remained low, but increased markedly after 3 hr incubation and remained high thereafter (Fig. 1B).

FIT2 and *FIT3* have been identified as genes that are transcriptionally activated by the transcription factor Aft1p during iron deprivation (Protchenko *et al.*, 2001). Aft1p is usually located in the cytoplasm, but insufficient cellular iron uptake causes it to enter the nucleus, where it enhances the expression of genes involved in the maintenance of iron homeostasis (Yamaguchi-Iwai *et al.*, 1995; Yamaguchi-Iwai *et al.*, 2002). It is therefore possible that cisplatin might increase Fit2p and Fit3p mRNA levels by activating Aft1p.

The marked increase in Fit2p and Fit3p expression suggests that the proteins might have a defensive function against the toxic effects of cisplatin. We therefore investigated the cisplatin susceptibility of yeasts overexpressing or lacking the *FIT2* or *FIT3* genes. As shown in Figs. 2 and 3, the susceptibilities of these yeasts to cisplatin were almost comparable with that of the wild-type strain, indicating that Fit2p and Fit3p are not directly involved in protecting against the toxic effects of cisplatin. However, the fact that cisplatin markedly increases Fit2p and Fit3p mRNA levels strongly suggests the existence of an activation mechanism of gene expression in response to cisplatin within the cell. A detailed further investigation of the effects of cisplatin on the activation of Aft1p is expected to elucidate the cell response mechanism to cisplatin.

ACKNOWLEDGMENT

The authors thank Nippon Kayaku Co., Ltd. (Tokyo, Japan) for providing the cisplatin used in this study.

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The Journal of Toxicological Sciences,
Vol.31, No.4, 391-398, 2006

CHARACTERIZATION OF AN IMMORTALIZED HEPATIC STELLATE CELL LINE ESTABLISHED FROM METALLOTHIONEIN-NULL MICE

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(Received June 26, 2006; Accepted August 14, 2006)

ABSTRACT — Hepatic stellate (HS) cells were isolated from the livers of metallothionein (MT)-null and control mice and used to establish IMS/MT(-) and IMS/N cell lines, respectively, using SV40 virus transformation. Cellular morphology, incorporation of vitamin A and expression of α-SMA, desmin and SV40 T-antigen were used to confirm that both cell lines were immortal HS cells. The growth rates of both cell lines were similar and there was little difference between cell line sensitivity to zinc. MT-null IMS/MT(-) cells were more sensitive to cadmium and mercury, although both cell lines accumulated almost equal amounts of cadmium during a 24-hr culture period. As HS cells play an important role in hepatic fibrosis and are activated by heavy metals such as cadmium or reactive oxygen, the MT-null HS cell line derived in this study should be a useful experimental model for examination of the role of MT in HS cell activation.

KEY WORDS: Metallothionein-null cells, Hepatic stellate cells, Cadmium, Immortalization

INTRODUCTION

Metallothionein (MT) is a unique protein consisting of 30% cysteine but lacking the usual cysteine S-S bonds (Webb, 1979; Lazo and Bahnson, 1989; Kägi, 1991). Various physiological effects of MT have been reported such as detoxification of heavy metals, scavenging of active oxygen and maintenance of zinc (Zn) homeostasis (Bremner, 1987; Lazo and Bahnson, 1989; Kägi, 1993; Sato and Bremner, 1993). Its role in detoxification has been particularly well studied and was originally examined using MT-inducing agents.

More recently, MT-transgenic cell lines and mice have been produced and used to demonstrate that MT provides protection against the toxicity of heavy metals such as cadmium (Cd) and mercury (Hg) (Kaina *et al.*, 1990; Dalton *et al.*, 1996) and the oxidative stress from radiation or reactive oxygen species (Lohrer and Robson, 1989; Kang *et al.*, 1997; Pitt *et al.*, 1997). Moreover, transgenic mice deficient in MT-I and -II, major molecular species of MT, have elucidated the basal level functions of MT (Michalska and Choo, 1993; Masters *et al.*, 1994). Model experimental systems using MT-null cell lines are of use in examining the precise physiological functions of MT. To this end, we have established and characterized an immortalized hepatic stellate (HS) cell line from the livers of MT-null mice.

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MATERIALS AND METHODS

Materials

All reagents were purchased from Wako Pure Chemical unless otherwise stated.

Isolation and immortalization of HS cells

HS cells isolated from MT-null mice (Masters *et al.*, 1994) and normal 129/Sv mice were immortalized by infection of SV40 virus. Cells were maintained in Dulbecco's modified minimum essential medium (DMEM, Nissui Pharmaceutical) supplemented with 10% fetal calf serum and 60 mg/ml kanamycin and cultured at 37°C in a humidified atmosphere containing 5% CO_2. These cells were designated IMS/MT(-) (immortalized mouse stellate cells from MT-null mice) and IMS/N (immortalized mouse stellate cells from normal mice), respectively (Miura and Naganuma, 2000).

Reverse transcription-PCR of SV40 large T-antigen

Total RNA from IMS/N and IMS/MT(-) cells was extracted and reverse transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen), AmpliTaq Gold (Applied Biosystems), oligo (dT) primers (Invitrogen) and a specific primer pair for the SV40 large T-antigen: forward, 5'-TAA CAC TGC CAT CCA AAT AAT CC-3'; reverse, 5'-TGT GTT GTT ATT GCT TGG GAT AT-3'. The reaction included an initial incubation at 95°C for 10 min followed by 30 cycles of 96°C for 30 sec, 65°C for 15 sec and 72°C for 60 sec. The PCR product was 667 bp.

Vitamin A accumulation

IMS/N and IMS/MT(-) cells (1×10^4 cells) were seeded in 8-well glass chambers (No 4118; Falcon) and incubated in cell medium containing 25 mM retinol acetate (Sigma-Aldrich) dissolved in dimethyl sulfoxide for three days (Matsuura *et al.*, 1989). After washing the cells with phosphate buffered saline (PBS), vitamin A autofluorescence in the cytoplasm was observed under a fluorescence microscope using a broad-range wavelength (330 to 385 nm) excitation filter (U-MWU; Olympus).

Cell growth rate and cell survival rate

IMS/N and IMS/MT(-) cells were seeded at 1×10^5 cells per well in 6-well plates and cultured for 24, 48, 72 or 96 hr. Cells were harvested by trypsinization and the cell number was counted by a hemocytometer. The survival rate against various types of external stress was estimated using AlamarBlue reagent (Becton Dickinson) as previously described (Miura *et al.*, 1999). Briefly, IMS/N and IMS/MT(-) cells (1×10^4 cells/well) were seeded in 96-well plates, and different concentrations of stimulants were applied 24 hr later. Cell survival rates were estimated after a further 24 hr.

Western blotting analysis

IMS/N and IMS/MT(-) cells were harvested, collected and sonicated in PBS for 15 s on ice. The samples were mixed with an equal volume of 2 × sample buffer (100 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 12% β-mercaptoethanol, 20% glycerol, 0.2% bromophenol blue) and boiled for 5 min followed by centrifugation at 12,000 × *g* for 10 min to remove insoluble cell debris. Cellular proteins (approximately 45 mg) were resolved on a 7.5% SDS-polyacrylamide gel, and electroblotted onto Immobilon polyvinylidene fluoride membrane (Millipore). Membranes were incubated overnight at room temperature with a rabbit polyclonal anti-desmin antibody (1:100 dilution; PC-10570, Cosmo Bio), followed by a 1 hr incubation with an alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG secondary antibody (1:3,000 dilution; Santa Cruz Biotechnology). Proteins were visualized by chemiluminescence using the ECL kit (Amersham) with α-smooth muscle actin (SMA) expression determined in a similar manner using the monoclonal anti-α-SMA antibody (1:100 dilution; A2547, Sigma-Aldrich) and the AP-conjugated goat anti-mouse IgG secondary antibody (1:3,000 dilution; Santa Cruz Biotechnology).

Accumulation and distribution of Cd in cells after $CdCl_2$ treatment

IMS/N and IMS/MT(-) cells (3×10^5 cells) were seeded in 6-well plates in triplicate and incubated overnight. Medium was changed to fresh DMEM containing 30 mM $CdCl_2$ and incubated for 8 hr or 24 hr. Cells were scraped, collected and sonicated in PBS. Cd concentrations in the sonicated sample were measured by atomic absorption spectrometry. Cellular total protein levels were determined by the Bradford reagent (Sigma-Aldrich).

To investigate Cd distribution, 2×10^6 cells were seeded in 100 mm dishes (four dishes per cell line). Cells were incubated with $CdCl_2$ (30 mM) for 24 hr, harvested and all cells collected into one tube. The cell concentration was adjusted by addition of PBS. Cells (4×10^6 cells in 400 ml PBS) were sonicated for 1 min on ice followed by centrifugation at 105,000 *g* for 1 hr

at 4°C. Supernatants were collected and filtered (0.45 mm pore size). The distribution profile of Cd was measured by high-performance liquid chromatography/inductively coupled plasma mass spectrometry (HPLC/ICP-MS). A 100 ml aliquot of supernatant was loaded on the TSK GEL SW3000 column (Tosoh Corporation) and eluted with 50 mM Tris-HCl (pH 8.4) at a flow rate of 0.8 ml/min (HP1100; Yokokawa Analytical Systems). The eluate was introduced directly into the ICP-MS instrument (HP4500; Yokokawa Analytical Systems). Cd concentrations were determined at mass numbers of 111 and 114 m/z.

RESULTS

Characterization of immortalized HS cells

IMS/MT(-) and IMS/N cell lines were grown for more than 30 passages under standard culture conditions supplemented with 10% FCS. Expression of SV40 large T-antigen, an index of cell immortalization, was identified in both cell lines by RT-PCR (Fig. 1a). Both cell lines assumed a similar morphology with elongated asteroidal multicellular nodules, which are characteristic of HS cells (Fig. 1b). Growth rates of both cells were similar, with doubling times of approximately 27 hr, as calculated from their growth curves (Fig. 1c). Both cell lines also lifted off the base of the dish in sheets at high confluency.

The contractile proteins desmin and α- SMA can be used as markers for HS cells as they are specifically expressed in this cell type (Yokoi *et al.*, 1984; Rockey *et al.*, 1992; Shimizu *et al.*, 1999). We therefore investigated the expression of these proteins using western blotting and identified 42 kDa and 50 kDa bands corresponding to the molecular weights of α-SMA and desmin, respectively, in both cell lines (Fig. 2).

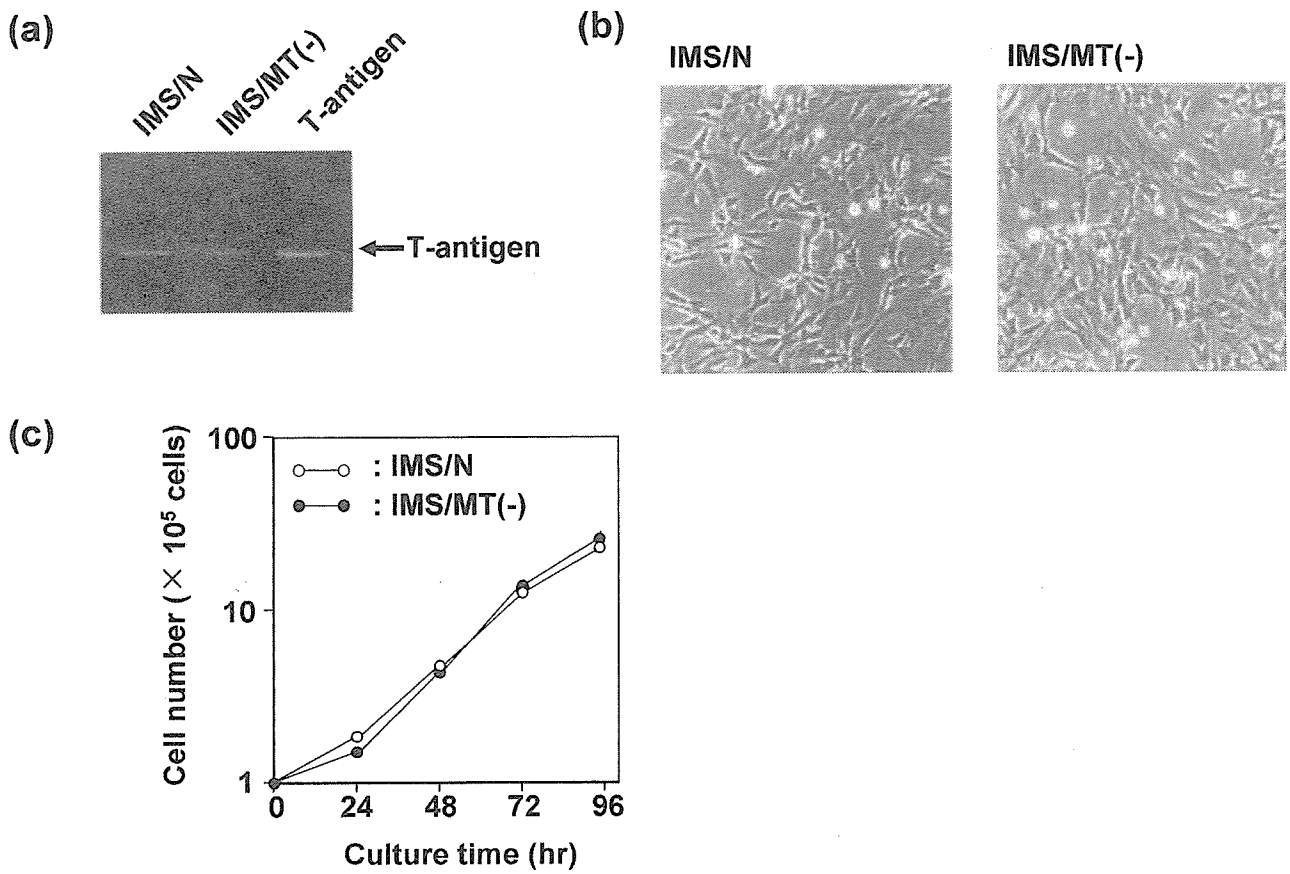

Fig. 1. Biochemical features of IMS cells. (a) SV40 T-antigen mRNA expression determined by RT-PCR. (b) Morphological characteristics of IMS/N and IMS/MT(-) cells shown by phase contrast microscopy (magnification × 100). (c) Cell proliferation curve of IMS/N (open circles) and IMS/MT(-) (closed circle) cells.

HS cells play important roles in vitamin A metabolism and are known to store vitamin A in ester form intracellularly (Hendriks *et al.*, 1985; Matsuura *et al.*, 1989). To investigate the incorporation of vitamin A into both established cell lines, we cultured them for three days with 25 μM all-trans retinol acetate. Fluorescence microscopy revealed clear droplet-like blue-green vitamin A autofluorescence in the cytoplasm of both cell lines, confirming their incorporation and accumulation of vitamin A (Fig. 3). Little morphological differences were observed between the cell lines after culture with retinol. These results suggest that IMS/MT(-) and IMS/N cells are immortalized HS cell lines and therefore suitable cell models for the examination of MT deficiency.

Sensitivity to environmental stress

Cell survival rates after 24-hr culture with different concentrations of $CdCl_2$ were determined to compare Cd sensitivity. MT-null IMS/MT(-) cells were shown to be more sensitive to Cd than normal IMS/N cells (Fig. 4-a). In addition, the cell lines demonstrated morphological differences after 24-hr culture with 30 μM Cd: IMS/N cells were elongated, while IMS/

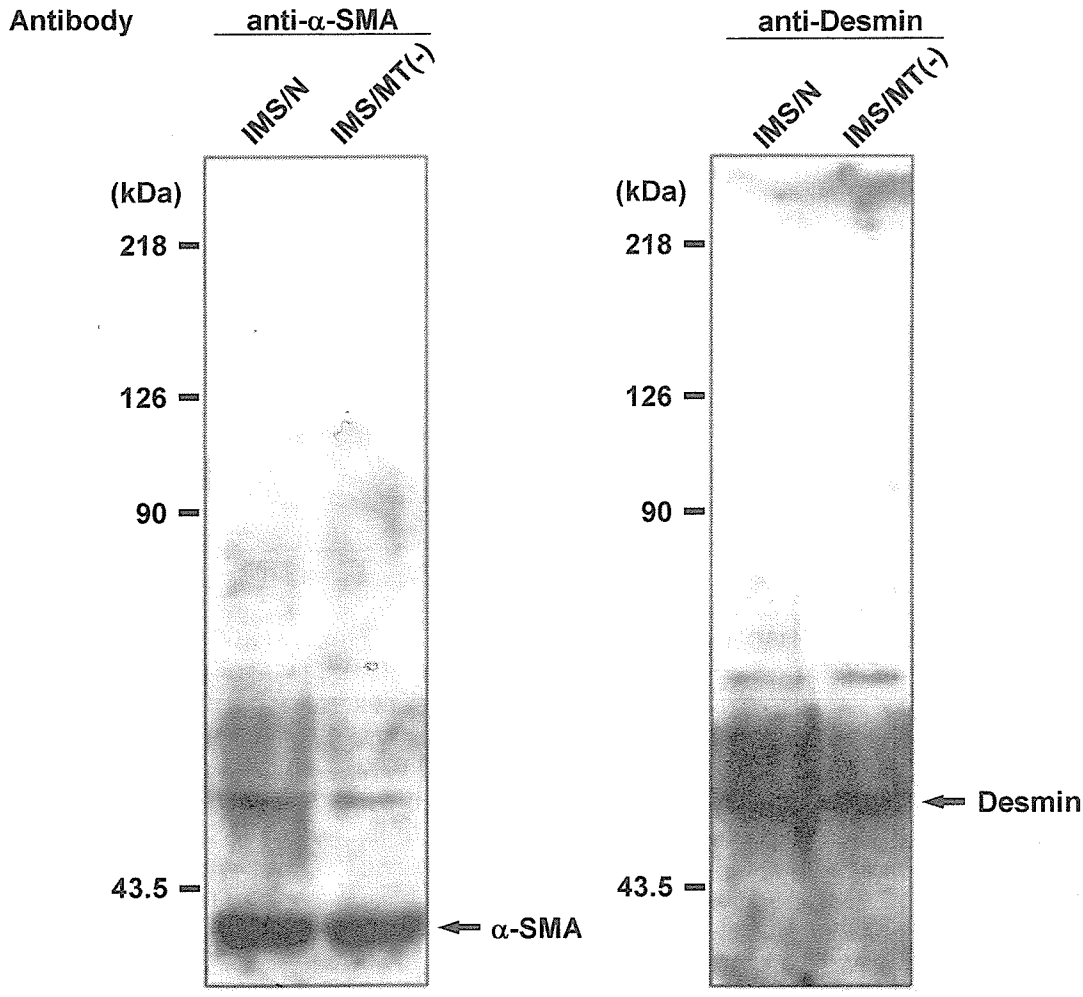

Fig. 2. Expression of α-SMA and desmin proteins as HS cell markers. Cellular proteins (approximately 45 mg) from IMS/N and IMS/MT(-) cells were resolved on 7.5% SDS-PAGE. Western blotting analysis was performed using an anti-α-SMA antibody or an anti-desmin antibody.

MT(-) cells were rounder in appearance and floated off the dish (Fig. 4b). IMS/MT(-) cells also showed a higher sensitivity to $HgCl_2$ than IMS/N cells, although their sensitivity to $ZnCl_2$ and retinol acetate was comparable (Fig. 4c, 4d, 4e). Little difference was observed in cell line sensitivity to paraquat and tert-butylhydroperoxide (TBH), both of which generate reactive oxygen and hydrogen peroxide (data not shown).

Intracellular Cd accumulation

Because of the distinct differences observed in Cd sensitivity between the two cell lines, we determined the intracellular Cd accumulation 8 hr and 24 hr after addition of $CdCl_2$. Cd accumulation increased with time in both cell types without significant difference in rate (Fig. 5a). The distribution of Cd as a soluble fraction was then examined after 24 hr culture with CdC_2 using HPLC/ICP-MS. This analysis showed that 41% Cd existed as a MT fraction in IMS/N cells (Fig. 5b, left), while almost all Cd existed as a high-molecular-weight fraction in MT-null IMS/MT(-) cells. In MT-null cells Cd appeared to be conjugated with high-molecular-weight protein(s), but not low-molecular-weight peptides or proteins (Fig. 5b).

DISCUSSION

Immortalized fibroblast cell lines from MT-null mice have been previously established from the kidney (Butcher *et al.*, 2004), lung (Jiang *et al.*, 2002) and fetus (Kondo *et al.*, 1999; Himeno, 2002; Mahboobi *et al.*, 2003). By contrast, the IMS/MT(-) and IMS/N cells established in this study are HS cells from the liver. HS cells are also known as Ito cells, fat-storing cells, perisinusoidal cells and lipocytes, and are non-parenchymal cells of mesenchymal origin, located in the hepatic Disse space. They are involved in the storage and metabolism of vitamin A and accumulate 80-90% of intrahepatic vitamin A as fat droplets (Hendriks *et al.*, 1985; Blomhoff and Wake, 1991).

HS cells play a major characteristic role in hepatic fibrosis, where they are activated to transform

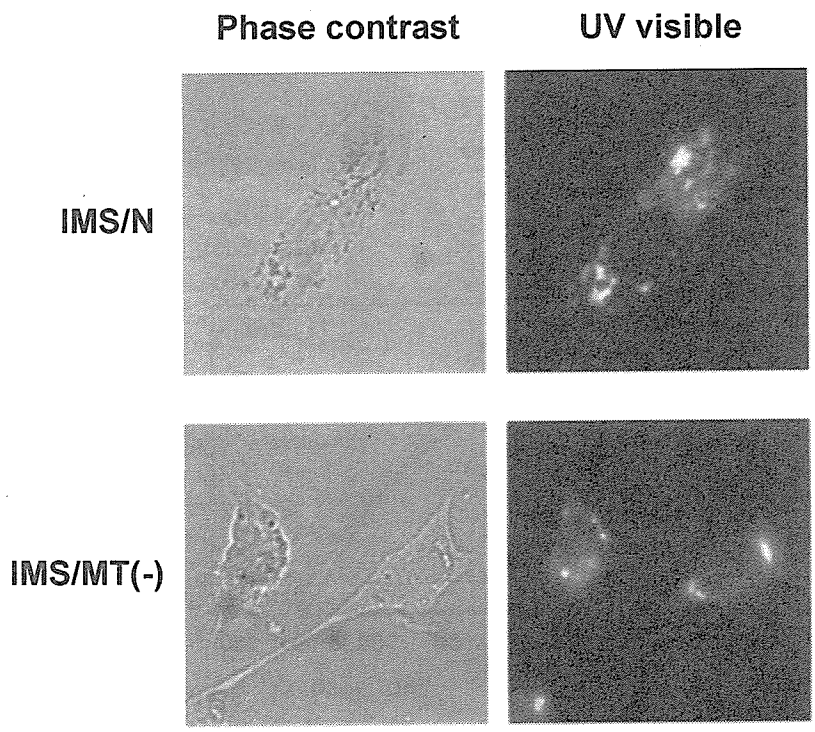

Fig. 3. Accumulation of vitamin A in IMS cells. IMS/N and IMS/MT(-) cells were incubated with 25 mM retinol acetate for three days and visualizedunder UV light (right) and phase contrast (left) (magnification × 400).

into myofibroblast-like cells, generating contractile proteins such as α-SMA and extracellular matrices such as collagen (mainly type I) or fibronectin (de Leeuw *et al.*, 1984; Rockey *et al.*, 1992). Previously-known factors that activate HS cells include the active oxygen generated by chronic alcohol drinking and viral infection (Schuppan *et al.*, 2003), while experimental HS cell activation by carbon tetrachloride has also been reported (Greenwel *et al.*, 1991). Del Carmen *et al.* (2002) reported that Cd induces expression of collagen type I in HS cells of rat liver. Increases in hepatic collagen contents by metals such as Hg, Pb, Mo, Cu and Cr have also been reported (Rana and Prakash, 1986). Moreover, HS cells help regulate sinusoidal bloodflow; in this way the flow rate of portal blood into the sinusoid is controlled by endothelin-1 contraction and nitric oxide relaxation (Rockey, 2001).

In these varied ways, the physiological activity of HS cells is controlled by many factors. Recent reports also revealed a relationship between increased intrahepatic MT and suppression of hepatic fibrosis (Cheng *et al.*, 2002, 2004), suggesting that increasing or decreasing the amount of MT protein could lead to HS cell activation. The immortal HS cell lines established in this study demonstrated varied sensitivity to heavy metals such as Cd and Hg. A more precise investigation into the effects of xenobiotics and oxidative stress on the function of both cell lines, rather than on their cytotoxicity, might elucidate the role of MT in hepatic disorders.

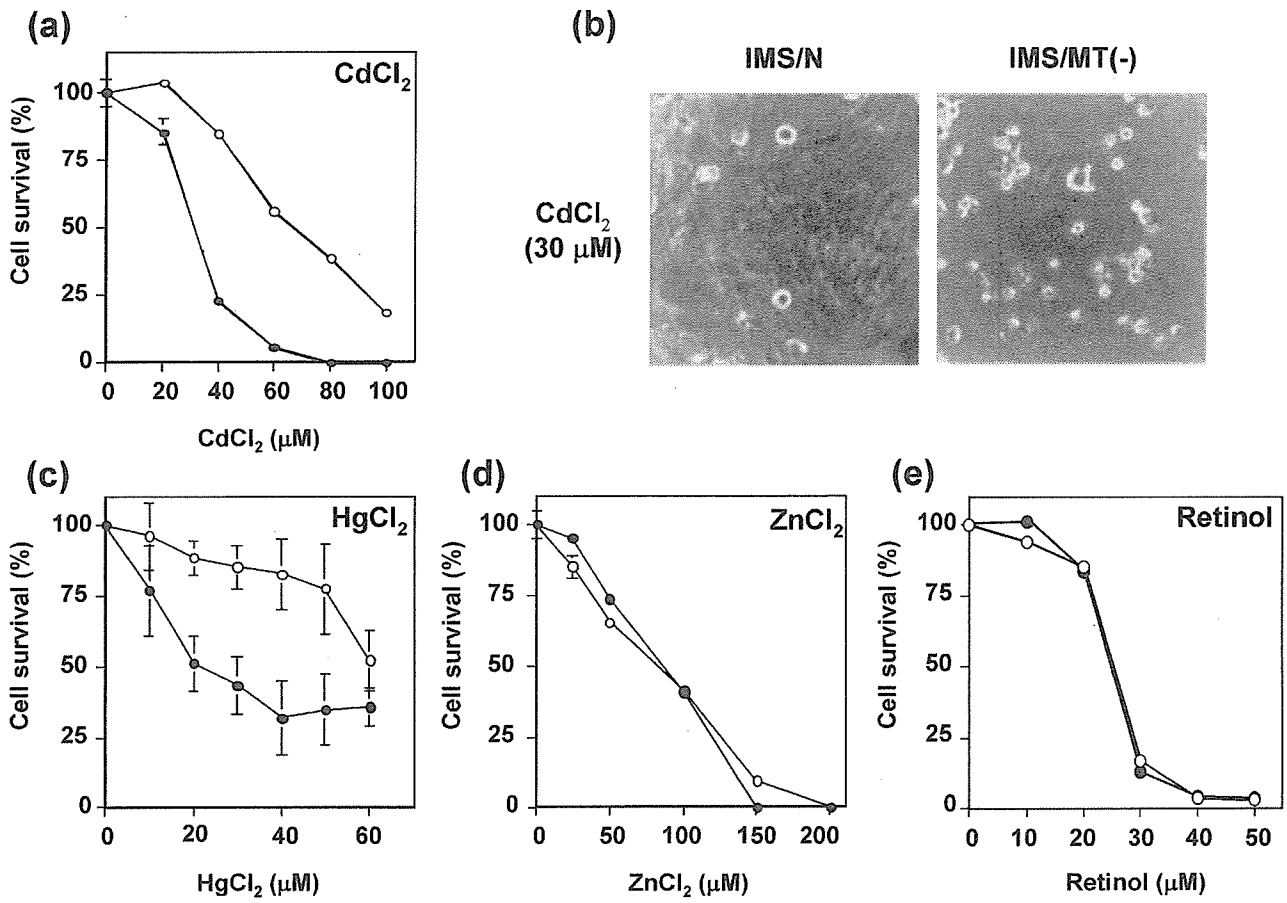

Fig. 4. Sensitivities of IMS cells to heavy metals and retinol. (a) IMS/N and IMS/MT(-) cells (1×10^4 cells/well) were incubated with the indicated concentrations of $CdCl_2$. Cell viability was estimated by addition of AlamarBlue. (b) Morphological changes of IMS/N and IMS/MT(-) cells after incubation with 30 mM $CdCl_2$ were observed by phase contrast microscopy (magnification × 100). Cell survival rates of other heavy metals: $HgCl_2$ (c), $ZnCl_2$ (d), and retinol acetate (e) were also estimated. IMS/N and IMS/MT(-) cells are represented by open and closed circles, respectively.

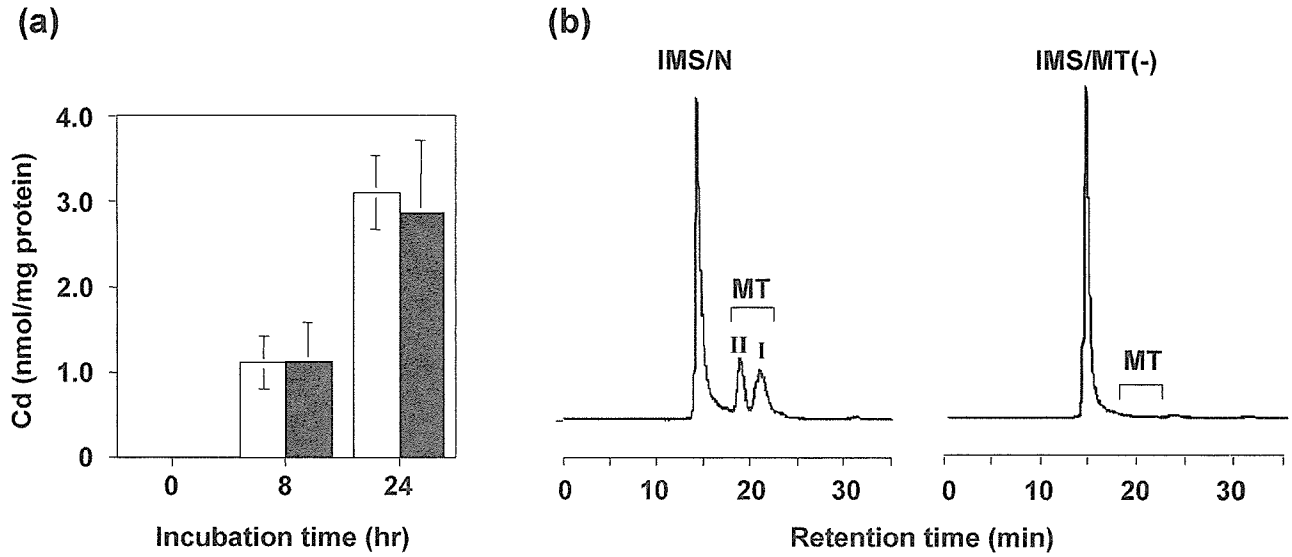

Fig. 5. Accumulation and distribution of Cd in IMS cells. (a) IMS/N and IMS/MT(-) cells (n=3) were incubated in medium containing 30 mM $CdCl_2$ for 8 hr or 24 hr and intracellular Cd concentrations determined by atomic absorption spectrometry. (b) IMS/N and IMS/MT(-) cells were incubated with 30 mM $CdCl_2$ and the Cd distribution profile was analyzed by HPLC/ICP-MS.

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Hum Genet (2006) 120:553–560
DOI 10.1007/s00439-006-0238-6

ORIGINAL INVESTIGATION

Potential effect on cellular response to cadmium of a single-nucleotide A → G polymorphism in the promoter of the human gene for metallothionein IIA

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Received: 10 May 2006 / Accepted: 25 July 2006 / Published online: 23 August 2006

Abstract Most people generally ingest cadmium in their food. Cadmium that has accumulated in tissues induces the synthesis of metallothioneins (MTs) which are metal-binding proteins that bind tightly to cadmium to inhibit its renal toxicity. Individuals whose ability to induce the synthesis of MTs is low seem likely to be particularly susceptible to the toxic effects of cadmium. In this study, we analyzed the polymorphism of the promoter region of the gene for MT-IIA, the major species of MT in humans, in 119 adult Japanese subjects. We found that about 18% of the subjects had an A → G single-nucleotide polymorphism in the core region of the promoter near the TATA box. A reporter-gene assay using HEK293 cells showed that replacement of A by G at position −5 reduced the efficiency of the cadmium-induced transcription of the gene for MT-IIA. This single-nucleotide polymorphism inhibited the binding of nuclear proteins to the core promoter region of the gene for MT-IIA. When the promoter region upstream of the TATA box was replaced by a sequence that contained three dioxin-responsive elements, the reporter-gene assay demonstrated that the A → G single-nucleotide polymorphism resulted in a marked reduction in the rate of dioxin-induced transcription. These results suggest that the A → G single-nucleotide polymorphism reduces the efficiency of those aspects of the transcription of the gene for MT-IIA that are controlled by general transcription factors.

Introduction

Metallothioneins (MTs) are proteins of low molecular weight whose synthesis is induced by heavy metals, such as cadmium (Cd), zinc (Zn) and mercury (Hg), and which bind tightly to these heavy metals to suppress

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their toxicity (Kägi 1991; Webb 1979). Treatment of mice with inducers of the expression of MT, such as Cd and Zn, to promote the synthesis of MT markedly reduces the toxicity of subsequently administered Cd (Webb 1979). Moreover, mice with defective genes for MT are ten times more sensitive than normal mice to Cd toxicity (Liu et al. 2000). Many researchers have reported similar data and it seems clear that MTs are the main determinants of the sensitivity of mammals and cultured mammalian cells to Cd.

Humans generally ingest Cd in their food, and people who ingest excessive amounts of Cd have a high incidence of renal tubular dysfunction (Friberg and Vahter 1983; Saito et al. 1977). The half-life of Cd in humans is estimated to range from 10 to 30 years (Webb 1979) and Cd that has accumulated in the body is excreted in very small amounts. Thus, the amount of Cd in the human body increases with age (Yoshida et al. 1998). Furthermore, since Cd induces the synthesis of MT, the concentration of MT in tissues also increases with age (Yoshida et al. 1998). Much of the Cd that accumulates in tissues is bound to MT with resultant suppression of the heavy metal's toxicity. It has been postulated that the accumulation of Cd in the kidney exceeding the rate of synthesis of MT induces renal tubular dysfunction.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has estimated that the critical concentration of renal cortical Cd, namely, the concentration that causes renal tubular dysfunction in 10% of individuals exposed to Cd, is 200 μg/g tissue (WHO 1993). The mean renal cortical concentration of Cd in the general Japanese population of 41–60 years of age has been reported to be 68.9 μg/g tissue (Yoshida et al. 1998), being one-third of the estimated critical concentration. However, in MT-deficient mice, renal dysfunction can be detected even at renal concentrations of Cd below 10 μg/g tissue (Liu et al. 2000). These findings suggest that individuals with abnormalities in the synthesis of MT might be prone to renal dysfunction due to exposure to Cd. In this study, we analyzed the polymorphism of the promoter of the gene for MT-IIA, the main species of MT in humans, and found that approximately 18% of the subjects examined had an A → G single-nucleotide polymorphism near the TATA box, which reduced the level of expression of the gene for MT-IIA.

Materials and methods

Cells

Human embryonic kidney 293 (HEK293) cells were maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM) that was supplemented with 10% fetal bovine serum and 60 μg/ml kanamycin.

Isolation of DNA

DNA was obtained from samples of blood from 119 unrelated Japanese individuals (mean age 58 ± 11 years, range 34–79 years; 28.6% men) and was stored at −20°C prior to analysis. Written informed consent was obtained from all individuals, as required by the research protocol that had been approved by the Medical Ethics Committee of Tohoku University.

Amplification of the promoter of the gene for MT-IIA

A 222-bp fragment of the promoter of the gene for MT-IIA was amplified from 200 ng of high-molecular-weight DNA by PCR. This fragment extended from a site that was 202 bases upstream of the site of initiation of transcription (including MRE-a, -b, -c, -d and -e) to the 5′-end of exon 1 (20 bases downstream of the site for initiation of transcription; see Fig. 1). Amplification by PCR was performed in a buffered solution that contained 10 μM each primer (forward, 5′-GGG CCG CCT TCA GGG AAC TG-3′; and reverse, 5′-GGA CTT GGA GGA GGC GTG GT-3′), 200 μM dNTP mix, 10% dimethylsulfoxide and 0.025 units of AmpliTaq Gold (PE Applied Biosystems, Foster, CA) in a total volume of 100 μl. Samples were heated at 95°C for 10 min and then subjected to 30 cycles of incubation at 96°C for 30 s, at 61°C for 30 s and at 72°C for 30 s. The products of PCR were purified with a High Pure PCR Product Purification Kit (Boehringer Mannheim GmbH, Mannheim, Germany) according to manufacturer's instructions. The samples were stored at −20°C prior to analysis.

Analysis of single-strand conformation of polymorphism (SSCP)

Eight microliters of a solution of the product of PCR were mixed with 15 μl of F-dye solution (95% formamide, 10 mM EDTA, pH 8.0, 0.05% xylene cyanole and 0.05% bromophenol blue), denatured by boiling for 5 min, and snap-chilled in iced water for 5 min. Samples were then loaded onto a 10% polyacrylamide gel that had been prepared in 1× TBE and subjected to electrophoresis at 180 V for approximately 90 min in 0.5× TBE. DNA was silver-stained with PlusOne DNA Silver-Staining Kit (Amersham Pharmacia Biotech, Buckinghamshire, England) according to manufacturer's instructions.

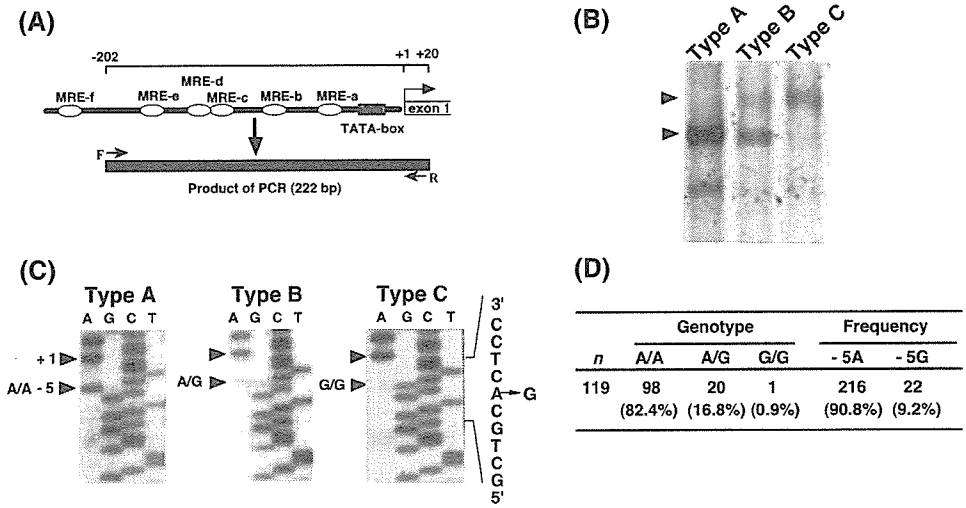

Fig. 1 Identification of polymorphism in the promoter region of the gene for MT-IIA. **a** The region of the gene for MT-IIA that was amplified for SSCP analysis. *F* and *R* indicate positions of primers. **b** Representative results of SSCP analysis. The patterns of bands after electrophoresis were of three types, as indicated. **c** Nucleotide sequencing of the DNA isolated from each band indicated in **b**. Re-amplified samples of the DNA were subjected to direct sequencing. **d** Genotypes and allele frequencies of the core promoter region of the gene for MT-IIA

Analysis of restriction fragment length polymorphism (RFLP) by PCR

The core promoter region of the gene for MT-IIA was amplified by PCR with the primers; forward, 5′-CGC CTG GAG CCG CAA GTG AC-3′; and reverse, 5′-TGG GCA TCC CCA GCC TCT TA-3′. Amplified fragments of 185 bp were digested with *Bsg*I (New England Biolabs, Hertfordshire, UK), fractionated on a 2% agarose gel, and visualized by staining with ethidium bromide. The lengths of fragments obtained by digestion of each 185-bp fragment by *Bsg*I were 144 bp and 41 bp for the A/A type (see below), 185 and 144 bp for the A/G type, and 185 bp for the G/G type.

Direct sequencing

The bands of products of PCR were excised from a silver-stained dried gel (see above) and transferred to microtubes. After addition of 50 μl of molecular-grade water, tubes were incubated at 37°C for 10 min and then at 85°C for 15 min to elute the DNA from each slice of gel. To amplify these samples of DNA, we performed PCR as described above, using 1 μl of the solution of eluted DNA as template. We purified the products of PCR and subjected an aliquot (350 fmol) to sequencing reactions using a Themo Sequenase Cycle Sequencing Kit (Amersham Pharmacia Biotech) and the ^{32}P-labeled forward or reverse primer (see above) as the sequencing primer. Samples were mixed with loading buffer, heat-denatured, and resolved on a 6% sequencing polyacrylamide gel at 3,000 V in 0.6× TBE buffer. The gel was dried on 3 MM paper (Whatman, Middlesex, UK) and bands were visualized by autoradiography.

Construction of plasmids

A bacterial expression vector, pcDNA3.1/*lac*Z (Invitrogen, Carlsbad, CA), in which expression of the gene for β-galactosidase is driven by the CMV promoter, was used to construct a reporter plasmid. The vector was first double-digested with *Nru*I and *Nhe*I to eliminate the CMV promoter, blunted and ligated to yield a promoter-less vector, namely, pcDNA3.1a/*lac*Z. Then the A/A type or the G/G type promoter region of the gene for MT-IIA including the 5′-end of exon 1 (positions −202 to +20; Fig. 1) was amplified by PCR using the following primers: common 5′ primer, 5′-GGG CCG CCT TCA GGG AAC TG-3′; A/A-type 3′ primer, 5′-GGA CTT GGA GGA GGC GTG GTG GAG TGC AGC GCG-3′; and G/G-type 3′ primer, 5′-GGA CTT GGA GGA GGC GTG GTG GAG CGC AGC GCG-3′. The two products of PCR were blunted and cloned into the *Eco*RV-digested pcDNA3.1a/*lac*Z vector. The resultant plasmids were designated simply −5A (A/A type) and −5G (G/G type), and each was sequenced with the Thermo Sequenase Cycle-Sequencing Kit to confirm the sequence of the inserted promoter.

Assay of reporter-gene expression

To assess the transcriptional activities of the A/A- and G/G-type promoters, we plated HEK293 cells (3.2×10^4/well) in a 24-well plate and cultured them overnight. We then transfected cells with the A/A-type or G/G-type plasmid (0.13 μg) and a reference plasmid (pRSV/Luc; 0.13 μg) using the lipofection reagent FuGene™6 (0.2 μl; Roche Diagnostics, Tokyo, Japan). After a 12-h incubation, cells were washed once with DMEM and incubated with fresh DMEM that contained non-lethal concentration of $ZnCl_2$ (100, 150, 200 or 250 μM) or $CdCl_2$ (0.125, 0.25, 0.5 or 1.0 μM) for a further 24 h. Cells were then washed with cold PBS (−) and combined with 100 μl of Reporter Lysis Buffer (Promega, Madison, WI) and lysed by freezing (−80°C for 30 min) and thawing (25°C for 20 min). To determine the activity of β-galactosidase as measure of the transcriptional activity of each MT-IIA promoter, we combined aliquots of lysed cells (45 μl) with an equal volume of a 0.2% solution of *o*-nitrophenyl-β-D-galactopyranoside, as substrate, and incubated the mixture at 37°C for 2–6 h. Each reaction was terminated by addition of 100 μl of 1 M Na_2CO_3 and then absorbance were measured at 405 nm with a microplate reader. For measurements of luciferase activity, the same samples as described above (45 μl) were combined with 10 μl of luciferin (Reconstituted Substrate; Promega) and the mixture was shaken on a vortex mixer for 10 s. Then luminescence was estimated with a luminescent plate reader.

Preparation of nuclear extract

HEK293 cells were plated at 1.2×10^6 cells per well of a 6-well plate with 2 ml of medium per well and incubated at 37°C for 24 h. Cells were then incubated with $ZnCl_2$ (final concentrations, 100 and 250 μM) for another 4 h. Nuclear extracts were prepared as described by Muller et al. (Muller et al. 1989) with slight modifications. In brief, cells were harvested with ice-cold PBS (−) and collected by centrifugation (2,000×*g*, 1 min, 4°C). The cell pellet was resuspended in 350 μl of buffer A (10 mM HEPES–KOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.25% (v/v) NP–40, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 2 μg/ml pepstatin A) and mixed vigorously for 10 s. The suspension was then centrifuged as described above to pellet the nuclei. Nuclei were resuspended in 35 μl of buffer C (50 mM HEPES–KOH (pH 7.8), 420 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 20% (v/v) glycerol, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 2 μg/ml pepstatin A) and mixed with gentle shaking for 30 min at 4°C. Then the suspension was centrifuged at 20,000 × *g* for 15 min at 4°C. The supernatant was collected and frozen in aliquots at −80°C. Concentrations of protein were determined by Bradford's protein assay method with a kit from Bio-Rad (Hercules, CA) and bovine serum albumin as the standard.

Electrophoretic mobility shift assay (EMSA)

Synthesized oligonucleotide probes are shown in Fig. 3a. The double-stranded probes were end-labeled with [γ−^{32}P] ATP by T4 polynucleotide kinase for 30 min at 37°C, and then they were purified on Micro-Spin G-50 columns (Amersham Pharmacia). Each reaction mixture (23 μl) contained 12 mM HEPES–KOH (pH 7.6), 50 mM KCl, 5 mM NaCl, 5 mM $MgCl_2$, 12% (v/v) glycerol, 0.6 mM DTT, 100 μM $ZnSO_4$, 3 μg poly(dI–dC)-(dI–dC), and 10 μg of nuclear extract. The binding reaction was started by addition of a [γ-^{32}P]-labeled probe (10,000 cpm/μl; 2 μl), and reaction mixtures were incubated for 20 min at room temperature. Then they were loaded on a 4% non-denaturing polyacrylamide gel that contained 1× TGE (25 mM Tris–HCl (pH 8.5), 190 mM glycine, 0.5 mM EDTA) and 100 μM $ZnSO_4$, and fractionated by electrophoresis at 120 V for 100 min at room temperature in a running buffer that consisted on 1× TGE plus 100 μM $ZnSO_4$. The gel was dried and autoradiographed on Scientific Imaging Film (Kodak).

Results and discussion

We subjected DNA that had been isolated from the leukocytes of 119 Japanese subjects to SSCP analysis in an attempt to identify polymorphisms in the upstream region of the gene for MT-IIA (from position −202 to position +20; Fig. 1a). We classified the resulting electrophoretic patterns into three types (Fig. 1b). Direct sequencing of several products of PCR of each type revealed the presence of an A → G single-nucleotide polymorphism at the fifth nucleotide (−5) from the site of initiation of transcription (+1; Fig. 1C). This single-nucleotide polymorphism was located in the core promoter between the TATA box and the site of initiation of transcription (Fig. 2). Since the A → G substitution abolished the *Bsg*I restriction site, we were able to easily determine the frequency of the A/A genotype. We examined the corresponding region of nucleotide sequence in all samples from individuals with genotypes other than A/A to identify the nucleotide at position −5. We found that the A/A, A/G, and G/G

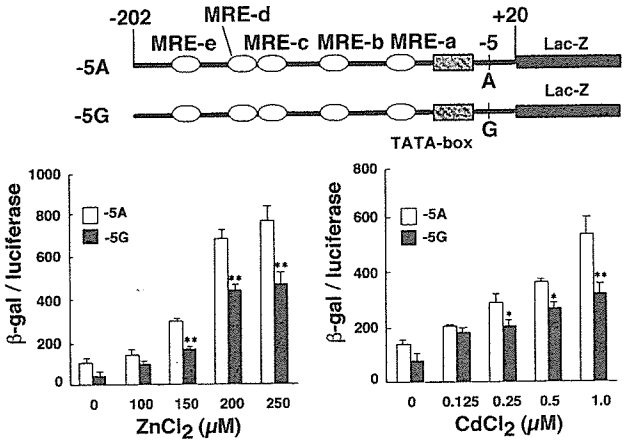

Fig. 2 Effects of the single-nucleotide A → G polymorphism on the promoter activity of the gene for MT-IIA. HEK293 cells were transfected with a plasmid with the A/A type of promoter (−5A) or the G/G type of promoter (−5G). After a 12-h incubation, cells were treated with $ZnCl_2$ or $CdCl_2$ and incubated for a further 24 h. The activities of β-galactosidase (an index of the activity of the promoter derived from the gene for MT-IIA) and of luciferase (an index of the efficiency of transcription) were measured. Results are mean ± SD ($n = 5$). A schematic representation of the relevant regions of the reporter plasmids is shown above the histograms. *, **Significantly different from the −5A type (*$P < 0.001$, **$P < 0.0001$, Student's t test)

genotypes accounted for 98 (82%), 20 (17%), and 1 (0.9%), respectively, of the 119 samples (Fig. 1d).

To examine the effects of the single-nucleotide polymorphism in the core promoter on the induction of transcription of the gene for MT-IIA by Zn and by Cd, we performed reporter-gene assays using HEK293 cells. We found that, although the transcription from both the −5A type and the −5G type promoter increased in a dose-dependent manner upon treatment of cells with Zn and with Cd, transcription from the −5G type of promoter was less efficient than that from the −5A type at all concentrations of metal ions tested (Fig. 2). The almost same results are also observed in HepG2 cells (data not shown). These findings suggested that the A → G single-nucleotide polymorphism in the core region of the promoter reduced the efficiency of the induction of transcription of the gene for MT-IIA by heavy metals. The extent of inhibition of the induction of the transcription of the gene for MT-IIA due to the single-nucleotide polymorphism was not very large. However, since people ingest Cd on their food and very little of the Cd that accumulates in their bodies is excreted, the ratio of the concentration of MT to the concentration of tissue Cd (MT/Cd) might become significantly lower in individuals with the G/G variant, who have a limited ability to induce the synthesis of MT, and somewhat lower in those with the A/G variant than it is in those with the A/A type during a human lifetime of more than 70 years. In short, the possibility cannot be excluded that the A → G single-nucleotide polymorphic variant renders an individual more prone to Cd toxicity than does the A/A type.

The main transcription factor involved in the regulation of expression of the gene for MT-IIA is metal transcription factor-1 (MTF-1) (Kita et al. 2001; Otsuka et al. 1994; Westin and Schaffner 1988). This factor is activated by Zn and by Cd and it binds to the consensus sequence (TGCRCNC) of the metal-responsive element (MRE) within the promoter of the gene for MT-IIA gene, thereby promoting transcription of this gene (Carter et al. 1984; Karin et al. 1984; Stuart et al. 1985). There are six MREs (MRE-a through MRA-f) upstream of the TATA box of the promoter of the gene for MT-IIA, and MTF-1 binds most strongly to MRA-a (Koizumi et al. 1999). A sequence identical to that of MRE-a has been found in the region between the site of initiation of transcription and the TATA box (Stuart et al. 1985). The single-nucleotide polymorphism that we found in the present study is an A → G substitution located in the center of the consensus sequence TGCACTC (from position −2 to −8; Fig. 3a). Therefore, we examined the binding of MTF-1 to this MRE-like sequence in the core promoter region. We radio labeled an oligonucleotide that corresponded to the MRE-a-containing region (from position −61 to −36) and used it as a probe (^{32}P-MRE-a oligo). We mixed the probe with a nuclear extract from Zn-treated cells and subjected the mixture to an electrophoretic mobility shift assay (EMSA). Our results confirmed previous reports that Zn dose-dependently increased the binding of MTF-1 to the ^{32}P-MRE-a probe (Fig. 3b, lanes 1–3). Treatment of the nuclear extract with a 250-fold molar excess of unlabeled MRE-a probe prior to be addition of the labeled probe almost completely abolished this binding (Fig. 3, lane 4). However, formation of the MTF-1/MRE-a complex was barely inhibited by prior treatment of the nuclear extract with an unlabeled oligonucleotide (−5A oligo or −5G oligo) that corresponded to the region (from −15 to +11) that included the MRE-like sequence with a single-nucleotide polymorphism (Fig. 3b, lanes 5–8). These findings suggested that MTF-1 might not bind to the MRE-like sequence in the core promoter region and is, thus, uninvolved in the difference in transcriptional activity that was due to the single-nucleotide substitution. However, since a number of basic transcription factors bind to the polymorphic core promoter region, we postulated that the single-nucleotide substitution might inhibit such binding. Therefore, we allowed the ^{32}P-labeled −5A and

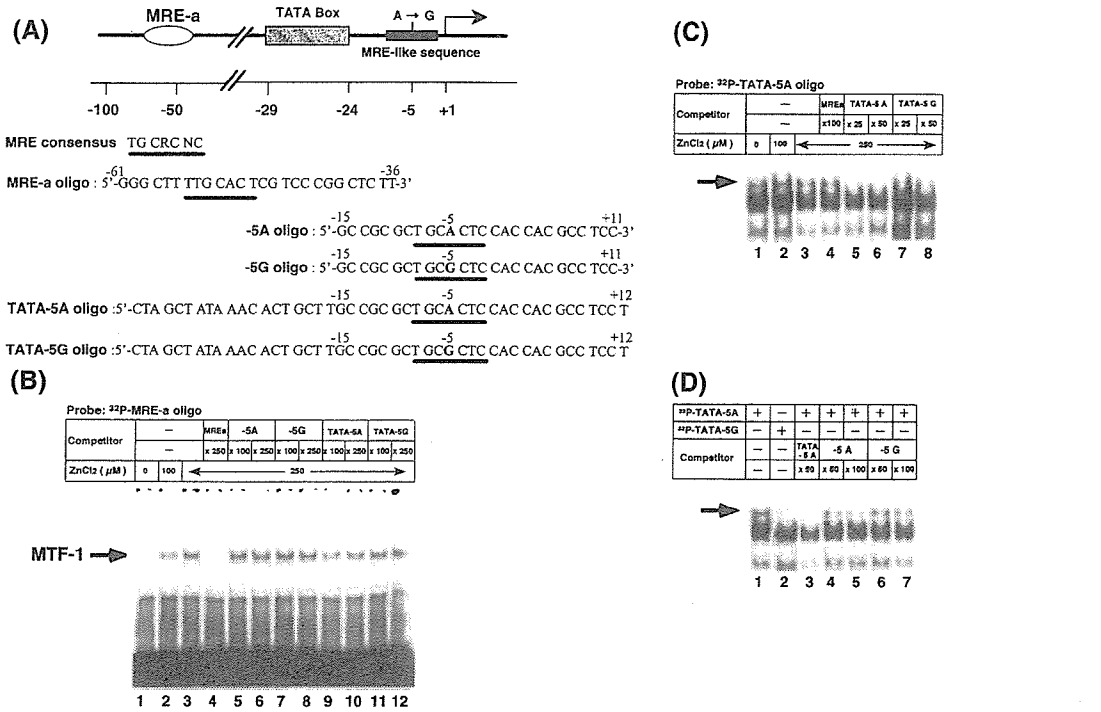

Fig. 3 Effects of the single-nucleotide A → G polymorphism on the binding of nuclear proteins to the core promoter region of the gene for MT-IIA. **a** Oligonucleotide probes for the electrophoretic mobility shift assay. The MRE core sequence is *underlined*. An *arrow* indicates the site of initiation of transcription. The numbers above sequences refer to positions relative to this site. **b** The ^{32}P-labeled MRE-a probe was incubated with a nuclear extract and the mixture was fractionated on non-denaturing polyacrylamide gel. For competition assays, unlabeled oligonucleotide probes were incubated with the nuclear extract prior to the addition of ^{32}P-labeled MRE-a. The *arrow* indicates the MTF-1/MRE complex. **c**, **d** Results of electrophoretic mobility shift assays with ^{32}P-labeled TATA–5A as probe. *Arrows* indicate complexes of transcriptional factor(s) and the TATA–5A probe

−5G oligonucleotides to react with the nuclear extract. However, no specific factors bound to either probe (data not shown).

A number of basic transcription factors are known to bind to the TATA box-containing core region of the promoter (Robert et al. 1996). Since the −5A and −5G oligonucleotides that we tested in the EMSA did not contain a TATA box, we extended them to include the TATA box and prepared TATA −5A and TATA −5G oligonucleotides (from position −15 to +34; Fig. 3a) and tested them in the EMSA. We found that proteins that bound to the ^{32}P-labeled TATA −5A oligonucleotide were present in the nuclear extract of HEK293 cells (Fig. 3c, lanes 1–3). In addition, a competition assay using unlabeled TATA −5A or TATA −5G oligonucleotides showed that the addition of a 25-fold molar excess of TATA −5A oligonucleotide inhibited binding, whereas a 50-fold molar excess of TATA −5G had a negligible inhibitory effect (Fig. 3C, lanes 5–8). The addition of 100-fold molar excess of MRE-a oligonucleotide, which bound to MTF-1 (as shown in Fig. 3b), did not inhibit the binding (Fig. 3c, lane 4). We also confirmed that neither the TATA −5A oligonucleotide nor the TATA −5G oligonucleotide bound MTF-1 (Fig. 3b, lanes 9–12).

The addition of Zn did not affect the binding of the ^{32}P-labeled TATA −5A oligonucleotide to nuclear proteins. This result suggests that the single-nucleotide substitution influenced the efficiency of basal transcription. Therefore, we labeled the TATA −5G oligonucleotide with ^{32}P and allowed it to react with a nuclear extract from Zn-untreated HEK293 cells. The extent of binding of the TATA −5G oligonucleotide to nuclear proteins (Fig. 3d, lane 2) was significantly lower than that of the TATA −5A oligonucleotide (Fig. 3d, lane 1). Furthermore, a competition assay in which a 100-fold molar excess of unlabeled −5A oligonucleotide or −5G oligonucleotide competed with the binding of the ^{32}P-labeled TATA -5A oligonucleotide for binding to nuclear proteins demonstrated an absence of competition (Fig. 3D, lanes 4–7). These results suggest that the single-nucleotide polymorphism in the promoter of the gene for MT-IIA might reduce the binding, to the core promoter region, of a nuclear protein that is involved in basic transcription, with a marked resultant

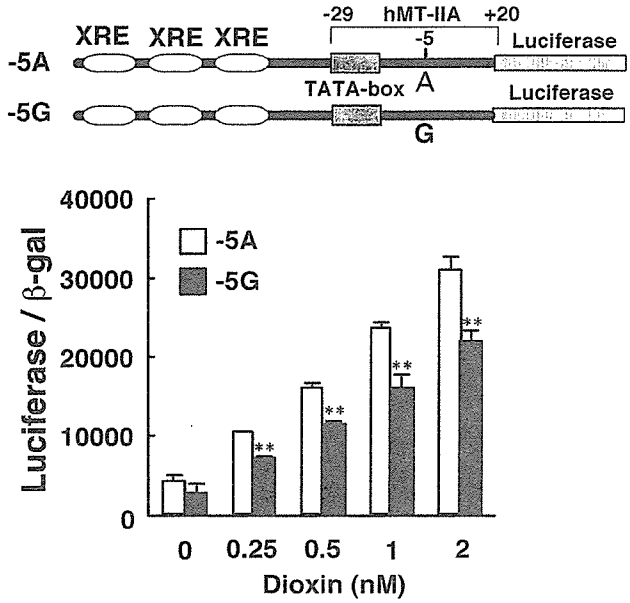

Fig. 4 Effects of the single-nucleotide A → G polymorphism on the promoter activity of dioxin-responsive elements. The region upstream of the TATA box of the gene for MT-IIA was replaced by a sequence that contained three dioxin-responsive elements (XREs) and performed a reporter-gene assay. After a 12-h incubation, HEK293 cells were treated with dioxin and incubated for a further 24 h. The activities of luciferase (an index of the activity of the promoter) and of β-galactosidase (an index of the efficiency of transcription) were measured. Results are mean ± SD ($n = 5$). A schematic representation of the relevant regions of the reporter plasmids is shown above the histograms. **Significantly different from the −5A type ($P < 0.0001$, Student's t test)

reduction in the efficiency of induction of transcription of the gene by heavy metals via the activation of MTF-1. If our hypothesis is correct, the A → G polymorphism should reduce the efficiency of induction of transcription even if the promoter region upstream of the TATA box is replaced by a region derived from the promoter of other genes. Therefore, we replaced the region upstream of the TATA box by a sequence that contained three dioxin-responsive elements (XREs) and performed a reporter-gene assay. We observed, as predicted, a marked reduction in the efficiency of induction by dioxin (Fig. 4). Point mutations in the core promoter region are known to inhibit the binding of nuclear proteins to this region (Chalkley and Verrijzer 1999; Yean and Gralla 1997). Yanai et al. (1997) reported that a polymorphism in the core promoter region of the gene for angiotensinogen reduced the binding of the nuclear factor AGCEI to this region. Similarly, in the case of the gene for a MT, the single-nucleotide polymorphism reduced basic transcriptional activity, thereby reducing the extent of induction of the synthesis of MT by heavy metals. The nuclear protein, whose binding to the core promoter region of MT-IIA gene is influenced by the A → G substitution, should be identified by future studies.

The MTs are characterized by free cysteine residues that account for approximately one-third of the total number of amino acid residues but do not form S–S bonds (Kägi 1991; Webb 1979). Exploiting its abundant intramolecular SH groups, MT reduces the toxicity of alkylating agents (Cagen and Klaassen 1979), γ-irradiation (Satoh et al. 1989), and pesticides (Satoh et al. 1992), as well as that of heavy metals such as Cd, and acts as a scavenger of free radicals to protect cells and animals against various types of oxidative stresses (Sato and Bremner 1993). In addition, MT is known to reduce the side effects of many anticancer drugs (Okazaki et al. 1998; Satoh et al. 1993b), such as cisplatin (Naganuma et al. 1987) and adriamycin (Naganuma et al. 1988), and MT-deficient mice have been shown to be particularly susceptible to toxicity of anticancer drugs (Zhang et al. 1998). Furthermore, MT has been shown to inhibit spontaneous or chemical carcinogenesis in mice (Satoh et al. 1993a). Thus, the A → G substitution in the core promoter region of the gene for MT-IIA might reduce the protective role of MT-IIA against such various stresses and carcinogenesis.

The single-nucleotide polymorphism found in this study raises a serious issue with respect to the effect of Cd on human health. In the population of middle-aged and elderly Japanese, the mean concentration of Cd in the kidney exceeds one-third of the JFCFA's estimated critical concentration (Yoshida et al. 1998). Olsson et al. (2002) reported that the level of ingested Cd in farmers in Sweden is not as high. However, a higher urinary concentration of Cd was associated with lower renal function (Olsson et al. 2002). It has also been reported that Cd, at the levels at which people routinely ingest it, has a potent estrogen-like activity and acts as an endocrine disruptor (Johnson et al. 2003). These reports suggest that the concentration of Cd that accumulates in the bodies of ordinary people is close to the toxic level. The A → G genetic polymorphism that influences the efficiency of synthesis of MT, which plays such an important role in reducing Cd toxicity, might be one of the major factors that determine individual differences in human sensitivity to Cd.

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

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Research Article

Endocytic Ark/Prk Kinases Play a Critical Role in Adriamycin Resistance in Both Yeast and Mammalian Cells

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Abstract

To elucidate the mechanism of acquired resistance to Adriamycin, we searched for genes that, when overexpressed, render *Saccharomyces cerevisiae* resistant to Adriamycin. We identified *AKL1*, a gene of which the function is unknown but is considered, nonetheless, to be a member of the Ark/Prk kinase family, which is involved in the regulation of endocytosis, on the basis of its deduced amino acid sequence. Among tested members of the Ark/Prk kinase family (Ark1, Prk1, and Akl1), overexpressed Prk1 also conferred Adriamycin resistance on yeast cells. Prk1 is known to dissociate the Sla1/Pan1/End3 complex, which is involved in endocytosis, by phosphorylating Sla1 and Pan1 in the complex. We showed that Akl1 promotes phosphorylation of Pan1 in this complex and reduces the endocytic ability of the cell, as does Prk1. Sla1- and End3-defective yeast cells were also resistant to Adriamycin and overexpression of Akl1 in these defective cells did not increase the degree of Adriamycin resistance, suggesting that Akl1 might reduce Adriamycin toxicity by reducing the endocytic ability of cells via a mechanism that involves the Sla1/Pan1/End3 complex and the phosphorylation of Pan1. We also found that HEK293 cells that overexpressed AAK1, a member of the human Ark/Prk family, were Adriamycin resistant. Our findings suggest that endocytosis might be involved in the mechanism of Adriamycin toxicity in yeast and human cells. (Cancer Res 2006; 66(24): 11932-7)

Introduction

Adriamycin is an anticancer drug that is widely used in a clinical setting (1). The mechanism of action of Adriamycin, via inhibition of nucleic acid synthesis by intercalation into DNA and promotion of the cleavage of DNA by inhibition of DNA topoisomerase II, is well known, as is the mechanism of cytotoxicity, which involves the production of free radicals, but many other effects of the drug have also been reported, with many issues remaining to be clarified (2, 3). In cancer chemotherapy with Adriamycin, both the natural and the acquired resistance of cancer cells pose serious problems, as do the adverse effects of the drug (4). The acquisition of Adriamycin resistance involves promotion of the excretion of the drug from cells by overexpressed ATP-binding cassette transporters, such as P-glycoprotein and multidrug-resistance protein (5–7), and, in addition to the transporters involved in drug excretion, many other factors seem to be related to the mechanism of Adriamycin resistance. However, the properties of some Adriamycin-resistant cells cannot be explained by these phenomena, suggesting the presence of as yet unknown mechanisms of acquired resistance (8, 9). To shed further light on mechanisms of Adriamycin toxicity and the acquisition of Adriamycin resistance, we have identified genes involved in Adriamycin resistance in *Saccharomyces cerevisiae*. *S. cerevisiae* is widely used as a model eukaryote and its entire genome has been sequenced. Because many products of yeast genes have functions similar to those of human genes, information obtained from yeast cells might help to clarify the mechanism of acquisition by human cells of resistance to Adriamycin. We previously searched for genes that conferred Adriamycin resistance on yeast cells when such genes were overexpressed, using a yeast DNA genomic library, and we identified the *SSL2* (10, 11) and *BSD2* (12) genes. In the present study, we identified *AKL1* as a gene of which the overexpression rendered yeast cells resistant to Adriamycin. Akl1 is considered to be a member of the Ark/Prk kinase family and to be involved in the regulation of endocytosis on the basis of certain characteristics of its deduced amino acid sequence (13), but, to our knowledge, its function has not been fully analyzed and its relationship to drug resistance has not been investigated.

This study revealed the involvement of Akl1 in the regulation of endocytosis via phosphorylation of Pan1 in the Sla1/Pan1/End3 complex, as is Prk1 (14, 15). In addition, our results suggest that overexpressed Akl1 inhibits the internalization step of endocytosis, leading to Adriamycin resistance in yeast cells. Because human cells that overexpressed AAK1 (16), which is a member of the human Ark/Prk kinase family that is known to be involved in the regulation of endocytosis, were also Adriamycin resistant, it is possible that the Ark/Prk kinase family might be involved in Adriamycin toxicity via the regulation of endocytosis not only in yeast cells but also in human cells.

Materials and Methods

Yeast strains and media. The wild-type yeast strains used in this study were W303B (*MATα his3 can1-100 ade2 leu2 trp1 ura3*; ref. 17) and BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*). The deletion strains (*akl1Δ, ark1Δ, prk1Δ, end3Δ, sla1Δ, sla2Δ, vrp1Δ, rvs161Δ, vps23Δ*, and *vps27Δ*), derived from the parent BY4742 strain, were obtained from Euroscarf (Frankfurt, Germany). Yeast cells were grown in synthetic dextrose medium with or without leucine. For the characterization of cells that harbored the expression vector pYES2 (Invitrogen, Carlsbad, CA) or genes expressed under the control of the *GAL1* promoter, cells were grown in synthetic galactose medium that contained 2% galactose and 4% raffinose as the carbon source instead of dextrose.

Quantification of the toxicity of Adriamycin in yeast cells. Yeast cells were cultured (1×10^4/200 μL) in 96-well plates in synthetic dextrose medium that contained Adriamycin at various concentrations. After incubation for 48 hours, absorbance at 620 nm was determined spectrophotometrically. For the colony-formation assay, a suspension of yeast cells was incubated for 3 hours in liquid medium that contained

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doi:10.1158/0008-5472.CAN-06-3220

Adriamycin. After washing with synthetic dextrose liquid medium, the cells were resuspended in synthetic dextrose liquid medium and spotted onto a plate of agar-solidified medium.

Construction of a point mutant of *AKL1* (*AKL1* D181Y). Construction of a point mutant of *AKL1* (*AKL1*D181Y) was done as described by Hwang et al. (18) with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). For mutagenesis of *AKL1*, PCR was done using plasmid pRS315-*AKL1* as the template and the following oligonucleotides as the mutagenic primers: 5′-CCTTGATCCATCGCTATATCAAGATTG-3′ and 5′-CAATCTTGATATAGCGATGGATCAAGG-3′. The mutation in *AKL1* was verified with an automated DNA sequencer (LI-COR, Lincoln, NE).

Construction of an expression vector for various genes. The *ARK1* and *PRK1* genes were cloned separately by PCR with chromosomal DNA of *S. cerevisiae* as template. The following oligonucleotides were used as primers: 5′-GCGGCTCGCCAACTTGGAAG-3′ and 5′-CAGTTAAAGCCAC-TAGTTCCT-3′ for *ARK1*, and 5′-AGTGGTCTCTAGCGGGATCG-3′ and 5′-GCTGTTCAGAGAACCACAATG-3′ for *PRK1*. Each product of PCR was inserted into the pGEM-T easy vector (Promega, Madison, WI). Each insert was digested with the restriction endonuclease *Not*I and fragments were ligated into the single-copy plasmid pRS315. For the expression of each gene, we used the respective homologous promoter.

Construction of a *PAN1*-HA-expressing plasmid. For construction of the *PAN1*-hemagglutinin (HA) vector, the *PAN1* gene was amplified by PCR with chromosomal DNA of *S. cerevisiae* as template and primers 5′-GCTAATCTGTACAACTGAATATG-3′ and 5′-TCAAGCGTAATCTGGAA-CATCGTATGGGTATGGAAGGGGTGGGGGTGGAGGAATA-3′. The amplified DNA was inserted into the pGEM-T easy vector to produce plasmid pGEM-*PAN1*-HA. The insert was digested with the restriction endonuclease *Not*I and fragments were ligated into the pYES2 expression vector.

Phosphorylation of Pan1-HA. Yeast cells (3×10^7) expressing HA-tagged Pan1 (Pan1-HA) were cultured in 10 mL of synthetic galactose (−Ura, −Leu) medium for 4 hours and harvested. After washing with ice-cold water, cells were lysed by grinding with glass beads at 4°C. An aliquot of cell lysates (100 μg of protein) was immunoprecipitated with anti-HA agarose beads (Sigma, St. Louis, MO). Immunoprecipitates were fractionated by SDS-PAGE (7.5% polyacrylamide) and transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA). Immunoblotting was carried out using rabbit antibodies against phosphothreonine (Zymed, South San Francisco, CA) or rat antibodies against HA (Roche, Basel, Switzerland) and peroxidase-conjugated second antibodies (Dako A/S, Glostrup, Denmark). Immunoreactive proteins were detected with the enhanced chemiluminescence system from Amersham Pharmacia (Uppsala, Sweden).

Endocytic uptake of lucifer yellow. Fluid-phase endocytosis was assayed using lucifer yellow carbohydrazide (Molecular Probes, Eugene, OR). Yeast cells were incubated in synthetic dextrose medium in the presence of 4 mg/mL lucifer yellow carbohydrazide. After a 2-h incubation, cells were collected and washed thrice with PBS. Cells were resuspended in PBS and observed under a light microscope.

Construction of the AAK1-expression vector. The cDNA for human full-length AAK1 (clone KIAA1048) in pBluescript was provided by the Kazusa Research Institute (Kisarazu, Chiba, Japan). For construction of the AAK1-expression vector, the full-length AAK1 cDNA insert was amplified by PCR with primers 5′-CTCGAGACCATGTACCCATACGACGTGCCAGAC-TACGCTATGAAGAAGTTTTTCGACTCCCGG-3′ and 5′-GAATTCTTAAA-TAGCCTTGGCTTCTGGGGTGG-3′, and then the product of PCR was subcloned into the pcDNA3.1/Hygro expression vector (Invitrogen).

Measurement of the viability of AAK1-expressing HEK293 cells. Human embryonic kidney cells (HEK293 cells) were cultured at 37°C in DMEM supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine. Transfection of HEK293 cells with the plasmid that expressed AAK1 was done as described elsewhere (19) with TransIT-LT1 (Mirus, Madison, WI) according to the manufacturer's protocol. After incubation for 8 hours, cells (5×10^3) were plated in 96-well plates and cultured in 90-μL aliquots of medium. One day after plating, Adriamycin was added and cells were cultured for a further 3 days. Cell viability was assessed by the Alamar Blue assay (Biosource, Camarillo, CA). Each experiment was repeated at least thrice and representative results are presented.

Immunoblotting of HA-AAK1. Whole-cell extracts were prepared from individual transfectants by freezing and thawing. Immunoblotting with HA-specific antibody was done as described above.

Results

In a previous study, we searched for genes related to Adriamycin resistance using *S. cerevisiae* transfected with a chromosomal DNA library, and we obtained plasmids AR13 and AR17 that included genes that conferred resistance to Adriamycin on yeast cells when they were overexpressed (12). In the present study, we analyzed the DNA sequences of the genomic DNA fragments that had been

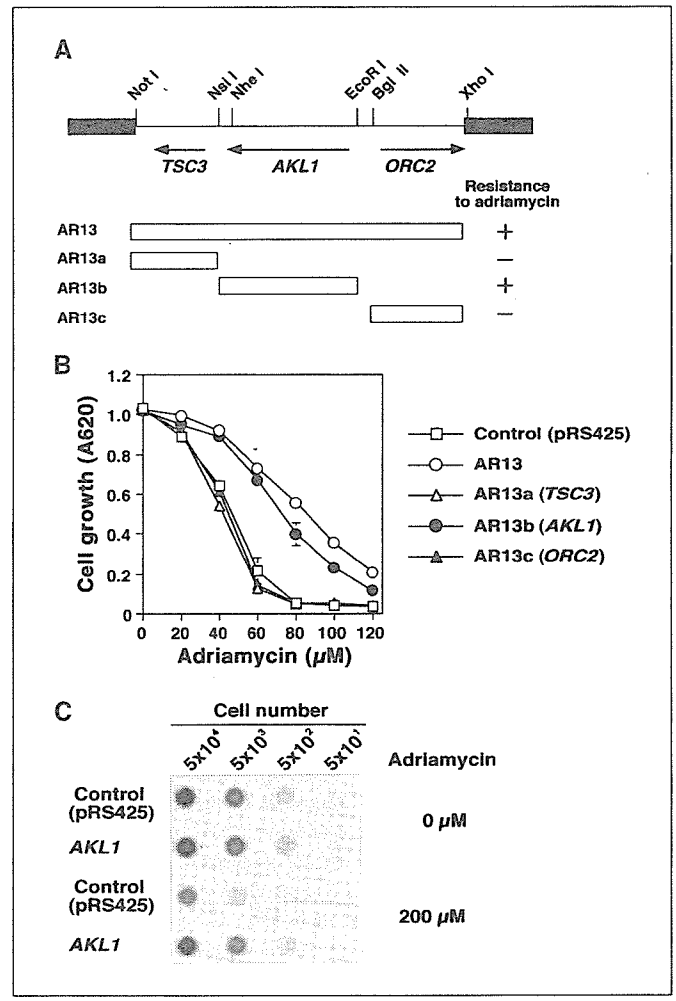

Figure 1. Identification of *AKL1* as an Adriamycin resistance gene. *A*, restriction map of the genomic DNA insert in plasmid AR13. *Thick black line*, vector pRS425; *thin line*, genomic DNA insert. Vertical lines above the genomic DNA insert indicate the restriction sites used to generate different subclones. The ability of three subclones (AR13a, AR13b, and AR13c) to confer resistance to Adriamycin is indicated (+, conferred resistance; −, did not confer resistance). ORFs are indicated by arrows that point in the direction of transcription, with the name of each ORF given below the respective arrow. *B*, sensitivity to Adriamycin of yeast cells that harbored plasmids with the indicated inserts. Yeast strains (W303B) carrying plasmids AR13a, AR13b, AR13c, or pRS425 were grown in synthetic dextrose (−Leu) medium that contained Adriamycin. After incubation for 48 hours at 30°C, absorbance was measured spectrophotometrically at 620 nm. *Points*, mean of results from three cultures; *bars*, SD. The absence of a bar indicates that the SD falls within the symbol. *C*, effects of overexpression of Akl1 on the sensitivity of yeast cells to Adriamycin. Yeast cells were incubated in the presence of Adriamycin for 3 hours and washed with synthetic dextrose (−Leu) medium. Then yeast cells were grown on a plate of agar-solidified synthetic dextrose (−Leu) for 24 hours.

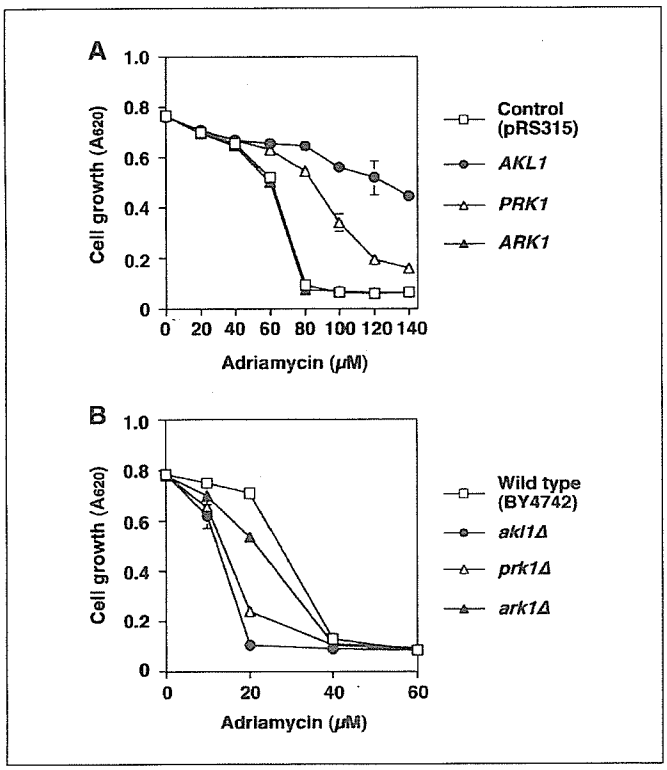

Figure 2. Involvement of members of the Ark/Prk kinase family in the sensitivity of yeast cells to Adriamycin. *A*, yeast cells (BY4742) harboring pRS315, pRS315-*AKL1*, pRS315-*ARK1*, or pRS315-*PRK1* were grown in synthetic dextrose (−Leu) medium that contained Adriamycin. *B*, yeast (BY4742) cells lacking Ark1, Prk1, or Akl1 (*ark1Δ*, *prk1Δ*, and *akl1Δ*, respectively) were grown in synthetic dextrose medium that contained Adriamycin. For further details, see Fig. 1*B* legend.

inserted into these plasmids, referring to the *Saccharomyces* Genome Database. A region of ~5 kbp derived from yeast chromosome no. 2 was present in both AR13 and AR17, and the region contained three open reading frames (ORF), designated *TSC3*, *AKL1*, and *ORC2* (Fig. 1*A*). To identify the gene involved in Adriamycin resistance, we cleaved AR13 with restriction enzymes and subcloned the resultant fragments (AR13a, AR13b, and AR13c) in pRS425. We introduced the three new plasmids into yeast W303B cells and examined the Adriamycin sensitivity. Only the yeast cells transfected with AR13b, which contained the *AKL1* ORF, exhibited Adriamycin resistance, showing that overexpression of *AKL1* conferred Adriamycin resistance on the yeast cells (Fig. 1*B* and *C*). Overexpression of the *AKL1* gene from a single-copy plasmid, pRS315, also led to Adriamycin resistance, suggesting that a severalfold increase in the intracellular level of Akl1 is sufficient to allow wild-type yeast cells to become Adriamycin resistant (Fig. 2).

Akl1 is classified as a member of Ark/Prk kinase family, which consists of serine/threonine kinases involved in the regulation of endocytosis, on the basis of its deduced amino acid sequence (13). However, to our knowledge, its function has not yet been analyzed. To investigate the involvement of the activity of Akl1 kinase in the Akl1 overexpression–induced reduction of Adriamycin toxicity, we prepared plasmids that expressed mutants of Akl1 with point mutations in the Akl1 kinase domain. All members of the Ark/Prk kinase family have a kinase domain in the NH_2-terminal half of the amino acid sequence, and the abolition of the kinase activity of Prk1 by substituting tyrosine (Y) for aspartic acid (D) at position 158 in this domain has been reported (14). Because the corresponding amino acid in Akl1 is aspartic acid at position 181 (Asp^{181}), we generated a mutant (Akl1D181Y) with tyrosine at this position. When Akl1D181Y was overexpressed, the sensitivity to Adriamycin of the yeast cells was similar to that of the control strain (Fig. 3). As described below, yeast cells had Akl1 kinase activity, and Asp^{181} was essential for this kinase activity (Fig. 4*A*). In view of these findings, we postulated that Akl1 kinase activity might be necessary for the Akl1 overexpression–induced acquisition of resistance to Adriamycin.

Akl1 is considered to function similarly to Ark1 and Prk1 because the kinase domain of Akl1 is strongly homologous to those of Ark1 and Prk1, two members of the Ark/Prk kinase family (13). Therefore, we investigated the sensitivity to Adriamycin of yeast cells that overexpressed Ark1 and Prk1, respectively. The sensitivity of Ark1-overexpressing yeast cells was similar to that of the control yeast cells, but Prk1-overexpressing yeast cells were apparently resistant to Adriamycin, although the level of resistance was lower than that of Akl1-overexpressing cells (Fig. 2*A*). When we prepared yeast cells with defective members of the Ark/Prk kinase family and investigated their Adriamycin sensitivity, we found that Ark1-defective yeast cells were slightly sensitive and Akl1- and Prk1-defective yeast cells were markedly sensitive to Adriamycin (Fig. 2*B*). These observations suggested that Akl1 and Prk1 might be intimately involved in the mechanism of Adriamycin toxicity, whereas the involvement of Ark1 might be limited.

Prk1 seems to promote the dissociation of the Sla1/Pan1/End3 complex via phosphorylation of both Sla1 and Pan1 (14, 15, 20). The Sla1/Pan1/End3 complex loses its function on dissociation, and Sla1- and End3-defective cells have low endocytic capacity (21, 22). When we investigated the sensitivity to Adriamycin of *SLA1*- and *END3*-defective yeast cells, we found that both defective lines of yeast cells were markedly resistant to Adriamycin (Fig. 5). Moreover, overexpression of Akl1 in these *SLA1*- and *END3*-defective cells did not increase the level of resistance (Fig. 5), suggesting that the presence of Sla1 and End3 is essential for the Akl1 overexpression–induced reduction in Adriamycin toxicity. In this series of experiments, we did not examine the effects of deletion of the *PAN1* gene because Pan1 is a protein that is essential for the proliferation of yeast cells.

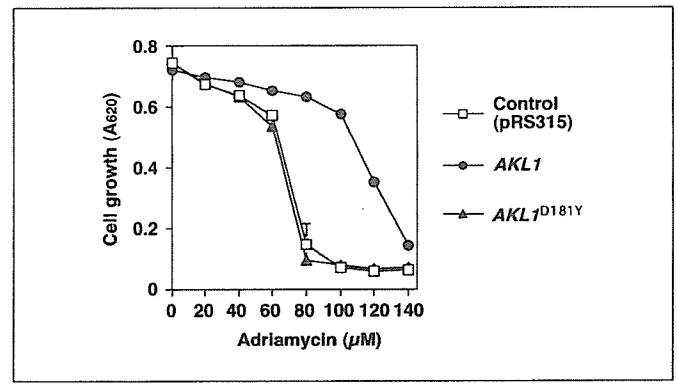

Figure 3. Akl1 kinase activity is required for the acquisition of Adriamycin resistance. Yeast cells (BY4742) harboring pRS315, pRS315-*AKL1*, or pRS315-*AKL1*D181Y were grown in synthetic dextrose (−Leu) medium that contained Adriamycin. For further details, see Fig. 1*B* legend.