Methylthioadenosine Phosphorylase, a Gene Frequently Codeleted with p16\(^{cdkN2a/ARF}\), Acts as a Tumor Suppressor in a Breast Cancer Cell Line

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ABSTRACT

The human methylthioadenosine phosphorylase (MTAP) gene is located on 9p21 and is frequently homozygously deleted, along with p16\(^{cdkN2a/ARF}\), in a wide variety of human tumors and human tumor-derived cell lines. The function of MTAP is to salvage methylthioadenosine, which is produced as a byproduct of polyamine metabolism. We have reintroduced MTAP into MCF-7 breast adenocarcinoma cells and have examined its effect on the tumorigenic properties of these cells. MTAP expression does not affect the growth rate of cells in standard tissue culture conditions but severely inhibits their ability to form colonies in soft agar or collagen. In addition, MTAP-expressing cells are suppressed for tumor formation when implanted into SCID mice. This suppression of anchorage-independent growth appears to be because of the enzymatic activity of MTAP, as a protein with a missense mutation in the active site does not exhibit this phenotype. MTAP expression causes a significant decrease in intracellular polyamine levels and alters the ratio of putrescine to total polyamines. Consistent with this observation, the polyamine biosynthesis inhibitor \(o\)-difluoromethylornithine inhibits the ability of MTAP-deficient cells to form colonies in soft agar, whereas addition of the polyamine putrescine stimulates colony formation in MTAP-expressing cells. These results indicate that MTAP has tumor suppressor activity and suggest that its effects may be mediated by altering intracellular polyamine pools.

INTRODUCTION

A quarter century ago, Toohey (1) first recognized that certain murine malignant hematopoietic cell lines lacked MTAP activity. MTAP is a key enzyme in the methionine salvage pathway (see Fig. 1). This pathway functions to salvage MTA, which is formed as a by-product of polyamine metabolism. Phosphorylation of MTA by MTAP results in the conversion of MTA into adenine and MTR1P. A series of reactions then salvages the methyl-thio group from MTR1P to form methionine. This pathway has been most extensively studied in Klebsiella pneumoniae (2–5), but has also been shown to exist in rat liver (6–8) and in Saccharomyces cerevisiae (9, 10). MTAP, the first enzyme in the pathway, is found in cells derived from a variety of different tissues and appears to be expressed in all normal human tissues (11, 12). These facts suggest that the entire salvage pathway is present in all of the cells in the human body.

Early studies on human leukemia cell lines revealed that loss of MTAP activity was relatively common (~30%; Refs. 13, 14). This loss does not appear to be an artifact of cell culture as primary leukemic cells also lack MTAP activity (15). These enzymatic studies also reported MTAP deficiency in some cell lines derived from solid tumors including melanoma and breast cancer (11, 16). These early studies were hampered by the lack of commercial availability of the radiochemical substrate. However, this problem was partially overcome with the development of polyclonal MTAP antiserum. Using this antibody, it was shown that loss of MTAP was a very common occurrence in glioblastomas and non-small cell lung cancer (17, 18). Because loss of MTAP expression in tumors appears to be mostly the result of homozygous deletions of the MTAP gene (see below), more recent studies have examined loss of MTAP DNA in various cancers. About one-third of all acute lymphoblastic leukemia patients have a homozygous deletion for the gene encoding MTAP (19, 20). Genetic studies show high rates of MTAP loss in non-small cell lung cancer, melanoma, bladder cancer, pancreatic, osteosarcoma, and endometrial cancer (21–24).

MTAP was initially mapped to human chromosome 9p, and later studies refined this region to 9p21–22 (see Fig. 1B; Refs. 25, 26). This region of the human genome is especially interesting because it is homozygously deleted in a variety of cancers, including gliomas, melanomas, pancreatic, non-small cell lung cancers, mesothelioma, and acute leukemias (26–28). These deletions are unusual in that the majority of them are quite large, eliminating multiple genes including MTAP. A detailed study of >500 primary tumors by Cairns et al. (29) found that almost all of the deletions remove a 170 kb region, which includes at least three transcripts: MTAP, CDKN2A, and p14\(^{ARF}\). CDKN2A (also known as p16\(^{ink4A}\)/MTS1/INK4) encodes p16, an inhibitor of the cyclin D-dependent kinase cdk4 (30). Germ line point mutations in CDKN2A are associated with familial multiple melanoma (31, 32), indicating that it is a tumor suppressor gene. The p14\(^{ARF}\) gene is an alternatively spliced transcript of CDKN2A (33). The first exon (1B) is not shared with CDKN2A, but exons two and three are read in an alternative reading frame. Thus, the p14\(^{ARF}\) protein is entirely unrelated to the p16 protein. Molecular studies have established that p14\(^{ARF}\) binds to and inactivates MDM2, preventing the degradation of p53 (34). Thus in the absence of p14\(^{ARF}\), p53 is destabilized, and unable to function in growth arrest and apoptosis.

The deletion of these two genes causes dysregulation of the two pathways, Rb and p53, that are thought to be important in most cancers, and has therefore led to the assumption that loss of MTAP activity is incidental and not of pathogenic consequence (35). However, there are several reasons to suspect that this is not the case. First, homozygous deletion is an unusual mechanism for inactivation of a tumor suppressor gene. Most tumor suppressor genes are inactivated by point mutation of one allele followed by loss of the other allele (LOH). This is rarely observed for CDKN2A (29). Why is this the case?

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2 Supported by United States Army Grant DAMD17-97-1-7707, USPHS Grant CA-22153, and Core Grant CA-06927 from the NIH, and by an appropriation from the Commonwealth of Pennsylvania.
3 The abbreviations used are: MTAP, methylthioadenosine phosphorylase; MTA, methylthioadenosine; MTR1P, methylthioribose-1-phosphate; ODC, ornithine decarboxylase; DFMO, \(o\)-difluoromethylornithine; LOH, loss of heterozygosity; BrdUrd, bromodeoxyuridine.

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non-small cell lung cancer that homozygous deletion of MTAP occurred in 38% (19 of 50) of samples compared with only 18% (9 of 50) for p16. In another report, it was found that 3 of 7 primary astrocytomas were deleted for MTAP, but only 2 of 7 were deleted for p16 (36). The fact that MTAP is lost independently of p16 hints that loss of MTAP may have some functional basis in tumor biology.

In this paper we examine the effect of reintroduction of MTAP to a MTAP-deleted tumor cell line. Our results indicate that MTAP acts as a tumor suppressor and may exert its effect through altering polyamine metabolism.

**MATERIALS AND METHODS**

**Plasmids and Site-directed Mutagenesis.** The plasmid pTRE2:MTAP was created by cloning the MTAP containing BamHI/EcoRV fragment from pCR: sMTAP (37) into pTRE2 (Clontech, Palo Alto, CA). The MTAP D220A mutant was created from pTRE2:MTAP by site-directed mutagenesis using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers used to engineer the mutation were: 5'-ATGGCGACAGCTTATGACTGC-3' and 5'-GCAAGTCTATAACGTTGCTGCCCCAT-3'. The plasmid pTRE2:MTAP was cotransfected in 16-gram athymic SCID mice implanted with 0.25 mg 21-day release s.c. β-estadiol pellets (Innovative Research of America, Sarasota, FL). Tumors were harvested and processed as described. MTAP activity was measured by measuring BrdUrd incorporation into cellular DNA using an enzyme immunoassay kit according to the manufacturer’s instructions (Roche Biomedical).

**Cell Line and Stable Transfectants.** MCF-7 Tet-off cells (Clontech) were cultured in Clontech-recommended medium using heat-inactivated fetal bovine serum and supplemented with 250 μg/ml G418. Transfections were performed using Fugene reagent (Roche, Indianapolis, IN), a nonliposomal transfection agent according to the manufacturer’s instructions. A total of 3 μg of plasmid DNA in a 10:1 ratio of pTK-Hyg to pTRE2-MTAP was used for each transfection. Clones were selected using 250 μg/ml G418 and were used in all subsequent experiments. In one experiment, clones were grown either in the presence or absence of doxycycline for 48 h. In another experiment, clones were grown for 7 days in the presence of doxycycline and Western analysis was performed using an Agilent 8453 multidiode array spectrophotometer at 37°C with ceuvettes containing a total volume of 1 ml of 40 mM potassium phosphate (pH 7.4), 0.8 units of xanthine oxidase, 0.5 mM MTA, and crude cell extract (100–250 μg protein). Before the addition of xanthine oxidase, the reaction was preincubated to remove trace contaminants of adenine. Adenine release rates were linear with protein concentrations up to 250 μg protein per assay well. The enzyme yields from the reaction were measured by the formation of 1 μmol of TTP/min at 37°C. Cell pellets with 0.6 N perchloric acid, dansylated, and measured by reverse-phase HPLC/MS/MS.

**Western Analysis and Enzyme Assays.** Cell lysates were prepared by three cycles of freeze-thawing at −80°C in 20 mM KH2PO4 (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (Sigma) and 1 mM DTT (Sigma). Cell lysates were suspended in 1× SDS sample buffer and separated by SDS-PAGE. Proteins were then transferred onto an Immobilon P membrane (Millipore Corp., Bedford, MA). The primary antibody used in this study was MTAP antibody produced from chicken yolk (from the laboratory of Dennis Carson, University of San Diego, San Diego, CA).

MTA phosphorylase activity was measured by a modification of a method described previously by Savarese et al. (38). In this assay the liberation of adenine by MTAP is monitored by its conversion to 8-dihydroxyadenine by xanthine oxidase. This results in an increased absorbance at 305 nm. Assays were performed using an Agilent 8453 multidiode array spectrophotometer at 37°C, with cuvettes containing a total volume of 1 ml of 40 mM potassium phosphate (pH 7.4), 0.8 units of xanthine oxidase, 0.5 mM MTA, and crude cell extracts (100–250 μg protein). Before the addition of xanthine oxidase, the reaction was preincubated to remove trace contaminants. Adenine release rates were linear with protein concentrations up to 250 μg protein per assay well. The enzyme yields from the reaction were measured by the formation of 1 μmol of adenine/min at 37°C. Cell pellets with 0.6 N perchloric acid, dansylated, and measured by reverse-phase high-performance liquid chromatography as described.

**RESULTS**

It was shown previously by our group that MCF-7 cells lack MTAP activity and are deleted for markers in 9p21 (37). We transfected MCF-7 cells with a construct that would express MTAP under a Tet-repressible promoter and isolated several MTAP expressing clones (Fig. 2A; Table 1). These clones expressed MTAP at levels that were similar to a control fibroblast cell line. When the tetracycline analogue, doxycycline, was added, two of the lines showed a decrease in MTAP activity.
in enzyme levels and activity, but MTAP was still expressed at detectable levels. We also transfected a construct expressing the luciferase gene as a negative control.

We compared the growth rates in standard tissue culture conditions (anchorage-dependent growth) of three MTAP-expressing and nonexpressing subclones using BrdUrd labeling. We observed no significant difference in BrdUrd uptake between MTAP-expressing and nonexpressing cells (Fig. 3). We next tested the ability of MTAP-expressing and nonexpressing cells to exhibit anchorage-independent growth by examining their ability to form colonies in soft agar (Table 2). Approximately 20% of the cells from the three MTAP+ control lines formed colonies on soft agar, whereas we observed almost no colonies forming from three different MTAP− lines. The MTAP-expressing lines also failed to form colonies when doxycycline was added to the medium, indicating that low levels of MTAP were sufficient to suppress anchorage-independent growth. These experiments show that MTAP suppresses anchorage-independent growth but does not affect anchorage-dependent growth.

We next tested whether MTAP enzymatic activity was necessary for suppression of anchorage-independent growth or whether the MTAP protein might have some additional nonenzymatic function. On the basis of the crystal structure of human MTAP, aspartate 220 is hypothesized to play a key role in the catalytic mechanism (41). Therefore, we mutated this residue to alanine (D220A) and isolated a MTAP protein that is catalytically inactive.

MTAP enzymatic activity was determined as described in Materials and Methods. The 10,000 cells were plated on 60-mm dishes in soft agar, and colony formation was assessed after 21 days by screening under a microscope with ×40 magnification. A grid pattern was placed in the back of each plate to aid in the counting process. The rows below the dark line are from a separate experiment.

Table 2. Suppression of colony formation by MTAP on soft agar

<table>
<thead>
<tr>
<th>Cells</th>
<th># Plated</th>
<th># Colonies</th>
<th>% Colony formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7 parent</td>
<td>10,000</td>
<td>2118</td>
<td>21.2</td>
</tr>
<tr>
<td>Luc1</td>
<td>10,000</td>
<td>1979</td>
<td>19.8</td>
</tr>
<tr>
<td>Luc2</td>
<td>10,000</td>
<td>2214</td>
<td>22.1</td>
</tr>
<tr>
<td>MTAP1</td>
<td>10,000</td>
<td>15</td>
<td>0.15</td>
</tr>
<tr>
<td>MTAP2</td>
<td>10,000</td>
<td>13</td>
<td>0.13</td>
</tr>
<tr>
<td>MTAP4</td>
<td>10,000</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MTAP4 + Dox</td>
<td>10,000</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MTAP8</td>
<td>10,000</td>
<td>2</td>
<td>0.2</td>
</tr>
<tr>
<td>MTAP8 + Dox</td>
<td>10,000</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>WS1 (fibroblast)</td>
<td>10,000</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MTAP4</td>
<td>3610</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MTAP8</td>
<td>3020</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MTAP9</td>
<td>3660</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D220A4</td>
<td>2410</td>
<td>214</td>
<td>8.9</td>
</tr>
<tr>
<td>D220A5</td>
<td>4000</td>
<td>502</td>
<td>12.6</td>
</tr>
<tr>
<td>D220A6</td>
<td>1670</td>
<td>181</td>
<td>11.6</td>
</tr>
</tbody>
</table>

The D220A mutant cell lines were next tested for their ability to form colonies on soft agar (Table 2) and on collagen (Fig. 4). All three of the cell lines were able to form colonies in both soft agar and collagen, whereas the MTAP-expressing cells were not. In addition, we tested the ability of three MTAP-expressing and two D220A-expressing cell lines to form tumors in SCID mice (Fig. 5). After 30 days, measurable tumors appeared on the mice implanted with the D220A-expressing cells, and these tumors grew rapidly until the mice had to be sacrificed. No measurable tumors appeared in animals injected with the MTAP-expressing cells. These results show that MTAP enzymatic activity is required for the suppression of anchorage-dependent growth and that MTAP can suppress tumor formation in vivo.

Cells lacking MTAP would be expected to build up the polyamine byproduct MTA (Fig. 1). MTA has been shown in vitro to be an inhibitor of spermine synthase and spermidine synthase, two key enzymes in polyamine biosynthesis. Inhibition of these enzymes would be expected to cause an increase in putrescine inside MTAP-deficient cells. Therefore, we tested the hypothesis that MTAP expression might affect polyamine levels and distribution. We measured intracellular putrescine, spermidine, and spermine levels in three D220A and three MTAP-expressing cell lines (Table 3). As expected putrescine levels were elevated in D220A cells relative to MTAP-expressing cells (3285 versus 571; P < 0.00001). However, unexpectedly we found that spermine and spermidine levels were also significantly elevated (3225 versus 1126; P < 0.00001), although the ratio of putrescine to the total polyamine pools was still significantly different between the MTAP+ and MTAP− cells (0.19 versus 0.32; P < 0.001). These findings show that cells lacking MTAP have increased polyamine pools and suggests that there may be feedback regulation of polyamine biosynthesis by a downstream product in the salvage pathway.

We next tested the effects of putrescine (2 mM) and DFMO (50 μM) on anchorage-independent growth of three MTAP+ and three MTAP− cell lines (Fig. 4; Table 4). DFMO is an inhibitor of ODC and has been shown to cause depletion of intracellular polyamines (42). At the concentrations used there was no effect on growth rate of either MTAP+ or MTAP− cells as judged by BrdUrd uptake in standard tissue culture conditions (anchorage-dependent growth; data not shown). However, DFMO severely inhibited colony formation MTAP− cells on soft agar, and this inhibition could be reversed by the addition of putrescine. Putrescine alone did not appear to have any additional effect on colony formation in the MTAP− cells. Addition
of putrescine to MTAP− cells resulted in the stimulation of colony formation and anchorage-independent growth. Taken together, these experiments demonstrate that elevated putrescine is required for anchorage-independent growth and are consistent with the hypothesis that at least some of the tumorigenic effects of MTAP are because of its influence on polyamine levels.

**DISCUSSION**

In this report we demonstrate that when MTAP is expressed in MTAP-deficient MCF-7 cells, it results in a suppression of anchorage-independent growth in vitro and tumor formation in vivo. We saw no effect of MTAP expression on growth rate under standard tissue culture conditions, indicating that this effect is not a general effect on cell-cycle, but rather appears to be specific to growth in an anchorage-independent environment. This phenomenon is unusual, as most cell cycle-related tumor suppressor genes affect growth under both conditions (43–45). However, our observation is not unprecedented, because certain oncogenes, such as Rho and Bcr-Abl, allow anchorage-independent growth but have limited effect on cell proliferation (46).

On the basis of our data, MTAP is the third protein identified in the 9p21–22 region with tumor suppressor activity. The two other confirmed suppressor proteins are p16 and p14ARF. Each protein appears to affect different cellular functions important in transformation: p16 affecting cell-cycle, p14ARF affecting apoptosis, and MTAP affecting anchorage dependence. Our findings may help explain why homozygous deletions are so common in this chromosomal region in tumors. Most tumor suppressor genes are inactivated by mechanisms involving point mutation of one copy followed by LOH of the other allele. The 9p21–22 region is unusual because LOH is relatively rare, but homozygous deletion is not. To inactivate these genes by point mutation would be difficult, but all of the three genes could be inactivated by a large deletion event. Consistent with this idea, a large mapping study of 545 primary tumors shows that tumors containing homozygous deletions of 9p21 have a minimal 170-kb region deleted that includes both MTAP and p16 (29).

Our data suggest that MTAP may suppress anchorage-independent growth via its effect on polyamine metabolism. We found that MCF-7 cell lines expressing MTAP had significantly lower levels of all three major polyamines and had a reduced ratio of putrescine to total polyamines. We also found that addition of putrescine to MTAP-expressing MCF-7 cells stimulated their ability to grow on soft agar. Conversely, the ODC inhibitor, DFMO, inhibited anchorage-independent growth of MTAP− cells. These results suggest that elevated levels of polyamines, especially putrescine, are correlated with anchorage-independent growth.

Another way to elevate polyamine pools is to overexpress ODC.

**Table 3 Polyamine levels in MTAP− and MTAP+ cells**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Putrescine (μmoles/106 cells)</th>
<th>Spermidine (μmoles/106 cells)</th>
<th>Spermine (μmoles/106 cells)</th>
<th>Putrescine/total polyamines</th>
</tr>
</thead>
<tbody>
<tr>
<td>D220A5 (MTAP−)</td>
<td>4.0</td>
<td>4.8</td>
<td>4.1</td>
<td>0.31</td>
</tr>
<tr>
<td>D220A5 (MTAP+)</td>
<td>2.9</td>
<td>3.5</td>
<td>2.7</td>
<td>0.31</td>
</tr>
<tr>
<td>D220A6 (MTAP−)</td>
<td>3.0</td>
<td>3.3</td>
<td>2.9</td>
<td>0.33</td>
</tr>
<tr>
<td>MTAP+       /Avg.</td>
<td>3.3</td>
<td>3.9</td>
<td>3.2</td>
<td>0.32</td>
</tr>
<tr>
<td>MTAP4</td>
<td>0.7</td>
<td>1.7</td>
<td>0.9</td>
<td>0.21</td>
</tr>
<tr>
<td>MTAP8</td>
<td>0.4</td>
<td>1.1</td>
<td>1.2</td>
<td>0.16</td>
</tr>
<tr>
<td>MTAP9</td>
<td>0.6</td>
<td>1.6</td>
<td>1.3</td>
<td>0.21</td>
</tr>
<tr>
<td>MTAP+       Avg.</td>
<td>0.6</td>
<td>1.5</td>
<td>1.1</td>
<td>0.19</td>
</tr>
</tbody>
</table>

**Table 4 Effect of putrescine and DFMO on colony formation**

<table>
<thead>
<tr>
<th>Cell line + treatment</th>
<th>Colonies formed</th>
<th>% Colonies/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7 + none</td>
<td>2198</td>
<td>22</td>
</tr>
<tr>
<td>MCF7 + DFMO</td>
<td>221</td>
<td>2.2</td>
</tr>
<tr>
<td>MCF7 + PUT</td>
<td>2126</td>
<td>21</td>
</tr>
<tr>
<td>MTA4 + none</td>
<td>91</td>
<td>0.9</td>
</tr>
<tr>
<td>MTA4 + DFMO</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MTA4 + PUT</td>
<td>397</td>
<td>4.0</td>
</tr>
<tr>
<td>MTA8 + none</td>
<td>77</td>
<td>0.77</td>
</tr>
<tr>
<td>MTA8 + DFMO</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MTA8 + PUT</td>
<td>458</td>
<td>4.58</td>
</tr>
</tbody>
</table>
ODC activity is up-regulated frequently in a variety of animal and human tumors. Several groups have shown that overexpression of ODC in nontransformed fibroblasts results in transformation and anchorage-independent growth (47–49). In addition, transgenic mice overexpressing ODC have increased frequency of spontaneous skin tumors and are more susceptible to tumors induced by mutagens (50, 51). Taken together, these observations suggest that elevated polyamine levels are tumorigenic. The mechanism by which polyamines promote tumorigenicity is not well understood, but polyamines can stimulate a variety of cellular processes and enzymes including those involved in transcription, translation, and signal transduction (52).

Our findings may also have clinical significance. DFMO is currently undergoing clinical trials for use as an adjunct therapy for a number of different cancers (53, 54). Our data suggest that MTAP–tumors may be particularly sensitive to DFMO. We found that DFMO inhibited anchorage-independent growth at concentrations that had no effect on growth rates in standard tissue culture conditions. It may be worthwhile stratifying the ongoing trials for MTAP status of the primary tumor to determine whether DFMO treatment might be more effective in MTAP-deleted tumors.

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