The Identification of a Novel Gene, MAPO2, That Is Involved in the Induction of Apoptosis Triggered by O6-Methylguanine

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Abstract

O6-Methylguanine, one of alkylated DNA bases, is especially mutagenic. Cells containing this lesion are eliminated by induction of apoptosis, associated with the function of mismatch repair (MMR) proteins. A retrovirus-mediated gene-trap mutagenesis was used to isolate new genes related to the induction of apoptosis, triggered by the treatment with an alkylating agent, N-methyl-N-nitrosourea (MNU). This report describes the identification of a novel gene, MAPO2 (O6-methylguanine-induced apoptosis 2), which is originally annotated as C1orf201. The MAPO2 gene is conserved among a wide variety of multicellular organisms and encodes a protein containing characteristic PxPxxY repeats. To elucidate the function of the gene product in the apoptosis pathway, a human cell line derived from HeLa MR cells, in which the MAPO2 gene was stably knocked down by expressing specific miRNA, was constructed. The knockdown cells grew at the same rate as HeLa MR, thus indicating that MAP2O2 played no role in the cellular growth. After exposure to MNU, HeLa MR cells and the knockdown cells underwent cell cycle arrest at G2/M phase, however, the production of the sub-G1 population in the knockdown cells was significantly suppressed in comparison to that in HeLa MR cells. Moreover, the activation of BAK and caspase-3, and depolarization of mitochondrial membrane, hallmarks for the induction of apoptosis, were also suppressed in the knockdown cells. These results suggest that the MAPO2 gene product might positively contribute to the induction of apoptosis triggered by O6-methylguanine.

Citation: Fujikane R, Sanada M, Sekiguchi M, Hidaka M (2012) The Identification of a Novel Gene, MAPO2, That Is Involved in the Induction of Apoptosis Triggered by O6-Methylguanine. PLoS ONE 7(9): e44817. doi:10.1371/journal.pone.0044817

Editor: Thomas G. Hofmann, German Cancer Research Center, Germany

Received January 10, 2012; Accepted August 14, 2012; Published September 24, 2012

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Funding: This work was supported by the Cancer Research Fund from Fukuoka Foundation for Sound Health (to RF), MEXT-Grants-in-Aid and MEXT-Supported Program for the Strategic Research Foundation at Private Universities, 2008–2012. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

SN1 type alkylating agents, such as N-methyl-N-nitrosourea (MNU) and N-methyl-N-nitro-N-nitrosoguanidine (MNNG), produce various methylated bases, most of which are believed to be efficiently repaired by base excision repair (BER) [1,2]. O6-methylguanine is especially important, since it allows the progression of replication fork and can mispair with thymine during DNA replication [3,4,5], thus leading to a GC to AT transition. To preserve genome integrity from the mutagenic insult, organisms possess a specific repair protein, O6-methylguanine-DNA methyltransferase (MGMT), which transfers a methyl group from O6-methylguanine to a methyl-acceptor cytosine residue in the molecule [6,7,8]. O6-methylguanine that is not repaired by MGMT has chance to pair with thymine, and such a mispair is recognized by a mismatch repair (MMR) protein complex, composed of MSH2, MSH6, MLH1 and PMS2 [9,10,11]. Thereafter, a series of proteins are activated and a signal is delivered to induce apoptosis [12]. Reflecting the distinct roles of MGMT and MMR proteins, Mgmt−/− mice are hypersensitive to the killing effect of alkylating agents and display a decrease in size of the thymus and hypocellular bone marrow after MNU administration [13,14,15]. Furthermore, mice with mutations in both the Mgmt gene and one of the mismatch repair genes, such as Mlh1, are as resistant to MNU as are wild-type mice, in terms of survival, but do develop numerous tumors after receiving MNU [16]. These findings clearly indicate that the MMR proteins-dependent apoptosis contributes to the suppression of cells predisposed to form tumors [16,17].

Following the recognition of O6-methylguanine-thymine mispairs by MMR proteins, ATR kinase is activated, which in turn phosphorylates CHK1 protein, thus leading to the activation of a cell cycle checkpoint [18]. The depolarization of mitochondrial membranes followed by the activation of caspase-3 is induced, through the regulation of the activities of the BCL-2 family of proteins [19,20]. However, the precise molecular mechanism that activates the signaling cascade leading to apoptosis has been elusive.

The gene-trap method is one of the elaborate techniques used for the study of the gene function and has thus helped us to gain a better understanding of the many processes through the capture of actively transcribed endogenous genes [21]. By using this method, we previously isolated a new gene, Mapo1, which is involved in the O6-methylguanine-induced apoptosis [22]. The mouse cell line, KH101, carries a single insertion of the vector sequence in one of alleles of the Mapo1 gene, and is unable to induce apoptosis...
properly after treatment with MNU. Therefore, the gene-trapping method is considered to be a powerful tool to identify new genes functioning in the O6-methylguanine-induced apoptosis pathway.

By extending the gene-trap mutagenesis screening, we isolated a new gene, Mapo2, which is highly conserved among a wide range of multicellular organisms and encodes a protein with characteristic repetitive motifs. This study found that the gene product may be involved in the execution of apoptosis induced by O6-methylguanine.

## Results

### Isolation of a mouse cell line defective in the Mapo2 gene

Retrovirus-mediated gene-trap mutagenesis was performed to identify new genes functioning in the process of apoptosis triggered by O6-methylguanine, as described previously [22]. MNU-sensitive mouse-derived cells, due to the defect in MGMT activity, were infected with the gene-trap vector pLHAU3L-Neo, carrying a promoterless hygromycin B resistance gene, and hygromycin-resistant cells were selected. From Hygr cells, which carry the vector sequence within actively transcribed genes, MNU-resistant clones were isolated as candidates that were defective in genes related to MNU-induced apoptosis. The genes disrupted in the mutant clones were identified by an inverse PCR, which amplifies sequences spanning the junctions between the genomic DNA and the integrated vector sequences, followed by the determination of the DNA sequences. A database search revealed that one of the mutants had an insertion of the vector in a sequence corresponding to the fifth intron of an uncharacterized gene. The gene LOC78806, encoding 341 amino acids, was located in the D3 locus of mouse chromosome 4, and corresponds to the human gene C1orf201, the function of which is not known. The gene is novel, and thus, we named it MAPO2 for O6-methylguanine-induced apoptosis.

A homology search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that the related amino acid sequences to be present in a wide range of organisms, ranging from humans to some lower multicellular organisms, including Trichoplax adhaerens. The prospective proteins carry characteristic 7 PxPxxY repeats, which are evenly distributed among the sequences (Fig. 1). The human MAPO2/C1orf201 is located in the p36.11 locus of chromosome 1. The mouse-derived mutant cells might have mutations not only in the hypothetical gene but also in other unidentified gene, thus the cells are not suitable for further analysis of the effects by the disruption of the hypothetical gene. For the functional analysis of this hypothetical gene, the current study used the human-derived HeLa MR cell line, which is defective for MGMT activity and readily undergoes apoptosis after treatment with MNU.

### Construction of a MAPO2-knockdown human cell line

To analyze the function of the MAPO2 gene in the induction of apoptosis triggered by MNU, a stable human cell line defective in both MAPO2 and MGMT was constructed. A vector that expressed miRNA specific for MAPO2 was introduced to HeLa MR cells,
which are defective in MGMT expression, and the strain was named RF101. A quantitative real-time PCR analysis showed that the expression level of the MAPO2 gene in RF101 cells is reduced to about 5% of that of HeLa MR (Fig. 2A). The RF101 cells grew at almost same rate as did HeLa MR cells (Fig. 2B). The doubling times for HeLa MR and RF101 were both about 16 h, thus suggesting that the function of MAPO2 is not required for cell growth under normal conditions. Moreover, an immunoblotting analysis revealed that MAPO2-knockdown does not affect the expression levels of MMR proteins, which are required for the initial step of damage-recognition for MNU-induced apoptosis (Fig. 2C).

Suppression of MNU-induced apoptosis by MAPO2 knockdown

In order to investigate whether the MAPO2 gene is involved in the MNU-induced apoptosis, HeLa MR and MAPO2-knockdown RF101 cells were treated with 0.4 mM MNU and, then were subjected to a flow cytometric analysis after incubation for 3, 4, and 5 days. The cell cycle checkpoint was activated 3 days after MNU treatment, thus resulting in the accumulation of cells at G2/M phase (Fig. 3A). The sub-G1 cell population increased in both HeLa MR and RF101 cells. However, the degree of apoptotic induction in RF101 cells (10%) was significantly lower than that obtained in HeLa MR cells (17%; Fig. 3A and B). The suppression of the production of sub-G1 cells in RF101 samples was also observed at later time points and about 30% of the cell population in the RF101 still remained at the G2/M phase even at day 5, whereas only 19% of the cell population was there in the HeLa MR. This decrease of the sub-G1 cell population in RF101 cells after treatment with MNU was not observed when the cells were exposed to a DNA interstrand-cross-linker, 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU), and a DNA double-strand break inducer, etoposide (data not shown). These results imply that MAPO2 might be involved in the induction of apoptosis caused by MNU-induced O6-methylguanine.

Effects of MAPO2-knockdown on apoptosis-related events

The status of phosphorylation of CHK1, which is a downstream target of ATR, a DNA damage sensor kinase, was analyzed to determine whether cell cycle checkpoint is indeed induced in MAPO2-knockdown RF101 cells after MNU treatment. The phosphorylation of S317 of CHK1 was clearly detected by immunoblotting analysis in RF101 as well as HeLa MR cells after treatment with MNU (Fig. 4). These data indicated that the function of MAPO2 is dispensable for the activation of the cell cycle checkpoint.

BAK, a member of the pro-apoptotic BCL-2 family of proteins, plays a crucial role in the mitochondria-dependent apoptosis pathway [23]. As shown in Fig. 5, the activation of BAK was observed in HeLa MR cells during the process of MNU-induced apoptosis, as was evident by the finding that the amount of the active form of BAK homodimer, stabilized by the introduction of disulphide bonding, increased gradually. The total amount of BAK, detected as the monomer form under reducing conditions, was not altered. In MAPO2-knockdown RF101 cells, however, the formation of active BAK dimers was significantly inhibited, thus suggesting that the cells were unable to induce apoptosis effectively even after the treatment with MNU. To obtain further evidence supporting that MAPO2-knockdown cells are defective in the induction of apoptosis, the effect of MAPO2-knockdown was investigated on the depolarization of the mitochondrial membrane and the activation of caspase-3, which are known to occur during the process of apoptosis [20,24]. The two types of cells were treated with 0.4 mM MNU for 1 h and were then subjected to both assays. In the flow cytometric analysis, the depolarization of mitochondria was gradually induced in HeLa MR (MAPO2-positive) cells after treatment with MNU, whereas such depolarization was significantly suppressed in RF101 (MAPO2-knockdown) cells, especially at day 2 and 3 (Fig. 6A and B). The processing of caspase-3 was examined by immunoblotting and the results were shown in Fig. 6C and D. The bands corresponding to cleaved caspase-3 were clearly detected at day 3 after the MNU treatment in HeLa MR cells and the intensity of the bands further

Figure 2. Construction of a HeLa MR-derived MAPO2-knockdown cell line. A) The relative expression levels of the MAPO2 gene in HeLa MR and its derivative RF101 cells, as measured by quantitative real-time RT-PCR. B) The growth rate of the two types of cells without MNU treatment. The numbers of cells grown under normal conditions were counted every 24 h and plotted. The experiments were performed 3 times, independently, and average values from the 3 experiments were plotted. Diamonds, HeLa MR; triangles, RF101. (C) The levels of MMR proteins. The expression levels of MMR proteins in HeLa MR and RF101 cells were analyzed using immunoblotting with anti-MSH2, anti-MSH6, anti-MLH1 and anti-PMS2 antibodies. β-Actin was used as a loading control. doi:10.1371/journal.pone.0044817.g002
increased at day 4 and 5. However, the bands were significantly weaker in RF101 cells and at day 5 the level of cleavage was 40% of that of HeLa MR cells, indicating the inefficient activation of caspase-3 due to the knockdown of the \textit{MAPO2} gene. Taken together, it is strongly suggested that \textit{MAPO2} might play an important role in the induction of apoptosis triggered by O\textsuperscript{6}-methylguanine.

\section*{Discussion}

The gene-tap mutagenesis method was used to isolate mouse-derived mutant cell lines that acquired resistance to MNU even in the absence of MGMT activity. One of the mutant cell lines carried an insertion in the \textit{Mapo2/C1orf201} gene, which had been recognized only by sequence analogies of the human genome. Knocking down the \textit{MAPO2} gene in human HeLa MR cells, which readily undergo apoptosis after the treatment with MNU, was performed to reveal the function of the \textit{MAPO2} gene in MNU-induced apoptosis. Although the DNA damage-induced cell cycle checkpoint at G\textsubscript{2}/M phase was little affected by the \textit{MAPO2}-knockdown, the production of the sub-G\textsubscript{1} cell population, the depolarization of the mitochondrial membrane and the activation of BAK and caspase-3, all of which are hallmarks for the induction of apoptosis, were significantly suppressed in the knockdown cells. Thus, we conclude that \textit{MAPO2} may be a new gene that is involved in the O\textsuperscript{6}-methylguanine-induced apoptosis.
Figure 5. The effect of MAPO2 knockdown on the activation of BAK proteins. Soluble protein fractions were extracted from HeLa MR and RF101 cells harvested 2, 3, 4 and 5 days after MNU treatment. The extracts were treated with disulphide bonding inducer to form intermolecularly linked active BAK dimers. The samples were boiled with (reduced) or without (CuPhe) 2-mercaptoethanol and subjected onto SDS-PAGE followed by immunoblotting analysis to detect the total amounts of BAK monomers and active BAK dimers, respectively, using anti-BAK monoclonal antibody ab-1. β-Actin was used as a loading control. The molecular weights are shown on the left.

doi:10.1371/journal.pone.0044817.g005

Figure 6. The involvement of MAPO2 in MNU-induced apoptosis. HeLa MR and RF101 cells were treated with or without 0.4 mM MNU for 1 h. (A) Depolarization of the mitochondrial membrane. The cells harvested 2, 3, 4, and 5 days after MNU treatment were subjected to a flow cytometric analysis to monitor mitochondrial membrane depolarization. The representative patterns of the assay at 2 days after Mock- and MNU-treatment are shown. (B) The levels of mitochondrial membrane depolarization. The mean values of mitochondrial membrane depolarized cells at day 2, 3, 4 and 5, and the standard deviations are presented. Light gray bars, HeLa MR; dark gray bars, RF101. (C) The activation of caspase-3. The whole cell extracts prepared from the cells were used for immunoblotting with an anti-caspase-3 antibody, to detect pro-caspase-3 and cleaved caspase-3. (D) The levels of caspase-3 activation. The intensity of the bands, corresponding to pro- and cleaved caspase-3, detected in (C) were measured. The mean values of the ratio of cleaved caspase-3 to total caspase-3 (sum of pro-caspase-3 and cleaved caspase-3), and the standard deviations calculated from four independent experiments are shown. Light gray bars, HeLa MR; dark gray bars, RF101.

doi:10.1371/journal.pone.0044817.g006
The human MAPO2 encodes a protein with a calculated molecular mass of 36,786, and the gene is conserved throughout various multicellular organisms. A notable feature of the protein is that the amino acid sequence is rich in proline, which alone accounts for 12.5% of the total number of residues and that it contains evenly distributed 7 PxPxY repeats in its polypeptides composed of 254 amino acids [25]. The SHIPPO 1 localizes on the sperm flagella outer dense fibers and is likely to associate with different components that characterize the domains of the sperm tail. Proline is an α-helix breaker residue, thus the Pro-Gly-Pro repeats of SHIPPO 1 might play an important role in the final conformation of the protein [25]. Since the predicted secondary structure of MAPO2 is a highly coiled protein, as is observed in SHIPPO 1, it may be inferred that the PxPxY repeats in the protein may contribute to the formation of its tertiary structure, through which MAPO2 would associate with other apoptosis-related proteins. The identification of the interacting proteins would therefore help to achieve a better understanding of the molecular function of MAPO2 in MNU-induced apoptosis. The subcellular localization of the MAPO2 protein has not been determined to date, because of the unavailability of specific antibodies that recognize the endogenous protein. Therefore, FLAG-tagged mouse MAPO2 was expressed in mouse YT102 cells to determine the localization of the protein. The indirect immunofluorescent analysis using an anti-FLAG antibody showed that mMAPO2 was mainly present in the cytoplasm and also in the nucleus (Fig. S1). The FLAG-tagged protein was also detected by immunoblotting analysis in the chromatin-enriched fraction as well as in the cytosolic fraction (data not shown).

The knockdown of the MAPO2 expression could effectively suppress the induction of apoptosis triggered by MNU. However, the survival fraction of the knockdown cells, as measured by counting the number of colonies formed 10 days after MNU treatment, was comparable to that of MNU-positive HeLa MR cells, indicating that the knockdown of the MAPO2 expression does not prevent ultimate cell death (data not shown). There could be at least two possible explanations as follows: i) The small amount of MAPO2 protein expressed under the knockdown condition (about 5% of mRNA level in comparison to the wild-type) would belatedly induce apoptosis and lead to cell death. ii) An alternative cell death pathway, e.g. caspase-independent apoptosis or necrotic cell death, might be employed, which could bypass the dysfunction of the MAPO2-dependent apoptosis pathway. The establishment of Mapo2-knockout cells would be worthwhile to gain clues to elucidate the role of the protein in the induction of apoptosis triggered by O6-methylguanine.

Materials and Methods

Ethics Statement

N/A

Cell lines and cultivation of cells

Mouse cell lines were established from the lung tissue of mice [17]. Human cell line, HeLa MR, which was originally obtained from H. Hayakawa [26], was used from our laboratory stocks. All the cell lines used are defective in O6-methylguanine-DNA methyltransferase activity. The cells were cultured in Dulbecco’s modified Eagle’s medium (D-MEM, Wako Pure Chemical Industries Ltd.) containing 10% fetal bovine serum at 37°C in 5% CO2.

Gene-trap mutagenesis and isolation of MNU-resistant clones

Gene-trap mutagenesis was performed as described previously with some modifications [22]. MNU-sensitive mouse-derived cells, due to a defect in MGMT activity, were infected with a modified pLHAu3L retrovirus vector [22], pLHA3L-Neo, carrying a promoterless hygromycin B resistance gene and a neomycin resistance gene under the control of a polIII promoter. The cells were sequentially selected in a medium containing 0.3 mg/ml of hygromycin B (Sigma) and in a medium with 0.5 mg/ml of neomycin (Sigma), treated with 0.4 mM MNU for 1 h and further incubated in the drug-free medium. The colonies that formed were isolated as MNU-resistant clones.

Construction of MAPO2-knockdown cell line

A miRNA expression vector, pMAPO2KD1, was constructed using the BLOCK-iT Pol II miR RNAi Expression Vector Kit with EmGFP (Invitrogen, Life Technologies Corp.), according to the manufacturer’s instruction manual. The oligonucleotides to express the MAPO2-specific miRNA, 5’-TGCGTGGAGGTT-TCAAACTGAGCCTTTGCCCCATGACCTG-TCGAATTTGAAACTCCT-3’, were purchased from Invitrogen, Life Technologies Corp. HeLa MR cells were transfected with pMAPO2KD1 using Lipofectamine 2000 (Invitrogen, Life Technologies Corp.), and selected in medium containing 5 μg/ml blasticidin (Sigma). The stable transfectants were isolated and a line, in which the MAPO2 gene expression was knocked down, was obtained and named RF101.

Quantitative real-time PCR analysis

Total RNA was prepared from RF101 cells using the RNaseasy Mini Kit (QIAGEN) and used to synthesize cDNAs by using PrimeScript Reverse Transcriptase (Takara Bio Inc.). Real-time PCR was performed with the 7500 Real Time PCR System (Applied Biosystems) using SYBR Premix Ex Taq II (Takara Bio Inc.). The PCR primers for a MAPO2 gene, 5’-CTTGTGAGAG-CAGTCTGGCCAAATACAT-3’ and 5’-AGAGATCTACTC- GACCGGACCT-3’, and for the GAPDH gene as a reference, 5’-GCACCGTCGAGGCTGAGAAC-3’ and 5’-ATGGTGGTG- GAAGACGCCAGT-3’, were purchased from Takara Bio Inc.

Analyses for apoptosis-related activities

The cells grown on dishes were washed with Dulbecco’s phosphate-buffered saline (PBS) and treated with 0.4 mM MNU in a serum-free medium buffered with 0.02 M Hepes-HCl (pH 6.0), at 37°C for 1 h. The cells were further incubated in complete medium for 2, 3, 4 and 5 days and then were harvested for the assays. The preparation of the cell extracts to detect the active BAK dimer was basically followed as described earlier [27]. Briefly, the soluble fraction extracted from the cells with 1% digitonin-containing buffer (20 mM Tris-HCl (pH 7.5), 100 mM sucrose, 2.5 mM MgCl2, 100 mM KCl and protein inhibitor cocktail (Roche)) was treated with the redox catalyst copper(II)(1,10-phenanthroline)3 on ice for 30 minutes. The reaction was quenched by adding 20 mM of EDTA, and the unreacted SH groups were blocked by 20 mM N-ethylmaleimide. The samples, with or without 2-mercaptoethanol treatment, were analyzed using SDS-PAGE followed by immunoblotting. The cells were treated using MitoProbe DiOC2 (3) Assay kit (Invitrogen, Life Technologies Corp.) for the mitochondrial membrane depolarization assay, as described previously [20], and analyzed using a FACS Calibur flow cytometer (Becton Dickinson). The cells collected were washed with PBS and resuspended in PBS.
containing 0.1% Triton X-100, 25 µg/ml propidium iodide and 0.1 ng/ml RNaseA. The samples were analyzed by the FACS Calibur flow cytometer to detect the sub-G1 population.

Immunoblotting

Whole cell extracts were prepared by the direct lysis of cells on culture dishes with 2x SDS polyacrylamide gel electrophoresis sample buffer, and then separated by SDS-PAGE, followed by electrophoretic transfer onto a PVDF membrane (Bio-Rad). Anti-phosphoCHK1 (S317) (Bethyl), anti-CHK1 (Santa Cruz), anti-BAK ab-1 (Millipore), anti-MSH2 (Invitrogen, Life Technologies Corp.), anti-MSH6 (BD Biosciences), anti-PMS2 (BD Biosciences), anti-MLH1 (BD Biosciences), anti-caspase-3 (Cell Signaling) and anti-β-actin (Sigma) were used as primary antibodies.

Supporting Information

Figure S1 The localization of FLAG-tagged mMAPO2 protein. Mouse-derived YT102 (Mgmt-/-) cells were transfected with pMAPO2CMV10, in which a p3xFLAG-CMV-10 vector (Sigma) contains mouse Mapo2 cDNA, using Lipofectamine 2000 (Invitrogen, Life Technologies Corp.). The cells were incubated for 24 h, washed with PBS and fixed in methanol at −20°C for 15 min. Anti-FLAG M2 antibody (Sigma) and Alexa488 conjugated anti-mouse-IgG Goat antibody (Invitrogen, Life Technologies Corp.) were used to detect the FLAG-tagged mMAPO2 protein, and Hoechst33342 (Invitrogen, Life Technologies Corp.) to stain nuclei, for the analysis using fluorescent microscopy. The images for FLAG-tagged mMAPO2, Hoechst33342 and merged signals are represented at the top, middle and bottom, respectively. (TIF)

Acknowledgments

The authors thank Drs Y. Takagi, H. Hayakawa, D. Setoyama, and R. Ito at Fukuoka Dental College for their helpful discussions.

Author Contributions

Conceived and designed the experiments: RF M. Sanada M. Sekiguchi MH. Performed the experiments: RF M. Sanada. Analyzed the data: RF M. Sanada MH. Contributed reagents/materials/analysis tools: RF M. Sanada. Wrote the paper: RF M. Sekiguchi MH.

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