cells

Yeast cells (10^4 cells/200 μl) were grown in SD (−Ura) liquid medium that contained methylmercuric chloride at various concentrations. After incubation for 48 h, we measured the absorbance of each culture at 600 nm to quantify cell growth. For the colony-formation assay, we cultured yeast cells (10^6 cells/ml) in SD (−Ura) liquid medium that contained methylmercuric chloride (1 μM). After incubation for 3 h at 30 °C, cells were pelleted by centrifugations and each pellet was suspended and diluted in 0.1 ml sterilized water to yield 10^7, 10^6 and 10^5 cells/ml. Five microliters of each suspension of yeast cells were spotted on agar-solidified SD (−Ura) medium and formation of colonies was examined after culture for 48 h at 30 °C. Yeast cells transformed with the plasmid pKT10-GAPDH were used as controls.

2.4. Site-directed mutagenesis

Site-directed mutagenesis of the genes for Hrt3 or Ylr224w was performed, as described elsewhere [18], with a kit for site-directed mutagenesis from Stratagene (Cedar Creek, TX) according to the manufacturer's instructions. We constructed the FLAG-*HRT3ΔF* and FLAG-*YLR224WΔF* genes by creating pairs of *Kpn*I sites in the open reading frames (ORFs) of the FLAG-*HRT3* or FLAG-*YLR224W* genes and excising the fragments between the respective pairs of *Kpn*I sites. We amplified fragments by PCR using plasmids pKT10-FLAG-*HRT3* and pKT10-FLAG-*YIL224W* as templates and the following oligonucleotides as primers: HRT3ΔF-F and HRT3ΔF-R for deletion of the F-box domain of Hrt3; and YLR224WΔF-F and YLR224WΔF-R for deletion of the F-box domain of Ylr224w. After creation of each pair of *Kpn*I sites, the plasmid was cleaved with *Kpn*I and self-ligated. All mutations were confirmed by DNA sequencing. The resultant plasmids were designated pKT10-FLAG-*HRT3ΔF* and pKT10-FLAG-*YLR224W ΔF*.

2.5. Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed as described elsewhere [19]. Yeast cells (10^7 cells/ml) were grown in 40 ml of SD (−Ura, −Trp) liquid medium for 5 h and harvested. Approximately 0.1 ml of cell pellet was suspended in 0.4–0.5 ml of buffer C [20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 5 mM $MgCl_2$, 50 mM KCl, 5% glycerol, 3 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin A] and lysed with glass beads (425–600 μm; Sigma, St. Louis, MO), in a cell disruptor (Micro Smash™ MS-100R; Tomy, Tokyo, Japan). Cell extracts were adjusted to equal volumes and concentrations of protein, and analyzed either by immunoprecipitation, or directly by immunoblotting. The Skp1-HA protein was immunoprecipitated from cell extracts (1 mg of protein) using the monoclonal HA-specific affinity matrix clone 3F10 (Roche, Indianapolis, IN). After incubation with cell extract overnight at 4 °C, beads were washed five times with Tris-buffered saline [20 mM Tris–HCl (pH 7.4), 500 mM NaCl] and then the protein was eluted by boiling in sample buffer for SDS–PAGE. Eluates and cell extracts (20 μg of protein) were fractionated by SDS–PAGE (12.5%), and then bands of protein were transferred to an Immobilon-P membrane (Millipore, Bedford, MA). FLAG-Hrt3 and FLAG-Ylr224w were visualized with FLAG-specific monoclonal antibodies (Sigma) and peroxidase-conjugated goat antibodies against mouse immunoglobulins (Dako A/S, Glostrup, Denmark) as primary and secondary antibodies, respectively.

3. Results

3.1. Search for F-box proteins involved in protection of yeast cells against methylmercury toxicity

We generated 17 yeast strains that overexpressed each of 17 different proteins, which have F-box domain, in an attempt to identify the F-box proteins involved in protection of yeast cells

against methylmercury toxicity, and we examined the sensitivity of each of these strains to methylmercury by monitoring colony formation on agar-solidified medium and rates of cell proliferation in liquid medium. We treated yeast cells that overexpressed each F-box protein with 1 μM methylmercury for the colony-formation assay, and we found that the sensitivity of yeast cells that overexpressed Cos111, Ctf13, Dia2, Flm1, Met30, Ydr219c, Ydr306c or Yjl149w was similar to that of control cells (Fig. 1A). By contrast, yeast cells that overexpressed Cdc4, Ela1, Rcy1 or Ynl311c were somewhat

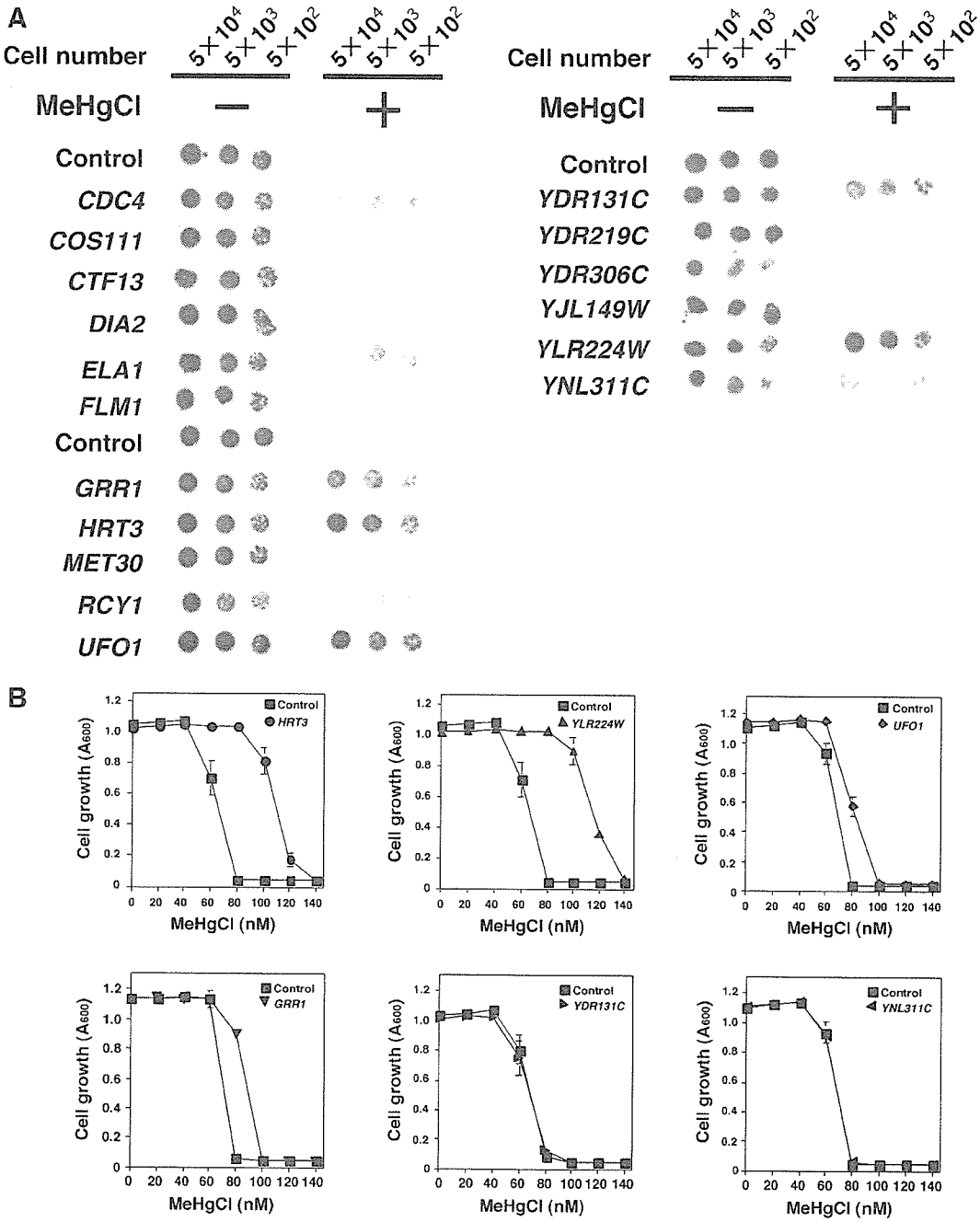

Fig. 1. Sensitivity to methylmercury (MeHgCl) of yeast cells that overexpressed genes for various F-box proteins. (A) Yeast cells (10^6 cells/ml) that harbored pKT10, pKT10-*CDC4*, pKT10-*COS111*, pKT10-*CTF13*, pKT10-*DIA2*, pKT10-*ELA1*, pKT10-*FLM1*, pKT10-*GRR1*, pKT10-*HRT3*, pKT10-*MET30*, pKT10-*RCY1*, pKT10-*UFO1*, pKT10-*YDR131C*, pKT10-*YDR219C*, pKT10-*YDR306C*, pKT10-*YJL149W*, pKT10-*YLR224W* or pKT10-*YNL311C* were grown in SD (−Ura) liquid medium with or without methylmercury (1 μM). After incubation for 3 h at 30 °C, cells of each strain were diluted in sterilized water to 10^7, 10^6 and 10^5 cells/ml. Five microliters of each resultant suspension of cells were spotted on agar-solidified SD (−Ura) medium. Plates were photographed after incubation for 48 h at 30 °C. Three separate experiments were performed and the results were reproducible. (B) Yeast cells (10^4 cells/200 μl/well) that harbored pKT10, pKT10-*HRT3*, pKT10-*YLR224W*, pKT10-*UFO1*, pKT10-*GRR1*, pKT10-*YDR131C* or pKT10-*YNL311C* were grown in SD (−Ura) liquid medium that contained methylmercury at the indicated concentration. After incubation for 48 h at 30 °C, absorbance was measured spectrophotometrically at 600 nm. Each point represents the mean value of results from three cultures with SD (bars). The absence of a bar indicates that the SD falls within the symbol.

resistant to methylmercury (Fig. 1A). However, yeast cells that overexpressed Grr1, Hrt3, Ufo1, Ydr131c or Ylr224w were strongly resistant to methylmercury, as compared with the control cells (Fig. 1B). We next examined the sensitivity of the 17 yeast strains to methylmercury monitoring by growth rates in liquid medium. Only yeast cells that overexpressed Grr1, Hrt3, Ufo1 or Ylr224w exhibited clear resistance to methylmercury, as compared with the control cells, and the cells that overexpressed Hrt3 or Ylr224w were particularly resistance to methylmercury (Fig. 1B). These results suggested that Cdc4, Ela1, Grr1, Hrt3, Rcy1, Ufo1, Ydr131c, Ylr224w and Ynl311c might be F-box proteins that are involved in protection against methylmercury and that Hrt3 and Ylr224w, in particular, might play a major role in such protection. The yeast cells that overexpressed Ydr131c or Ynl311c were not resistant to methylmercury in liquid medium, perhaps because proteins that were expressed at high levels only when yeast cells were cultured on agar-solidified medium might have been involved in the protective mechanism. Therefore, we focused, in the present study, on Hrt3 and Ylr224w, both of which rendered yeast cells strongly resistant to methylmercury.

3.2. Characterization of Hrt3 and Ylr224w

It has been proposed that F-box proteins bind to Skp1, a constituent of the E3 complex, via their respective F-box domains to form an SCF complex (E3) [20,21]. Both Hrt3 and Ylr224w have an F-box domain and both were identified in this study as proteins involved in protection against methylmercury toxicity. However, their functions as F-box proteins have not yet been confirmed. Therefore, we examined whether formation of an SCF complex is necessary for acquisition of resistance to methylmercury when Hrt3 and Ylr224w, respectively, are overexpressed in yeast cells. First, we expressed fusion proteins in which a FLAG tag was fused to the amino terminus of each protein (FLAG-Hrt3 and FLAG-Ylr224w) and proteins in which a FLAG tag was fused to the amino terminus of derivatives of Hrt3 and Ylr224w with deleted F-box domains (FLAG-Hrt3ΔF and FLAG-Ylr224wΔF) in yeast cells in which we also expressed Skp1 fused to an HA tag at its carboxyl terminus (Skp1-HA). We immunoprecipitated extracts of these cells with HA-specific antibodies and then immunoblotted the immunoprecipitates with anti-FLAG-specific antibodies. We confirmed that wild-type FLAG-Hrt3 and FLAG-Ylr224w bound to Skp1-HA, while binding of the derivatives with deleted F-box domains (FLAG-Hrt3ΔF and FLAG-Ylr224wΔF) and Skp1-HA was undetectable (Figs. 2A and B). These results are the first, to our knowledge, to demonstrate the binding of Hrt3 and Ylr224w to Skp1 through their F-box domains and they suggest that both proteins bind to Skp1 to form an SCF complex.

The yeast cells that overexpressed wild-type FLAG-Hrt3 or FLAG-Ylr224w were resistant to methylmercury, while yeast cells that overexpressed the proteins with deleted F-box domains (FLAG-Hrt3ΔF and FLAG-Ylr224wΔF) were not

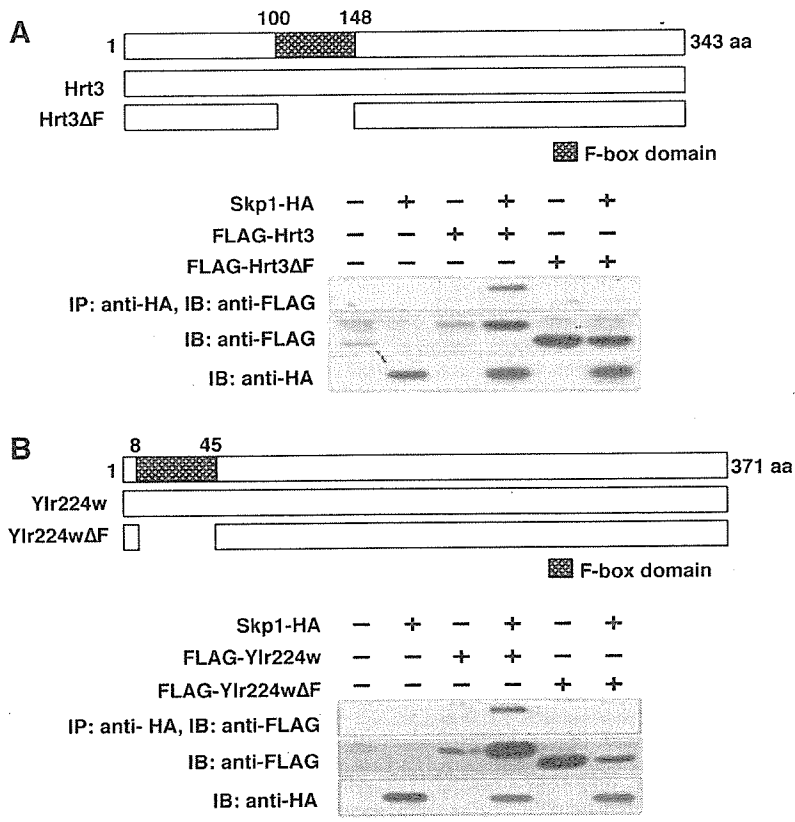

Fig. 2. Effects of deletion of the F-box domains of Hrt3 and Ylr224w on their binding to Skp1. A yeast strain expressing Skp1 fused to the HA tag (Skp1-HA) was transformed with pKT10, pKT10-FLAG-*HRT3* or pKT10-FLAG-*HRT3ΔF* (A) and pKT10, pKT10-FLAG-*YLR224W* or pKT10-FLAG-*YLR224wΔF* (B). Cell extracts were prepared from the various strains and analyzed by immunoprecipitation (IP) or directly by immunoblotting (IB). The Skp1-HA protein was immunoprecipitated from cell extracts with the monoclonal HA-specific affinity matrix (anti-HA). Immunoblotting analysis was performed with the FLAG-specific monoclonal antibodies (anti-FLAG).

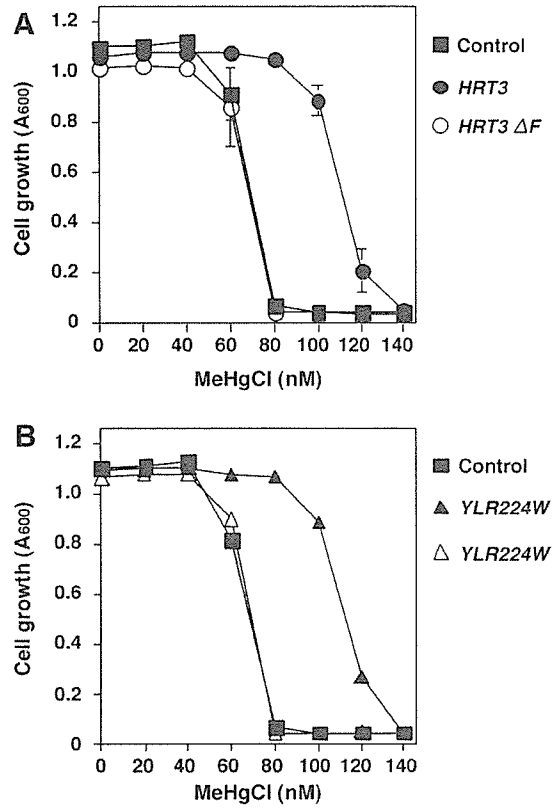

Fig. 3. Effects of deletion of the F-box domains of Hrt3 and Ylr224w on the sensitivity of yeast cells to methylmercury. Yeast cells (10^4 cells/200 μl/well) that harbored pKT10, pKT10-FLAG-*HRT3* or pKT10-FLAG-*HRT3ΔF* (A) or pKT10, pKT10-FLAG-*YLR224W* or pKT10-FLAG-*YLR224WΔ* (B) were grown in SD (−Ura) liquid medium that contained methylmercury at the indicated concentration. For other details, see legend to Fig. 1B.

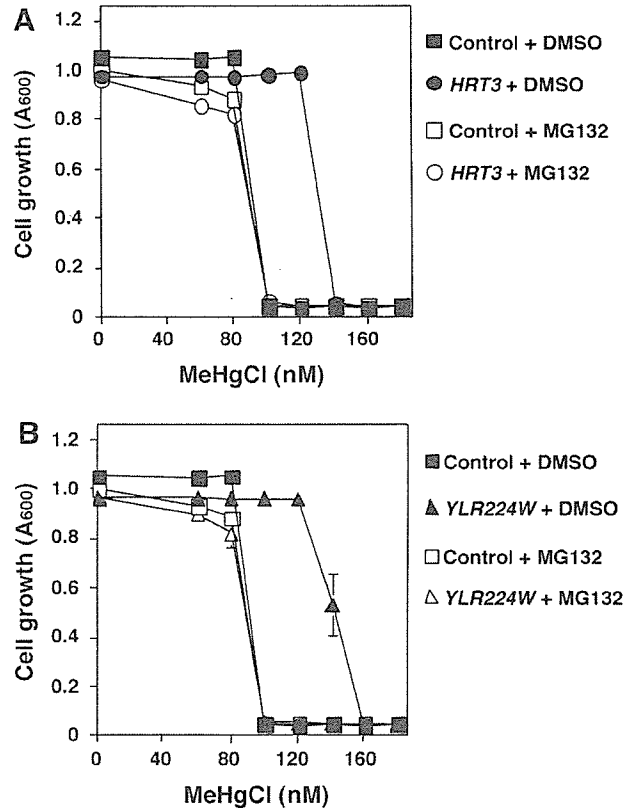

Fig. 4. Effects of a proteasome inhibitor on the Hrt3- and Ylr224w-mediated resistance of yeast cells to methylmercury. Yeast *erg6Δ* cells (10^4 cells/200 μl/well) that harbored pKT10 or pKT10-*HRT3* (A) or pKT10 or pKT10-*YLR224W* (B) were grown in SD (−Ura) liquid medium, with or without the proteasome inhibitor MG132 (50 μM), which had been dissolved in DMSO, and methylmercury at the indicated concentration. After incubation for 72 h at 30 °C, absorbance was measured spectrophotometrically at 600 nm. For other details, see legend to Fig. 1B.

(Fig. 3). Similar results were obtained when derivatives of both F-box proteins without FLAG tags were overexpressed in yeast cells (data not shown). Our results suggested that formation of an SCF complex might be necessary if yeast cells are to exhibit resistance to methylmercury upon overexpression of Hrt3 and Ylr224w and that both proteins act as F-box proteins in yeast cells.

Proteins that are polyubiquitinated via the ubiquitin system should be recognized and degraded by proteasomes. Therefore, we examined the possible role of proteasomes in the acquisition of resistance to methylmercury upon overexpression of Hrt3 and Ylr224w using MG132, a proteasome inhibitor [22]. Wild-type yeast cells are unable to incorporate MG132. Thus, we used an *ERG6*-deleted (*erg6Δ*) yeast strain whose cell membrane is more permeable than that of wild-type strains to MG132 [10,23]. Overexpression of both F-box proteins resulted in resistance to methylmercury in *ERG6*-deleted yeast cells, as anticipated. However, the resistance to methylmercury of *ERG6*-deleted yeast cells that overexpressed Hrt3 or Ylr224w disappeared in the presence of MG132 (Fig. 4). These results suggested that degradation of polyubiquitinated proteins by proteasomes was necessary for the resistance to methylmercury that resulted from the overexpression of the two F-box proteins. Therefore, a protein(s) that reinforces the toxicity of methylmercury might be included among those proteins that are recognized by Hrt3 or Ylr224w and are degraded by proteasomes after their polyubiquitination.

4. Discussion

The results obtained in this study demonstrate that Hrt3 and Ylr224w, two proteins with F-box domains, act as F-box proteins to reduce the toxicity of methylmercury through the formation of SCF complexes. Numerous proteins that are polyubiquitinated by the actions of E2 and the SCF complex are degraded by proteasomes. However, the transcriptional activity of Met4, for example, a transcription factor that is recognized and subsequently polyubiquitinated by the SCF complex that includes Met30 as its F-box protein, is suppressed upon its polyubiquitination, without subsequent degradation by proteasomes [24,25]. Therefore, polyubiquitination appears to be involved not only in protein degradation but also in the regulation of the activities of enzymes and other cellular factors. The present study revealed that overexpression of Hrt3 and of Ylr224w rendered yeast cells resistant to methylmercury, and resistance almost disappeared upon treatment of permeable yeast cells with the proteasome inhibitor MG132

(Fig. 4). Our results suggest that the resistance to methylmercury conferred on yeasts by overexpression of these two F-box proteins might be derived from enhanced degradation in proteasomes after enhanced polyubiquitination of a protein or proteins (hereafter collectively designated X-protein) that reinforce the toxicity of methylmercury.

In this study, disruption of the genes for Hrt3 and/or Ylr224w did not affect the sensitivity of yeast cells to methylmercury (data not shown). Thus, it is possible that X-protein might also be recognized and polyubiquitinated by some F-box protein(s) other than Hrt3 and Ylr224w.

Multiple E2s coexist with multiple F-box proteins in yeast cells. Therefore, it is possible that not only F-box proteins but also E2 might be involved in the selection of proteins as substrates for degradation. As described above, we found previously that overexpression of Cdc34, an E2, also rendered yeast cells resistant to methylmercury and, thus, the F-box protein related to this phenomenon might be Hrt3 or Ylr224w. However, when we overexpressed Cdc34 in yeast cells in which the Hrt3 and/or the Ylr224w gene had been deleted, the resistance of all three lines of cells to methylmercury was almost as strong as that of wild-type yeast cells that overexpressed Cdc34 (data not shown). This result suggests that Cdc34 does not confer resistance to methylmercury in cooperation only with SCF complexes that contain Hrt3 or Ylr224w as the F-box protein. However, we have also found that some types of E2 other than Cdc34 confer resistance to methylmercury when overexpressed in yeast cells [4]. Therefore, it is possible that types of E2 other than Cdc34 or multiple types of E2, including Cdc34, might play a role in the polyubiquitination of X-protein by SCF complexes that contain Hrt3 or Ylr224w as the F-box protein.

Their functions of Hrt3 and Ylr224w as F-box proteins were confirmed for the first time in the present study, but no further information is currently available, to our knowledge, about the possible proteins that might be polyubiquitinated after being recognized by these F-box proteins. In the preliminary study, we have already identified some candidates of X-proteins, which play a role in the enhancement of the toxicity of methylmercury and which are degraded by proteasomes after its polyubiquitination, by searching for proteins that can bind to both F-box proteins. The SCF complex, an E3 that includes F-box proteins, is present not only in yeast cells but also in mammalian cells, including human cells, and it has the same function in mammals as in yeast [26,27]. The identification of X-protein should help to clarify the mechanism of damage to the central nervous system that is caused by methylmercury.

Acknowledgement: This work was supported by Health and Labour Sciences Research Grants for Research on Risk of Chemical Substances from Ministry of Health, Labour and Welfare Japan.

References

[1] Boening, D.W. (2000) Ecological effects, transport, and fate of mercury: a general review. Chemosphere 40, 1335–1351.
[2] Clarkson, T.W. (2002) The three modern faces of mercury. Environ. Health Perspect. 110 (Suppl. 1), 11–23.
[3] Hwang, G.W., Furuoya, Y., Hiroshima, A., Furuchi, T. and Naganuma, A. (2005) Overexpression of Bop3 confers resistance to methylmercury in Saccharomyces cerevisiae through interaction with other proteins such as Fkh1, Rts1, and Msn2. Biochem. Biophys. Res. Commun. 330, 378–385.
[4] Furuchi, T., Hwang, G.W. and Naganuma, A. (2002) Overexpression of the ubiquitin-conjugating enzyme Cdc34 confers resistance to methylmercury in *Saccharomyces cerevisiae*. Mol. Pharmacol. 61, 738–741.
[5] Miura, N., Kaneko, S., Hosoya, S., Furuchi, T., Miura, K., Kuge, S. and Naganuma, A. (1999) Overexpression of L-glutamine:D-fructose-6-phosphate amidotransferase provides resistance to methylmercury in *Saccharomyces cerevisiae*. FEBS Lett. 458, 215–218.
[6] Goebl, M.G., Yochem, J., Jentsch, S., McGrath, J.P., Varshavsky, A. and Byers, B. (1988) The yeast cell cycle gene CDC34 encodes a ubiquitin-conjugating enzyme. Science 241, 1331–1335.
[7] Glickman, M.H. and Ciechanover, A. (2002) The ubiquitin–proteasome proteolytic pathway: destruction for the sake of construction. Physiol. Rev. 82, 373–428.
[8] Hershko, A. and Ciechanover, A. (1998) The ubiquitin system. Annu. Rev. Biochem. 67, 425–479.
[9] Pickart, C.M. (2001) Ubiquitin enters the new millennium. Mol. Cell 8, 499–504.
[10] Hwang, G.W., Furuchi, T. and Naganuma, A. (2002) A ubiquitin–proteasome system is responsible for the protection of yeast and human cells against methylmercury. FASEB J. 16, 709–711.
[11] Jackson, P.K. and Eldridge, A.G. (2002) The SCF ubiquitin ligase: an extended look. Mol. Cell 9, 923–925.
[12] Deshaies, R.J. (1999) SCF and Cullin/Ring H2-based ubiquitin ligases. Annu. Rev. Cell. Dev. Biol. 15, 435–467.
[13] Kipreos, E.T. and Pagano, M. (2000) The F-box protein family. Genome Biol. 1, (Reviews 3002).
[14] Feldman, R.M., Correll, C.C., Kaplan, K.B. and Deshaies, R.J. (1997) A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. Cell 91, 221–230.
[15] Skowyra, D., Craig, K.L., Tyers, M., Elledge, S.J. and Harper, J.W. (1997) F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell 91, 209–219.
[16] Patton, E.E., Willems, A.R. and Tyers, M. (1998) Combinatorial control in ubiquitin-dependent proteolysis: don't Skp the F-box hypothesis. Trends Genet. 14, 236–243.
[17] Furuchi, T., Nitta, K., Takahashi, T. and Naganuma, A. (2004) Overexpression of Ssl2p confers resistance to adriamycin and actinomycin D in Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. 314, 844–848.
[18] Furuchi, T., Takahashi, T., Tanaka, S., Nitta, K. and Naganuma, A. (2004) Functions of yeast helicase Ssl2p that are essential for viability are also involved in protection from the toxicity of adriamycin. Nucl. Acid. Res. 32, 2578–2585.
[19] Takahashi, T., Furuchi, T. and Naganuma, A. (2005) A novel role for Bsd2 in the resistance of yeast to adriamycin. J. Cell. Physiol. 202, 100–104.
[20] Craig, K.L. and Tyers, M. (1999) The F-box: a new motif for ubiquitin dependent proteolysis in cell cycle regulation and signal transduction. Prog. Biophys. Mol. Biol. 72, 299–328.
[21] Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J.W. and Elledge, S.J. (1996) SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell 86, 263–274.
[22] Lee, D.H. and Goldberg, A.L. (1998) Proteasome inhibitors: valuable new tools for cell biologists. Trends Cell Biol. 8, 397–403.
[23] Lee, D.H. and Goldberg, A.L. (1996) Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in *Saccharomyces cerevisiae*. J. Biol. Chem. 271, 27280–27284.
[24] Flick, K., Ouni, I., Wohlschlegel, J.A., Capati, C., McDonald, W.H., Yates, J.R. and Kaiser, P. (2004) Proteolysis-independent regulation of the transcription factor Met4 by a single Lys 48-linked ubiquitin chain. Nat. Cell Biol. 6, 634–641.
[25] Kaiser, P., Flick, K., Wittenberg, C. and Reed, S.I. (2000) Regulation of transcription by ubiquitination without proteolysis: Cdc34/SCF(Met30)-mediated inactivation of the transcription factor Met4. Cell 102, 303–314.
[26] Cardozo, T. and Pagano, M. (2004) The SCF ubiquitin ligase: insights into a molecular machine. Nat. Rev. Mol. Cell. Biol. 5, 739–751.
[27] Ang, X.L. and Wade Harper, J. (2005) SCF-mediated protein degradation and cell cycle control. Oncogene 24, 2860–2870.

The Journal of Toxicological Sciences,
Vol.31, No.5, 537-538, 2006

– Toxicogenomics/proteomics Report –

DNA MICROARRAY ANALYSIS OF TRANSCRIPTIONAL RESPONSES OF HUMAN NEUROBLASTOMA IMR-32 CELLS TO METHYLMERCURY

Gi-Wook HWANG[1] and Akira NAGANUMA[1,2]

[1]*Laboratory of Molecular and Biochemical Toxicology,*
Graduate School of Pharmaceutical Sciences, Tohoku University,
Aoba-ku, Sendai 980-8578, Japan
[2]*Tohoku University 21st Century COE Program "Comprehensive Research and Education Center for Planning of Drug Development and Clinical Evaluation",*
Aoba-ku, Sendai 980-8578, Japan

(Received September 19, 2006; Accepted October 4, 2006)

ABSTRACT — We used DNA microarrays to monitor the transcriptional responses of IMR-32 neuroblastoma cells to methylmercury. Methylmercury increased levels of expression of seven genes, including genes for ETV5 and ID4, and reduced those of two genes.

KEY WORDS: Methylmercury, DNA microarray, IMR-32 cells, ETV5, ID4

INTRODUCTION

Methylmercury is an environmental pollutant that damages the central nervous system, and many cases of methylmercury poisoning have been reported (Castoldi *et al.*, 2003). However, little is known about the molecular mechanism of methylmercury poisoning. We postulated that treatment of neuroblastoma cells with methylmercury would induce various intracellular reactions and, in particular, changes in the levels of expression of certain genes. To examine our hypothesis, we examined levels of transcripts in IMR-32 cells before and after treatment for 6 and 24 hr with methylmercury using DNA microarrays.

MATERIALS AND METHODS

Exposure of cells to methylmercury

IMR-32 human neuroblastoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 mg/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 10% CO_2 in air. IMR-32 cells (1×10^6 cells) were distributed in six-well plates and cultured in 1-ml aliquots of medium. After incubation for 24 hr, 1 μM methymercury chloride was added to the medium and cells were cultured for 6 hr, during which the cell-survival rate was close to 100%, and for 24 hr, during which fewer than 10% of cells ceased to be viable.

Eextraction of RNA

Control IMR-32 cells and cells that had been treated with methylmercury chloride were lysed with TRIzol (Life Technologies Inc., Rockville, MD, USA) according to the manufacturer's protocol and total RNA was extracted by the standard method and stored at –80°C.

Microarray analysis

Purified RNA (8 μg) was reverse transcribed by Superscript II reverse transcriptase (Life Technologies Inc.) with T7-$(dT)_{24}$, which contains a promoter recognized by T7 RNA polymerase, as primer. Then complementary DNA (cDNA) was synthesized and used as template for transcription *in vitro* of biotinylated complementary RNA (cRNA). Fifteen micrograms of fragmented cRNA were allowed to hybridize to an HG-Focus microarray (Affymetrix, Santa Clara, CA, USA) for 16 hr at 45°C with constant rotation at 60 rpm according to the protocol from Affymetrix. This high-density oligonucleotide-based array is specific for approximately 8,500 human genes selected from the

Correspondence: Akira NAGANUMA (E-mail: naganuma@mail.pharm.tohoku.ac.jp)

Table 1. Genes whose levels of expression changed upon treatment of IMR-32 cells with methylmercury.

Gene name	Accession number	Changes in level of transcript		Function of gene product
		6 hr	24 hr	
[Increased]				
BAIAP2	NM_017450	4.5	2.7	Signal transduction
CDKN1A	NM_000389	3.9	2.2	Signal transduction
DKK1	NM_012242	2.8	2.5	Signal transduction
ETV5	NM_004454	22.6	2.6	Transcription
FZD1	NM_003505	3.8	2.4	Signal transduction
ID4	NM_001546	6.6	2.2	Transcription
NQO1	NM_000903	4.7	2.1	Signal transduction
[Decreased]				
CPO1	NM_173077	0.4	0.5	Tumor progression
MLF1	NM_022443	0.5	0.3	Tumor progression

IMR-32 cells were treated with methylmercury for 6 hr and 24 hr, and then gene expression was analyzed with DNA microarrays. The genes presented in the Table include only those whose levels of expression changed (with an increase of more than two-fold or a decrease of more than 50%) not only when IMR-32 cells were treated with methylmercury for 6 hr but also when they were treated for 24 hr.

Gene Bank database of the National Center for Biotechnology Information (NCBI, USA). A separate microarray was used for the analysis of each individual sample. After hybridization, the microarray was washed and stained on an Affymetrix Fluidics Station and scanned with a confocal argon laser, with emission of 488 nm and detection at 570 nm. Data were analyzed with Genespring software, version 7.0 (Silicon Genetics, San Carlos, CA, USA).

RESULTS AND DISCUSSION

We used DNA microarrays to identify for genes whose levels of expression chaged when IMR-32 cells were treated with methylmercury (1 μM) for 6 hr (cell viability rate, approximately 100%) and 24 hr (cell viability rate, greater than 90%). We found that treatment for 6 hr and for 24 hr more than doubled the levels of expression of seven genes and more than halved those of two genes (Table 1). Among the first set of genes, the gene for CDKN1A has already been identified as a gene whose level of expression increases when cells from rat embryonic limb buds and the midbrain central nervous system are treated with methylmercury (Ou *et al.*, 1999). However, to our knowledge, the remaining eight genes that we identified have not previously been reported to be associated with exposure of cells to methylmercury. Our findings provide useful clues to the nature of the response of neural cells to methylmercury.

ACKNOWLEDGMENT

This work was supported by Health and Labour Sciences Research Grants for Research on Risk of Chemical Substances from the Ministry of Health, Labour and Welfare Japan.

REFERENCES

Castoldi, A.F., Coccini, T. and Manzo, L. (2003): Neurotoxic and molecular effects of methylmercury in humans. Rev. Environ. Health, **18**, 19-31.

Ou, Y.C., Thompson, S.A., Ponce, R.A., Schroeder, J., Kavanagh, T.J. and Faustman, E.M. (1999): Induction of the cell cycle regulatory gene p21 (Waf1, Cip1) following methylmercury exposure *in vitro* and *in vivo*. Toxicol. Appl. Pharmacol., **157**, 203-212.

The Journal of Toxicological Sciences,
Vol.31, No.3, 287-290, 2006

– Letter –

CISPLATIN-INDUCED EXPRESSION OF IRON-RETAINING GENES *FIT2* AND *FIT3* IN *SACCHAROMYCES CEREVISIAE*

Akiko KIMURA[1], Kazuaki OHASHI[1,2], Reiko YAMAMOTO[3] and Akira NAGANUMA[1,4]

[1]*Laboratory of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba-ku, Sendai 980-8578, Japan*

[2]*Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan*

[3]*Department of Human Health and Nutrition, Faculty of Comprehensive Human Sciences, Shokei Gakuin University, Yurigaoka, Natori 981-1295, Japan*

[4]*Tohoku University 21st Century COE Program "Comprehensive Research and Education Center for Planning of Drug Development and Clinical Evaluation", Aoba-ku, Sendai 980-8578, Japan*

(Received December 26, 2005; Accepted February 18, 2006)

ABSTRACT — cDNA microarray analysis indicated that mRNA levels of Fit2p and Fit3p, proteins involved in iron retention within the yeast cell wall, were markedly increased by treatment of *Saccharomyces cerevisiae* with cisplatin. Expression of *FIT2* and *FIT3* is known to be transcriptionally regulated by Aft1p. Northern blotting demonstrated a time- and concentration-dependent increase in the mRNA levels of both proteins following treatment with cisplatin. However, overexpression or disruption of the *FIT2* or *FIT3* genes had little effect on the susceptibility of yeast to cisplatin. Although Fit2p and Fit3p do not appear to be directly involved in protecting against the toxic effects of cisplatin, the present results suggest the existence of an activation system of gene expression in response to cisplatin within yeast cells.

KEY WORDS: Cisplatin, Induction, Gene expression, Fit2, Fit3

INTRODUCTION

Cisplatin is an anticancer agent that has been shown to be effective against various malignant tumors (Einhorn and Williams, 1979). The mechanism of acquired resistance to cisplatin has been widely studied (Siddik, 2003), but few investigations into cellular responses to cisplatin have been reported. We carried out a study of genes whose expression levels were altered by cisplatin in budding yeast (*Saccharomyces cerevisiae*). Since many yeast genes share common functions with those of mammals, including humans, the findings obtained from investigations using yeast are considered useful for elucidating the functions of human proteins.

MATERIALS AND METHODS

Yeast strain and media

The wild-type yeast strain BY4742 (*MATα*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*) and yeast strains with disrupted *FIT2* or *FIT3* (Euroscarf, Frankfurt, Germany) were grown in synthetic dextrose (SD) medium supplemented with amino acids.

Construction of gene expression vectors

FIT2 and *FIT3* were amplified by PCR using yeast genomic DNA as a template with the following primers:

FIT2-F, 5'- TCATAAAGCCATCACAAAACA-3'
and
FIT2-R, 5'-TTTCTGGTTTGAAACCGAGC-3'
for the *FIT2* gene
FIT3-F, 5'-AACTAATAACAATAATCCC-3' and

Correspondence: Akira NAGANUMA (E-mail: naganuma@mail.pharm.tohoku.ac.jp)

FIT3-R, 5'-AAATCGGATATCCCGCATGA-3' for the *FIT3* gene.

The amplified fragments were ligated into the pKT10-GAPDH (*URA3*) expression vector. BY4742 cells were transformed with each plasmid according to the lithium acetate procedure (Naganuma *et al.*, 2000).

Northern blotting

Yeast cells (5×10^6 cells/ml) were precultured in SD medium for 3 hr at 30°C and then cultured in SD medium containing cisplatin. Total RNA was prepared as described elsewhere (Furuchi *et al.*, 2002). The probes for *FIT2*, *FIT3*, and *ACT1* were amplified by PCR from yeast genomic DNA using the following primers:

FIT2-1-F, 5'-ACAGTTATGACTGCCGTCTCG-3' and
FIT2-1-R, 5'-ACACTTGCTCCTTGGAATGCA-3' for the *FIT2* probe
FIT3-1-F, 5'-AACTAATAACAATAATCCC-3' and
FIT3-1-R, 5'-AAATCGGATATCCCGCATGA-3' for the *FIT3* probe
ACT1-1-F, 5'-GGTGATGAAGCTCAATCCAA-3' and
ACT1-1-R, 5'-AGAAGATTGAGCAGCGGTTT-3' for the *ACT1* probe

Northern blotting was performed using the digoxigenin system (Takara Bio Inc., Shiga, Japan), in accordance with the manufacturer's instructions.

Quantitation of cisplatin toxicity in yeast.

Yeast cells were cultured (1×10^4 cells/200 µl) in SD medium containing cisplatin at various concentrations. After 48 hr, the absorbance of the culture at 620 nm was measured to quantify cell growth.

RESULTS AND DISCUSSION

We examined the effect of cisplatin on gene expression in *S. cerevisiae* by cDNA microarray. The results showed that the mRNA levels of Fit2p and Fit3p, proteins involved in the retention of iron within the cell wall (Protchenko *et al.*, 2001), were the most markedly increased (>30 fold) by the cisplatin treatment (data not shown). In contrast to our findings, previous cDNA microarray studies into altered gene expression levels in cisplatin-treated *S. cerevisiae* did not reveal Fit2p or Fit3p to be affected (Birrell *et al.*, 2002). We therefore investigated the effects of cisplatin on Fit2p and Fit3p by Northern blotting and showed that the mRNA levels of both proteins increased after cisplatin treatment in a dose-dependent manner (Fig. 1A). After 1 hr incubation with cisplatin, mRNA levels

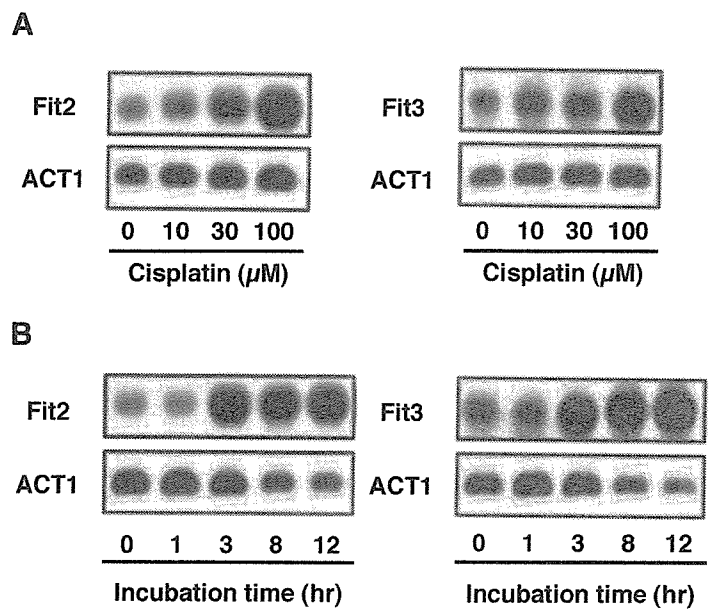

Fig. 1. Effect of cisplatin on Fit2p and Fit3p mRNA levels in yeast cells. *S. cerevisiae* was cultured with various concentrations of cisplatin for 3 hr (A), or with 100 µM cisplatin for various incubation times (B). Each lane contains 10 µg total RNA.

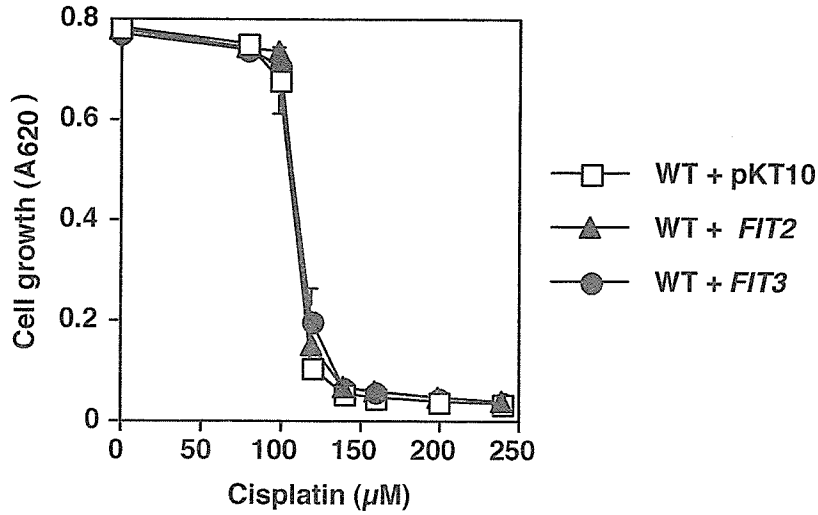

Fig. 2. Effect of *FIT2* or *FIT3* overexpression on sensitivity of yeast cells to cisplatin.
Yeast strains (BY4742) carrying pKT10 (open square), pKT10-*FIT2* (closed triangle) or pKT10-*FIT3* (closed circle) were grown in SD (-Ura) medium containing cisplatin. After 48 hr incubation, the absorbance at 620 nm was measured spectrophotometrically. Each point and bar represents the mean value and standard deviation (S.D.), respectively, of the results from three cultures. The absence of a bar indicates that the S.D. falls within the symbol.

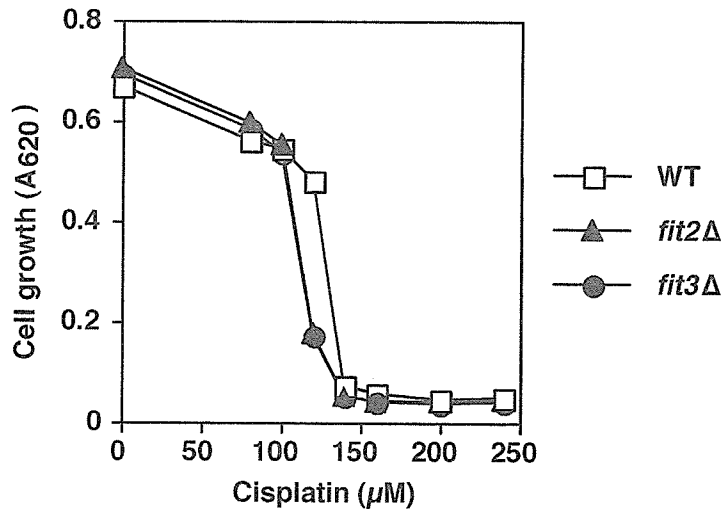

Fig. 3. Effect of *FIT2* or *FIT3* disruption on the sensitivity of yeast cells to cisplatin.
Yeast cells (BY4742 (open square), *fit2Δ* (closed triangle) or *fit3Δ* (closed circle)) were grown in SD medium containing cisplatin. After 48 hr incubation, the absorbance at 620 nm was measured spectrophotometrically. Each point and bar represents the mean value and S.D., respectively, of the results from three cultures. The absence of a bar indicates that the S.D. falls within the space occupied by a symbol.