Mutator Phenotype of MUTYH-null Mouse Embryonic Stem Cells

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A mutant mammalian mutY homolog, named the Mutyh gene, which encodes adenine DNA glycosylase excising adenine misincorporated opposite 8-oxoguanine in the template DNA, was isolated from a 129/Sv mouse genomic library, using a mouse Mutyh cDNA probe (8). Genomic fragments of 4.3 and 3 kb flanked by exons 8–15 of murine Mutyh were used to generate a targeting construct in CCE28 mouse embryonic stem (ES) cells. In the Mutyh-null ES cells carrying no adenine DNA glycosylase activity, the spontaneous mutation rate increased 2-fold in comparison with wild type cells. The expression of wild type Mutyh or mutant mutYH protein with an amino acid substitution at the proliferating cell nuclear antigen binding motif restored the increased spontaneous mutation rates of the Mutyh-null ES cells to the wild type level. The expression of a mutant Mutyh protein with an amino acid substitution (G365D) that corresponds to a germ-line mutation (G392D) found in patients with multiple colorectal adenomas could not suppress the elevated spontaneous mutation rate of the Mutyh-null ES cells. Although the recombinant Mutyh(G365D) purified from Escherichia coli cells had a substantial level of adenine DNA glycosylase activity as did wild type Mutyh, no adenine DNA glycosylase activity was detected in the Mutyh-null ES cells expressing the Mutyh(G365D) mutant protein. The germ-line mutation (G382D) of the human MUTYH gene is therefore likely to be responsible for the occurrence of a mutator phenotype in these patients.

Cellular DNA is at high risk of being oxidized by reactive oxygen species, which are inevitably generated by normal metabolic functions such as mitochondrial respiration or by environmental exposure to ionizing radiation and chemicals. The oxidation of DNA appears to result in either spontaneous mutagenesis or cell death, thus being implicated in various age-related diseases such as cancer and neurodegeneration (1). Among the various types of oxidized damage in DNA, 8-oxo-G, an oxidized form of guanine, can pair with adenine as well as cytosine during DNA replication and is thus considered to be one of the spontaneous causes of G:C to T:A transversion mutation (2).

Mutator mutants in Escherichia coli revealed that the mutM (pgp) gene encoding 8-oxo-G DNA glycosylase, which excises 8-oxo-G opposite cytosine in DNA, and the mutY gene encoding the adenine DNA glycosylase, which removes adenine incorporated opposite 8-oxo-G in template DNA, play important roles in the prevention of such spontaneous mutagenesis (3). It has been established that most eukaryotic cells also possess either a MutM homolog or its functional homolog, OGG1 for the repair of 8-oxo-G, and a MutY homolog (MUTYH or MYH) for the repair of adenine opposite 8-oxo-G (4).

In E. coli and fission yeast, the absence of these repair enzymes resulted in significant increases in the spontaneous mutation rate, especially of G:C to T:A transversion mutation (3, 5). Recently, familial alterations in the human MUTYH gene have been reported to be possible causative mutations for certain types of familial colorectal tumors without a germ-line mutation in the APC gene, thus suggesting that the absence of the MUTYH function in human cells might also result in a mutator phenotype (6, 7).

To evaluate the role of MUTYH in the prevention of mutagenesis in mammals, we generated MUTYH-null mouse ES cells and characterized their mutator phenotype.

EXPERIMENTAL PROCEDURES

Generation of the MUTYH-null ES Cell Lines—Genomic fragments spanning the sequence encoding exons 3–17 of murine Mutyh was isolated from a 129/Sv mouse genomic library, using a mouse Mutyh cDNA probe (8). Genomic fragments of 4.3 and 3 kb flanked by exons 8–15 of Mutyh gene were used to generate a targeting construct in which exons 8–15 were replaced by a RNA polymerase II-neo-poly(A) cassette (9). To increase the frequency of gene targeting, a pair of HSV-1 tk cassettes (tk1 and tk2) was placed flanking the Mutyh genomic sequence in the targeting vector (9). CCE28 ES cells were electroporated with the Sall-linearized targeting vector, and then Mutyh−/− clones were selected in the presence of 0.25 mg/ml G418 (Sigma) and 5 μM ganciclovir (Japan Syntechs). Among the 89 ES cell colonies, 23 correctly targeted clones were identified by a Southern blot analysis. Subsequently, two Mutyh−/−/− clones (YDK15, YDK32) were independently obtained by the selection of two Mutyh−/− cells (YSK25, YSK38) in higher concentrations of G418 (2.0 mg/ml). For mutagenesis assay, we used YDK15 and YDK32 cells at the passage number 7 to 8 after single colony isolation of each line, and wild type CCE28 cells were similarly passaged before the experiment.

RT-PCR Analysis—Total RNA was prepared from the cultured cells using ISOGEN (Nippon Gene). First-strand cDNA was synthesized using a first-strand cDNA synthesis kit (Amersham Biosciences) as described in the manufacturer’s instructions. Subsequently, PCR was performed with primers mY3-4A (5′-CTCTGGTGCAAAAGCCCTGTA-3′) corresponding to nucleotides 1071–1088 of Mutyh mRNA (GenBank accession number NM_133250) and mY3-4A (5′-ATTCCTCCCAGGTAC-3′) corresponding to nucleotides 1345–1326 of the Mutyh mRNA to confirm the deletion of Mutyh. A RT-PCR analysis of GAPDH

The abbreviations used are: 8-oxo-G, 8-oxoguanine; hMUTYH, mouse MUTYH; hMUTYH, human MUTYH; BER, base excision repair; ES, embryonic stem; neo, neomycin resistance gene; HSV, herpes simplex virus; tk, thymidine kinase; RT, reverse transcriptase; Trx, thioredoxin; DNA, thioguanine; PCNA, proliferating cell nuclear antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAT, hypoxanthine, aminopterin, and thymidine; FAM, N-(3-fluoranthyl)maleimide.

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mRNA was also performed as a control with primers mGAS–1 (5′-CTGCCATTGCTGGGCAAAG-3′) corresponding to nucleotides 126–146 of GAPDH mRNA (GenBank™ accession number XM_194302) and mGA9–1 (5′-TGTTACCAAGAAGGAG-3′) corresponding to nucleotides 1230–1210 of the GAPDH mRNA. These oligonucleotides were obtained from the Hokkaido System Science Co. Ltd.

Western Analysis—Protein samples were separated by SDS-PAGE and then were transferred onto Immobilon P membrane (Millipore Inc.), and Western blotting analyses were performed as described previously by Tsuchimoto et al. (10), using anti-hMUTYH (11).

Expression of mMUTYH Protein—For expression of mMUTYH in E. coli, mouse Mutyh cDNA was subcloned into pET32a(+) (Novagen). A fusion protein with thioredoxin (Trx-mMUTYH) was expressed and purified using cobalt beads (TALON™) and metal affinity resin, followed by gel filtration on Superose 12/30 (Amersham Biosciences), as described previously (9, 10). For the expression of MUTYH protein in mouse cells, mouse Mutyh cDNA was subcloned into pcDNA3.1/Hygro(+) (Invitrogen), and plasmid was electroporated into YDK15 cells, and stable transfectants grown in the presence of hygromycin B (140 and 160 μg/ml, Wako Pure Chemical Industries, Ltd.) were established. YDK15 cells expressing wild type mMUTYH were designated as YDKWT cells. YDK15 cells expressing a mutant mMUTYH(G356D) were designated as YDKG365D cells. YDK15 cells, which received only pCDNA3.1/Hygro(+) (Invitrogen), were designated as YDKV cells. All cell lines, YDKWT, YDKAA, YDKG365D, and YDKV, were used for the mutagenesis assay at the passage number 7 to 8 after single colony isolation of each line.

PCNA Pull-down Assay—Trx-mMUTYH fusion proteins or Trx protein immobilized on S-protein-agarose (Novagen) were used for PCNA pull-down assays as described previously by Tsuchimoto et al. (10).
Mutator Phenotype of MUTYH-null ES Cells

RESULTS

To understand the physiological role of MUTYH in vivo, we disrupted the Mutyh gene in mouse ES cells (Fig. 1A). Mutyh<sup>−/−</sup> ES cell lines were obtained by homologous recombination, and subsequently two independent Mutyh<sup>−/−</sup> ES cell lines (YDK15, YDK32) were established (Fig. 1, B and C). As shown in Fig. 1D, several bands were detected in the whole cell extract prepared from parental CCE28 cells by Western blotting with anti-hMUTYH, and a major 50-kDa and minor 47-kDa band disappeared in the Mutyh<sup>−/−</sup> ES cells (YDK15, YDK32).

A MUTYH activity introducing alkaline-labile site opposite 8-oxo-G, namely DNA glycosylase activity excising adenine opposite 8-oxo-G, was detected in the extract from CCE 28, but such activity was not detected in the extracts from YDK15 and YDK32 cells. All extracts examined contained comparable levels of nicking activity toward 8-oxo-G opposite cytosine in oligonucleotide (Fig. 2B), thus confirming that YDK15 and YDK32 cells are MUTYH-null mutants.

The MUTYH-null cells grew normally in ES medium, and their average doubling times (9.2 h) at 37 °C were essentially the same as that of CCE28 cells. We did not observe any hypersensitivity to H<sub>2</sub>O<sub>2</sub> or menadione challenge in the MUTYH-null ES cells. Chimeric mice were generated from the Mutyh<sup>−/−</sup> ES cells to establish MUTYH-null mice. The MUTYH-null mice were viable and fertile, and those are now under long term observation for carcinogenesis (data not shown).

The mutation rates toward 6-TG resistance were determined by a fluctuation test using three cell lines, CCE28, YDK15 and YDK32. A 2-fold higher mutation rate was observed in the two independently isolated MUTYH-null cells, compared with the value of parental CCE28 cells (Table I). Essentially the same levels of spontaneous mutation rates were obtained by a mutation analysis utilizing HAT-cleansed cultures: CCE28, 2.99 × 10<sup>−8</sup>; YDK15, 6.20 × 10<sup>−8</sup> mutations/locus/generation, respectively.

We introduced an expression vector carrying Mutyh cDNA into the YDK15 cells and established a stable cell line (YDKWT). The levels of adenine DNA glycosylase activity as well as the 50-kDa mMUTYH protein in the cells were comparable with the levels seen in CCE28 cells (Fig. 2, A and B). The spontaneous mutation rate of YDKWT cells determined by using HAT-cleansed cells was as low as that of CCE28 cells, but YDKV cells, in which only the vector plasmid was introduced, exhibited a 2-fold higher mutation rate than YDKWT cells (Fig. 3).

We introduced amino acid substitutions (500/501FF to 500/501AA) in the PCNA binding motif of mMUTYH, and an expression vector encoding the mutant mMUTYH(AA) protein was introduced into YDK15 cells. An established cell line (YDKAA) expressed mutant mMUTYH(AA) protein with a substantial level of adenine DNA glycosylase activity (Fig. 2, A and B). YDKAA cells exhibited a slightly lower level of spontaneous mutation rate in comparison with YDKWT cells (Fig. 3). Recombinant Trx-mMUTYH efficiently bound to PCNA in vitro.

| Number of samples (C) | 21 | 21 | 21 | 21 | 21 | 21 |
|----------------------|----|----|----|----|----|----|
| Final cell number (Nt) × 10<sup>6</sup> | 4.83 | 6.13 | 7.99 | 7.20 | 5.18 | 4.14 |
| Average number of mutant cells (r) | 0.24 | 0.14 | 0.95 | 1.19 | 0.71 | 0.43 |
| Cr<sup>−</sup> | 5.04 | 2.94 | 19.95 | 24.99 | 14.91 | 9.03 |
| CaNt<sup>a</sup> | 3.77 | 2.83 | 9.05 | 10.59 | 7.43 | 5.47 |
| Variance | 0.29 | 0.13 | 3.65 | 13.5 | 5.31 | 3.86 |
| Mutation rate (a)<sup>b</sup> × 10<sup>−9</sup>/locus/generation | 3.72 | 2.20 | 5.40 | 7.00 | 6.83 | 6.29 |

<sup>a</sup> Derived by linear interpolation from the table of CaNt as a function of Cr (13).

<sup>b</sup> Mutation rate, a = CaNt/CNt.

DISCUSSION

The spontaneous mutation rate in the Hprt locus increased 2-fold in MUTYH-null ES cells in comparison with wild type CCE28 ES cells. We previously reported that MTH1-null ES cells also exhibited a 2-fold increase in the spontaneous mutation rate (16). Mice lacking MTH1 spontaneously develop cancer more frequently in the liver, lung, or stomach than do wild type, thus suggesting that these proteins play a tissue-specific role in the suppression of tumorigenesis (16). Mice lacking MUTYH protein are now under long term observation for spontaneous carcinogenesis, and the moderate mutator phenotype of MUTYH-null ES cells suggests that such mice are also mildly predisposed to certain types of tumors.

The increased spontaneous mutation rate in the MUTYH-null ES cells was completely suppressed by the expression of a mouse Mutyh cDNA encoding a 50-kDa mMUTYH protein with

Fig. 3. Mutation analysis of MUTYH-null cells expressing exogenous mMUTYH proteins. HAT-cleansed cells were grown in ES medium for 2 days and subjected to 6-TG selection. Mutation rates were calculated as described under "Experimental Procedures." Each experiment was performed in triplicate, and the data are shown with S.E. WT, YDKWT; AA, YDKAA; G365D, YDKG365D; Vector, YDKV cells.

while no binding of Trx-mMUTYH(AA) to PCNA was observed (data not shown).

Finally, we examined the effect of a G365D mutation in mMUTYH, which corresponds to a germ-line mutation (G382D) of the human MUTYH gene found in patients with multiple colorectal adenomas (6, 7). Recombinant Trx-mMUTYH(G365D) had a closely similar level of adenine DNA glycosylase activity to that of wild type Trx-mMUTYH (Fig. 2, C and D). The mutant mMUTYH(G365D) was stably expressed in the MUTYH-null ES cells; however, no adenine DNA glycosylase activity was detected in the cell-free extract prepared from the established cell line (YDKG365D) (Fig. 2, A and B), and the cells exhibited an increased spontaneous mutation rate which was as high as that of MUTYH-null cells (YDKV) (Fig. 3), thus indicating that the G365D mutation indeed causes a loss of the MUTYH function in vivo.
the restoration of adenine DNA glycosylase activity, thus confirming that the 50-kDa mMUTYH protein is a functional form of mouse MUTYH. Based on this finding, we examined exogenously expressed mutant mMUTYH proteins to determine whether or not they can suppress the increased spontaneous mutagenesis in MUTYH-null ES cells. We and others (8, 17, 18) showed that MUTYH may interact with PCNA to exert post-replicative BER for adenine opposite 8-oxo-G. After introduction of a plasmid containing the A:8-oxo-G pair into differentiated MUTYH-null ES cells with a plasmid encoding wild type but not the mutant mMUTYH(AA) protein, which had amino acid substitutions at the PCNA binding motif, the A:8-oxo-G pair was efficiently repaired to C:G pair (8), thus indicating that mMUTYH requires an interaction with PCNA to exert the post-replicative BER. In the present study, the mutant mMUTYH(AA) significantly suppressed the increased spontaneous mutation rate in the MUTYH-null ES cells, and we concluded that MUTYH may not require PCNA to exert its repair function. In the previous study (8), only the post-replicative BER for A:8-oxo-G pair was assayed using a replication-competent plasmid. In contrast, the spontaneous mutagenesis analyzed in the present study reflects all existing mechanisms involved in the process, including both the pre-replicative and post-replicative BER, as well as the mismatch repair. This may be a reason that the contribution of PCNA-dependent BER is very low in the endogenous target gene, Hprt, or it is still possible that the transient transfection experiment of the reporter plasmid may not fully reproduce the chromosomal context of endogenous genes, thus exhibiting only a part of facts.

If mMUTYH(AA) protein without its PCNA interaction can excise adenine in the template DNA to which 8-oxo-G was inserted from nucleotide pool, A:T to C:G transversion mutation might be highly induced. Our results suggest that mMUTYH(AA) or wild type mMUTYH can also interact with other components of post-replicative BER, as reported previously (19), thus ensuring the strand recognition. Alternatively, 8-oxo-dGTP produced in the nucleotide pool is efficiently sanitized by MTH1, thus preventing the incorporation of 8-oxo-G opposite adenine in the template DNA. As a result, mMUTYH(AA) could efficiently suppress spontaneous mutagenesis by simply excising adenine opposite 8-oxo-G. If this is the case, then MUTYH-null cells expressing mMUTYH(AA) might increase their spontaneous mutation rate in the absence of the MTH1 function.

A germ-line mutation (G382D) of the human MUTYH gene found in patients with multiple colorectal adenomas (6, 7) is a yeast MutY mutant with a corresponding amino acid substitution (G253D) was reported to possess a substantial level of adenine DNA glycosylase (6). We confirmed that it is also true for mMUTYH mutant with G365D substitution. In contrast, MUTYH-null cells expressing the mMUTYH(G365D) mutant exhibited no adenine DNA glycosylase activity in the cell-free extract, and their spontaneous mutation rate was as high as that of MUTYH-null cells, thus indicating that the mMUTYH(G365D) expressed in mouse ES cells could not demonstrate repair capacity either in vivo or in the cell-free extract.

Currently, it is not clear why the mutant protein expressed in mouse ES cells was not active as adenine DNA glycosylase. The mMUTYH(G365D) protein exhibited a slightly decreased mobility on SDS-PAGE in comparison with wild type or mMUTYH(AA) mutant proteins (Fig. 2A), indicating that there may be a difference in post-translational modifications of these proteins. Since a serine residue in the amino acid sequence containing the G365 residue, RPDSGKLL was predicted as a possible phosphorylation site by calcium/calmodulin-dependent protein kinase II or protein kinase A using a software program PhosphoBase V 2.0 (www.cbs.dtu.dk/databases/PhosphoBase/) (20), it may be likely that the G365D substitution alters such a modification in vivo, thus resulting in both the decreased mobility on SDS-PAGE and the loss of its catalytic activity. This is the first observation of a functional abnormality in the mMUTYH protein with an amino acid substitution corresponding to the germ-line mutation (G382D) of the human MUTYH gene in patients with multiple colorectal adenomas. Our findings and MUTYH-null cells are thus considered to provide a clue to elucidate the molecular basis of mutagenesis and carcinogenesis which can be suppressed by MUTYH.

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