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MRP5b/SMRP mRNA is Highly Expressed in Metallothionein- Deficient Mouse Liver

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To investigate whether multidrug resistance protein 5 (MRP5) functions as a xenobiotic detoxification factor, we measured MRP mRNA expression levels in metallothionein (MT)-I, II knockout mouse, and the results showed that MRP5b/SMRP, a splicing variant of MRP5, was highly expressed in the liver of MT-I, -II null mice.

Key words —— metallothionein, multidrug resistance protein, multidrug resistance protein 5

INTRODUCTION

Metallothionein (MT) is a small protein that plays a role in protecting against the toxic effects of heavy metals and xenobiotics as well as in drug resistance and free radical scavenging,[1–4] and MT knockout mice exhibit hypersensitivity to heavy metals, such as a cadmium[5,6] and mercury.[7] MT is known to be one of the factors involved in resistance to cisplatin, and Sato *et al.* reported that MT knockout mice are more sensitive to cisplatin than normal mice.[8]

We previously cloned multidrug resistance protein 5 (MRP5)/SMRP, a member of the MRP family, from cisplatin-resistant lung cancer cell line PC-14/CDDP, and the expression levels of MRP5/SMRP have been found to be enhanced by cisplatin in lung cancer patients.[9–11] Since McAleer *et al.* reported that MRP5 confers resistance to cadmium and antimony,[12] we speculated that MRP5 plays a role in heavy metal detoxification in MT-I, II knockout mice instead of MT, and in this study we investigated MRP5 mRNA expression levels in the tissues of MT-I, II knockout mice.

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MATERIALS AND METHODS

Animals —— Homozygous MT-I and MT-II knockout mice (MT-null), and the corresponding wild-type (parental strain), 129/sv mice, were used.[13] The animals were housed in a temperature-controlled and light-controlled animal room.

RNA Isolation and RT-PCR Analysis —— Total RNA was extracted from the mouse tissues with ISOGEN reagent according to the protocol provided by the manufacturer (Nippongene, Toyama, Japan). cDNA synthesis and the polymerase chain reaction were carried out by using a RevaTra Dash RT-PCR kit according to the protocol provided by manufacturer (TOYOBO, Tokyo, Japan). The amplification primer pairs were: MRP1, MRP2, MRP3, MRP4, MRP5 and G3PDH, 5′-CTG GGT TTG TTC CGG ATC AAT G-3′ and 5′-TGA CAC AAA GCC CTT TAG GTG A-3′; 5′-CTG CCT CTT CAG AAT CTT AG-3′ and 5′-CCC AAG TTG CAG GCC AGC CAC-3′; 5′-CCA TGA CTC TTT GCC TGT TCC G-3′ and 5′-TCA TCT AGG CAA GTC CCG CAT C-3′; 5′-AGT GCA AGT AGC GCC CAC CC-3′ and 5′-AGC TCC TCG TCC GTG TGC TC-3′; 5′-GTG GTG ACC TTC TCT GTT CA-3′ and 5′-ATA CTG GAG TGG GAG GAG TC-3′; 5′-ACC ACA GTC CAT GCC ATC AC-3′ and 5′-TCC ACA CCC TGT TGC TGT A-3′. PCR was cycled 30 times (98°C for 30 sec, 60°C 2 sec, 74°C 30 sec) and followed by incubation at 74°C for 2 min. The PCR products were separated by electrophoresis in 8% TBE polyacrylamide gel or 1% TAE agarose gel, stained with ethidium bromide, and visualized under an ultraviolet transilluminator.

RESULTS AND DISCUSSION

To investigate MRP5 function as a detoxification factor for xenobiotics, we investigated the mRNA expression levels of MRP5 in the MT-I, II

knockout mouse by performing RT-PCR analysis of brain, lung, heart, liver, and kidney tissue from three animals each (Fig. 1). The MRP5 expression patterns were similar to those we obtained in humans, and no marked difference in MRP5 expression levels between any of the tissues of the MT-null and wild-type mice was observed. However, the MRP5 expression pattern in the liver differed slightly from its pattern of expression in the other tissues. To clarify the expression pattern in the liver, we performed a second PCR analysis (Fig. 2), and the results showed that the long fragment was the major product in the MT-I, II knockout mouse. We previously reported that MRP5 has two variants, MRP5a/MRP5 and MRP5b/SMRP, and since MRP5b was preferentially expressed in liver and skeletal muscle, we speculated that the long fragment expressed in MT-null mouse liver was MRP5b/SMRP. We recently found that adriamycin inducees both MRP5a/MRP5 and MRP5b/SMRP in lung cancer cell lines and that these MRP5s are over-expressed in adriamycin-resistant cell lines,[14] and in this study we found that SMRP is expressed in the liver of the MT-I, -II null mice. Although the physiological function of MRP5s remains unknown, MRP5b/SMRP may play a role in the detoxification of xenobiotics by these mice instead of MT. We also investigated the expression levels of MRP family members other than MRP5 in the tissues obtained from the same mice. The results showed no differences in expression levels of MRP1, 2, 3, and 4 between the brain, lung, heart, and kidney tissues of the MT-null mice and wild type mice, and the tissues distribution patterns were similar to those in humans. Typical expression patterns are shown in Fig. 3.

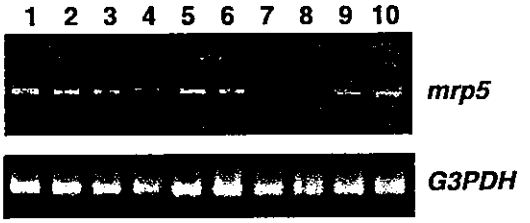

Fig. 1. Expression Analysis of MRP5 in MT-Null Mouse Tissue
PCR was performed using primer sets for MRP5 and G3PDH. The total RNA templates were prepared from MT-null mouse tissue (lane 2, 4, 6, 8, 10) and wild-type mouse tissue (lane 1, 3, 5, 7, 9). The bands of G3PDH provide an indication of the amount of total RNA loaded in each lane. Lane 1, 2: brain; lane 3, 4: lung; lane 5, 6: heart; lane 7, 8: liver; lane 9, 10: kidney.

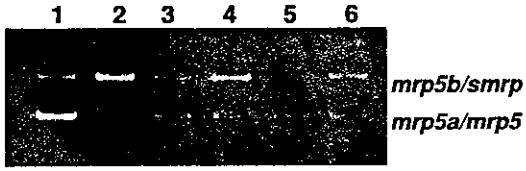

Fig. 2. Expression Analysis of MRP5 Variant in MT-Null Mouse Liver
First PCR products obtained from MT-null mouse liver (lane 2, 4, 6) and wild-type mouse liver (lane 1, 3, 5) were used as a template. Other experimental conditions were same as those of Fig. 1.

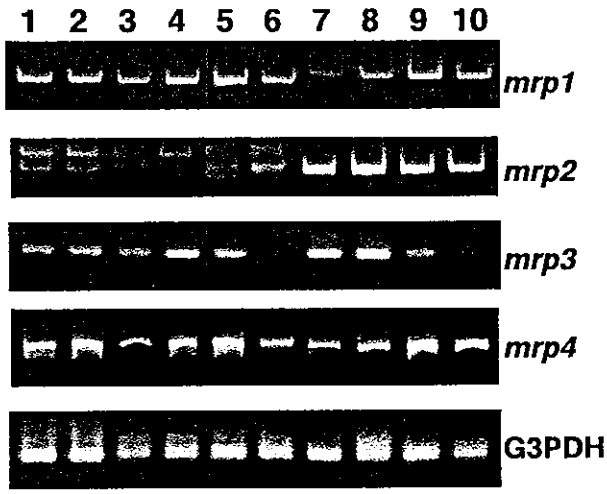

Fig. 3. Expressi on Pattern of MRPs on MT-Null Mouse Tissues
MRP1, -2, -3, -4 were detected by RT-PCR. The total RNA templates were prepared from MT-null mouse tissue (lane 2, 4, 6, 8, 10) and wild-type mouse tissue (lane 1, 3, 5, 7, 9). The bands of G3PDH provide an indication of the amount of total RNA loaded in each lane. Lane 1, 2: brain; lane 3, 4: lung; lane 5, 6: heart; lane 7, 8: liver; lane 9, 10: kidney.

In conclusion, our results suggest that the expression level of SMRP may be increased by MT deficiency in the liver and that SMRP or MRP5 may contribute to heavy metal detoxification in the liver of MT-null mice instead of MT.

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Intracellular factors involved in methylmercury toxicity in yeast

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Using yeast cells, we searched for the genes involved in the expression of methylmercury toxicity, and found that genes encoding L-glutamine.D-fructose-6-phosphate amidotransferase (GFAT) and ubiquitin transferase (Ubc3) confer methylmercury resistance on the cells. These findings provide important clues about the mechanism underlying methylmercury toxicity in mammals.

Introduction

Methylmercury is a known pollutant that causes severe central nervous system disorders. Despite the efforts of many researchers, including ourselves, the mechanisms involved in methylmercury toxicity and the defense against this toxicity remain unknown. Although more than 40 years have passed since the first outbreak of Minamata disease, we still only know that the causative agent is methylmercury which is highly cytotoxic, but how it exerts its toxicity at the molecular level remains to be determined.

We focused on the fact that drug resistance is sometimes involved in elevation of the concentration of the intracellular target of the drug. Screening for the genes conferring methylmercury resistance on cells transformed with individual genes would indicate the genes encoding the target molecules of methylmercury together with the genes for the defense against its toxicity.

Methods

We investigated yeast genes obtained from a library, since yeast genes can be easily identified because the nucleotide sequences of the entire genome has been clarified. Plasmids carrying a chromosome fragment (usually containing 2–4 genes) were transfected into yeast cells, and genes contained in the chromosome fragments were expressed at high levels in the cells. Among such yeast cells, those that could grow on a medium containing methylmercury at a concentration that would not permit the growth of normal yeast cells were selected. Since these yeast cells acquired methylmercury resistance following the introduction of the gene fragments, genes conferring methylmercury resistance must be contained in the introduced gene fragments. Plasmids were isolated from the yeast cells that had acquired methylmercury resistance, and the chromosome fragments carried in the plasmids were investigated.

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Results

We identified *GFA1*[1,2] and *CDC34* as the genes involved in methylmercury resistance.[3,4] *GFA1* is the gene coding for L-glutamine·D-fructose-6-phosphate amidotransferase (GFAT), which is a catalytic enzyme involved in the production of glucosamine 6-phosphate from glutamine and fructose. *CDC34* is the gene encoding ubiquitin transferase (Ubc3), which is involved in the ubiquitination of intracellular proteins.

1. GFAT

Glucosamine 6-phosphate generated by the reaction catalyzed by GFAT is the precursor of all amino sugars synthesized intracellularly. Yeast cells cannot survive without amino sugars, because glycoproteins cannot be produced in their absence. Therefore, GFAT is an essential enzyme for the growth of yeast cells.[5] Since GFAT is an SH enzyme, methylmercury inhibits the GFAT activity. The inhibitory effects of methylmercury on various SH enzymes were determined.[2] The activity of GFAT was almost completely inhibited by 4 μM methylmercury, while those of other SH enzymes were hardly affected by methylmercury at this concentration. The inhibition constant (Ki) of methylmercury was 4 μM for GFAT, while the Ki values for other SH enzymes were higher than 10-fold this value. These results indicate that methylmercury has a high affinity for GFAT and specifically inhibits its activity, suggesting that GFAT is the target molecule of methylmercury.

Yeast cells transfected with the *GFA1* gene are highly resistant to methylmercury, and a relatively large amount of GFAT is synthesized by the cells. Therefore, it remains possible that strong binding of GFAT at a high concentration to methylmercury reduced the concentration of free methylmercury and suppressed methylmercury toxicity, inducing methylmercury resistance in the yeast cells. Therefore, we determined the effects of increasing the intracellular concentration of glucosamine 6-phosphate induced by the reaction catalyzed by GFAT on methylmercury toxicity. Since glucosamine 6-phosphoric acid added to media is not taken up by cells, glucosamine was added to media. Glucosamine is not synthesized by cells, but extracellularly added glucosamine is taken up by cells and transformed to glucosamine

6-phosphate by hexokinase. The toxicity of methylmercury toward the yeast cells was markedly reduced depending on the concentration of added glucosamine.[2]

In conclusion, (1) yeast cells with high level GFAT expression are resistant to methylmercury, (2) methylmercury specifically inhibits GFAT activity, (3) methylmercury toxicity is markedly reduced by addition of glucosamine 6-phosphate, the product of the GFAT reaction, to cells, and (4) GFAT is an essential enzyme in yeast. These results suggest that GFAT is the main target molecule of methylmercury in yeast.[2]

2. Ubiquitin system

As described above, we showed that the gene encording Ubc3, in addition to that encording GFAT, confers methylmercury resistance on yeast. Ubc3 is an important enzyme in the ubiquitination of intracellular proteins. The ubiquitin system, which consists of a ubiquitin activation enzyme (E1), ubiquitin transferase (E2) and ubiquitin ligase (E3), is involved in the degradation of abnormal intracellular proteins. In this system, ubiquitin is activated by E1 and then binds to E2, while E3 recognizes target proteins such as abnormal proteins. E2 bound to ubiquitin binds to E3, and transfers ubiquitin to the target protein. Finally, the target protein ubiquitinated by these reactions is recognized by proteasomes and rapidly degraded.[6,7]

E2 proteins belong to the family of ubiquitin transferases, and the UBC domain is preserved as the catalytic domain in all E2 proteins. It is known that cysteine residues involved in the binding to ubiquitin, which is essential to the expression of E2 activity, are present in the UBC domain. To clarify the mechanism by which Ubc3 confers methylmercury resistance on yeast cells, we produced yeast cells overexpressing a mutant Ubc3 by substituting this cysteine residue with alanine, and found that these yeast cells were not methylmercury-resistant. Therefore, the ubiquitin transfer activity of Ubc3 is considered essential to the acquisition of methylmercury resistance.

Thirteen enzymes of the E2 family of yeast have been identified, each considered to exihibit substrate specificity. Therefore, we produced yeast cells overexpressing Ubc2, Ubc4, Ubc5 or Ubc7 of the E2 family, and determined their methylmercury resistance. Methylmercury resistance was observed in the yeast cells overexpressing Ubc4, Ubc5, and Ubc7.[3] Our study was the first to show that high-level expression of E2 family enzymes confers resistance to toxic chemicals.[4] Among the yeast cells overexpressing E1 (Uba1) or E3 (CDC53, SKP1, HRT1) proteins, only the yeast cells overexpressing Uba1 of the E1 family exhibited weak methylmercury resistance. These results suggest that E2 is the rate-limiting enzyme in the ubiquitination reaction, and the amount of ubiquitinated protein within cells was markedly increased by the overexpression of Ubc3.

It is considered that protein denatured by active oxygen is ubiquitinated by the ubiquitin system and degraded. It is hypothesized that cytotoxicity results when abnormal protein do not undergo normal degradation and accumulate in cells. Therefore, it is suggested that some modification of a specific protein within cells by methylmercury causes cytotoxicity, and enhancement of the degradation of the protein by ubiquitination reduces this toxicity.[4] In this case, the ubiquitin system acts to protect cells against methylmercury toxicity, and the protein ubiquitinated following modification by methylmercury would be the target molecule of methylmercury toxicity.

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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

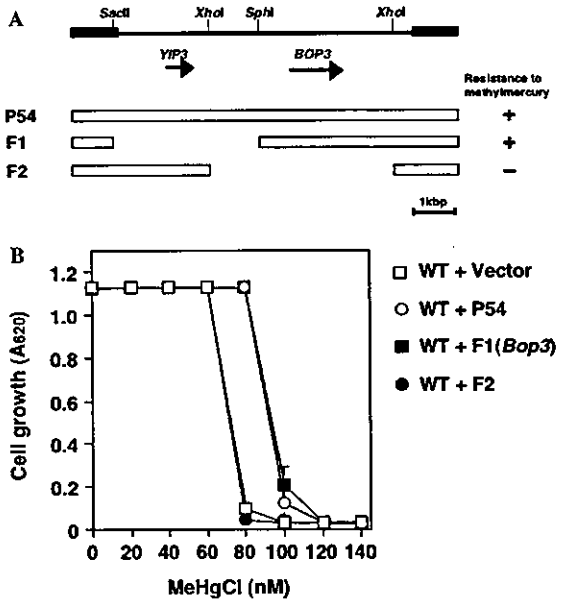

Fig. 1. Identification of a gene that confers resistance to methylmercury. (A) Restriction map of the genomic DNA insert (P54) that conferred resistance to methylmercury. The thick black lines represent the vector YEp13; the thin line represents the genomic DNA inserts. The restriction sites used to 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.

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

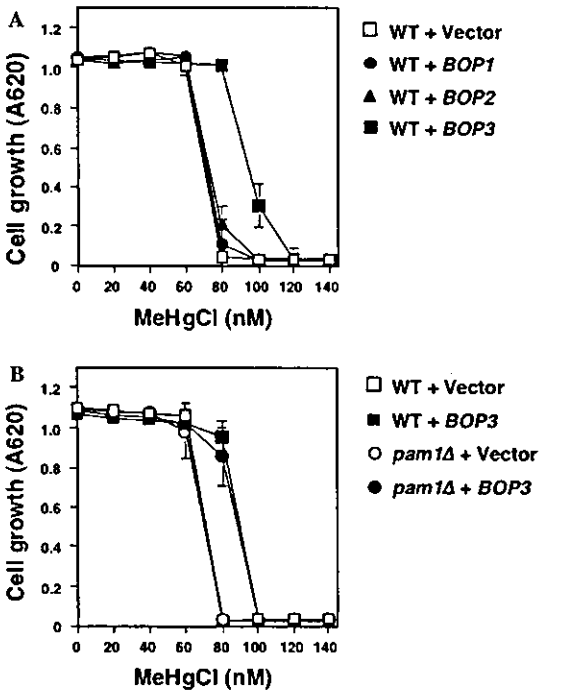

Fig. 2. Sensitivity of yeast cells that overexpressed each Bop family protein to methylmercury (A), and effect of disruption of *PAM1* gene on sensitivity to methylmercury of yeast that overexpressed Bop3 (B). (A) Yeast cells that harbored pKT10 (control), pKT10-BOP1, pKT10-BOP2 or pKT10-BOP3 were grown 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.

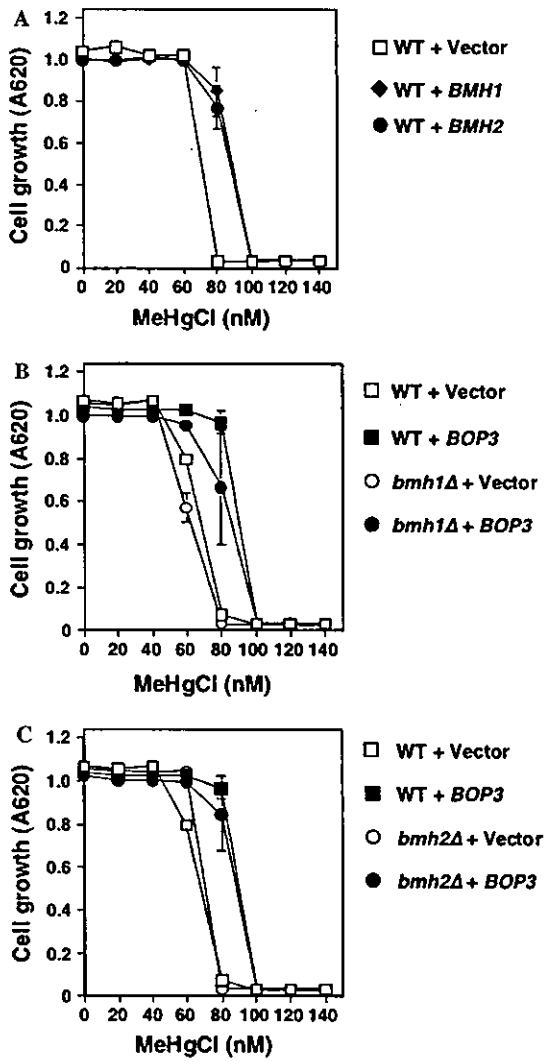

Fig. 3. Sensitivity of yeast cells overexpressing Bmh1 or Bmh2 to methylmercury (A), and effect of disruption of *BMH1* or *BMH2* gene on sensitivity to methylmercury of yeast that overexpressed Bop3 (B,C). (A) Yeast cells that harbored pKT10 (control), pKT10-BMH1 or pKT10-BMH2 were grown in SD (−Ura) 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

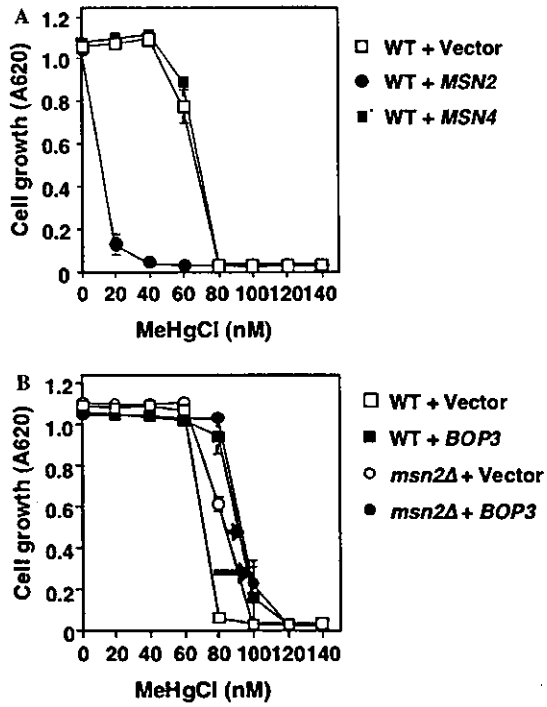

Fig. 4. Sensitivity of yeast cells that overexpressed Msn2 or Msn4 to methylmercury (A), and effect of disruption of *MSN2* gene on sensitivity to methylmercury of yeast that overexpressed Bop3 (B). (A) Yeast cells that harbored pKT10 (control), pKT10-MSN2 or pKT10-MSN4 were grown in SD (−Ura) liquid 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 Fkh1 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.

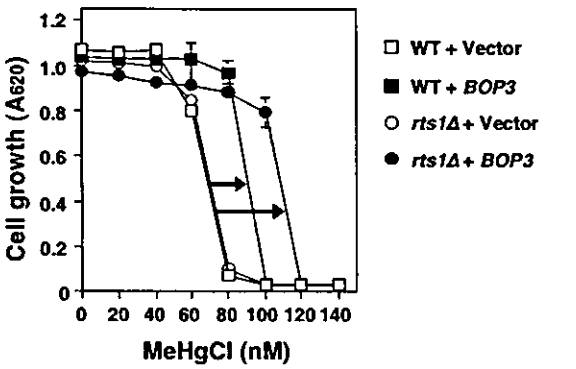

Fig. 5. Effect of disruption of *RTS1* gene on sensitivity to methylmercury of control yeast or Bop3-overexpressing yeast. Yeast *rts1Δ* cells, harboring pRS425 (control) or pRS425-BOP3, were grown in SD (−Leu) liquid medium that contained methylmercuric chloride. Arrows in the figure represent the magnitude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

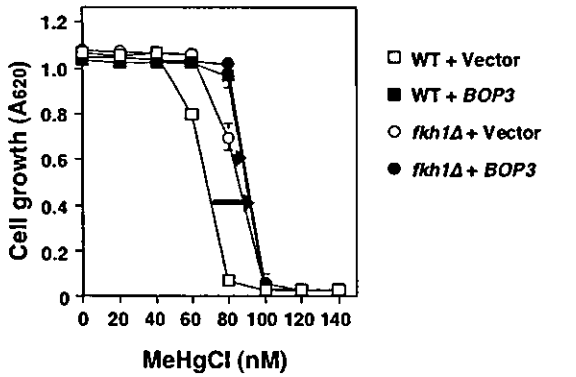

Fig. 6. Effect of disrupting the *FKH1* gene on sensitivity to methylmercury of control yeast or Bop3-overexpressing yeast. Yeast *fkh1Δ* cells, harboring pRS425 (control) or pRS425-BOP3, were grown in SD (−Leu) liquid medium that contained methylmercuric chloride. Arrows in the figure represent the 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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