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Overexpression of Rad23 Confers Resistance to Methylmercury in *Saccharomyces cerevisiae* via Inhibition of the Degradation of Ubiquitinated Proteins

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ABSTRACT

We report here that overexpression of Rad23, a protein related to the ubiquitin-proteasome system, renders yeast cells resistant to methylmercury. Rad23 has three domains: two ubiquitin-associated (UBA) domains that bind to the multiubiquitin chain of ubiquitinated proteins and a single ubiquitin-like (UbL) domain that binds to proteasomes. To examine the mechanism of acquisition of methylmercury resistance that is induced by overexpression of Rad23, we expressed variants of Rad23 in which one or the other of the two types of domain was defective in yeast cells. In cells that overexpressed full-length intact Rad23, we detected elevated levels of intracellular ubiquitinated proteins, and the cells were resistant to methylmercury. In contrast, cells that overexpressed Rad23 with a defective UBA domain were not resistant to methylmercury and contained control levels of ubiquitinated proteins. Yeast cells that overexpressed Rad23 with a defective UbL domain exhibited enhanced resistance to methylmercury and contained even higher levels of ubiquitinated proteins than cells that overexpressed intact full-length Rad23. Rad23 is known to have two mutually contradictory functions. It suppresses the degradation of ubiquitinated proteins by proteasomes via a mechanism mediated by the UBA domains, and it enhances the degradation of ubiquitinated proteins via a mechanism that is mediated by the UbL domain. Therefore, our findings suggest that Rad23 might induce resistance to methylmercury in yeast cells by suppressing the degradation of proteins that reduce the toxicity of methylmercury via a UBA domain-mediated mechanism.

Methylmercury is an important environmental pollutant, causing severe damage to the central nervous system as a result of passage across the blood-brain barrier (Clarkson, 2002; Castoldi et al., 2003; Sanfeliu et al., 2003). Many reports on methylmercury poisoning have been published, but mechanisms of methylmercury toxicity, as well as bio-defense mechanisms against this toxicity remain to be clarified.

We have been studying the genes that are involved in resistance to methylmercury in yeast, a eukaryotic unicellular organism whose gene products have many functional similarities to those of mammals, including humans (Miura et al., 1999; Naganuma et al., 2000, 2002; Furuchi et al., 2002; Hwang et al., 2002). We demonstrated that overexpression of Cdc34, a ubiquitin-conjugating enzyme (E2) that is a component of the ubiquitin-proteasome system, induces a resistance to methylmercury toxicity in yeast cells (Furuchi et al., 2002).

The ubiquitin-proteasome system is involved in the intracellular degradation of proteins (Hershko and Ciechanover, 1998; Pickart, 2001, 2004). In this system, multiple ubiquitin molecules are linked to substrate proteins by sequential reactions that are catalyzed by ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3). The multiubiquitin chain that has been attached to a protein in this way allows the ubiquitinated protein to be recognized by the 26S proteasome and degraded. When Cdc34 is overexpressed in cells, ubiquitination reactions are activated, and the degradation of certain proteins by the ubiquitin-proteasome system is enhanced (Hwang et al., 2002). The acquisition of resistance to methylmercury in Cdc34-overexpressing yeast cells requires the ubiquitin-conjugating activity of Cdc34 and the proteolytic activity of proteasomes (Hwang et al., 2002). Therefore, it seems likely that

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ABBREVIATIONS: UBA domain, ubiquitin-associated domain; UbL domain, ubiquitin-like domain; SD, synthetic dextrose; PAGE, polyacrylamide gel electrophoresis; F, forward; R, reverse.

certain as-yet-unidentified proteins that increase methylmercury toxicity might exist in cells and that toxicity might be reduced by the enhanced degradation of such proteins, mediated by the ubiquitin-proteasome system, when Cdc34 is overexpressed (Hwang et al., 2002).

In the present study, we examined the effects of Rad23, which is known to interact with ubiquitinated proteins, on the acquisition of resistance to methylmercury by yeast cells. It has been reported that Rad23 has two mutually contradictory functions. One of the functions of Rad23 is the inhibition of elongation of the ubiquitin chain via binding to the ubiquitin moiety of ubiquitinated proteins (Ortolan et al., 2000; Bertolaet et al., 2001; Chen et al., 2001; Raasi and Pickart, 2003). Because proteasomes recognize multiubiquitinated proteins as substrates when more than a certain number of ubiquitin molecules have been attached (Pickart, 2001, 2004), it has been postulated that proteins can elude degradation by proteasomes when the elongation of ubiquitin chains is inhibited. The second function of Rad23 is the transportation of ubiquitinated proteins to proteasomes (Chen and Madura, 2002; Rao and Sastry, 2002; Lambertson et al., 2003). Rad23 binds to proteasomes, thereby enhancing the degradation of ubiquitinated proteins. Thus, Rad23 seems to regulate the degradation of ubiquitinated proteins via the mutually contradictory enhancement and suppression of such degradation. In this study, we found that overexpression of Rad23 renders yeast cells resistant to methylmercury, and, in contrast to overexpression of Cdc34, the resistance might be induced by suppression of the degradation of proteins that is mediated by the ubiquitin-proteasome system.

Materials and Methods

Culture and Transformation of Yeast Cells. The strain of *Saccharomyces cerevisiae* used in this study was W303B (*MATα his3 can1-100 ade2 leu2 trp1 ura3*) (Naganuma et al., 2000). Yeast cells were cultured at 30°C in synthetic dextrose (SD) medium. Plasmid DNA was introduced into W303B cells by the high-efficiency lithium acetate transformation method (Miura et al., 1999).

Construction of Plasmids. The *RAD*23 and FLAG-*RAD23* genes were amplified by the polymerase chain reaction from yeast genomic DNA as template with the following oligonucleotides as primers: RAD23-F, 5′-CACAGAGCACACAAAGACAAC-3′, and RAD23-R, 5′-GTGAAGATACTTCAAGCCA-3′ for the *RAD23* gene; and RAD23-FLAG- F, 5′-CATACAATAGAAAAATGGACTACAAGGATGACGATGACAAGGTTAGCTTAACCTTTAA-3′ and RAD23-R for the FLAG-*RAD23* gene. The amplified fragments of DNA were ligated into the pKT10 yeast expression vector. Sequences of constructs were verified with an automated sequencer.

Quantification of the Toxicity of Methylmercury in Yeast Cells. Yeast cells (10^5 cells/200 μl) were cultured in SD (-Ura) liquid medium that contained methylmercuric chloride at various concentrations. To quantify cell growth, we measured the absorbance of the culture at 600 nm every 2 h for 48 h. For the colony-formation assay, we cultured yeast cells (10^6 cells/ml) in SD (-Ura) liquid medium that contained methylmercuric chloride (800 nM) for 3 h at 30°C. After treatment with methylmercuric chloride, yeast cells were seeded at a density of 10^3 cells per plate on agar-solidified SD (-Ura) medium, and formation of colonies was examined after culture for 2 days at 30°C. Yeast cells transformed with the pKT10 empty vector were used as controls.

Site-Directed Mutagenesis. Site-directed mutagenesis of the gene for Rad23 was performed as described elsewhere (Furuchi et al., 2004) with a kit for site-directed mutagenesis from Stratagene (Cedar Creek, TX) according to the manufacturer's instructions. We constructed a variety of mutant *RAD23* or FLAG-*RAD23* genes by creating pairs of SacI sites in the open reading frame of the *RAD23* or FLAG-*RAD23* genes and excising the fragments between the respective pairs of SacI sites. We amplified fragments by polymerase chain reaction using plasmid pKT10-*RAD23* or pKT10-FLAG-*RAD23* as the template and the following oligonucleotides as primers: 5′UbL-F, 5′-CAACATACAATAGAAAAATGGAGCTCTTAACCTTTAAAAATTTCAAG-3′; 5′UbL-R, 5′-CTTGAAATTTTTAAAGGTTAAGAGCTCCATTTTTCTATTGTATGTTG-3′; 3′UbL-F, 5′-CATGGTTTCTCAAAAAGAGCTCACGAAGACCAAAGTAAC-3′; and 3′UbL-R, 5′-GTTACTTTGGTCTTCGTGAGCTCTTTTTGAGAAACCATG-3′ for deletion of the UbL domain; 5′UBA1-F, 5′-CGGGATTCGTGGTGGGAGAGCTCAGGAACGAGACCATCGAG-3′; 5′UBA1-R, 5′-CTCGATGGTCTCGTTCCTGAGCTCTCCCACCACGAATCCCG-3′; 3′UBA1-F, 5′-GAATATCTACTGATGGAGCTCCCAGAAAATCTGCGTC-3′; and 3′UBA1-R, 5′-GACGCAGATTTTCTGGGAGCTCCATCAGTAGATATTC-3′ for deletion of the UBA1 domain; and 5′UBA2-F, 5′-CTTTCCAAGTTGACTATACCGAGCTCGACGATCAAGCTATTTCGC-3′; 5′UBA2-F, 5′-GCGAAATAGCTTGATCGTCGAGCTCGGTATAGTCAACTTGGAAAG-3′; 3′UBA2-F, 5′-CAAATATTCTATTCAGCGAGCTCGCCGACTGAGATTGTAG-3′; and 3′UBA2-R, 5′-CTACAATCTCAGTCGGCGAGCTCGCTGAATAGAATATTTG-3′ for deletion of the UBA2 domain. After creation of each pair of SacI sites, the plasmid was cleaved with SacI and self-ligated. All mutations were confirmed by DNA sequencing. The resultant plasmids were designated pKT10-FLAG-*RAD23*$^{UbL\Delta}$, pKT10-FLAG-*RAD23*$^{UBA1\Delta}$, pKT10-FLAG-*RAD23*$^{UBA2\Delta}$, and pKT10-FLAG-*RAD23*$^{UBA1\Delta+UBA2\Delta}$.

Immunoblotting. Cell extracts were prepared, and immunoblotting was performed as described elsewhere (Hwang et al., 2002). To quantify the total ubiquitinated proteins, we cultured yeast cells that overexpressed FLAG-Rad23 or FLAG-mutant Rad23 to the midlogarithmic phase of growth in SD (-Ura) liquid medium and collected them by centrifugation. We suspended the cells in buffer C (20 mM Tris-HCl , pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 50 mM KCl, 5% glycerol, 3 mM dithiothreitol, and the protease inhibitors phenylmethylsulfonyl fluoride and pepstatin A) and lysed them using glass beads. The cell extracts were fractionated by SDS-polyacrylamide gel (12.5%) electrophoresis (PAGE), and then bands of protein were transferred to an Immobilon-P membrane (Millipore Corporation, Billerica, MA) and subjected to immunostaining with FLAG-specific monoclonal antibodies (anti-FLAG M2; Sigma Chemical, St. Louis, MO) or multiubiquitin-specific monoclonal antibodies (clone FK2; MBL, Nagoya, Japan), which recognize multiubiquitin chains of the polyubiquitinated proteins but not free ubiquitin or the protein moieties (Fujimuro et al., 1994).

Results

We investigated the effects of overexpression of Rad23, which is a protein that interacts with the ubiquitin-proteasome system, on the sensitivity of yeast cells to methylmercury. As shown in Fig. 1, yeast cells that overexpressed Rad23 were much more resistant to methylmercury than were control yeast cells, which harbored the empty vector.

Suppression of the elongation of ubiquitin chain by Rad23 is mediated by two ubiquitin-associated (UBA) domains (UBA 1 and UBA2) (Fig. 2a). Rad23 binds to the ubiquitin moiety of ubiquitinated proteins via these domains, inhibiting the further elongation of ubiquitin chains (Ortolan et al., 2000; Chen et al., 2001; Raasi and Pickart, 2003). By contrast, the region of the Rad23 molecule that is required for the transportation of ubiquitinated proteins to proteasomes is the ubiquitin-like (UbL) domain that is located at the amino terminus (Elsasser et al., 2002; Saeki et al., 2002;

Walters et al., 2003). The UbL domain is strongly homologous to ubiquitin, and it has been postulated that Rad23 acts as a role as a shuttle that transports multiubiquitinated proteins to the proteasome by binding to the proteasome via this domain (Chen and Madura, 2002; Rao and Sastry, 2002).

To clarify the relationship between the toxicity of methylmercury and the two contradictory functions of Rad23, we investigated the sensitivity to methylmercury of yeast cells in which we overexpressed truncated variants of Rad23 that were defective in either the UbL domain or one or both of the UBA domains as FLAG-fusion proteins (Fig. 2a). We confirmed the expression of each truncated protein in the respective lines of yeast cells by Western blotting with FLAG-specific monoclonal antibodies (Fig. 2b). We found that yeast cells which overexpressed the Rad23 with a defective UbL domain (UbLΔ) were more resistant to methylmercury than were the cells that overexpressed intact full-length Rad23 (Fig. 2c). In contrast, yeast cells that overexpressed Rad23 with a defective UBA1 domain (UBA1Δ) were less resistant to methylmercury than cells that overexpressed intact full-length Rad23. Furthermore, yeast cells that overexpressed Rad23 with a defect only in UBA2 (UBA2Δ) or with a defect in both UBA1 and UBA2 (UBA1Δ + UBA2Δ) were resistant only to a very low level of methylmercury (Fig. 2c). We obtained similar results with yeast cells that overexpressed the respective Rad23 mutants without the FLAG tag (data not shown). Our findings suggest that the UbL domain in Rad23 might be involved in the enhancement of methylmercury toxicity, whereas both the UBA1 and UBA2 domains might be involved in the acquisition of resistance to methylmercury toxicity. Moreover, the UBA2 domain might be more intimately involved than the UBA1 domain. The observation that yeast cells which overexpressed intact full-length Rad23 were resistant to methylmercury (Fig. 1) indicates that the functions mediated by the UBA1 and UBA2 domains might dominate those mediated by the UbL domain with respect to the acquisition of resistance to methylmercury, at least when Rad23 is overexpressed.

Chen and Madura (2002) reported that overexpression of Rad23 increased the total amount of cellular multiubiquitinated proteins. Therefore, we examined the total amounts of ubiquitinated proteins in yeast cells that overexpressed the various truncated mutant forms of Rad23 as FLAG-fusion proteins. We found a marked increase in the total amount of ubiquitinated proteins in the yeast cells that overexpressed the intact full-length Rad23, as reported previously, and a still more marked increase in the total amount of ubiquitinated proteins in the yeast cells that overexpressed Rad23 with defect in the UbL domain (Fig. 3). In addition, we detected a marked reduction in the total amount of ubiquitinated proteins in the yeast cells that overexpressed Rad23 with a defect in either the UBA1 or the UBA2 domain. The extent of the reduction was higher when the defect was in UBA2 than when it was in UBA1 (Fig. 3). These results show clearly that the UbL domain of Rad23 plays a role in reducing the cellular level of ubiquitinated proteins, whereas the UBA1 and UBA2 domains play a role in the opposite phenomenon and increase levels of these proteins. Thus, our findings support the reported contradictory mechanisms of action of Rad23 in the degradation of ubiquitinated proteins.

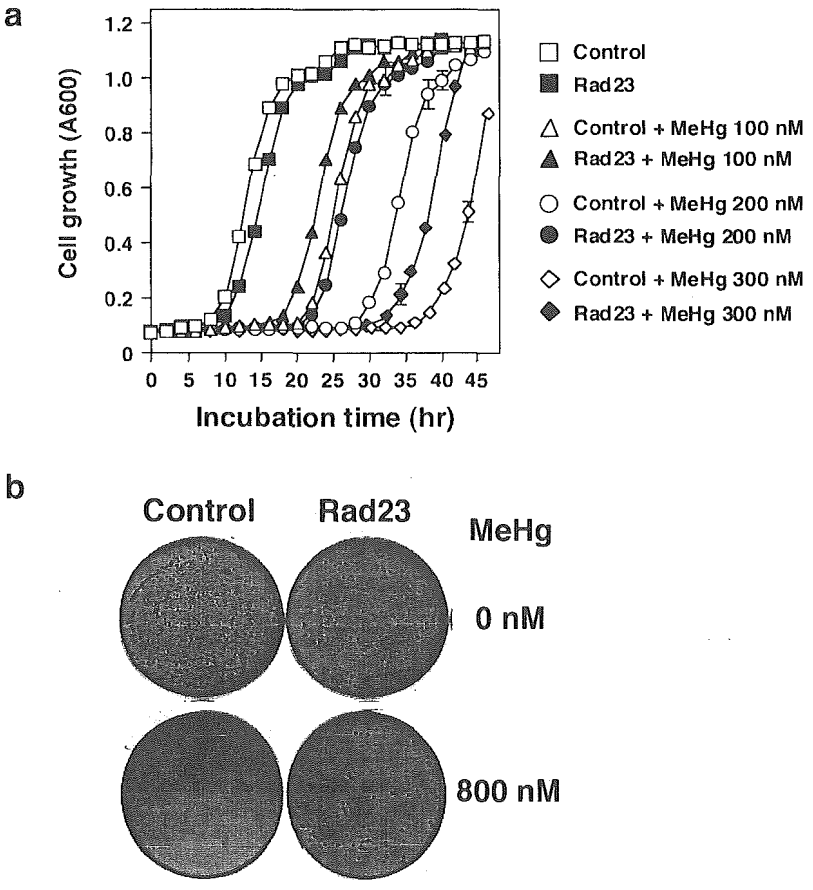

Fig. 1. Sensitivity of yeast cells that overexpressed Rad23 to methylmercury. a, yeast cells (10^5 cells/200 μl/well) that harbored pKT10 or pKT10-*RAD23* were cultured at 30°C in SD (-Ura) liquid medium that contained methylmercuric chloride (MeHg) at the indicated concentrations. Absorbance at 600 nm was measured spectrophotometrically every 2 h for 48 h. Each point and bar represents the mean value and S.D. of results from three cultures. The absence of a bar indicates that the S.D. falls within the symbol. b, yeast cells (10^6 cells/ml) that harbored pKT10 or pKT10-*RAD23* were cultured in SD (-Ura) liquid medium that contained 800 nM MeHg for 3 h at 30°C. After treatment with MeHg, yeast cells were seeded at a density of 10^3 cells per plate on agar-solidified SD (-Ura) medium, and formation of colonies was determined after incubation for 2 days at 30°C.

The degradation of ubiquitinated proteins by the proteasome is enhanced by UbL-mediated transport of ubiquitinated proteins to the proteasome (Elsasser et al., 2002; Saeki et al., 2002; Walters et al., 2003), whereas suppression of the degradation of ubiquitinated proteins results from inhibition of the elongation of the ubiquitin chains of ubiquitinated proteins, which is mediated by the UBA1 and UBA2 domains of Rad23 (Ortolan et al., 2000; Chen et al., 2001; Raasi and Pickart, 2003).

Discussion

With respect to the two functions of Rad23, it is likely that methylmercury toxicity is reduced by suppression of the degradation of ubiquitinated protein via the UBA domains, whereas toxicity is increased by enhancement of the degradation of ubiquitinated proteins via the UbL domain. In the yeast cells that overexpressed Rad23, the activity of Rad23 that suppresses the degradation of ubiquitinated proteins might dominate the activity that enhances the degradation of ubiquitinated proteins (Fig. 3), explaining, perhaps, the acquisition of the resistance to methylmercury by Rad23-overexpressing yeast cells. Therefore, we propose that certain protein(s) in yeast cells are involved in the reduction of methylmercury toxicity and are degraded by the ubiquitin-proteasome system and that Rad23 might play a role in enhancing the protective actions of these proteins against methylmercury toxicity by suppressing their degradation.

Heretofore, studies of each of the two contradictory functions of Rad23 have been performed independently. There have been few investigations to determine whether the two functions involve the same ubiquitinated proteins as substrates. The results in Figs. 2 and 3 indicate that sensitivity to methylmercury (Fig. 2) decreases with increases in the amounts of ubiquitinated cellular proteins (Fig. 3). Thus, it is possible that Rad23 might mediate both the enhancement and the inhibition of the degradation of a single set of proteins that is involved in protection against methylmercury toxicity. An elaborate mechanism must exist to regulate the two functions of Rad23 whereby cellular concentrations of ubiquitinated proteins are controlled, perhaps via the involvement of Rad23-related proteins, which respond flexibly to various physiological conditions.

We showed previously that overexpression of Cdc34, a ubiquitin-conjugating enzyme, induced resistance to methylmercury (Furuchi et al., 2002) and enhanced the ubiquitina-

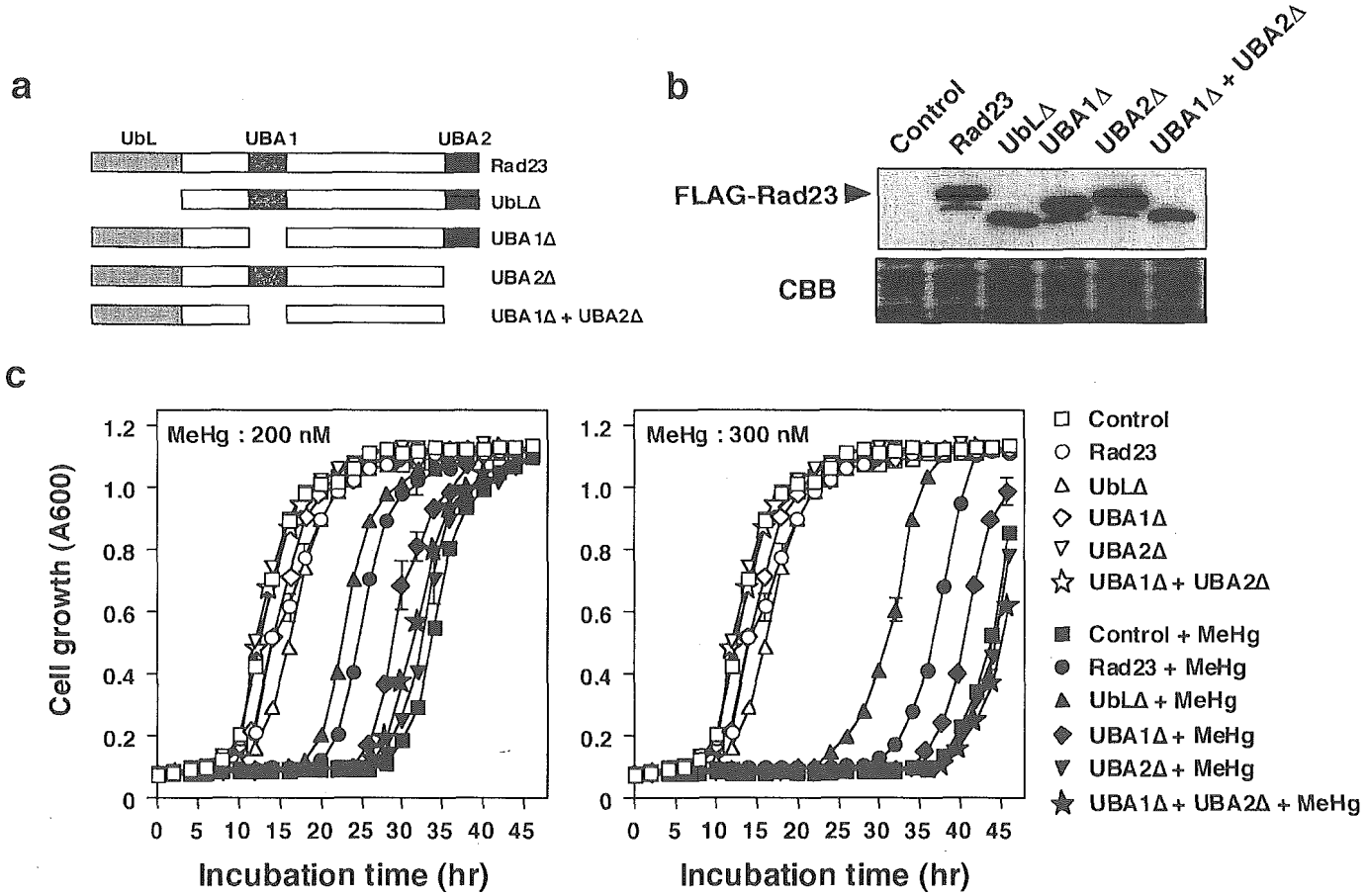

Fig. 2. Effects of overexpression of mutant forms of Rad23 on the sensitivity of yeast cells to methylmercury. a, schematic representation of the structural domains of Rad23 and the mutant proteins generated in this study. Rad23 contains a UbL domain and two UBA domains. b, lysates (20 μg per lane) of yeast cells that harbored pKT10, pKT10-FLAG-*RAD23*, pKT10-FLAG-*RAD23*$^{UbL\Delta}$, pKT10-FLAG-*RAD23*$^{UBA1\Delta}$, pKT10-FLAG-*RAD23*$^{UBA2\Delta}$, or pKT10-FLAG-*RAD23*$^{UBA1\Delta+UBA2\Delta}$ were fractionated by SDS-PAGE. Immunoblotting analysis was performed with FLAG-specific monoclonal antibodies. Staining with Coomassie Brilliant Blue (CBB; bottom) provides an indication of the amount of total protein loaded in each lane. c, yeast cells (10^5 cells/200 μl/well) that overexpressed FLAG-Rad23 or mutant derivatives were cultured at 30°C in SD (-Ura) liquid medium that contained MeHg at the indicated concentrations. Absorbance at 600 nm was measured spectrophotometrically every 2 h for 48 h. Each point and bar represents the mean value and S.D. of results from three cultures. The absence of a bar indicates that the S.D. falls within the symbol.

tion of proteins in yeast cells (Hwang et al., 2002). Cdc34 might confer the resistance to methylmercury by accelerating the degradation of some protein(s) that enhances methylmercury toxicity (Hwang et al., 2002). However, the present study revealed that the activation of ubiquitin-proteasome system is not necessarily effective in preventing methylmercury toxicity. Unlike Cdc34, Rad23 reduces methylmercury toxicity by suppressing the degradation of the proteins that might reduce methylmercury toxicity. Nevertheless, we cannot rule out the possibility that both Cdc34 and Rad23 recognize, as substrate, the same proteins that are indirectly involved in methylmercury toxicity, because Cdc34 is involved in protein ubiquitination and Rad23 binds to the ubiquitin chain of ubiquitinated proteins. However, when we overexpressed Cdc34 in normal and Rad23-defective yeasts, resistance to methylmercury was enhanced to almost the same extent in both lines of yeast cells (data not shown). 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 as a ubiquitin-conjugating enzyme. Multiple proteins that reduce or enhance methylmercury toxicity and are ubiquitinated might be present in cells. The ubiquitin-proteasome system and related proteins might determine the extent of methylmercury toxicity by regulating the cellular concentrations of these various proteins.

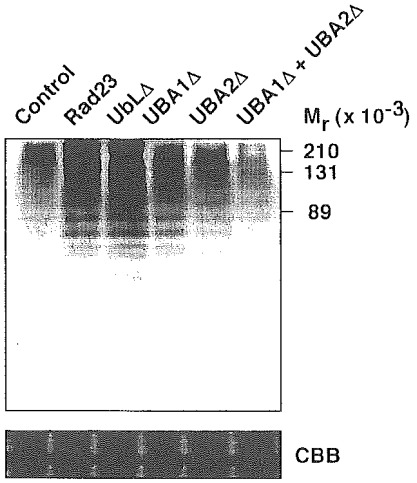

Fig. 3. Effects of overexpression of Rad23 and of mutant derivatives of Rad23 on cellular levels of total ubiquitinated proteins. Lysates (20 μg per lane) of yeast cells that overexpressed FLAG-Rad23 or mutant derivatives of this FLAG-tagged protein were fractionated by SDS-PAGE. Immunoblotting analysis was performed with multiubiquitin-specific monoclonal antibodies. Staining with CBB (bottom) provides an indication of the amount of total protein loaded in each lane.

References

Bertolaet BL, Clarke DJ, Wolff M, Watson MH, Henze M, Divita G, and Reed SI (2001) UBA domains of DNA damage-inducible proteins interact with ubiquitin. *Nat Struct Biol* **8**:417–422.

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

Chen L and Madura K (2002) Rad23 promotes the targeting of proteolytic substrates to the proteasome. *Mol Cell Biol* **22**:4902–4913.

Chen L, Shinde U, Ortolan TG, and Madura K (2001) Ubiquitin-associated (UBA) domains in Rad23 bind ubiquitin and promote inhibition of multi-ubiquitin chain assembly. *EMBO (Eur Mol Biol Organ) Rep* **2**:933–938.

Clarkson TW (2002) The three modern faces of mercury. *Environ Health Perspect* **110 (Suppl 1)**:11–23.

Elsasser S, Gali RR, Schwickart M, Larsen CN, Leggett DS, Muller B, Feng MT, Tubing F, Dittmar GA, and Finley D (2002) Proteasome subunit Rpn1 binds ubiquitin-like protein domains. *Nat Cell Biol* **4**:725–730.

Fujimuro M, Sawada H, and Yokosawa H (1994) Production and characterization of monoclonal antibodies specific to multi-ubiquitin chains of polyubiquitinated proteins. *FEBS Lett* **349**:173–180.

Furuchi T, Hwang GW, and Naganuma A (2002) Overexpression of the ubiquitin-conjugating enzyme Cdc34 confers resistance to methylmercury in *Saccharomyces cerevisiae*. *Mol Pharmacol* **61**:738–741.

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. *Nucleic Acids Res* **32**:2578–2585.

Hershko A and Ciechanover A (1998) The ubiquitin system. *Annu Rev Biochem* **67**:425–479.

Hwang GW, 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.

Lambertson D, Chen L, and Madura K (2003) Investigating the importance of proteasome-interaction for Rad23 function. *Curr Genet* **42**:199–208.

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.

Naganuma A, Furuchi T, Miura N, Hwang GW, and Kuge S (2002) Investigation of intracellular factors involved in methylmercury toxicity. *Tohoku J Exp Med* **196**:65–70.

Naganuma A, Miura N, Kaneko S, Mishina T, Hosoya S, Miyairi S, Furuchi T, and Kuge S (2000) GFAT as a target molecule of methylmercury toxicity in *Saccharomyces cerevisiae*. *FASEB J* **14**:968–972.

Ortolan TG, Tongaonkar P, Lambertson D, Chen L, Schauber C, and Madura K (2000) The DNA repair protein Rad23 is a negative regulator of multi-ubiquitin chain assembly. *Nat Cell Biol* **2**:601–608.

Pickart CM (2001) Ubiquitin enters the new millennium. *Mol Cell* **8**:499–504.

Pickart CM (2004) Back to the future with ubiquitin. *Cell* **116**:181–190.

Raasi S and Pickart CM (2003) Rad23 ubiquitin-associated domains (UBA) inhibit 26 S proteasome-catalyzed proteolysis by sequestering lysine 48-linked polyubiquitin chains. *J Biol Chem* **278**:8951–8959.

Rao H and Sastry A (2002) Recognition of specific ubiquitin conjugates is important for the proteolytic functions of the ubiquitin-associated domain proteins Dsk2 and Rad23. *J Biol Chem* **277**:11691–11695.

Saeki Y, Sone T, Toh-e A, and Yokosawa H (2002) Identification of ubiquitin-like protein-binding subunits of the 26S proteasome. *Biochem Biophys Res Commun* **296**:813–819.

Sanfeliu C, Sebastia J, Cristofol R, and Rodriguez-Farre E (2003) Neurotoxicity of organomercurial compounds. *Neurotox Res* **5**:283–305.

Walters KJ, Lech PJ, Goh AM, Wang Q, and Howley PM (2003) DNA-repair protein hHR23a alters its protein structure upon binding proteasomal subunit S5a. *Proc Natl Acad Sci USA* **100**:12694–12699.

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Overexpression of Bop3 confers resistance to methylmercury in *Saccharomyces cerevisiae* through interaction with other proteins such as Fkh1, Rts1, and Msn2

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Abstract

We found that overexpression of Bop3, a protein of unknown function, confers resistance to methylmercury in *Saccharomyces cerevisiae*. Bmh2, Fkh1, and Rts1 are proteins that have been previously shown to bind Bop3 by the two-hybrid method. Overexpression of Bmh2 and the homologous protein Bmh1 confers resistance to methylmercury in yeast, but overexpression of either Fkh1 or Rts1 has a minimal effect. However, the increased level of resistance to methylmercury produced by overexpression of Bop3 was smaller in Fhk1-deleted yeast as compared with that of the wild-type strain. In contrast, the degree of resistance was significantly elevated in Rts1-deleted yeast. Msn2 and Msn4 were previously reported as proteins that bind to Bmh1 and Bmh2. Overexpression of Msn2 conferred a much greater sensitivity to methylmercury in yeast, while deletion of the corresponding gene lowered the degree of resistance to methylmercury induced by overexpression of Bop3. These results suggest that multiple proteins are involved in minimizing the toxicity of methylmercury induced by overexpression of Bop3.

Keywords: Methylmercury; Resistance; Yeast; Bop3; Rts1; Fkh1

Methylmercury, the predominant form of organic mercury present in the environment, is a toxic compound producing severe disorders in the human central nervous system [1–3]. Fish are susceptible to the bioaccumulation of methylmercury, which may then be ingested by humans in their daily diet. In Japan in the 1950s thousands of people developed central nervous system disorders through eating large amounts of fish and shellfish polluted with methylmercury [4]. Recent epidemiological studies have indicated that ingestion of methylmercury in fish during pregnancy can result in neuroethological effects in the offspring [5]. A warning was issued in several countries recommending a restriction of fish in the diet of pregnant women and infants. Given the severe nature of this poison, methods for both the prophylaxis and treatment of methylmercury intoxication are required. Unfortunately the mechanism underlying the toxicity of methylmercury is not fully understood. Furthermore, the nature of the protective mechanism that exists in some organisms has not been elucidated.

To elucidate the mechanism of methylmercury toxicity, we examined intracellular factors involved in yeast. Yeast is a genetically well-characterized eukaryotic

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organism that shares many genes common with mammals, including humans. Thus, the factors found in yeast are expected to function similarly in mammalian cells. We have already identified and reported two proteins, GFAT [6–8] and Cdc34 [8–10], which confer resistance to methylmercury in yeast. Genes encoding similar proteins have been found in humans. However, it is anticipated that many additional factors are involved in the development of methylmercury toxicity. In the present study, we have conducted further screening for proteins capable of conferring resistance to methylmercury in yeast and succeeded in identifying a new protein of unknown function, Bop3. Bop3 is a protein that was identified as a multicopy suppressor that could compensate for deletion of Pam1 [11], which was identified as a multicopy suppressor on the deletion of serine/threonine phosphatase 2A (PP2A) [12]. Although the function of Bop3 is unclear, extensive investigations conducted with the two-hybrid method demonstrated that Bop3 is capable of binding to Bmh2 [13], Fkh1 [14], and Rts1 [14]. Our results suggest that although Pam1 is not directly involved in resistance to methylmercury induced by overexpression of Bop3, these binding proteins and their homologues are involved, at least partially, in the resistance mechanism. The results of the present study indicate the involvement of Bop3 in the regulation of some important intracellular functions that exert protective effects against methylmercury toxicity through interaction with multiple proteins.

Materials and methods

Selection of genes that confer resistance to methylmercury. Methylmercury resistance genes were screened as described previously [9,10]. Briefly, *Saccharomyces cerevisiae* W303B (*MAT* α his3 can1-10 ade2 leu2 trp1 ura3) was transformed with a genomic DNA library that had been constructed by inserting a fragment of yeast chromosomal DNA into the *LEU2*-based multicopy plasmid Yep13 [10,15–17]. Transformants were grown in synthetic dextrose (SD) liquid medium without leucine (−Leu) at 30 °C. The Leu^+ transformants were cultured (10^5 cells/200 μl per well) in SD (−Leu) medium for 24 h at 30 °C in the presence of a subtoxic concentration of methylmercury (0.4 μM) in 96-well plates. Transformed cells that had proliferated rapidly and had formed aggregates in the presence of methylmercury were isolated from individual wells and plated on agar-solidified SD (−Leu) medium at a cell density of 5×10^3 cells per 10-cm plate. After incubation for 24 h at 30 °C, colonies were collected and their sensitivity to methylmercury was examined. The sensitivity of yeast cells from each colony was determined by culturing cells (10^5 cells/200 μl per well) in SD (−Leu) medium that contained various concentrations of methylmercury for 48 h. We chose eight colonies that exhibited strong resistance to methylmercury and isolated plasmids from them as described previously. The plasmids were amplified in *Escherichia coli* and then reintroduced into W303B cells to confirm the phenotype. We selected the plasmid that conferred the strongest resistance to methylmercury for further study. The nucleotide sequence of the genomic insert in the selected plasmid was determined with an automated DNA sequencer (Li-Cor, Lincoln, NE). After mapping, the genomic insert was excised and subcloned into the pRS425 vector. Subclones were introduced into W303B yeast cells and the sensitivity to methylmercury of each resultant cell line was determined.

Quantification of growth inhibition by methylmercury and other compounds. The toxic effects of various agents on yeast cells were quantified during growth of cells in SD liquid medium (−Leu or −Ura). A suspension of cells (10^4 cells per well) was grown in a 200 μl aliquot of fresh liquid medium that contained one of the respective compounds at the concentration indicated. After incubation for 48 h, the absorbance at 620 nm (A_{620}) was measured spectrophotometrically as an index of cell growth.

Construction of gene expression vectors. The genes from yeast genomic DNA were amplified by PCR with the following oligonucleotides as primers:

5′-GACCAAAAACGGCGTTAAGA-3′ and
5′-CCTTTATCCCATCGACAAAGC-3′ for the *BOP1* gene;
5′-TCTCCGGGTAGAAGTGAAAAG-3′ and
5′-TGCTAGTACAACACGTTTGG-3′ for the *BOP2* gene;
5′-GTTGCTCTTCTAGTGCAGTTG-3′ and
5′-TACATACGTAGGGGGAACATC-3′ for the *BOP3* gene;
5′-CCTACTTTTCAAAATTGAGAG-3′ and
5′-GAACTACAAATTATTACACCC-3′ for the *BMH1* gene;
5′-CAAATCAACAAAAAGTACCCG-3′ and
5′-CTTCATTTCCCCTTGTATTTC-3′ for the *BMH2* gene;
5′-GCTCATAGAAGAACTAGATC-3′ and
5′-AGCCGTAAGCTTCATAAGTC-3′ for the *MSN2* gene;
5′-TAACGCCTTTATCAGTTCGGC-3′ and
5′-TTGTCATACCGTAGCTTGTC-3′ for the *MSN4* gene.

The resulting PCR products were ligated into the pGEM-T Easy or pTargeT vector (Promega, Madison, WI). Each insert was digested with restriction endonucleases as follows: *Kpn*I and *Xho*I for the *BOP1*, *BOP2*, and *BOP3* genes; *Not*I for the *MSN2* gene; and *Eco*RI for the *BMH1*, *BMH2*, and *MSN4* genes, and fragments were ligated into the pKT10-GAPDH (*URA3*) yeast expression vector. Sequences of constructs were verified with an automated sequencer.

Gene disruption. Genes of the W303B yeast strain were disrupted as described previously [9,17]. For constructions of the *bmh1::HIS3*, *bmh2::HIS3*, *fkh1::HIS3*, and *rts1::HIS3* vector, the *HIS3* gene was amplified by PCR with the following oligonucleotides as primers: BMH1-HIS-F (5′-ATGTCAACCAGTCGTGAAGATTCTGTGTA CCTAGCCAAGTTGGCTGAACACTCTTGGCCTCCTCTAG-3′) and BMH1-HIS-R (5′-TTACTTTGGTGCTTCACCTTCGGCGGC AGCAGGTGGCTGCTGTTGCTGATTCGTTCAGAATGACAC G-3′) for disruption of the *BMH1* gene; BMH2-HIS-F (5′-ATG TCCCAAACTCGTGAAGATTCTGTTTACCTAGCTAAATTAGC TGAACACTCTTGGCCTCCTCTAG-3′) and BMH2-HIS-R (5′-TTATTTGGTTGGTTCACCTTGAGTTTGTTCAGCTGGAGCTT GTTGTTGCTTCGTTCAGAATGACACG-3′) for disruption of the *BMH2* gene; MSN2-HIS-F (5′-ATGACGGTCGACCATGATTTC AATAGCGAAGATATTTTATTCCCCATAGACTCTTGGCCTCC TCTAG-3′) and MSN2-HIS-R (5′-GTGATAAATTAGTGTCATCA TCATCATCATTCAATAAGAGATCACTAGAATCGTTCAGAA TGACACG-3′) for disruption of the *MSN2* gene; FKH1-HIS-F (5′-ATGTCTGTTACCAGTAGGGAACAAAAATTTAGTGGTAAGT ATAGTTCGTACTCTTGGCCTCCTCTAG-3′) and FKH1-HIS-R (5′-ATTCCTCCTCTGGTGTAATTTTCATCTTCTTCATCTTCTT CCATCATAATTCGTTCAGAATGACACG-3′) for disruption of the *FKH1* gene; RTS1-HIS-F (5′-ATGATGCGTGGTTTCAAGCA AAGATTAATAAAGAAGACCACCGGGTCTTCCTCTTGGCCT CCTCTAG-3′) and RTS1-HIS-R (5′-TCGAATCTAGATGAAGAA TGACGTTGGGGAGTCTTAATTAACTCTAAATCTCGTTCAG AATGACACG-3′) for disruption of the *RTS1* gene. To disrupt the *PAM1* gene of W303B strain yeast, *pam1::KAN*^{r} was amplified from the *PAM1*-disrupted yeast (Complete Set of *S. cerevisiae* Gene Deletion Strains; Euroscarf, Frankfurt, Germany), which is a deletion mutant of BY4742 strain yeast, by PCR with primers 5′-

GGCGATTTGCTTCCATATTCT-3′ and 5′-GGCAACGTTTT CAATGGTCA-3′. The PCR products were introduced into yeast strain W303B to construct the corresponding disruptant. Disruption of the gene was verified by PCR analysis with primers:

5′-GGCATATCTCGCGATAGGTAT-3′ and
5′-TTGGATTGGAAAGACAAGGG-3′ for the *PAM1* gene;
5′-GAACTACAAATTATTACACCC-3′ and
5′-CAAATCAACAAAAAGTACCCG-3′ for the *BMH1* gene or
5′-CTTCATTTCCCCTTGTATTTC-3′ for the *BMH2* gene;
5′-GCTCATAGAAGAACTAGATC-3′ and
5′-AGCCGTAAGCTTCATAAGTC-3′ for the *MSN2* gene;
5′-GCAAAGAAAGGCTTGGAGAGA-3′ and
5′-ATACATATGGGTTCGACGACG-3′ for the *FKH1* gene;
5′-CGTGCTATTTTCGAACATCCA-3′ and
5′-TCCTCACTTCTTCGAGCTTGT-3′ for the *RTS1* gene.

Results and discussion

To identify genes whose overexpression confers resistance to methylmercury in *S. cerevisiae*, we introduced a library of yeast chromosomal DNA fragments in the 2-μm multicopy vector Yep13, into the yeast strain W303B. Eight transformants were obtained that could grow on SD (−Leu) medium containing an otherwise lethal amount of methylmercury (0.4 μM). The transformant P54, which exhibited the strongest resistance, was selected for further study. Partial sequencing of clone P54 and a subsequent search of the *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces/) revealed that the insert corresponded to region 544177–550829 on chromosome 14 of *S. cerevisiae*. Two open reading frames, *YIP3* and *BOP3*, were contained on the P54 fragment. Each open reading frame was excised from pRS425-P54 using appropriate restriction enzymes to give two fragments, F1 and F2 (Fig 1A). These two fragments were subcloned into pRS425 vector. Sensitivity to methylmercury was then examined after introduction of the respective plasmids into the parent yeast strain. Yeast harboring pRS425-F1 (pRS425-BOP3) showed stronger resistance to methylmercury as compared to yeast harboring pRS425 or pRS425-F2 (Fig. 1B). These results indicated that *BOP3* is involved in resistance to methylmercury.

First, we examined the sensitivity of Bop3-overexpressing yeast cells to heavy metal compounds other than methylmercury. Yeast cells overexpressing Bop3 did not show resistance to mercury compounds, such as inorganic mercury and *p*-chloromercuribenzoic acid (*p*CMB), nor other heavy metals, such as cadmium and copper (data not shown). Thus, overexpression of Bop3 in yeast seemed to impart a relatively specific resistance to methylmercury.

Bop3 (Bypass Of Pam1) is a protein with unknown function that was identified, together with Bop1 and Bop2, as a multicopy suppressor of Pam1 [11]. Therefore, we examined the effect of overexpression of either Bop1 or Bop2 on the sensitivity to methylmercury. Overexpression of Bop1 or Bop2 in yeast resulted in a similar sensitivity to methylmercury as that of the control (i.e., yeast harboring the vector, pKT10) (Fig. 2A). These results indicate that the function as a multicopy suppressor of Pam1 is not needed for the protective effects of Bop3 against methylmercury.

Pam1 was identified as a multicopy suppressor on PP2A deletion [12], but the mechanism of action of this protein remains unclear. We generated a PAM1-deleted strain (*pam1Δ*) and examined its sensitivity to methylmercury. Almost no difference was noted with regard to the sensitivity to methylmercury of the *pam1Δ* strain in comparison to the control yeast (Fig. 2B). Sensitivity to methylmercury was also found to be nearly the same when Bop3 was overexpressed in the wild-type strain and *pam1Δ* strain, respectively (Fig. 2B). Thus, Pam1

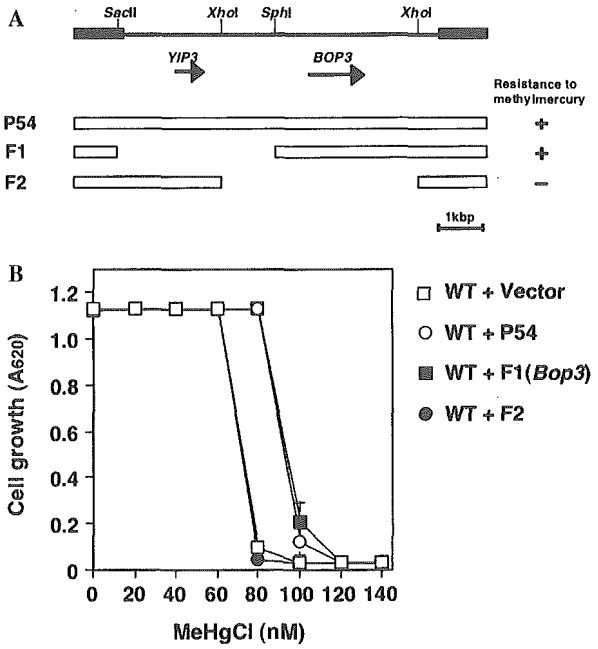

Fig. 1. Identification of a gene that confers resistance to methylmercury. (A) Restriction map of the genomic DNA insert (P54) that conferred resistance to methylmercury. The thick black lines represent the vector YEp13; the thin line represents the genomic DNA inserts. The restriction sites used to generate different subclones are indicated by vertical lines above the yeast genomic DNA insert (P54). The ability of two subclones (F1 and F2) to confer methylmercury resistance is indicated (+, conferred resistance; −, did not confer resistance). ORFs are indicated by black arrows that point in the direction of transcription, with the name of each ORF given below the respective arrow. (B) Sensitivity to methylmercury of yeast that harbored plasmids with the indicated inserts. Yeast cells transformed with pRS425-P54, pRS425-F1, pRS425-F2 or pRS425 were cultured in SD (−Leu) liquid medium that contained methylmercuric chloride at the indicated concentration. After 48-h incubation the absorbance of the culture was measured at 620 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.

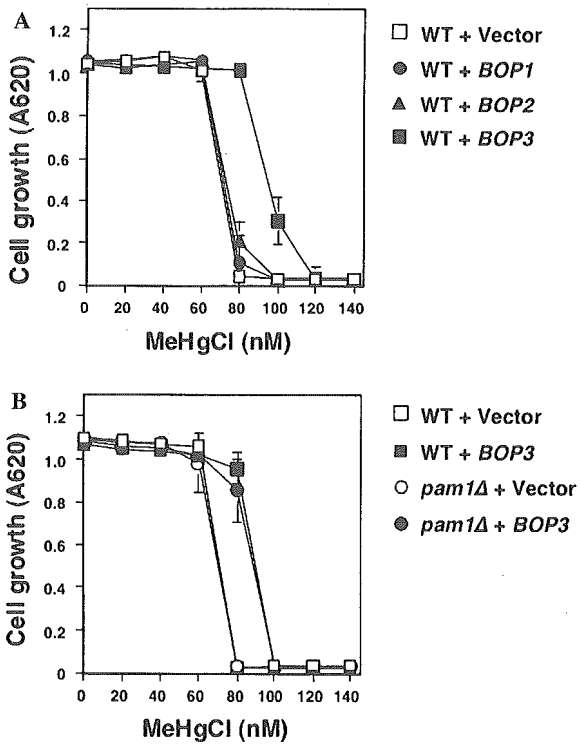

Fig. 2. Sensitivity of yeast cells that overexpressed each Bop family protein to methylmercury (A), and effect of disruption of *PAM1* gene on sensitivity to methylmercury of yeast that overexpressed Bop3 (B). (A) Yeast cells that harbored pKT10 (control), pKT10-BOP1, pKT10-BOP2 or pKT10-BOP3 were grown in SD (−Ura) liquid medium that contained methylmercuric chloride. (B) Yeast *pam1Δ* cells, harboring pRS425 (control) or pRS425-BOP3, were grown in SD (−Leu) liquid medium that contained methylmercuric chloride. For other details, see legend to Fig. 1B.

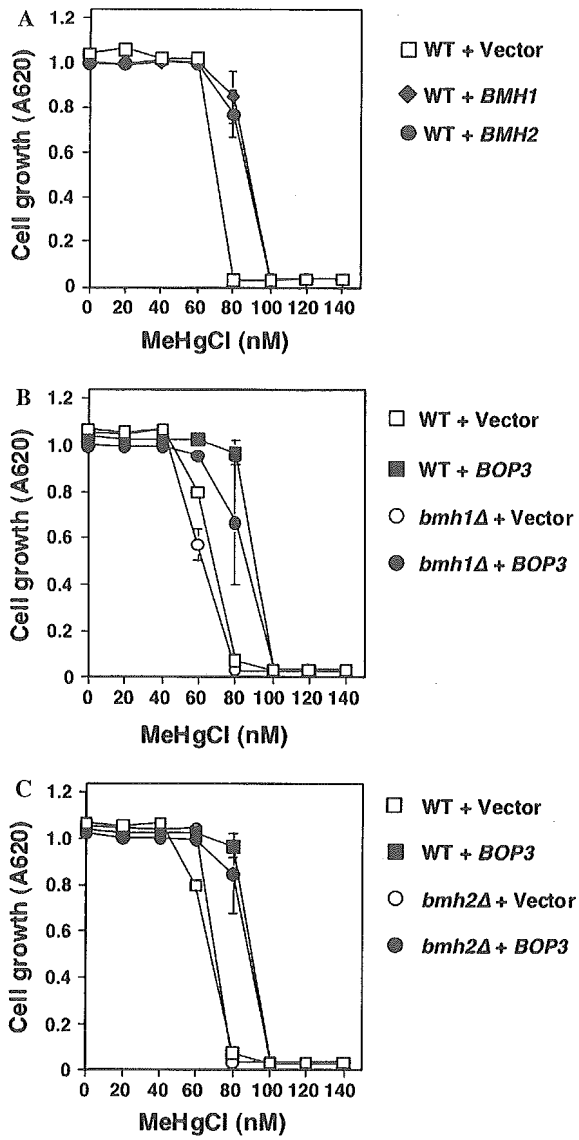

Fig. 3. Sensitivity of yeast cells overexpressing Bmh1 or Bmh2 to methylmercury (A), and effect of disruption of *BMH1* or *BMH2* gene on sensitivity to methylmercury of yeast that overexpressed Bop3 (B,C). (A) Yeast cells that harbored pKT10 (control), pKT10-BMH1 or pKT10-BMH2 were grown in SD (−Ura) liquid medium that contained methylmercuric chloride. (B,C) Yeast *bmh1Δ* (B) or *bmh2Δ* (C) cells, harboring pRS425 (control) or pRS425-BOP3, were grown in SD (−Leu) liquid medium that contained methylmercuric chloride. For other details, see legend to Fig. 1B.

appears to be unnecessary for the protective effects of Bop3 against methylmercury.

Bmh2 (yeast homologue of human 14-3-3 protein) [18,19], Fkh1 (transcription factor of forkhead type) [20], and Rts1 (subunit of protein phosphatase 2A) [21–23] have been identified as proteins that bind to Bop3. We next examined the possible role of these Bop3-binding proteins in the mechanism of acquisition of resistance to methylmercury by overexpression of Bop3. 14-3-3 is a protein conserved in most eukaryotes and is known to play a versatile role within the cell, such as regulation of apoptosis through binding to p53 and regulation of the cell cycle through binding to the Cdc2–CycB complex [24–26]. It was also reported that not only Bmh1 but also Bmh2, a homologous protein of Bmh1, was involved in RAS/MAPK cascade signalling in yeast [18,27]. We examined the effects of overexpression of Bmh1 and Bmh2 in yeast on the sensitivity to methylmercury. Yeast cells that overexpress Bmh1 or Bmh2 showed a significant resistance to methylmercury compared with the control harboring only the vector (Fig. 3A). However, there were differences with regard to the degree of resistance among the clones overexpressing Bmh1 or Bmh2—indeed, there were even clones that did not show resistance. It was reported previously that the growth of yeast overexpressing Bmh1 or Bmh2 was slow [18]. Thus, the level of Bmh mRNA in each clone was compared using quantitative PCR. It was found that the level of Bmh mRNA in the clones showing resistance to methylmercury was three to four times higher than in the controls. However, the mRNA level in clones not showing resistance to methylmercury was seven to eight times higher and the growth of the clones was found to be drastically reduced (data not

shown). On the basis of these findings, clones in which the Bmh1 or Bmh2 expression levels were three to four times higher than that of controls were used as yeast that overexpressed Bmh1 or Bmh2 for further study.

The effect of the deletion of *BMH1* or *BMH2* on the sensitivity of yeast to methylmercury was also examined. Virtually no change was observed in the sensitivity of the yeast to methylmercury when either *BMH1* or *BMH2* was disrupted (Fig. 3B). Upon overexpression of Bop3, the sensitivity of yeast deleted in either *BMH1* or *BMH2* to methylmercury was nearly the same as that of the wild-type strain after overexpression of Bop3 (Fig. 3B). These results seem to indicate that Bmh1 and Bmh2 are not directly related to the methylmercury resistance conferred by overexpression of Bop3. However, because Bmh1 and Bmh2 share high homology (93%) with each other, and the yeast with disruptions in both Bmh1 and Bmh2 cannot grow [19], the possibility that the functional defects induced by deletion of either one of these proteins may be complemented by the other cannot be ruled out.

In addition to Bop3, several other proteins, such as Msn2 and Msn4 [28], are also known to bind to Bmh1 and Bmh2. Because Msn2 and Msn4 are the transcription factors that respond to oxidative stress, changes in osmotic pressure, heat shock or malnutrition [29,30], it is conceivable that these proteins may exert some influence on the sensitivity of the cell to methylmercury. Therefore, the effect of overexpression of Msn2 or Msn4 on methylmercury sensitivity of yeast was examined. While sensitivity to methylmercury of yeast overexpressing Msn4 was similar to that of the control, yeast that overexpressed Msn2 showed a very high sensitivity to methylmercury (Fig. 4A). In contrast, although strains lacking Msn2 (*msn2Δ*) showed some resistance to methylmercury, the degree of resistance was quite low (Fig. 4B). These results indicate that Msn2 is not an essential factor for the development of resistance to methylmercury, although it is capable of augmenting the toxicity of this compound. To examine the involvement of Msn2 in the acquisition of resistance to methylmercury by overexpression of Bop3, Bop3 was overexpressed in the *msn2Δ* strain. The degree of resistance conferred by overexpression of Bop3 in the *msn2Δ* strain was relatively low compared with that of the wild-type yeast (Fig. 4B). This result suggests the partial involvement of Msn2 in the mechanism of resistance to methylmercury by overexpression of Bop3. Msn2 is a transcription factor of the Cys2His2 Zn-finger type that localizes from the cytosol to the nucleus in response to various types of stresses. In the nucleus, Msn2 activates transcription by binding to the stress response element (STRE) in the promoter region of the target gene [31]. High sensitivity to methylmercury brought about by overexpression of Msn2 may result from an induction of one or more of these target genes. If this were

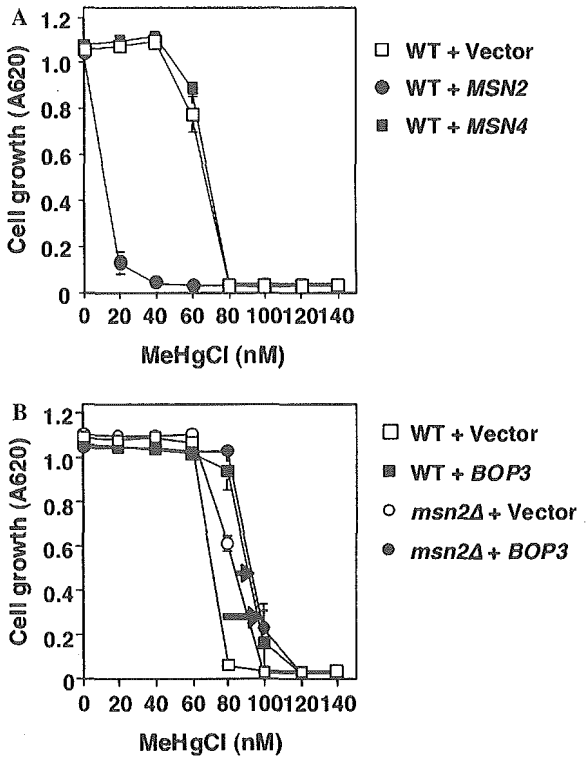

Fig. 4. Sensitivity of yeast cells that overexpressed Msn2 or Msn4 to methylmercury (A), and effect of disruption of *MSN2* gene on sensitivity to methylmercury of yeast that overexpressed Bop3 (B). (A) Yeast cells that harbored pKT10 (control), pKT10-MSN2 or pKT10-MSN4 were grown in SD (−Ura) liquid medium that contained methylmercuric chloride. (B) Yeast *msn2Δ* cells, harboring pRS425 (control) or pRS425-BOP3, were grown in SD (−Leu) liquid medium that contained methylmercuric chloride. Arrows in the figure represent the magnitude of acquired resistance by overexpression of Bop3 in control yeast or in *msn2Δ* yeast. For other details, see legend to Fig. 1B.

the case, inhibition of either Msn2 or its target protein(s) by Bop3 might contribute to the acquisition of resistance to methylmercury by overexpression of Bop3.

Recently, Fkh1 and Rts1 were identified as binding proteins of Bop3 by the two-hybrid method [14]. Forkhead homologue 1 (Fkh1) belongs to a family of transcription factors that have a DNA-binding domain of a winged-helix type called forkhead. It is known that transcription factors of the forkhead type participate in various intracellular responses such as early embryogenesis, differentiation, and the cell cycle [20]. Because the mRNA level of G_2/M cyclin is elevated in the Fkh1-deleted strain, participation of Fkhl in transcription silencing and pseudohyphal growth through cell cycle regulation is suggested [32]. Rts1 is known to represent one of the B-subunits of serine-threonine phosphatase 2A (PP2A) [21–23]. PP2A is well conserved from yeast to human, where it is involved in a variety of intracellular responses such as DNA replication, transcription, signal transduction, and intermediary metabolism [33].

When Fkh1 or Rts1 was overexpressed in wild-type yeast, sensitivity to methylmercury was almost the same as that of the control (data not shown). While the methylmercury sensitivity of the Rts1-disrupted strain (*rts1Δ*) was nearly the same as that of the control strain (Fig. 5), the Fkh1-disrupted strain (*fkh1Δ*) did show resistance to methylmercury (Fig. 6). When Bop3 was overexpressed in *fkh1Δ*, the increased level of resistance to methylmercury was lower than in the wild-type strain overexpressing Bop3 (Fig. 6). However, overexpression of Bop3 in *rts1Δ* markedly increased the level of resistance to methylmercury in comparison to the wild-type strain (Fig. 5). Fkh1, combined with other factors, may increase the toxic effects of methylmercury. However, Fkh1 might not be the rate-limiting factor in this scheme because overexpression of Fkh1 had little effect on the sensitivity of yeast to methylmercury. Fkh1 activity in the system might already be high in wild-type yeast, thereby nullifying the effect of its overexpression. Inhibition of Fkh1 activity by Bop3 may also be involved in the acquisition of resistance to methylmercury by overexpression of Bop3. In contrast, when Bop3 was overexpressed in *rts1Δ*, resistance to methylmercury was found to be greater than that observed when Bop3 was overexpressed in wild-type yeast (Fig. 5). This is despite the fact that deletion or overexpression of Rts1 did not affect the sensitivity of the yeast to methylmercury. Although the precise mechanism of action of Rts1 remains unknown, our results suggest that this protein suppresses the protective effect of Bop3 against methylmercury. It is conceivable that protein(s) with functions similar to those of Rts1 are present in the cell, and that the protein(s) exert inhibitory actions against the protective effects of Bop3 to methylmercury using the same mechanism. As the amount of the protein(s) present in wild-type yeast exceeded that necessary for inhibition of Bop3 activity, deletion or overexpression of Rts1 had no effect on the sensitivity to methylmercury. If this were the case, when the cellular amount of Bop3 exceeds the inhibitory activity of both Rts1 and the protein(s) with similar functions to Rts1, the excess Bop3 will elicit a protective effect against methylmercury. Furthermore, when Rts1 is deleted in Bop3-overexpressing cells, the resistance level of the cell to methylmercury is enhanced because the amount of Bop3 that evades the otherwise inhibitory activity of Rst1 is increased.

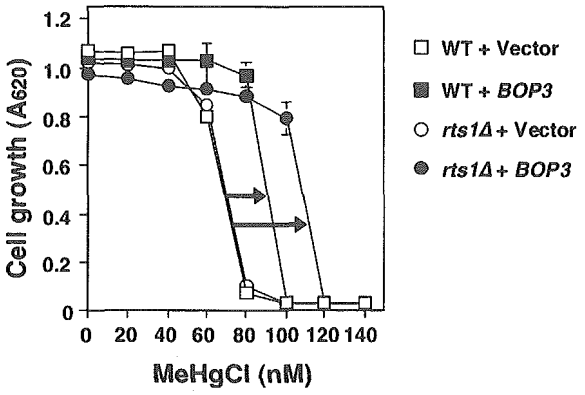

Fig. 5. Effect of disruption of *RTS1* gene on sensitivity to methylmercury of control yeast or Bop3-overexpressing yeast. Yeast *rts1Δ* cells, harboring pRS425 (control) or pRS425-BOP3, were grown in SD (−Leu) liquid medium that contained methylmercuric chloride. Arrows in the figure represent the magnitude of acquired resistance by overexpression of Bop3 in control yeast or in *rts1Δ* yeast. For other details, see legend to Fig. 1B.

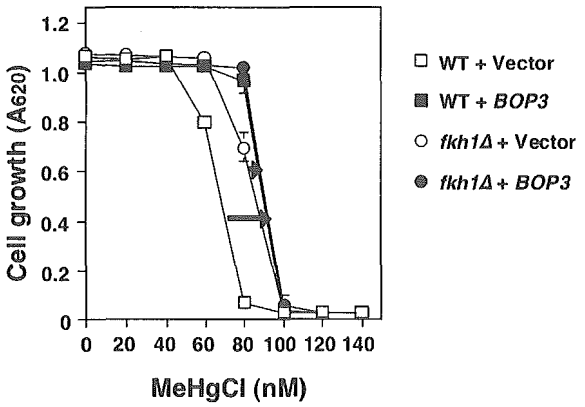

Fig. 6. Effect of disrupting the *FKH1* gene on sensitivity to methylmercury of control yeast or Bop3-overexpressing yeast. Yeast *fkh1Δ* cells, harboring pRS425 (control) or pRS425-BOP3, were grown in SD (−Leu) liquid medium that contained methylmercuric chloride. Arrows in the figure represent the magnitude of acquired resistance by overexpression of Bop3 in control yeast or in *fkh1Δ* yeast. For other details, see legend to Fig. 1B.

In the present study, overexpression of Bop3 was found to confer resistance to methylmercury in yeast. In addition, it was also found that proteins related to Bop3 (i.e., Bmh1, Bmh2, Msn2, Fkh1, and Rts1) participated in the augmentation or alleviation of methylmercury toxicity. Although Fkh1 and Rts1 are the proteins found to bind to Bop3 by screening with a two-hybrid method, there has been no report indicating the functional connection of these proteins to Bop3. The results of the present study show that Fkh1 and Rts1, as well as Msn2 (a Bmhs-binding protein), are involved in methylmercury toxicity. Disruption of the genes encoding each of these proteins influences the degree of acquisition of resistance to methylmercury in yeast overexpressing Bop3. This clearly demonstrates the involvement of these three proteins (Fkh1, Rts1, and Msn2) in the resistance mechanism to methylmercury brought about by overexpression of Bop3. It is conceivable that multiple systems are involved in the protective mechanism brought about by overexpression of Bop3 because deletion of Fkh1 or Msn2 only partially inhibits the protective effect of Bop3 against methylmercury. We believe that Bop3 participates in the regulation of an important intracellular function that exerts a protective effect

against methylmercury toxicity. It is anticipated that the protective effect of Bop3 against methylmercury, as reported for the first time in the present study, will contribute greatly to the elucidation of functions of this interesting protein.

Acknowledgments

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References

[1] K.R. Mahaffey, Recent advances in recognition of low-level methylmercury poisoning, Curr. Opin. Neurol. 13 (2000) 699–707.
[2] T.A. Slotkin, J. Bartolome, Biochemical mechanisms of developmental neurotoxicity of methylmercury, Neurotoxicology 8 (1987) 65–84.
[3] W.D. Atchison, M.F. Hare, Mechanisms of methylmercury-induced neurotoxicity, FASEB J. 8 (1994) 622–629.
[4] K. Eto, Minamata disease, Neuropathology 20 (Suppl.) (2000) S14–S19.
[5] P. Grandjean, P. Weihe, R.F. White, F. Debes, S. Araki, K. Yokoyama, K. Murata, N. Sorensen, R. Dahl, P.J. Jorgensen, Cognitive deficit in 7-year-old children with prenatal exposure to methylmercury, Neurotoxicol. Teratol. 19 (1997) 417–428.
[6] N. Miura, S. Kaneko, S. Hosoya, T. Furuchi, K. Miura, S. Kuge, A. Naganuma, Overexpression of L-glutamine:D-fructose-6-phosphate amidotransferase provides resistance to methylmercury in *Saccharomyces cerevisiae*, FEBS Lett. 458 (1999) 215–218.
[7] A. Naganuma, N. Miura, S. Kaneko, T. Mishina, S. Hosoya, S. Miyairi, T. Furuchi, S. Kuge, GFAT as a target molecule of methylmercury toxicity in *Saccharomyces cerevisiae*, FASEB J. 14 (2000) 968–972.
[8] A. Naganuma, T. Furuchi, N. Miura, G.W. Hwang, S. Kuge, Investigation of intracellular factors involved in methylmercury toxicity, Tohoku J. Exp. Med. 196 (2002) 65–70.
[9] G.W. Hwang, T. Furuchi, A. Naganuma, A ubiquitin–proteasome system is responsible for the protection of yeast and human cells against methylmercury, FASEB J. 16 (2002) 709–711.
[10] T. Furuchi, G.W. Hwang, A. Naganuma, Overexpression of the ubiquitin-conjugating enzyme Cdc34 confers resistance to methylmercury in *Saccharomyces cerevisiae*, Mol. Pharmacol. 61 (2002) 738–741.
[11] G.Z. Hu, H. Ronne, Multicopy suppressors of the yeast PAM1 gene, *Saccharomyces*, Genome Database (2002).
[12] G.Z. Hu, H. Ronne, Overexpression of yeast PAM1 gene permits survival without protein phosphatase 2A and induces a filamentous phenotype, J. Biol. Chem. 269 (1994) 3429–3435.
[13] P. Uetz, L. Giot, G. Cagney, T.A. Mansfield, R.S. Judson, J.R. Knight, D. Lockshon, V. Narayan, M. Srinivasan, P. Pochart, A. Qureshi-Emili, Y. Li, B. Godwin, D. Conover, T. Kalbfleisch, G. Vijayadamodar, M. Yang, M. Johnston, S. Fields, J.M. Rothberg, A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*, Nature 403 (2000) 623–627.
[14] T. Ito, T. Chiba, R. Ozawa, M. Yoshida, M. Hattori, Y. Sakaki, A comprehensive two-hybrid analysis to explore the yeast protein interactome, Proc. Natl. Acad. Sci. USA 98 (2001) 4569–4574.
[15] T. Furuchi, H. Ishikawa, N. Miura, M. Ishizuka, K. Kajiya, S. Kuge, A. Naganuma, Two nuclear proteins, Cin5 and Ydr259c, confer resistance to cisplatin in *Saccharomyces cerevisiae*, Mol. Pharmacol. 59 (2001) 470–474.
[16] T. Furuchi, K. Nitta, T. Takahashi, A. Naganuma, Overexpression of Ssl2p confers resistance to adriamycin and actinomycin D in *Saccharomyces cerevisiae*, Biochem. Biophys. Res. Commun. 314 (2004) 844–848.
[17] T. Takahashi, T. Furuchi, A. Naganuma, A novel role for Bsd2 in the resistance of *Saccharomyces cerevisiae* to adriamycin, J. Cell Physiol. 202 (2005) 100–104.
[18] D. Gelperin, J. Weigle, K. Nelson, P. Roseboom, K. Irie, K. Matsumoto, S. Lemmon, 14-3-3 proteins: potential roles in vesicular transport and Ras signaling in *Saccharomyces cerevisiae*, Proc. Natl. Acad. Sci. USA 92 (1995) 11539–11543.
[19] G.P. van Heusden, D.J. Griffiths, J.C. Ford, A.W.T.F. Chin, P.A. Schrader, A.M. Carr, H.Y. Steensma, The 14-3-3 proteins encoded by the BMH1 and BMH2 genes are essential in the yeast *Saccharomyces cerevisiae* and can be replaced by a plant homologue, Eur. J. Biochem. 229 (1995) 45–53.
[20] P.C. Hollenhorst, G. Pietz, C.A. Fox, Mechanisms controlling differential promoter-occupancy by the yeast forkhead proteins Fkh1p and Fkh2p: implications for regulating the cell cycle and differentiation, Genes Dev. 15 (2001) 2445–2456.
[21] Y. Shu, H. Yang, E. Hallberg, R. Hallberg, Molecular genetic analysis of Rts1p, a B′ regulatory subunit of *Saccharomyces cerevisiae* protein phosphatase 2A, Mol. Cell. Biol. 17 (1997) 3242–3253.
[22] Y. Zhao, G. Boguslawski, R.S. Zitomer, A.A. DePaoli-Roach, *Saccharomyces cerevisiae* homologs of mammalian B and B′ subunits of protein phosphatase 2A direct the enzyme to distinct cellular functions, J. Biol. Chem. 272 (1997) 8256–8262.
[23] H. Yang, W. Jiang, M. Gentry, R.L. Hallberg, Loss of a protein phosphatase 2A regulatory subunit (Cdc55p) elicits improper regulation of Swe1p degradation, Mol. Cell. Biol. 20 (2000) 8143–8156.
[24] T. Samuel, H.O. Weber, P. Rauch, B. Verdoodt, J.T. Eppel, A. McShea, H. Hermeking, J.O. Funk, The G2/M regulator 14-3-3sigma prevents apoptosis through sequestration of Bax, J. Biol. Chem. 276 (2001) 45201–45206.
[25] H. Hermeking, C. Lengauer, K. Polyak, T.C. He, L. Zhang, S. Thiagalingam, K.W. Kinzler, B. Vogelstein, 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression, Mol. Cell 1 (1997) 3–11.
[26] C. Laronga, H.Y. Yang, C. Neal, M.H. Lee, Association of the cyclin-dependent kinases and 14-3-3 sigma negatively regulates cell cycle progression, J. Biol. Chem. 275 (2000) 23106–23112.
[27] R.L. Roberts, H.U. Mosch, G.R. Fink, 14-3-3 proteins are essential for RAS/MAPK cascade signaling during pseudohyphal development in *S. cerevisiae*, Cell 89 (1997) 1055–1065.
[28] T. Beck, M.N. Hall, The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors, Nature 402 (1999) 689–692.
[29] A.P. Schmitt, K. McEntee, Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in *Saccharomyces cerevisiae*, Proc. Natl. Acad. Sci. USA 93 (1996) 5777–5782.
[30] M.T. Martinez-Pastor, G. Marchler, C. Schuller, A. Marchler-Bauer, H. Ruis, F. Estruch, The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE), EMBO J. 15 (1996) 2227–2235.
[31] W. Gorner, E. Durchschlag, M.T. Martinez-Pastor, F. Estruch, G. Ammerer, B. Hamilton, H. Ruis, C. Schuller, Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated

by stress and protein kinase A activity, Genes Dev. 12 (1998) 586–597.
[32] P.C. Hollenhorst, M.E. Bose, M.R. Mielke, U. Muller, C.A. Fox, Forkhead genes in transcriptional silencing, cell morphology and the cell cycle. Overlapping and distinct functions for FKH1 and FKH2 in *Saccharomyces cerevisiae*, Genetics 154 (2000) 1533–1548.
[33] V. Janssens, J. Goris, Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling, Biochem. J. 353 (2001) 417–439.

A Novel Role for Bsd2 in the Resistance of Yeast to Adriamycin

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In a search for undiscovered mechanisms of resistance to adriamycin, we screened a genomic library derived from *Saccharomyces cerevisiae* for genes related to adriamycin resistance. To our surprise, we found that overexpression of *BSD2* rendered yeast cells resistant to adriamycin. Downregulation of the metal transporters Smf1 and Smf2 is the only activity of Bsd2 reported to date, and Bsd2 deficiency increases intracellular levels of Smf1 and Smf2. *SMF2*-disrupted cells exhibited significantly greater resistance to adriamycin, whereas the resistance of *SMF1*-disrupted cells was only slightly improved. The sensitivity of the *SMF1*- and *SMF2*-disrupted yeast cell line overexpressing *BSD2* was almost the same as that of the *BSD2*-overexpressing parental yeast cell. Thus the overexpression of *BSD2* and the disruption of *SMF1* and *SMF2* might be involved in the same mechanism that confers resistance to adriamycin. Although both *SMF1*- and *SMF2*-disrupted cells were very sensitive to EGTA, overexpression of *BSD2* had little or no effect on sensitivity to EGTA. However, a partial decrease in the intracellular level of FLAG-Smf2 was observed by overexpression of *BSD2*. Thus, the resistance to adriamycin acquired by overexpression of *BSD2* might be partially explained by downregulation of Smf2, but in addition to Smf2, other as of yet unidentified targets of Bsd2 must also be responsible for the resistance. J. Cell. Physiol. 202: 100–104, 2005. © 2004 Wiley-Liss, Inc.

Adriamycin is an anticancer drug that is widely used in the treatment of leukemias, lymphomas, and various carcinomas (Benjamin et al., 1974; Hortobagyi, 1997). Adriamycin intercalates into DNA causing inhibition of nucleic acid synthesis, and the inhibition of topoisomerase II (Hortobagyi, 1997; Gewirtz, 1999). The cytotoxicity of the drug has also been reported to involve the production of free radicals (Sinha et al., 1989; Feinstein et al., 1993).

The emergence of adriamycin-resistant cancer cells has been an obstacle to successful therapy (Booser and Hortobagyi, 1994). Cancer cells acquire adriamycin resistance by promoting the extracellular release of the drug, mediated by overexpression of ABC transporters such as *p*-glycoprotein and multidrug resistance-associated protein (MRP) (Ueda et al., 1987; Lincke et al., 1990; Cole and Deeley, 1998). Qualitative and quantitative changes in the expression of topoisomerase II (Zwelling et al., 1989; Withoff et al., 1996) and overexpression of glutathione S-transferase (Singh et al., 1989) have also been reported. However, these mechanisms alone cannot explain the development of adriamycin resistance in many types of cancer cell. A more detailed understanding of the mechanisms responsible for the acquisition of resistance is necessary if improvements are to be made in chemotherapy with adriamycin. Therefore, we have been searching for genes that might be related to adriamycin resistance. Yeast is an ideal eukaryotic organism for identifying genes conferring drug resistance because the methodologies for genetic manipulation are well established (e.g., gene disruption or introduction of exogenous genes). In the budding yeast *Saccharomyces cerevisiae*, biochemical and genetic screenings have identified some genes that confer resistance to adriamycin (Schenk et al., 2002, 2003; Furuchi et al., 2004). In this study, we found that the overexpression of *BSD2* (Liu et al., 1992; Liu and Culotta, 1994) conferred resistance to adriamycin in the yeast. *BSD2* gene encodes a protein, Bsd2, involved in the downregulation of the metal transporter proteins, Smf1 and Smf2 (Liu et al., 1997; Liu and Culotta, 1999; Portnoy et al., 2000).

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MATERIALS AND METHODS

Yeast strains and media

Yeast cells (*S. cerevisiae*) were grown in yeast extract-peptone-adenine-dextrose (YAPD) medium or in synthetic dextrose (SD) medium without leucine.

Transformation

Yeast cells (W303B; a leucine-auxotrophic strain) were transformed with a yeast genomic DNA library as described previously (Naganuma et al., 2000). The yeast genomic DNA library (Furuchi et al., 2001) was prepared by cloning size-fractionated *Sau*3AI fragments (5- to 10-kbp in length) of the yeast genome into the *Bam*HI cloning site of the *LEU2*-based multicopy plasmid YEp13. Transformants were grown in SD medium without leucine at 30°C.

Selection of genes that confer resistance to adriamycin

We cultured the Leu^+ transformants in 96-well plates (1×10^4 cells/200 μl/well) for 24 h at 30°C in SD (-Leu) medium plus 100 μM adriamycin. This concentration of adriamycin inhibits the growth of W303B cells. We isolated transformed cells that had proliferated rapidly and had formed aggregates in the presence of adriamycin. We obtained 24 aggregates that exhibited resistance to adriamycin. We isolated plasmids from the cells as described previously (Furuchi et al., 2001). The plasmids were amplified in *Escherichia coli* (Miura et al., 1999; Furuchi et al., 2002). Each plasmid was reintroduced into W303B cells to confirm the phenotype. We then selected the plasmid that conferred the strongest resistance to adriamycin for further study. The nucleotide sequence of the genomic insert in the selected plasmid was determined with an automated DNA sequencer (LI-COR, Lincoln, NE). After mapping, the genomic insert was excised and subcloned into the pRS425 vector. Subclones were introduced into W303B yeast cells and the sensitivity to adriamycin of each resultant cell line was determined.

Construction of FLAG-Smf2 expressing plasmid

For construction of the FLAG-*SMF2* vector, the *SMF2* gene was cloned by PCR with chromosomal DNA from *S. cerevisiae* as the template. The following oligonucleotides were used as primers: 5′-CCGTATAGCGTTTATGTTTGATGGACTACAAGGATGACGATGACAAGACGTCCCAAGAATATGAACC-3′ and 5′-TTAGAGGTGTACTTCTTTGCCCGTAG-3′.

The amplified DNA was inserted into the pGEM-T easy vector (Promega, Madison, WI) to produce plasmid pGEM-FLAG-*SMF2*. The insert was digested with the restriction endonuclease *Eco*RI and fragments were ligated into the pKT10-GAPDH (*URA3*) expression vector.

Immunoblotting

Yeast cells (1×10^8 cells) were cultured in 10 ml of SD (-Ura, -Leu) medium for 4 h. Yeast cell lysates were prepared by glass beads homogenization. Samples (25 μg of protein) were separated by sodium dodecyl sulfate (SDS)–polyacrylamide (12.5%) gel electrophoresis and transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA). Immunoblotting was carried out using anti-FLAG M2 monoclonal antibody (Sigma, St. Louis, MO) and peroxidase-conjugated goat anti-mouse immunoglobulins (Dako A/S, Glostrup, Denmark) as primary and secondary antibodies, respectively. Immunoreactive proteins were detected by enhanced chemiluminescence system (Amersham Pharmacia, Uppsala, Sweden).

Gene disruption

Genes were disrupted as described previously (Baudin et al., 1993; Gonzalez et al., 1999; Hwang et al., 2002). For construction of the *smf1::HIS3* vector, the *smf2::HIS3* vector, the *smf3::HIS3* vector and, the *bsd2::HIS3* vector, the *HIS3* gene was amplified by PCR with the following respective primers: *SMF1-HIS3*-F (5′-CTAACTTTCTCAATTAGGTCAAAATGGTGAACGTTGGTCCTTCTCATGCTCTCTTGGCCTCCTCTAG-3′) and *SMF1-HIS3*-R (5′-CAAACATTTGGGCAGATGGCACAAATCCTCTGAACACTTGTTTAACGGACTCGTTCAGAATGACACG-3′); *SMF2-HIS3*-F (5′-CCGTATAGCGTTTATGTTTGATGACGTCCCAAGAATATGAACCTATTCAACTCTTGGCCTCCTCTAG-3′) and *SMF2-HIS3*-R (5′-GAATGAGGCATTACCGTGGCGCCCAGAATTGCCAGACTTAAGTATAACCCTCGTTCAGAATGACACG-3′); and *SMF3-HIS3*-F (5′-CTTTAAATATATCGTTCTTCCAAGCTAATTGATAACAGTAGTAGCATCACCTCTTGGCCTCCTCTAG-3′) and *SMF3-HIS3*-R (5′-GGCAATTAAGAACAAAGAAATGATTAGTTCAGCGTACGCATAGTTTAACGTCGTTCAGAATGACACG-3′) and *BSD2-HIS3*-F (5′-GTCTAGGAAACTAAGCGCTATGCCAGAGCAAGAACTACTTATAGGGCAAGCTCTTGGCCTCCTCTAG-3′) and *BSD2-HIS3*-R (5′-CGTTGATTGTGAATTTAGACGAACATCATCGAATTCATTTGGATCCTCTATCGTTCAGAATGACACG-3′). Each product of PCR was introduced into W303B cells.

For construction of the *smf2::URA3* vector, the *SMF2* gene was cloned by PCR with chromosomal DNA from *S. cerevisiae* as the template. The amplified DNA was inserted into the pGEM-T easy vector (Promega) to produce plasmid pGEM-*SMF2*. The region between the *Bgl*II site and the *Eco*RV site in the open reading frame (ORF) of the *SMF2* gene in pGEM-*SMF2* was removed and replaced by the *URA3* gene. Disruption of genes was verified by PCR.

Quantification of growth inhibition by adriamycin and other compounds

Yeast cells that harbored pRS425-*BSD2* or pRS425 were cultured in 96-well plates (1×10^4 cells/ 200 μl) in SD (-Leu) medium that contained the indicated compound at various concentrations. After 48 h, the absorbance at 620 nm (A_{620}) was determined spectrophotometrically to quantify the growth of each line of cells. For spot assays using agar-solidified medium, a suspension of yeast cells was spotted onto a plate of agar-solidified medium containing adriamycin. Plates were photographed after incubation for 48 h at 30°C.

RESULTS AND DISCUSSION

To identify genes whose overexpression confers resistance to adriamycin on *S. cerevisiae*, we transformed strain W303B with the multicopy vector Yep13 into which a yeast genomic library had been inserted. We

cultured the resultant transformants for 72 h in SD (-Leu) medium that contained 100 μM adriamycin. Under these culture conditions, the parent strain transformed with vector (Yep13) containing no insert failed to grow. We picked 24 yeast colonies that had grown in the presence of 100 μM adriamycin and isolated the plasmid DNA from each. When parent W303B cells were transformed with the 24 individual plasmids (AR1 through AR24), AR9, AR13, AR16, and AR17 were found to confer adriamycin resistance to the transformants. The cell line AR16, which exhibited the strongest resistance was selected for further study, and the inserted fragment of genomic DNA was sequenced. A search for this sequence in the *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces/) revealed that a region of approximately 5 kbp, derived from yeast chromosome II, had been inserted in plasmid AR16. This region contained the ORFs of three genes, namely, *BSD2*, *CTP1*, and *YBR292c* (Fig. 1). To identify genes involved in adriamycin resistance, we digested AR16 with appropriate restriction enzymes into three fragments (AR16a, AR16b, and AR16c), each encoding one of the three ORFs. The fragments were subcloned into the multicopy vector pRS425, and used to transform W303B cells. Only yeast cells re-transformed with the plasmid that included AR16a were resistant to adriamycin. Since AR16a contained only the ORF of *BSD2*, it was clear that overexpression of *BSD2* had conferred resistance to adriamycin on yeast cells (Fig. 2).

In addition to adriamycin, the *BSD2*-overexpressing yeast cells exhibited resistance to actinomycin D, but not to other anticancer drugs, such as aclarubicin, cisplatin, and 5-fluorouracil. There was no detectable increase in resistance to inducers of oxidative stress, such as hydrogen peroxide and paraquat (data not shown). Thus, *BSD2*-overexpressing yeast cells appear to exhibit relatively specific resistance to adriamycin and actinomycin D.

BSD2 was originally identified as the gene that counteracts the enhanced sensitivity to oxidative stress reported in yeast cells lacking Cu/Zn superoxide dismutase (Liu et al., 1992; Liu and Culotta, 1994).

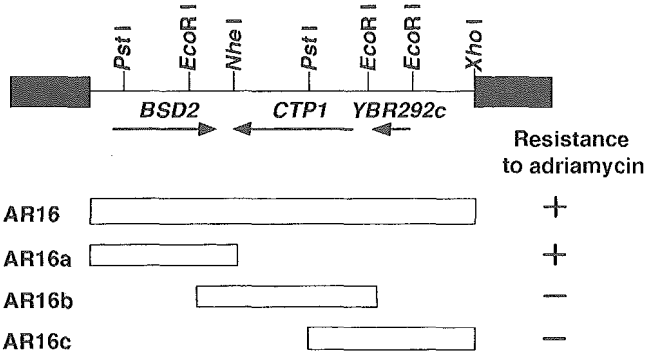

Fig. 1. Restriction map of the genomic DNA insert in plasmid AR16 and the ORFs that conferred resistance to adriamycin. The thick black line represents the vector YEp13; the thin line represents the genomic DNA insert. Vertical lines above the genomic DNA insert indicate the restriction sites used to generate various subclones. The ability of three subclones (containing fragments AR16a, AR16b, and AR16c) to confer resistance to adriamycin is indicated (+, confer resistance; −, did not confer resistance). ORFs are denoted by black arrows that indicate the direction of transcription.

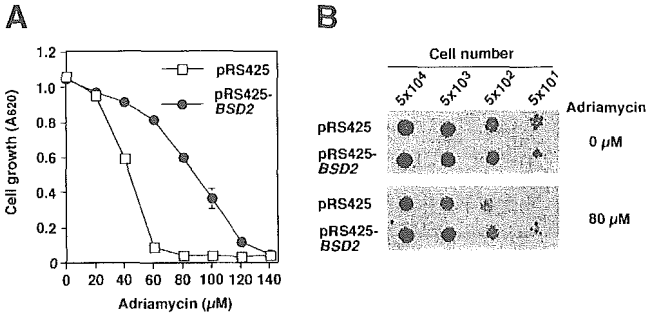

Fig. 2. Adriamycin sensitivity of yeast cells that overexpress *BSD2*. (**A**) Yeast cells that harbored pRS425 or pRS425-*BSD2* were grown in liquid SD (-Leu) medium that contained adriamycin at the indicated concentration. After incubation for 48 h at 30°C, absorbance was measured spectrophotometrically at 620 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. (**B**) Yeast cells that harbored pRS425 (control) or pRS425-*BSD2* were grown on a plate of agar-solidified SD (-Leu) medium with or without adriamycin. Plates were photographed after incubation for 48 h at 30°C.

Mutations in the *BSD2* gene causes upregulation of the metal transporter proteins, Smf1 and Smf2, allowing the accumulation of abnormally large amounts of metal ions (Liu et al., 1997; Liu and Culotta, 1999; Portnoy et al., 2000). Since the mechanism of resistance to adriamycin might plausibly involve Smf1 and Smf2, we constructed a number of yeast strains carrying a disrupted gene for Smf1 and/or Smf2 and examined their sensitivity to the drug. We found that *SMF2*-disrupted yeast cells (*smf2Δ*) exhibited a marked resistance to adriamycin, whereas *SMF1*-disrupted yeast cells (*smf1Δ*) displayed only a slight increase in resistance (Fig. 3). The resistance to adriamycin of yeast cells in which both genes had been disrupted (*smf1Δsmf2Δ*) was similar to that of *SMF2*-disrupted yeast cells. Smf1, Smf2, and Smf3 are all members of the SMF family but the intracellular level of Smf3 is not regulated by Bsd2 (Portnoy et al., 2000). Nonetheless, we examined the effects of disrupting the *Smf3* gene and found that Smf3-disrupted yeast cells (*smf3Δ*) exhibited similar sensitivity to adriamycin to that of the control parental strain (W303B) (Fig. 3). These results

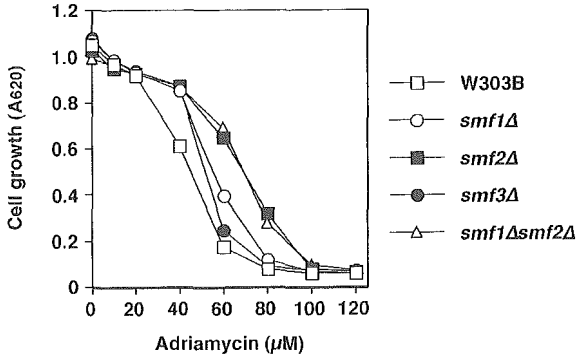

Fig. 3. Effects of disruption of genes in the SMF family on the sensitivity of yeast cells to adriamycin. Yeast cells, with a disrupted gene for *SMF1*, *SMF2*, or *SMF3* as indicated, were grown in SD medium containing adriamycin. For other details, see legend to Figure 2.

suggest that Smf3 plays no part in the mechanism of resistance to adriamycin mediated by the upregulation of Bsd2.

Disruption of *BSD2* is known to increase intracellular levels of both Smf1 and Smf2 posttranslationally (Liu and Culotta, 1999; Portnoy et al., 2000) but the effects of overexpression of *BSD2* on the intracellular levels of these factors have not been investigated. It has been reported that *SMF1*- or *SMF2*-disrupted yeast cells are very sensitive to the metal chelator EGTA (Cohen et al., 2000). If overexpression of *BSD2* were to depress the intracellular levels of Smf1 and Smf2, *BSD2*-overexpressing yeast cells would be expected to be very sensitive to EGTA. However, as shown in Figure 4, the overexpression of *BSD2* had little effect on sensitivity to EGTA. Yeast cells that lack Bsd2 are very sensitive to copper and cadmium (Liu et al., 1997), presumably because elevated intracellular levels of Smf1 and Smf2 promote the uptake of these metals. However, the sensitivity to copper and cadmium of *BSD2*-overexpressing yeast cells was the same as that of control yeast cells transformed with vector alone (data not shown). Our results suggest that elevated levels of Bsd2 lead to only a slight reduction in the intracellular levels of Smf1 and Smf2. To examine this issue in detail, we examined the effect of overexpression of *BSD2* on intracellular levels of Smf2 using a FLAG-specific antibody and a yeast strain expressing Smf2 fused to the FLAG tag (FLAG-Smf2). We found that overexpression of *BSD2* did not completely but partially decreased the intracellular level of FLAG-Smf2 (Fig. 5).

We also studied the effect of elevated levels of Bsd2 on adriamycin sensitivity in the *SMF1*- and *SMF2*-disrupted yeast cells. We found that adriamycin sensitivity of *BSD2*-overexpressing *SMF1*- and *SMF2*-disrupted yeast cells was almost the same as that of the *BSD2*-overexpressing parental yeast cell (Fig. 6). If the disruption of *SMF1* and *SMF2* and the overexpression of *BSD2* are operating by a separate mechanism to cause resistance, the combination effect should be additive or synergistic. Therefore, the overexpression of *BSD2* and the disruption of *SMF1* and *SMF2* might be involved in the same system to confer resistance to adriamycin. Our results suggest the possibility that the adriamycin resistance acquired by overexpression of *BSD2* might

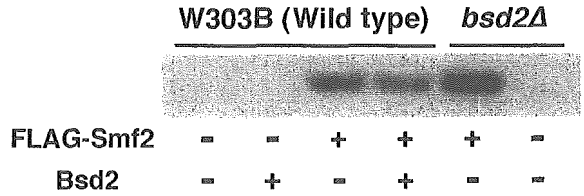

Fig. 5. Effects of overexpression of *BSD2* on intracellular levels of FLAG-Smf2. A yeast strain expressing Smf2 fused to the FLAG tag (FLAG-Smf2) was transformed with pRS425 or pRS425-*BSD2*. Lysates of indicated strains (W303B or *bsd2Δ*) expressing FLAG-Smf2 and/or Bsd2 were separated by SDS–PAGE. Immunoblotting was carried out using anti-FLAG M2 monoclonal antibody.

be partially explained by down-regulation of Smf1 and Smf2, but in addition to Smf1 and Smf2, other as of yet unidentified targets of Bsd2 must also be responsible for the resistance. Bsd2 might activate (or inhibit) a certain factor(s), which is/are involved in reduction (or enhancement) of the toxicity of adriamycin, independently of the downregulation of Smf1 and Smf2. Moreover, overexpression of *BSD2* might almost fully mask the effects of Smf1 and Smf2 on the toxicity of adriamycin.

In the *bsd2* mutant cell, a mutant variant of the H^+-ATPase Pma1 (pmal-7) is localized inappropriately (Luo and Chang, 1997). Thus, it has been suggested that Bsd2 might be involved in the stability of membrane proteins other than Smf1 and Smf2 (Luo and Chang, 1997). Various membrane proteins involved in the influx or the efflux of chemicals have been identified in yeast (Kolaczkowski et al., 1996; Mulet et al., 1999; Goossens et al., 2000; Wolfger et al., 2001; Ishida et al., 2002). Therefore, we cannot discount the possibility that overexpression of *BSD2* might confer adriamycin resistance on yeast cells by reducing the stability of transporter proteins. However, overexpression of *BSD2* had little or no effect on the intracellular accumulation of adriamycin (data not shown). Therefore, it seems likely that the mechanism of adriamycin resistance resulting from the overexpression of *BSD2* does not involve the regulation of membrane proteins that participate in the transport of adriamycin.

It has been reported that cells induced to express the endoplasmic reticulum (ER) stress-response protein GRP78, are resistant to adriamycin (Shen et al., 1987).

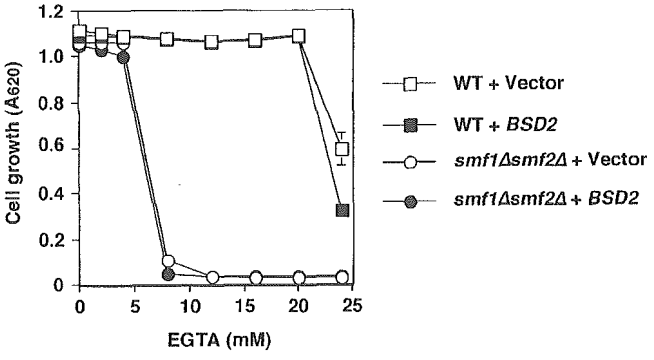

Fig. 4. Effects of overexpression of *BSD2* on the sensitivity to EGTA of yeast cells with disrupted *SMF* genes. Yeast cells with mutations as indicated, harboring pRS425 or pRS425-*BSD2*, were grown in SD (-Leu) medium containing EGTA. For other details, see legend to Figure 2.

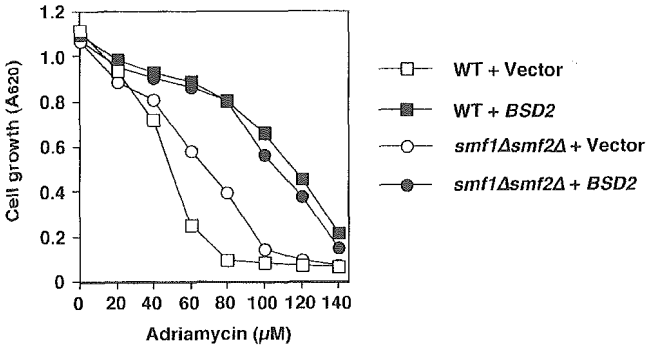

Fig. 6. Effects of overexpression of *BSD2* on the sensitivity to adriamycin of *SMF*-disrupted yeast cells. Yeast *smf1Δsmf2Δ* cells, harboring pRS425 or pRS425-*BSD2*, were grown in SD (-Leu) medium containing adriamycin as indicated. For other details, see legend to Figure 2.

Thus, the ER might be important in the development of adriamycin toxicity. Bsd2 is localized in the ER (Liu et al., 1997) and *BSD2*-overexpressing yeast cells might have elevated levels of Bsd2 in the ER.

On the other hand, our *BSD2*-overexpressing cells were resistant to actinomycin D, an inhibitor of RNA synthesis. Since adriamycin also inhibits RNA synthesis (Gewirtz, 1999) and overproduction of Ssl2 helicase confers resistance to both adriamycin and actinomycin D (Furuchi et al., 2004), the possible involvement of Bsd2 in RNA synthesis cannot be excluded.

In the present study, our results demonstrate the involvement of Bsd2 and Smf2 in the development of adriamycin toxicity. Although the function of these proteins remain to be elucidated, our findings indicate the existence of a novel mechanism that determines the sensitivity of yeast cells to adriamycin. Elucidation of the mechanism in yeast cells will be helpful for a full understanding of the various mechanisms responsible for the acquisition of resistance to and the cytotoxicity of adriamycin in human cells.

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LITERATURE CITED

Baudin A, Ozier-Kalogeropoulos O, Denouel A, Lacroute F, Cullin C. 1993. A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. Nucleic Acids Res 21:3329–3330.

Benjamin RS, Wiernik PH, Bachur NR. 1974. Adriamycin chemotherapy—efficacy, safety, and pharmacologic basis of an intermittent single high-dosage schedule. Cancer 33:19–27.

Booser DJ, Hortobagyi GN. 1994. Anthracycline antibiotics in cancer therapy. Focus on drug resistance. Drugs 47:223–258.

Cohen A, Nelson H, Nelson N. 2000. The family of SMF metal ion transporters in yeast cells. J Biol Chem 275:33388–33394.

Cole SP, Deeley RG. 1998. Multidrug resistance mediated by the ATP-binding cassette transporter protein MRP. Bioessays 20:931–940.

Feinstein E, Canaani E, Weiner LM. 1993. Dependence of nucleic acid degradation on in situ free-radical production by adriamycin. Biochemistry 32:13156–13161.

Furuchi T, Ishikawa H, Miura N, Ishizuka M, Kajiya K, Kuge S, Naganuma A. 2001. Two nuclear proteins, Cin5 and Ydr259c, confer resistance to cisplatin in *Saccharomyces cerevisiae*. Mol Pharmacol 59:470–474.

Furuchi T, Hwang GW, Naganuma A. 2002. Overexpression of the ubiquitin-conjugating enzyme Cdc34 confers resistance to methylmercury in *Saccharomyces cerevisiae*. Mol Pharmacol 61:738–741.

Furuchi T, Nitta K, Takahashi T, Naganuma A. 2004. Overexpression of Ssl2p confers resistance to adriamycin and actinomycin D in *Saccharomyces cerevisiae*. Biochem Biophys Res Commun 314:844–848.

Gewirtz DA. 1999. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. Biochem Pharmacol 57:727–741.

Gonzalez C, Perdomo G, Tejera P, Brito N, Siverio JM. 1999. One-step, PCR-mediated, gene disruption in the yeast *Hansenula polymorpha*. Yeast 15:1323–1329.

Goossens A, de La Fuente N, Forment J, Serrano R, Portillo F. 2000. Regulation of yeast H(+)-ATPase by protein kinases belonging to a family dedicated to activation of plasma membrane transporters. Mol Cell Biol 20:7654–7661.

Hortobagyi GN. 1997. Anthracyclines in the treatment of cancer: An overview. Drugs 54(Suppl 4):1–7.

Hwang GW, Furuchi T, Naganuma A. 2002. A ubiquitin-proteasome system is responsible for the protection of yeast and human cells against methylmercury. FASEB J 16:709–711.

Ishida S, Lee J, Thiele DJ, Herskowitz I. 2002. Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals. Proc Natl Acad Sci USA 99:14298–14302.

Kolaczkowski M, van der Rest M, Cybularz-Kolaczkowska A, Soumillion JP, Konings WN, Goffeau A. 1996. Anticancer drugs, ionophoric peptides, and steroids as substrates of the yeast multidrug transporter Pdr5p. J Biol Chem 271:31543–31548.

Lincke CR, van der Bliek AM, Schuurhuis GJ, van der Velde-Koerts T, Smit JJ, Borst P. 1990. Multidrug resistance phenotype of human BRO melanoma cells transfected with a wild-type human mdr1 complementary DNA. Cancer Res 50:1779–1785.

Liu XF, Culotta VC. 1994. The requirement for yeast superoxide dismutase is bypassed through mutations in *BSD2*: A novel metal homeostasis gene. Mol Cell Biol 14:7037–7045.

Liu XF, Culotta VC. 1999. Post-translation control of Nramp metal transport in yeast. Role of metal ions and the *BSD2* gene. J Biol Chem 274:4863–4868.

Liu XF, Elashvili I, Gralla EB, Valentine JS, Lapinskas P, Culotta VC. 1992. Yeast lacking superoxide dismutase. Isolation of genetic suppressors. J Biol Chem 267:18298–18302.

Liu XF, Supek F, Nelson N, Culotta VC. 1997. Negative control of heavy metal uptake by the *Saccharomyces cerevisiae BSD2* gene. J Biol Chem 272:11763–11769.

Luo W, Chang A. 1997. Novel genes involved in endosomal traffic in yeast revealed by suppression of a targeting-defective plasma membrane ATPase mutant. J Cell Biol 138:731–746.

Miura N, Kaneko S, Hosoya S, Furuchi T, Miura K, Kuge S, Naganuma A. 1999. Overexpression of L-glutamine:D-fructose-6-phosphate amidotransferase provides resistance to methylmercury in *Saccharomyces cerevisiae*. FEBS Lett 458:215–218.

Mulet JM, Leube MP, Kron SJ, Rios G, Fink GR, Serrano R. 1999. A novel mechanism of ion homeostasis and salt tolerance in yeast: The Hal4 and Hal5 protein kinases modulate the Trk1-Trk2 potassium transporter. Mol Cell Biol 19:3328–3337.

Naganuma A, Miura N, Kaneko S, Mishina T, Hosoya S, Miyairi S, Furuchi T, Kuge S. 2000. GFAT as a target molecule of methylmercury toxicity in *Saccharomyces cerevisiae*. FASEB J 14:968–972.

Portnoy ME, Liu XF, Culotta VC. 2000. *Saccharomyces cerevisiae* expresses three functionally distinct homologues of the nramp family of metal transporters. Mol Cell Biol 20:7893–7902.

Schenk PW, Boersma AW, Brok M, Burger H, Stoter G, Nooter K. 2002. Inactivation of the *Saccharomyces cerevisiae SKY1* gene induces a specific modification of the yeast anticancer drug sensitivity profile accompanied by a mutator phenotype. Mol Pharmacol 61:659–666.

Schenk PW, Brok M, Boersma AW, Brandsma JA, Den Dulk H, Burger H, Stoter G, Brouwer J, Nooter K. 2003. Anticancer drug resistance induced by disruption of the *Saccharomyces cerevisiae NPR2* gene: A novel component involved in cisplatin- and doxorubicin-provoked cell kill. Mol Pharmacol 64:259–268.

Shen J, Hughes C, Chao C, Cai J, Bartels C, Gessner T, Subjeck J. 1987. Coinduction of glucose-regulated proteins and doxorubicin resistance in Chinese hamster cells. Proc Natl Acad Sci USA 84:3278–3282.

Singh SV, Nair S, Ahmad H, Awasthi YC, Krishan A. 1989. Glutathione S-transferases and glutathione peroxidases in doxorubicin-resistant murine leukemic P388 cells. Biochem Pharmacol 38:3505–3510.

Sinha BK, Mimnaugh EG, Rajagopalan S, Myers CE. 1989. Adriamycin activation and oxygen free radical formation in human breast tumor cells: Protective role of glutathione peroxidase in adriamycin resistance. Cancer Res 49:3844–3848.

Ueda K, Clark DP, Chen CJ, Roninson IB, Gottesman MM, Pastan I. 1987. The human multidrug resistance (*mdr1*) gene. cDNA cloning and transcription initiation. J Biol Chem 262:505–508.

Withoff S, De Jong S, De Vries EG, Mulder NH. 1996. Human DNA topoisomerase II: Biochemistry and role in chemotherapy resistance. Anticancer Res 16:1867–1880.

Wolfger H, Mamnun YM, Kuchler K. 2001. Fungal ABC proteins: Pleiotropic drug resistance, stress response and cellular detoxification. Res Microbiol 152:375–389.

Zwelling LA, Hinds M, Chan D, Mayes J, Sie KL, Parker E, Silberman L, Radcliffe A, Beran M, Blick M. 1989. Characterization of an amsacrine-resistant line of human leukemia cells. Evidence for a drug-resistant form of topoisomerase II. J Biol Chem 264:16411–16420.