

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

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Received April 6, 2005; accepted July 5, 2005

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 con-

tained 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 overexpres-

sion 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

This work was supported by Grant-in-Aid for Scientific Research (A) 15209004 from the Japan Society for the Promotion of Science and by Health and Labor Sciences Research Grants for Research on Risk of Chemical Substances from Ministry of Health, Labor and Welfare Japan.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.105.013516.

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 (*MAT α 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 *RAD23* 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 μ l) 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 (Ce-

dar 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'-CAACATACAATAGAAAAATGGAGCTCTTAACCTTTAAAAAATTTCAAG-3'; 5'UbL-R, 5'-CTTGAAATTTTAAAGGTTAAGAGCTCCATTTTCTATTGTATGTTG-3'; 3'UbL-F, 5'-CATGGTTTCTCAAAAAGAGCTCACGAAGACCAAAGTAAC-3'; and 3'UbL-R, 5'-GTTACTTTGGTCTTCGTGAGCTCTTTTGAGAAACCATG-3' for deletion of the UbL domain; 5'UBA1-F, 5'-CGGGATTCGTGGTGGGAGAGCTCAGGAACGAGACCATCGAG-3'; 5'UBA1-R, 5'-CTCGATGGTCTCGTTCCTGAGCTCTCCCACCACGAATCCCG-3'; 3'UBA1-F, 5'-GAATATCTACTGATGGAGCTCCAGAAATCTGCGTC-3'; and 3'UBA1-R, 5'-GACGCAGATTTTCTGGGAGCTCCATCAGTAGATATTC-3' for deletion of the UBA1 domain; and 5'UBA2-F, 5'-CTTTCCAAGTTGACTATACCGAGCTCGACGATCAAGCTATTTTCGC-3'; 5'UBA2-R, 5'-GCCAAATAGCTTGATCGTCGAGCTCGGTATAGTCAACTTGGAAAAG-3'; 3'UBA2-F, 5'-CAAATATTCTATTACGCGAGCTCGCCGACTGAGATTGTAG-3'; and 3'UBA2-R, 5'-CTACAATCTCAGTCGCGGAGCTCGCTGAATAGAATA-TTTG-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 Δ} , pKT10-*FLAG-RAD23*^{UBA1 Δ} , pKT10-*FLAG-RAD23*^{UBA2 Δ} , and pKT10-*FLAG-RAD23*^{UBA1 Δ +UBA2 Δ} .

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 mid-logarithmic 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 MgCl₂, 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 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 (UbL Δ) 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 (UBA1 Δ) 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 (UBA2 Δ) or with a defect in both UBA1 and UBA2 (UBA1 Δ + UBA2 Δ) 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.

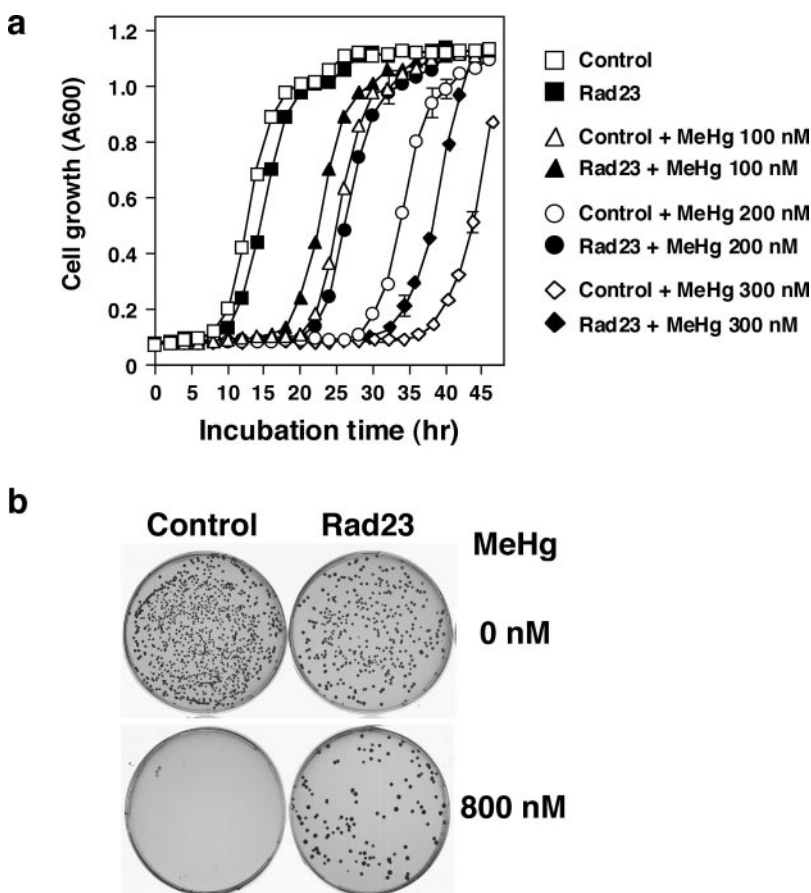


Fig. 1. Sensitivity of yeast cells that overexpressed Rad23 to methylmercury. **a**, yeast cells (10^5 cells/200 μ l/well) that harbored pKT10 or pKT10-RAD23 were cultured at 30°C in SD (-Ura) liquid medium that contained methylmercuric chloride (MeHg) at the indicated concentrations. Absorbance at 600 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-

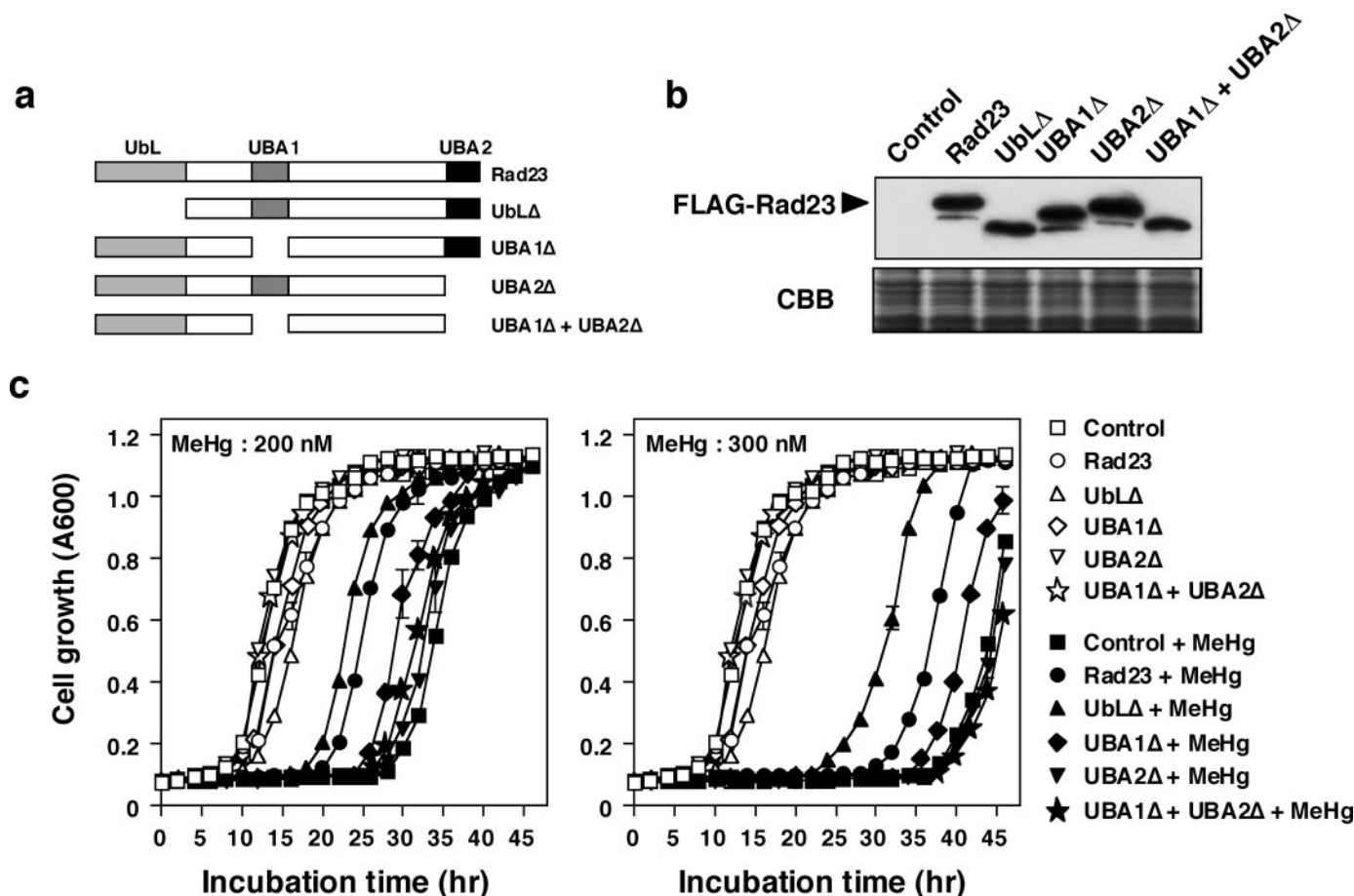


Fig. 2. Effects of overexpression of mutant forms of Rad23 on the sensitivity of yeast cells to methylmercury. **a**, schematic representation of the structural domains of Rad23 and the mutant proteins generated in this study. Rad23 contains a UbL domain and two UBA domains. **b**, lysates (20 μ g per lane) of yeast cells that harbored pKT10, pKT10-FLAG-RAD23, pKT10-FLAG-RAD23^{UbLΔ}, pKT10-FLAG-RAD23^{UBA1Δ}, pKT10-FLAG-RAD23^{UBA2Δ}, or pKT10-FLAG-RAD23^{UBA1Δ + UBA2Δ} 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 μ l/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 de-

termine the extent of methylmercury toxicity by regulating the cellular concentrations of these various proteins.

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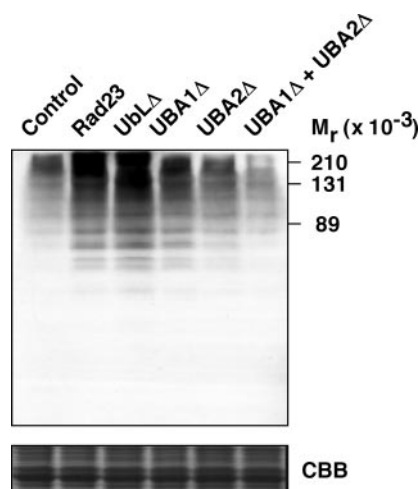


Fig. 3. Effects of overexpression of Rad23 and of mutant derivatives of Rad23 on cellular levels of total ubiquitinated proteins. Lysates (20 μ g per lane) of yeast cells that overexpressed FLAG-Rad23 or mutant derivatives of this FLAG-tagged protein were fractionated by SDS-PAGE. Immunoblotting analysis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

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