Molecular characterization of 5′-deoxy-5′-methylthioadenosine phosphorylase-deficient mutant clones of murine lymphoma cell line R1.1

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5′-Deoxy-5′-methylthioadenosine phosphorylase (MTAP), the polyamine and purine metabolic enzyme, is ubiquitously expressed in normal human tissues and cells, but is deficient in a variety of human and murine malignant cell lines. MTAP-deficient mutants were previously selected from murine lymphoma cell line R1.1 that contains abundant MTAP activity, to analyze the metabolic consequences of MTAP deficiency. Two mutants, one with partial deficiency (F clone) and the other with complete deficiency (H5 clone), were found to have the MTAP protein by immunoblotting. However, the molecular mechanism of enzyme deficiency in these mutants has not been established. In this study, we cloned the mouse cDNA and analyzed MTAP mRNA in the two mutants by reverse transcription-polymerase chain reaction (RT-PCR) followed by direct sequencing. Both mutants have a single nucleotide substitution at the third base of codon 223, which results in a change of cysteine to tyrosine (C223Y). The MTAP mRNA level determined by RT-PCR was significantly lower in the mutant with complete deficiency than in wild-type (WT) R1.1 cells, whereas the mRNA level in the mutant with partial deficiency was comparable to that in WT R1.1 cells. C223Y mutation may cause conformational alteration of the methylthioribose-binding site and decrease the substrate binding. Thus, C223Y may account for the partial deficiency in the F clone, but the complete deficiency with a remarkably low MTAP mRNA level in the H5 clone may be a result of transcriptional abnormality in addition to C223Y mutation. (Cancer Sci 2003; 94: 519–522)

5′-Deoxy-5′-methylthioadenosine phosphorylase (MTAP; EC 2.4.2.28) cleaves 5′-deoxy-5′-methylthioadenosine (MTA) produced during the synthesis of polyamines to adenine and 5-methylthio-D-ribose-1-phosphate, which are converted to adenine nucleotides and methionine, respectively.[1–4] Deficiency of this enzyme has been found in a variety of human cancer cell lines and primary tumors of various origins,[5–12] whereas all normal cells or tissues have abundant activity.[13] Recently, molecular analysis of MTAP-deficient human cell lines indicated that the deficiency was caused by partial or total gene deletion in all cell lines, except one lymphoma cell line, studied so far.[14] In the previous study, two MTAP-deficient mutants were selected from murine lymphoma cell line R1.1 by tritium suicide following ethyl methanesulfonate (EMS) treatment to determine the exact metabolic consequences of MTAP deficiency.[15] These mutants, one with partial deficiency and the other with complete deficiency, were found to have the MTAP protein by immunoblotting.[16] However, the molecular mechanism of the enzyme deficiency in these mutants was not established because of unavailability of the mouse MTAP cDNA. In the present study, we cloned the mouse MTAP cDNA and analyzed MTAP mRNA in the two mutants by reverse transcription-polymerase chain reaction (RT-PCR) followed by direct sequencing. Both mutants have a single nucleotide substitution at codon 223, which results in the change of cysteine to tyrosine. The MTAP mRNA level determined by RT-PCR was significantly lower in the mutant with complete deficiency than in wild-type (WT) R1.1 cells, whereas the mRNA level in the mutant with partial deficiency was comparable to that in WT R1.1 cells.

Materials and Methods

Cell lines. Mutant clones F and H5 derived from mouse lymphoma cell line R1.1 were selected by tritium suicide following EMS treatment as described previously.[16] Clone H5 was completely deficient in MTAP, whereas clone F had approximately 3.5% as much as MTAP activity as WT R1.1 cells. These cell lines were kindly provided by Dr. Dennis A. Carson, University of California at San Diego, and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 m*M* L-glutamine, 100 units of penicillin/ml and 100 μg of streptomycin/ml in a humidified atmosphere of 95% air, 5% CO_2.

Mouse MTAP cDNA cloning. Full-length cDNA for mouse MTAP was prepared by rapid amplification of cDNA ends (RACE) using mouse liver Marathon-ready cDNA (Clontech, Palo Alto, CA) as a template. The sequence of human MTAP cDNA was used to find the mouse sequence in the EST database by homology searching. Based on the partial sequences of truncated mouse cDNA found in the NCBI EST database (dbEST Id: 1673172, gi:3100259; 1874030, gi:3515835), the *MTAP* gene-specific primers 1 and 2 were synthesized. The 5′ end of MTAP cDNA was amplified with *MTAP* gene-specific primer 1 (5′-TGTGGCACACTCCTCTGGCACTGCA-3′) and adaptor primer 1 (5′-CCATCCTAATACGACTCACTATAGGGC-3′). For amplification of the 3′ end of MTAP cDNA, *MTAP* gene-specific primer 2 (5′-CATTCGGCAAGCCATCCGATGCCT-3′) and adaptor primer 1 were used. Following direct sequencing, these two PCR products were mixed and used for preparation of full-length MTAP cDNA by PCR amplification with RT sense primer (5′-AACTTCCCCGGTGCAGAC-3′) and RT antisense primer (5′-AACGTCTTCTTGTGTCAGCCA-3′) corresponding to the 5′- and 3′-noncoding sequences, respectively.

Western blot analysis. MTAP protein was detected by immunoblotting as described,[17, 18] with a minor modification. Briefly, crude extracts were prepared from WT and mutant R1.1 cells, separated in 7.5–15% gradient polyacrylamide gels containing 0.1% SDS, and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, MA). Blots were incubated with a rabbit anti-mouse MTAP antiserum diluted to 1:1000 for 2 h, followed by detection of a 32-kilodalton subunit of MTAP protein with an ECL detection system (Amersham Pharmacia Biotech, Uppsala, Sweden).

RT-PCR analysis. For detection of the MTAP transcript in the cells, total RNA was isolated with Sepa-Gene RV-R (Sanko-

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Junyaku, Tokyo) and transcribed to cDNA using the T-primed First Strand Kit (Amersham Pharmacia Biotech) followed by PCR amplification along with the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene as a control. The PCR amplification was carried out in a 50 μl reaction mixture containing 1 μg of the reverse-transcribed cDNA, 1× PCR buffer (10 m*M* Tris-HCl, pH 8.0, 50 m*M* KCl, 1.5 m*M* $MgCl_2$ and 0.001% gelatin), 200 mM each of four deoxynucleoside triphosphates, 200 mM each of RT sense and antisense primers, and 2.5 units of *Taq* polymerase (Perkin-Elmer, Norwalk, CT). Thirty cycles were performed with the programmable cyclic reactor (GeneAmp PCR system 9700; Perkin Elmer). Each cycle consists of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. RT-PCR products amplified from WT R1.1 cells and F and H5 clones were subcloned into the TA cloning vector (Invitrogen, San Diego, CA) according to the supplier's instructions. Several independent recombinant plasmids were sequenced by cycle sequencing according to the supplier's protocol (Perkin-Elmer).

Southern blot analysis. The RT-PCR products were separated by agarose gel electrophoresis and transferred to nylon membranes (Hybond-N nylon membrane, Amersham). The blot was hybridized with a digoxigenin-labeled MTAP cDNA probe in hybridization buffer at 42°C for 16 h using aa DIG DNA labeling and detection kit according to the manufacturer's instructions (Roche, Tokyo). After high stringency washing for 10 min at 60°C in 0.1× SSC containing 0.1% SDS, the membrane was incubated with an anti-digoxigenin Fab fragment conjugated to alkaline phosphatase at 0.75 units/ml. After washing, bands were detected using Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega, Madison, WI).

Chemicals. All other chemicals used in the present study were of the best quality commercially available.

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   1 ATGGCCTCGGGCTCCGCCTGCACGGCGGTGAAGATTGGAATAATTGGTGGAACAGGCTTG  60
     M  A  S  G  S  A  C  T  A  V  K  I  G  I  I  G  G  T  G  L

  61 GATGATCCCGAAATTTTAGAAGGAAGAACTGAGAAATATGTGGATACTCCATTCGGCAAG 120
     D  D  P  E  I  L  E  G  R  T  E  K  Y  V  D  T  P  F  G  K

 121 CCATCCGATGCCTTAATTTTGGGGAAGATAAAGAACGTTGATTGTGTACTTCTTGCAAGA 180
     P  S  D  A  L  I  L  G  K  I  K  N  V  D  C  V  L  L  A  R

 181 CACGGCAGACAACACACCATCATGCCTTCAAAAGTCAACTACCAGGCAAACATCTGGGCC 240
     H  G  R  Q  H  T  I  M  P  S  K  V  N  Y  Q  A  N  I  W  A

 241 TTGAAGGAAGAGGGCTGTACACATGTCATCGTGACCACAGCTTGCGGGTCCTTGAGAGAG 420
     L  K  E  E  G  C  T  H  V  I  V  T  T  A  C  G  S  L  R  E

 301 GAGATCCAGCCTGGTGACATGGTCATCATTGACCAATTCATTGACAGGACATCCCTAAGG 360
     E  I  Q  P  G  D  M  V  I  I  D  Q  F  I  D  R  T  S  L  R

 361 CCTCAGACCTTCTATGATGGGAGTCATTGCAGTGCCAGAGGAGTGTGCCACATCCCCATG 420
     P  Q  T  F  Y  D  G  S  H  C  S  A  R  G  V  C  H  I  P  M

 421 GCTGAACCGTTTTGCCCCAAAACAAGAGAGGTCCTCATAGAAACTGCTAAGAAGCTAGGA 480
     A  E  P  F  C  P  K  T  R  E  V  L  I  E  T  A  K  K  L  G

 481 CTTCGGTGCCATTCAAAGGGGACAATAGTCACAATTGAGGGGCCCCGTTTCAGCTCCCGG 540
     L  R  C  H  S  K  G  T  I  V  T  I  E  G  P  R  F  S  S  R

 541 GCAGAAAGCCTTATATTCCGTACTTGGGGCGCAGATGTTGTCAACATGACCACTGTTCCA 600
     A  E  S  L  I  F  R  T  W  G  A  D  V  V  N  M  T  T  V  P

 601 GAGGTGGTACTTGCTAAGGAGGCTGGAATTTGCTATGCAAGCATTGCCATGGCAACCGAC 660
     E  V  V  L  A  K  E  A  G  I  C  Y  A  S  I  A  M  A  T  D

 661 TATGATTGTTGGAAGGAGCATGAAGAAGCAGTGTCAGTGGATGGGGTTTTAAAGACCATG 720
     Y  D  C  W  K  E  H  E  E  A  V  S  V  D  G  V  L  K  T  M

 721 AAAGAAAATGCCAATAAAGCCAAAAGTTTACTCCTCACTACTATACCTCAGATAGGGTCG 780
     K  E  N  A  N  K  A  K  S  L  L  L  T  T  I  P  Q  I  G  S

 781 ATGGAATGGTCAGAAACGCTCCGTAACCTAAAGAACATGGCCCAGTTTTCCGTTTTACCA 840
     M  E  W  S  E  T  L  R  N  L  K  N  M  A  Q  F  S  V  L  P

 841 CCAAGACATTAA 852
     P  R  H  *
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Fig. 1. Mouse MTAP cDNA sequence and its deduced peptide sequence. This sequence was deposited in the DNA Data Bank of Japan under Accession Number AB056100.

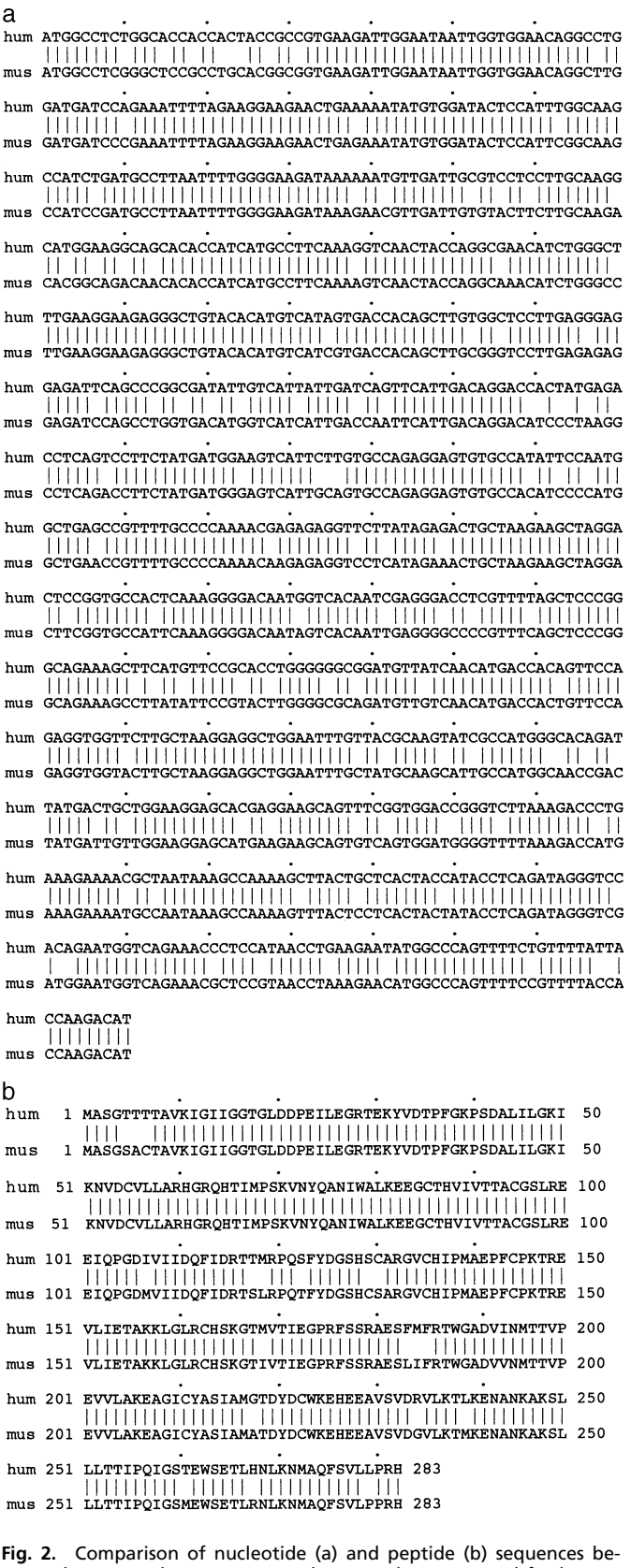

Fig. 2. Comparison of nucleotide (a) and peptide (b) sequences between human and mouse MTAP. "hum" and "mus" stand for human and mouse, respectively. Vertical lines indicate identical sequences.

Results

Mouse MTAP cDNA cloning and analysis of cDNA prepared from mutant clones. The cloned cDNA encodes a polypeptide consisting of 283 amino acids, as shown in Fig. 1. When the coding sequences were compared, mouse MTAP cDNA was 87.4% and 93.3% homologous to human MTAP cDNA at the nucleotide and peptide levels, respectively (Fig. 2). Crystallographic studies revealed that there are active, base-binding, methylthioribose-binding, and sulfate/phosphate-binding sites in human MTAP protein.[19] Since mouse MTAP shares approximately 90% identity with human MTAP, the high degree of structural homology between human and mouse MTAP is readily apparent from the primary peptide sequence data (Fig. 2b). The sequence amplified from mutant R1.1 cells had base substitutions at bases 243, 550, and 668 (Table 1). Base substitutions at bases 243 and 550 were found in WT R1.1 as well. Therefore, these base substitutions were thought to be polymorphic. The G to A transition at base 668 (G668A) in mutant cells was not found either in the cloned cDNA or in the sequence from WT R1.1, indicating that this change is a mutation which may account, at least in part, for the MTAP enzyme deficiency in mutant R1.1 cells. The G668A mutation occurred at the second nucleotide of codon 223 and resulted in a change of cysteine to tyrosine (C223Y) in the base-binding site of MTAP protein, as determined by crystallographic analysis. This mutation would presumably cause a change of the substrate binding and the catalytic rate in the mutant cells.

MTAP expression. MTAP expression was analyzed by immunoblotting and RT-PCR. The MTAP protein per unit total protein was significantly reduced in H5 clone, whereas the amount of immunoreactive MTAP protein in clone F was comparable to that in WT R1.1 cells (data not shown). RT-PCR analysis also revealed that MTAP mRNA level in H5 was significantly lower than that in F and WT (Fig. 3). These results suggested that the complete enzyme deficiency in H5 was attributable to transcriptional abnormality in addition to the mutation at codon 223.

Table 1. Amino acid and base changes found in wild-type and mutant R1.1 cells

No. of codon (No. of base in Fig. 1)	Change of amino acid (Base substitution)	Cell types
81 (243)	Phenylalanine→Leucine (TT**T**) (TT**G**)	WT, F, H5
184 (550)	Valine→Leucine (**G**TT) (**C**TT)	WT, F, H5
223 (668)	Cysteine→Tyrosine (T**G**T) (T**A**T)	F, H5

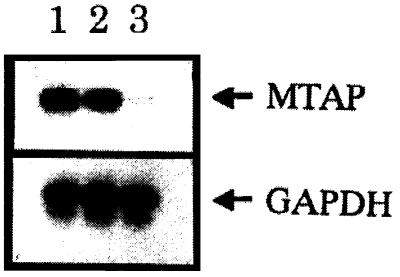

Fig. 3. MTAP mRNA expression in WT and mutant R1.1 cells by RT-PCR analysis. MTAP mRNA of WT and mutant F were expressed almost equally, whereas mutant H5 had a significantly lower level of MTAP mRNA. As a control, GAPDH was amplified with primers (RT sense, 5′-AACTTCCCCGGTGCAGAC-3′ RT antisense 5′-AACGTCTTCTTGTGTCAGCCA-3′) by RT-PCR as described in "Materials and Methods." Lanes are: 1, WT R1.1; 2, mutant F; 3, mutant H5. As an internal control, GAPDH was used for RT-PCR.

Discussion

MTAP deficiency has been found in a variety of human cancers. The molecular analysis of MTAP-deficient human cancer cell lines demonstrated that the gene deletion was the major mechanism of the enzyme deficiency. In addition to the gene deletion, hypermethylation of the MTAP promoter was found to cause the enzyme deficiency in certain cancers (manuscript in preparation). The mutant cell lines used in this study were generated by tritium suicide following EMS treatment and had been analyzed biochemically.[16] However, the molecular mechanism of MTAP deficiency in these mutant clones has not been determined since genetic information on the mouse *MTAP* gene was not available. We first cloned mouse MTAP cDNA based on the reported cDNA partial sequence and found that the nucleotide and peptide sequences were highly conserved between human and mouse *MTAP* gene. Mutant clones with partial or complete deficiency were then analyzed by RT-PCR amplification, followed by DNA direct sequencing. Three base-substitutions were found in both F and H5, which were T to G transversion at base 243 (T243G), G to C transversion at base 550 (G550C), and G to A transition at base 668 (G668A). The transversions found at bases 243 and 550 were thought to be single-nucleotide polymorphisms (SNPs), since WT R1.1 cells showed the same substitutions. In contrast, the G668A transition was thought to be a mutation that might be attributable in part to the decrease in the MTAP enzyme activity, since this was not found in WT R1.1.

These mutant clones were treated with EMS, whose ethyl group can be transferred to a variety of cellular, nucleophilic sites through an SN1 or an SN2 mechanism.[20] When DNA was exposed *in vitro* to EMS, the N-7 of guanine was reported to be most frequently ethylated.[21] Other sites included are: the O^6 and N-3 of guanine; the N-3, N-1 and N-7 of adenine; the N-3 of cytosine; the phosphate groups on the DNA backbone. Because of the high frequency of N-7-alkylguanine residues produced in the reaction of alkylating agents with DNA, mispairing of this modified purine was once believed to be an important cause of mutation. However, Loveless[22] suggested that mutagenicity might be correlated with the formation of O^6-alkylguanine rather than of N-7-alkylguanine. Chemicals reacting through an SN1 mechanism were reported to produce higher amounts of O^6-alkylguanine relative to N-7-alkylguanine.[23] Therefore, EMS, which can react via an SN1 mechanism as well as an SN2 mechanism, is expected to produce relatively more O^6-ethylation than that found with agents which react via an SN2 mechanism. Since N-7-methylguanine has similar base-pairing properties to guanine, the G668A mutation in F and H5 might be induced by EMS through the formation of O^6-ethylguanine, which causes the misincorporation of bases.

The G668A mutation causes a change of the amino acid residue at residue 223 from cysteine to tyrosine (C223Y). Crystallographic studies of human MTAP, which is highly homologous to mouse MTAP, demonstrated that the active sites of MTAP contain the base-, methylthioribose-, and sulfate/phosphate-binding sites.[19] C223Y mutation is adjacent to the base-binding site, which consists of F177, S178, D220, D222 and a water molecule. Generally speaking, cysteine plays a special role in proteins because its -SH group allows it to dimerize through an -S-S- bond to a second cysteine, thus covalently linking distinct regions of a polypeptide to one another. Human MTAP is a trimer consisting of three identical subunits. The subunit contacts within the trimer are extensive and primarily involve residues

from D112 to F145 of one subunit, and residues from I172 to T188 of a neighboring subunit. At the trimer interface, direct contacts between identical residues related by the threefold axis include a hydrophobic interaction of three W189 residues and a hydrogen-bond network involving three T118. Thus, C223 was not supposed to be involved in -S-S- bond formation and should be in a hydrophobic environment. In MTAP with a C223Y substitution, strongly hydrophobic tyrosine interacts with water through its hydroxyl group. This interaction between Y223 and a water molecule may cause conformational change of the methylthioribose-binding site and decrease the substrate binding. Accordingly, C223Y may account for the partial MTAP deficiency in F clone. Since the MTAP mRNA level was remarkably low in H5 compared to WT and F, the complete deficiency in H5 may be a result of transcriptional abnormality in addition to the C223Y mutation. In order to thoroughly elucidate the molecular mechanism of the MTAP deficiency in these mutant clones, promoter analysis would be worthwhile, and is now under way.

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