Toxicomics Report

CDC23 knockdown reinforces methylmercury sensitivity in HEK293 cells

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ABSTRACT — The ubiquitin-proteasome system is believed to play an important role in the determination of cell sensitivity to methylmercury. The ubiquitin ligase enzyme is involved in the recognition of substrate proteins that are degraded by the ubiquitin-proteasome system. In this study, the ubiquitin ligase species affecting methylmercury sensitivity was investigated by the gene interference method. We found that the inhibition of expression of the gene for Cell division cycle 23 (CDC23), a constitutional component of the ubiquitin ligase anaphase promoting complex/cyclosome, sensitized HEK 293 cells to methylmercury.

Key words: Methylmercury, CDC23, Ubiquitin ligase

INTRODUCTION

Methylmercury is an environmental pollutant that affects the central nervous system (Castoldi *et al.*, 2008; Grandjean and Herz, 2011). However, the mechanism of development of methylmercury toxicity or development of a defense mechanism against this toxicity is remain poorly understood. It has been previously determined that the proteins responsible for the decrease or increase in methylmercury toxicity are present within the cells; the intracellular concentration of some of these proteins is believed to be regulated by ubiquitin-proteasome system-mediated degradation (Hwang, 2011; Hwang *et al.*, 2002). Therefore, the ubiquitin-proteasome system is believed to play an important role in the determination of cell sensitivity to methylmercury. The ubiquitin ligase is involved in the recognition of substrate proteins degraded by the ubiquitin-proteasome system (Hershko and Ciechanover, 1998). Cells have a variety of ubiquitin ligase species. The determination of the ubiquitin ligase species affecting cell sensitivity to methylmercury could facilitate the identification of proteins involved in the methylmercury toxicity. In this study, we have attempted to determine the ubiquitin ligase species affecting the methylmercury sensitivity of the cells by suppression of gene expression.

RESULTS AND DISCUSSION

Over 600 types of ubiquitin ligase species are believed to exist in mammalian cells (Harper and Tan, 2012). Among these, approximately 100 species of enzymatically active ubiquitin ligases were selected for this study. Human embryonic kidney 293 (HEK293) cells were transfected with double-stranded siRNA that specifically recognize these ubiquitin ligase genes (2 different siRNAs with different sequences for each gene were used). The cells were treated with 4 μM methylmercuric chloride (a concentration believed to be sufficient to inhibit the growth of normal cells by approximately 50%) and incubated for 48 hr. Cell division cycle 23 (CDC23) was selected as a candidate of ubiquitin ligase effecting methylmercury hypersensitivity in HEK293 cells, by suppression of gene expression. The cells were transfected with two different CDC23 mRNA-targeting double-stranded siRNAs (siRNA #1 or siRNA #2) and treated with different concentrations of methylmercury. As a result, both sets

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of the cells showed higher sensitivity to methylmercury compared to the control cells (transfected with control siRNA) (Fig. 1A). We observed over 60% (approximately) decrease in the expression levels of CDC23 mRNA in both siRNA transfected cells compared to the control cells (Fig. 1B). These results indicated that CDC23 was responsible for the deterioration of methylmercury toxicity. However, the suppression of CDC23 gene expression did not significantly affect the cell sensitivity to metal compounds other than methylmercury and hydrogen peroxide (Fig. 2). These results indicated that CDC23 might selectively reduce methylmercury-mediated cytotoxicity.

CDC23 is a component of anaphase promoting complex/cyclosome (APC/C), a ubiquitin ligase species (Zhang *et al.*, 2014). APC/C is reported to regulate the development of mitosis by promoting the degradation of cyclin B1 and securin (Zhang *et al.*, 2014). In this study, we discovered a delay of approximately 20% in cell proliferation following suppression of CDC23 expression compared to the control cells (data not shown). Mitosis in neuronal cells of the brain is reported to be inhibited by methylmercury (Ochi, 2002; Rodier *et al.*, 1984), and it is hypothesized that this inhibitory activity is decreased by APC/C activity. On the other hand, APC/C is reported to play a role in the degradation of induced myeloid leukemia cell differentiation protein (Mcl-1) (Harley *et al.*, 2010) and modulator of apoptosis (MOAP)-1 (Huang *et al.*, 2012), which assist in the induction of apoptosis, or Tribbles homolog 3 (TRB3) (Ohoka *et al.*, 2010) that assists in the induction of cell death mediated by endoplasmic reticulum stress. These findings suggest that APC/C is an important ubiquitin ligase responsible for the regulation of a number of vital functions, in addition to the regulation of cell cycle. Future research focusing on APC/C-recognizing substrate proteins could aid in the identification of proteins involved in the development of methylmercury toxicity. This could aid in the elucidation of at least a part of the mechanism of development of methylmercury toxicity.

MATERIALS AND METHODS

Cell line and culture conditions

HEK293 cells, which are generally easy-to-transfect, were used for the specific selection by siRNA transfection of ubiquitin ligase that causes methylmercury hypersensitivity. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-

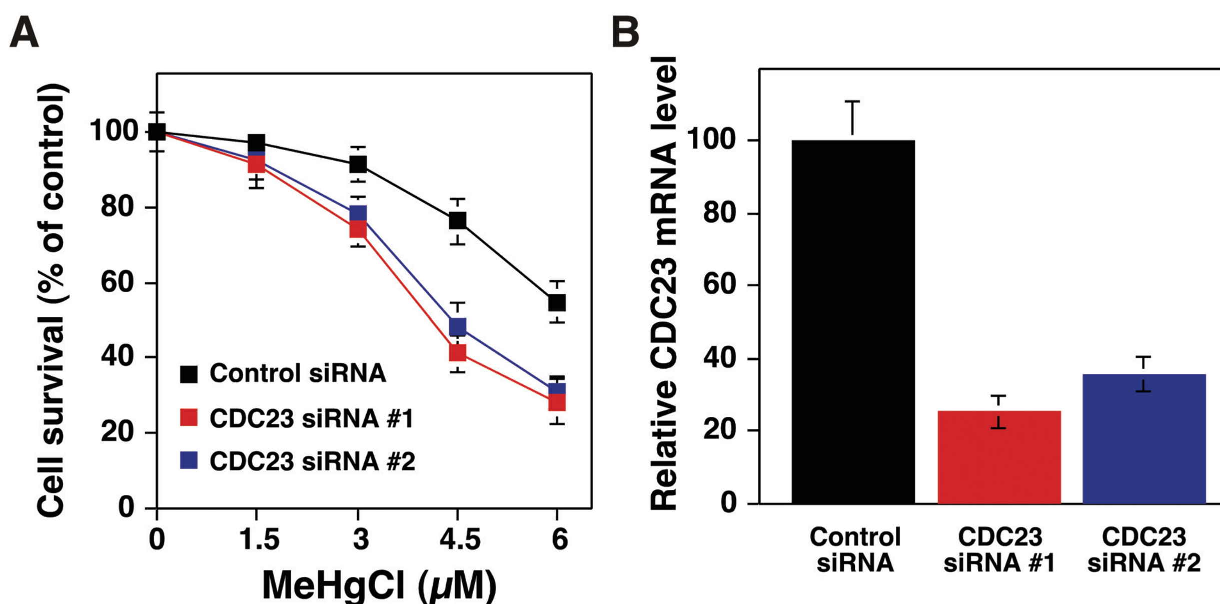

Fig. 1. Effect of CDC23 knockdown on methylmercury sensitivity in HEK293 cells. (A) HEK293 cells (5×10^3 cells/well) transfected with the CDC23 siRNAs were plated into 96-well plates, and cultured in 100 µL aliquots of the medium. The siRNA transfection was carried out for 48 hr. Methylmercuric chloride was then added to the cells, which were cultured for another 48 hr. Cell survival was determined using the Alamar Blue assay. Each point and bar represents the mean value and standard deviation of the results obtained from three cultures. The levels of CDC23 mRNA in HEK293 cells (transfected with double-stranded CDC23 siRNA) were analyzed by quantitative real-time PCR. CDC23 mRNA levels were normalized to those of GAPDH mRNA.

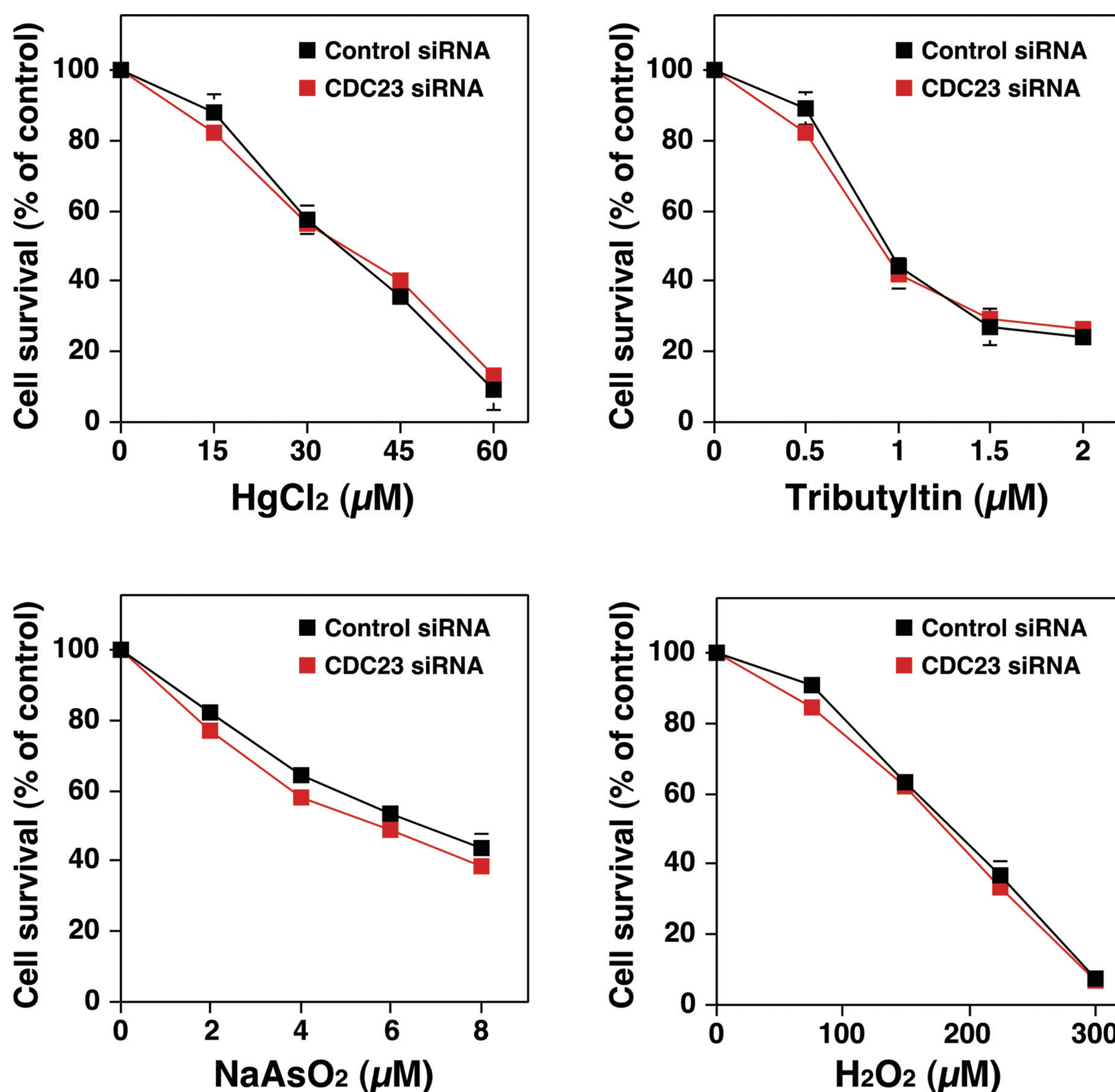

Fig. 2. Effect of CDC23 knockdown on sensitivity to various compounds in HEK293 cells. HEK293 cells (5×10^3 cells/well) transfected with double-stranded CDC23 siRNA #1 were plated into 96-well plates and cultured in 100 μL aliquots of the medium. For further details, see the legend to Fig. 1A.

inactivated fetal bovine serum, 100 IU/mL penicillin, and 100 mg/mL streptomycin , in a humidified 10% CO_2 incubator at 37°C.

Selection of ubiquitin ligase involved in methylmercury toxicity

AllStar® negative control siRNA and double-stranded siRNAs, each of which recognizes approximately 100 ubiquitin ligase, were purchased from Qiagen (Venlo, Netherlands). The siRNAs were transfected into HEK293 cells using the HiPerFect® transfection reagent (Qiagen) according to the manufacturer protocols. Briefly, 2.5 μL of siRNA mixture (final concentration, 10 nM) containing two different double-stranded siRNAs were spotted into each well in a 96-well plate. HiPerFect® transfection reagent (0.75 μL) diluted in 24.25 μL Opti-MEM (Invitrogen, Carlsbad, CA, USA) was added to the wells that were prespotted with the siRNA mixture. The plate

was incubated for 10 min at room temperature, in order to allow for the formation of transfection complexes. The cell suspension (65 µL) containing 5×10^3 cells was then added to each of the wells, and incubated for 48 hr. Ten microliters of 4 µM methylmercuric chloride was added to each of the wells, and the plate incubated for a further 48 hr. This concentration of methylmercuric chloride was believed to be sufficient to inhibit the growth of normal cells by approximately 50%. The cell viability was monitored using the Alamar Blue assay (Invitrogen), performed as per the manufacturer protocols.

Quantitative real-time polymerase chain reaction (PCR)

The siRNA sequences corresponding to the target sequences were as follows: FBXO6 siRNA No. 1, 5′-TACGAGAAACTCAATCAACTA-3′; FBXO6 siRNA No. 2, 5′-ACCCATCTTAATTAAGCCTTA ′. The siRNA transfected-cells (approximately 1×10^6 cells/well in 6-well plates) were incubated for 48 hr, and subsequently washed with phosphate buffer saline (PBS). Total RNA was isolated using ISOGEN II (Nippon Gene, Toyama, Japan), and first strand cDNA synthesis was performed using the PrimeScrip® RT reagent kit (TaKaRa Bio Inc., Otsu, Japan), according to the manufacturer instructions. Quantitative real-time PCR reactions were performed using the following primers for CDC23: forward, 5′-GA GCATGAAATCGGAGTTGAGTTATC-3′, and reverse, 5′-CCATTAGTGTCCAGGCACCAAG-3′; and GAPDH: forward, 5′-GCACCGTCAAGGCTGAGAAC-3′, and reverse, 5′-TGGTGAAGACGCCAGTGGA-3′. The fold decrease in the CDC23 mRNA levels was determined using the standard curves obtained by assay calibration.

Conflict of interest---- The authors declare that there is no conflict of interest.

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