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Expression of Methylthioadenosine Phosphorylase cDNA in *p16*$^{-}$, *MTAP*$^{-}$ Malignant Cells: Restoration of Methylthioadenosine Phosphorylase-Dependent Salvage Pathways and Alterations of Sensitivity to Inhibitors of Purine *de novo* Synthesis

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SUMMARY

5′-Deoxy-5′-methylthioadenosine phosphorylase (MTAP) is involved in the salvage of adenine and methylthio moieties of 5′-deoxy-5′-methylthioadenosine, a byproduct of polyamine synthesis, to adenine nucleotides and methionine, respectively. The gene encoding MTAP, *MTAP*, is frequently codeleted along with the tumor suppressor gene *p16* in malignant cells bearing homozygous deletions in the chromosome 9p21 region. *p16*$^{-}$, *MTAP*$^{-}$ malignant cells have been shown to be more susceptible to the purine *de novo* inhibitory actions of antifolates such as methotrexate than are *p16*$^{+}$, *MTAP*$^{+}$ cells. To understand the underlying mechanism, we reintroduced MTAP activity into two *p16*$^{-}$, *MTAP*$^{-}$ cell model systems, the MiaPaCa-2 and PANC-1 human pancreatic carcinoma cell lines, by transfection with *MTAP* cDNA. It was found that transfection with *MTAP* cDNA (i) restored both the MTAP-dependent adenine and methionine salvage pathways, (ii) decreased the rates of purine *de novo* synthesis (18–47% lower than the wild-type or sham-transfected counterparts), and (iii) decreased cellular sensitivity to the antipurine-related growth-inhibitory actions of methotrexate and azaserine. These data support the hypothesis that operation of the MTAP-dependent adenine salvage pathway renders *MTAP*$^{+}$ cells less dependent on *de novo* purine synthesis and hence less susceptible than *MTAP*$^{-}$ malignant cells to the growth-inhibitory actions of agents (e.g. antifolates) whose mechanism of action in part involves the *de novo* purine pathway. These findings provide a theoretical basis for the relatively selective action certain antifolates may have against MTAP-deficient malignancies.

MTAP (EC 24.2.28) catalyzes the phosphorolysis of the nucleoside MTA, a metabolite of SAM produced during the synthesis of the polyamines spermidine and spermine, to yield adenine and 5-methylthioribose-1-phosphate (1). Each of the products of the enzyme are reused: adenine is converted to adenine nucleotide pools via adenine phosphoribosyltransferase (2), and 5-methylthioribose-1-phosphate is metabolized to methionine and formate (3, 4). Thus, MTAP plays a crucial role in initiating the recycling the adenine and methylthio moieties of SAM back to the metabolites from which SAM is formed (i.e., ATP and methionine) (5). These salvage pathways are clearly active in that MTA can, in MTAP-containing cells, serve as a purine source in cells treated with purine *de novo* synthesis inhibitors (6–8) and as the sole methionine source for cells cultured in methionine-deficient media (9). Although virtually all normal tissues, including red blood cells, contain MTAP activity, many malignant cells lack MTAP activity (5, 6, 10–14). Cultured MTAP-deficient malignant cells do not metabolize MTA but instead simply excrete it (15).

The explanation for the selective loss of MTAP activity in malignant cells took a number of years to unravel. Seminal studies led by Diaz and Rowley (16) revealed that whole or portions of the *IFN* gene cluster undergoes frequent homozygous deletion in leukemia cell lines and certain primary leukemias and that in many cases, these deletions are associated with loss of MTAP activity (16). These findings suggested that the *MTAP* gene was located near the *INF* cluster,

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ABBREVIATIONS: MTAP, 5′-deoxy-5′-methylthioadenosine phosphorylase; 5′-dFAdo, 5′-deoxy-2-fluoroadenosine; FGAR, *N*-formylglycinamide ribonucleotide; FPGS, folylpolyglutamyl synthase; GARFT, glycinamide ribonucleotide transformylase; INF, interferon; MTA, 5′-deoxy-5′-methylthioadenosine; *MTAP*, the gene encoding 5′-deoxy-5′-methylthioadenosine phosphorylase; MTX, methotrexate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SAM, *S*-adenosylmethionine; DMEM, Dulbecco's modified Eagle's medium.

which had been mapped to the chromosome 9p21 region (17), and that loss of MTAP activity might be the result of a homozygous deletion of the gene. Deletion mapping studies using cell lines with chromosome 9p21 defects demonstrated that the minimal region of overlap of these deletions resides centromerically from the *IFN* cluster, at a site close to the suspected *MTAP* locus (18). Subsequently, Kamb *et al.* (19) used chromosome walking techniques to map and sequence this deleted region and discovered it contained the genes encoding $p16^{INK4}$ and $p15^{INK4B}$ (*p16/MTS-1* and *p15/MTS-2*, respectively), which, as inhibitors of cyclin-dependent kinase-4 and -6, are thought to have tumor suppressor activity (19–21). Homozygous deletion of *p16* has now been reported in subsets of a variety of human malignancies (22). Detailed mapping of this portion of the 9p21 region revealed that *MTAP* resides only ~100 kb in the telomeric direction from *p16* (23), accounting for the high incidence of codeletion of *MTAP* (>85%) in malignant cells bearing *p16* deletions (23, 24).

It has been suggested that the selective loss of *MTAP* in malignant cells might be exploited chemotherapeutically (6, 14). Exogenous MTA can be used *in vitro* as a purine source to selectively rescue MTAP-containing malignant cells but not MTAP-deficient malignant cells from the antipurine actions of MTX and related antifolates (6, 14). However, the presence of MTAP in the serum of many mammals, including humans (25), might limit the effectiveness of this strategy *in vivo*. Recently, we demonstrated that the antipurine action of MTX is more pronounced against $p16^-$, $MTAP^-$ pancreatic carcinoma cells than normal $p16^+$, $MTAP^+$ epithelial cells; it was suggested that the operation of the MTAP-dependent adenine salvage pathway in normal cells might render such cells less sensitive to antifolates whose mechanism of action involves inhibition of purine *de novo* synthesis (7). This same conclusion was reached in a study on a non-small cell lung carcinoma cell line transfected with *MTAP* cDNA (8).

To help determine the mechanism by which the MTAP-dependent salvage pathways might alter cell physiology and responsiveness to chemotherapeutic agents, we recently developed cell model systems in which *MTAP* cDNA was transfected and expressed in two human pancreatic carcinoma cell lines that are naturally MTAP deficient via homozygous deletion. Here, these model systems are used to determine whether reintroduction of MTAP activity in these deficient cell lines (i) restores the MTAP-dependent adenine and methionine salvage pathways, (ii) alters the rate of purine *de novo* synthesis, and (iii) decreases the sensitivity of these cells to the antiproliferative actions of agents that act as inhibitors of purine *de novo* synthesis.

Experimental Procedures

Materials. MTA, MTX, MTT, thymidine, and azaserine were purchased from Sigma Chemical (St. Louis, MO). [8-^{14}C]MTA (55 mCi/mmol) was obtained from Moravek Biochemicals (Brea, CA). [(U)-^{14}C]Glycine (106 mCi/mmol) was obtained from New England Nuclear Research Products (Boston, MA).

Cell culture and growth studies. The MiaPaCa-2 and PANC-1 wild-type lines were originally obtained from American Type Culture Collection (Rockville, MD). These cells were routinely cultured in DMEM supplemented with 50 units/ml penicillin G, 50 μg/ml streptomycin, 0.5 μg/ml Fungizone, 1 mM sodium pyruvate, and 10% fetal calf serum. Transfection of these cells with vectors containing the *neo* and/or *MTAP* sequences and selection of cell clones expressing these genes will be described elsewhere.[1] Cell lines expressing the *neo* genes, MiaPaCa-2/*neo* and PANC-1/*neo*, were grown in the above-mentioned DMEM medium containing 0.25 mg/ml geneticin (G418). Some MTAP-transfected cell lines, MiaPaCa-2/*MTAP*-AzG and PANC-1/*MTAP*-Az, were maintained in the the above-mentioned DMEM medium, except fetal calf serum was replaced with 10% donor horse serum and the medium was supplemented with 10 μM azaserine and 10 μM MTA. These conditions select for MTAP-expressing cells, which are able to use MTA as a purine source in the presence of the inhibitor of *de novo* purine biosynthesis, azaserine. Another set of MTAP-transfected cell lines, MiaPaCa-2/*MTAP*-G and PANC-1/*MTAP*-G, were cultured in methionine-deficient DMEM medium supplemented with 50 units/ml penicillin G, 50 μg/ml streptomycin, 0.5 μg/ml Fungizone, 1 mM sodium pyruvate, 1 mM glutamine, 3% donor horse serum, and 15 μM MTA. These conditions select for MTAP-expressing cells, which are able to grow using MTA as their sole methionine source.

For cell growth studies, cultured cells were harvested with trypsin-EDTA, washed twice in DMEM medium containing various antibiotics and 10% horse serum, and plated onto 12-well dishes (20,000 cells/well) in this medium. After 4–6 hr (to allow for cell attachment), the medium was replaced with horse serum-supplemented DMEM containing various concentrations of drugs or drug combinations. Unless indicated otherwise, the cells were incubated under these conditions for either 4 days (in experiments using MiaPaCa-2-derived cell lines) or 7 days (in experiments using PANC-1-derived cell lines). Cell numbers were quantified using the MTT-based colorimetric test (7).

Assessment of 9p21 markers and MTAP activity. All 9p21 markers except MTAP were assessed by polymerase chain reaction amplification of genomic DNA, as described previously(7, 24). MTAP activity was determined using the radiochemical assay reported previously (24).

Incorporation of [^{14}C]MTA into purine nucleotides pools. Control or *MTAP*-transfected cell lines were harvested in trypsin-EDTA and washed in phosphate-buffered saline, and 6×10^6 cells were plated onto 100-mm dishes in 6 ml of DMEM and 10% horse serum medium containing 5 μM [8-^{14}C]MTA (0.15 μCi/dish). After a 6-hr period at 37° in a humidified 95% air/5% CO_2 incubator, the cells were harvested in trypsin-EDTA and pelleted, and the supernatant was removed. Nucleotides were extracted by the addition of 100 μl of 6% perchloric acid onto the cell pellet; the samples were then vortexed and placed on ice for 10 min. The extracts are brought to neutral pH with 8 N KOH and centrifuged to remove perchlorate salts, and 50 μl of the supernatant was analyzed for incorporation into purine nucleotide pools using a modification of a previously described anion-exchange high performance liquid chromatography technique (26). A Waters model 510 liquid chromatograph equipped with a Whatman (Clifton, NJ) Partisil 5 SAX column (25 cm) was used, and the purine nucleotides were separated using a programmed gradient of 1 mM potassium phosphate, pH 4.5, as the low concentrate eluent, and 500 mM potassium phosphate, pH 4.5, as the high concentrate eluent. The gradient profile consisted of a linear increase of high concentrate eluent of 0–100% over a 40-min period, followed by a 20-min isocratic period at 100% high concentrate, at a flow rate of 2 ml/min. Absorbance was monitored at 259 nm, and fractions were collected at 1-min intervals. Each fraction was added to 10 ml of EcoScint A (National Diagnostics, Atlanta, GA) and counted in a liquid scintillation counter (model LS 6500; Beckman Instruments, Columbia, MD). Authentic adenosine and guanosine nucleotide standards (Sigma Chemical) were used to identify peaks based on retention times.

Rates of *de novo* purine biosynthesis. The rates of purine *de novo* biosynthesis in these cell lines were determined using a modification of a previously reported method (27). Here, *de novo* purine

[1] Z.-H. Chen, unpublished observations.

TABLE 1

Pattern of homozygous deletion of chromosome 9p21 markers in MTAP-transfected and control cell lines

All markers except MTAP were assessed by PCR amplification of genomic DNA, as described previously (24). Doubling time determined using DMEM containing 10% fetal bovine serum. MTAP was assessed using a previously described radiochemical assay (24). Enzyme activity, expressed as nmol of adenine formed/min/mg of protein, is given in parentheses (mean ± standard deviation of at least two determinations).-, MTAP activity in the cell extract was <0.01 nmol of adenine formed/min/mg of protien.

Cell line	Doubling time	*IFNB1*	*IFNA1*	*MTAP*	*p16 3′*	*p15 Exon 2*	*D9S171*	*D9S169*
	hr							
MiaPaCa-2 WT	23.9	−	−	−	−	−	+	+
MiaPaCa-2/neo	20.7	−	−	−	−	−	+	+
MiaPaCa-2/MTAP-AzG	26.5	−	−	0.30 ± 0.04	−	−	+	+
MiaPaCa-2/MTAP-G	24.2	−	−	0.21 ± 0.09	−	−	+	+
PANC-1 WT	31.9	+	+	−	−	−	+	+
PANC-1/neo	33.0	+	+	−	−	−	+	+
PANC-1/MTAP-Az	34.4	+	+	0.20 ± 0.10	−	−	+	+
PANC-1/MTAP-G	24.7	+	+	0.11 ± 0.02	−	−	+	+

+, Expected PCR product for the particular marker was observed, indicating the presence of at least one allele of the DNA segment in question. −, No PCR product was observed, indicating a homozygous deletion.

synthesis rates are assayed by measuring the incorporation of [(U)-^{14}C]glycine into FGAR in the presence of azaserine, which inhibits the further metabolism of FGAR. Cultured cells were harvested in trypsin-EDTA and washed twice in glycine-deficient RPMI 1640 medium containing 10% horse serum, and a cell suspension containing 4×10^6 cells in this medium was placed in a 37° shaking water bath. After a 15-min period to allow temperature equilibration, azaserine was added to final concentration of 0.6 mM and incubated for 15 min. At this time, glutamine was added to a final concentration of 2 mM, along with 5 μCi of [(U)-^{14}C]glycine (106.8 mCi/mmol), and the cell suspension was brought up to a total volume of 1 ml. After 3 hr of incubation at 37°, the cells were pelleted by centrifugation at 4°, and the supernatant fluid was removed. The cell pellet was washed once with cold 2 ml of glycine-free medium, and the washed pellet was extracted in 1 ml of 6% ice-cold perchloric acid. After 15 min of incubation on ice, the extract was alkalinized with 2 N KOH. After centrifugation to remove percholate salts, 1.3 ml of the neutralized supernatant was applied to a column of AG-1 × 8 anion-exchange resin (200–400 mesh, BioRad). Columns were washed with 20 ml of 0.5 M formic acid, and labeled FGAR was eluted with 15 ml of 4 M formic acid collected in three 5-ml aliquots. Fifteen milliliters of scintillant (EcoScint A; National Diagnostics, Atlanta, GA) was added to each aliquot and counted in a Beckman LS 6500 Scintillation System.

Results

Expression of MTAP cDNA in *p16*$^-$, *MTAP*$^-$ malignant cell lines. To develop model systems for studying the influence the MTAP-dependent salvage pathways have on cell physiology and drug sensitivity, *MTAP* cDNA was subcloned into the pSVL mammalian expression vector and transfected into two human pancreatic carcinoma cell lines, MiaPaCa-2 and PANC-1, both of which contain homozygous deletions of the *p16* and *MTAP* genes, as well as other other loci in the chromosome 9p21 region (7). As will be described elsewhere,[1] selection of MTAP-expressing cell clones were carried out using three methods: (i) selection based on ability to grow in the presence of the purine *de novo* inhibitor azaserine, with MTA used as a purine source; clones selected in this way were given the suffix *MTAP*-Az; (ii) after cotransfection with *MTAP* and *neo*- containing vectors, selection based on the ability to grow in medium containing G418, azaserine, and MTA; clones selected by this method were labeled *MTAP*-AzG; and (iii) after cotransfection with *MTAP* and *neo*-containing vectors, selection based initially on G418 resistance, followed by selection in methionine-deficient medium containing MTA as the sole source of this amino acid; these clones were labeled with the suffix, *MTAP*-G. Cell clones selected in this manner, PANC-1/*MTAP*-Az, PANC-1/*MTAP*-G, MiaPaCa-2/*MTAP*-AzG, and MiaPaCa-2/*MTAP*-G, but not the corresponding *neo*-transfected or wild-type cells, expressed MTAP because extracts of these cell clones were capable of converting [8-^{14}C]MTA to [8-^{14}C]adenine and contained MTAP mRNA.[2] The MTAP activities in these *MTAP*-transfected cell lines were 0.1–0.3 nmol/min/mg of protein (Table 1), which is at the low to middle end of the range of MTAP activity found in a survey of malignant cell lines (24). These *MTAP*-transfected lines as well as their *neo*-transfected counterparts display the same pattern of homozygous deletion of chromosome 9p21 markers as the parental (wild-type) lines, verifying their pedigrees and confirming that transfection has not restored any of the other deleted loci (Table 1). Finally, the doubling times of these transfected cell lines were found to be generally comparable to, or slightly longer than, those of the parental, wild-type cell lines (Table 1). Thus, these panels of cell lines are suitable as model systems to determine the effect of MTAP activity on cell physiology and drug sensitivity.

Expression of MTAP cDNA in *p16*$^-$, *MTAP*$^-$ malignant cell lines: restoration of MTAP-dependent salvage pathways. Our initial question was whether the expression of MTAP activity in these malignant cells bearing homozygous deletions of the *MTAP* locus would restore the salvage pathways that depend on this phosphorylase. If transfection and expression of MTAP restored the operation of the MTAP-dependent adenine salvage pathway in these cell clones, then they should be able to convert exogenous [8-^{14}C]MTA into their purine nucleotide pools. To test this, each of these MTAP-transfected cell lines and their sham-transfected counterparts were incubated for 6 hr in the presence of [8-^{14}C]MTA, the cells were extracted, and the nucleotide pools were analyzed by anion-exchange high performance liquid chromatography. As shown in Fig. 1A, cell lines in which the expression of MTAP was restored by transfection (e.g., PANC-1/*MTAP*-G and MiaPaCa-2/*MTAP*-AG) incorporate labeled MTA into both adenine and guanine nucleotide pools, whereas the corresponding cells transfected

[2] Z.-H. Chen, unpublished observations.

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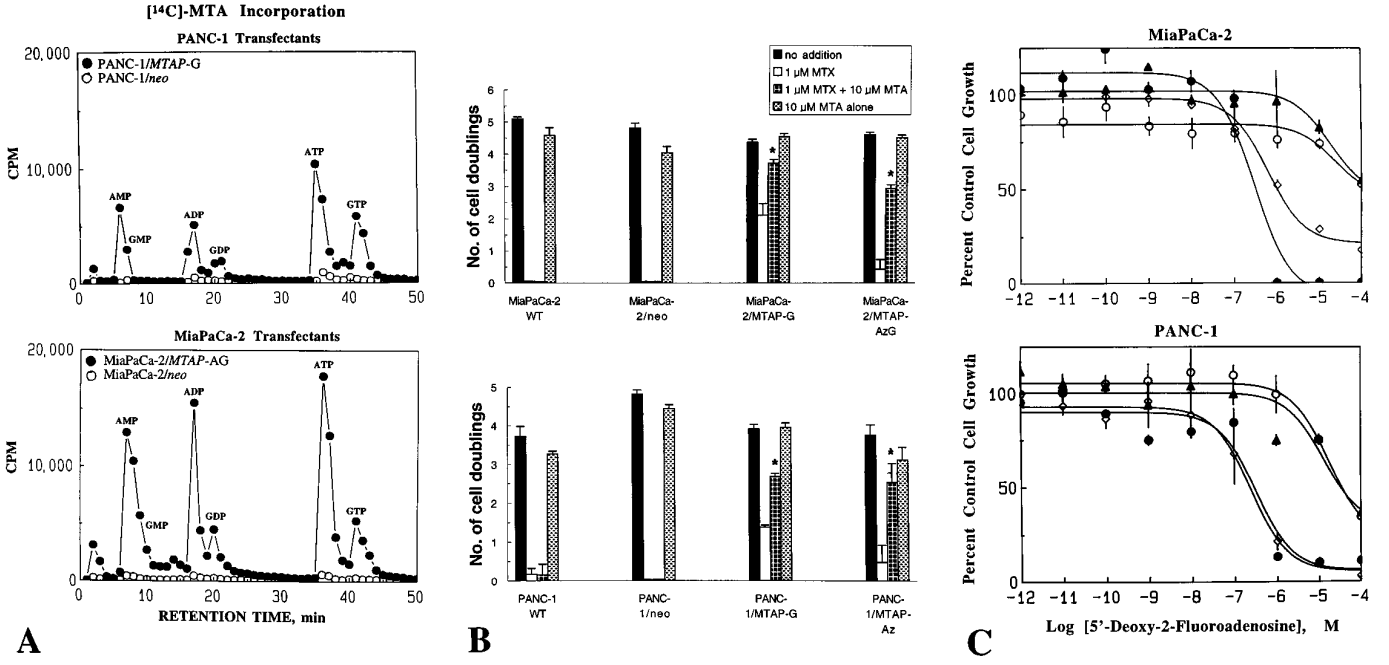

Fig. 1. Evidence for the restoration of the MTAP-dependent adenine salvage pathway in MTAP-deficient MiaPaCa-2 and PANC-1 human pancreatic carcinoma cell lines transfected with *MTAP* cDNA. A, Incorporation of [8-^{14}C]MTA into the purine nucleotide pools of *MTAP*-transfected and control cell lines derived from the PANC-1 (*top*) and MiaPaCa-2 (*bottom*) human pancreatic carcinomas. Cells were incubated with [8-^{14}C]MTA, cell extracts were obtained, purine nucleotides were separated by high performance liquid chromatography, and counts were assessed by liquid scintillation counting as described in the text. Authentic adenosine and guanosine nucleotide standards (Sigma Chemical) were used to identify purine nucleotide peaks on the basis of their retention times. B, Ability of *MTAP*-transfected and control MiaPaCa-2 and PANC-1 cell lines to grow using MTA as a purine source in the face of a blockade of purine *de novo* synthesis by MTX. Cells were plated onto 12-well dishes (20,000 cells/well) in DMEM supplemented with 10% horse serum, 20 μM thymidine, and with or without 1 μM MTX and/or 10 μM MTA. Cells were incubated at 37° in a 95% air/5% CO_2 atmosphere for either 5 days (MiaPaCa-2-derived cell lines) or 7 days (PANC-1-derived cell lines), and the cell number was determined using the MTT-based assay. Values are mean ± standard deviation of four independent determinations. *, Data are significantly different from samples treated with 1 μM MTX alone using a two-tailed paired *t* test ($p < 0.05$). C, Dose-response curves for the effect of the adenine-substituted MTA analog 5′-dFAdo on the growth of *MTAP*-transfected and control cells derived from the MiaPaCa-2 (*top*) and PANC-1 (*bottom*) human pancreatic carcinoma cell lines. Cells (20,000/well) were plated in DMEM containing 10% donor horse serum with the indicated concentration of 5′-dFAdo and incubated at 37° for 7 days. Cell number was determined using the MTT-based assay system (see Experimental Procedures). Values are mean ± standard deviation of three independent determinations. *Top*, MiaPaCa-2 wild-type, ○ (control cell doublings, 5.91 ± 0.03); MiaPaCa-2/*neo*, ▲ (control cell doublings, 5.52 ± 0.13); MiaPaCa-2/*MTAP*-AG, ● (control cell doublings, 4.55 ± 0.14); and MiaPaCa-2/*MTAP*-G, ◇ (control cell doublings, 4.40 ± 0.10). *Bottom*, PANC-1 wild-type, ○ (control cell doublings, 4.05 ± 0.20); PANC-1/*neo*, ▲ (control cell doublings, 3.91 ± 0.22); PANC-1/*MTAP*-Az, ● (control cell doublings, 3.08 ± 0.15); and PANC-1/*MTAP*-G, ◇ (control cell doublings, 4.49 ± 0.10).

with *neo*-containing vectors alone show virtually no incorporation. Additional evidence for restoration of the MTAP-dependent adenine salvage pathway is based on the fact that the *MTAP*-transfected cell lines, but not the sham-transfected or wild-type cell lines, are able to grow using MTA as a purine source in the face of a blockade of purine *de novo* synthesis effected by high concentrations (10^{-6} M) of MTX, in the presence of thymidine (Fig. 1B). The responsiveness of MTAP-transfected cell lines to the MTA analog 5′-dFAdo is further evidence for restoration of the adenine pathway. 5′-dFAdo is an alternative substrate of MTAP; the product of this reaction, 2-fluoroadenine, is converted to cytotoxic 2-fluoroadenosine-containing nucleotides via adenine phosphoribosyltransferase and the enzymes of adenine nucleotide salvage and is ~2 orders of magnitude more growth inhibitory to MTAP-containing leukemia cell lines than MTAP-deficient lines (28). In line with this, we found that expression of MTAP activity in the MiaPaCa-2 and PANC-1 cell lines markedly increased their sensitivity (ID_{50} = 0.2–0.9 μM) to the antiproliferative actions of 5′-dFAdo relative to the corresponding MTAP-deficient parental and *neo*-transfected lines (ID_{50} = 13–236 μM; Fig. 1C).

Another MTAP-dependent salvage pathway is conversion of MTA to methionine, in which the methylthio moiety of MTA, originally derived from SAM, is recycled back to methionine pools; the first step in this pathway is the cleavage of MTA to 5-methylthioribose-1-phosphate by MTAP (3, 4). We then examined whether reintroduction of MTAP activity via transfection would restore the ability of MTAP-deficient cells to metabolize MTA to methionine. One way to assess the activity of this salvage pathway in a given cell line is to plate the cells in a methionine-deficient medium and examine their ability to grow using exogenous MTA as their sole methionine source. As shown in Fig. 2, neither wild-type nor *neo*-transfected MiaPaCa-2 or PANC-1 cells, which are MTAP deficient, were able to grow in methionine-deficient culture medium supplemented with ≤100 μM MTA (Fig. 2, A and B, ♦ and ●). In contrast, the MTAP-transfected lines, including MiaPaCa-2/*MTAP*-AzG, MiaPaCa-2/*MTAP*-G, PANC-1/*MTAP*-Az, and PANC-1/*MTAP*-G, were each able to proliferate using MTA as a methionine source; this effect was dependent on MTA concentration (Fig. 2, A and B, △ and □). Together, these data indicate that transfection of *MTAP* cDNA into MTAP-deficient cell lines not only leads to expres-

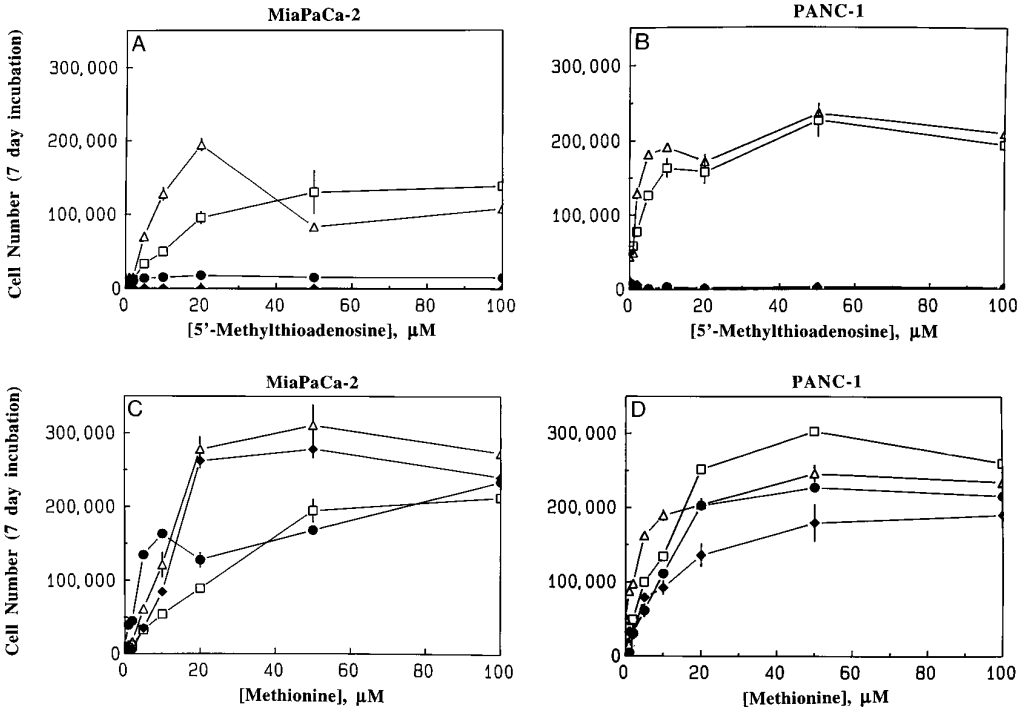

Fig. 2. Ability of MTA to serve as a methionine source for *MTAP*-transfected and control cell lines. Cultured *MTAP*- or sham-transfected Mia PaCa-2 cells (*left*) and *MTAP*- or sham-transfected PANC-1 cells (*right*) were harvested using trypsin-EDTA, washed twice in phosphate-buffered saline, and plated in 0.5 ml methionine-deficient (Met^-)MEM medium (made from Selectamine kits, GIBCO-BRL) supplemented with 6% undialyzed donor horse serum (20,000 cell/well, 12-well dishes); after a 4–6 hr period to allow cell attachment, an additional 0.5 ml of serum-free methionine-deficient medium was added to each well (final horse serum concentration, 3%), containing varying concentrations of MTA (*top*) or methionine (*bottom*). Cells were incubated 7 days at 37° in a humidified incubator in a 95% air/5% CO_2 environment. Cell counts were determined using the MTT cytotoxicity assay (7). Values are mean ± standard deviation of four independent determinations. A and C, ●, MiaPaCa-2 wild-type cells; ♦, MiaPaCa-2/*neo* cells; △, MiaPaCa-2/*MTAP*-AzG cells; and □, MiaPaCa-2/*MTAP*-G. B and D, ●, PANC-1 wild-type cells; ♦, PANC-1/*neo*; △, PANC-1/*MTAP*-Az; and □, PANC-1/*MTAP*-G.

sion of functional enzyme activity but also restores the role of this enzyme in cellular metabolism (i.e., salvaging of the adenine and methylthio moieties of MTA).

Influence of operation of the MTAP-dependent adenine salvage pathways on the rates of purine *de novo* synthesis. The cell model systems in which the MTAP-dependent salvage pathways have been restored or remain inoperative enabled us to study the influence these pathways might have on aspects of cellular metabolism. One metabolic pathway that might be affected by the salvage of adenine from MTA, for example, is purine *de novo* synthesis. Preformed purines dampen the rate of purine *de novo* synthesis by mechanisms that involve a competitive consumption of precursors such as 5-phosphoribosyl-1-pyrophosphate and feedback inhibition by purine nucleotides of the early steps in the *de novo* pathway (29). Thus, restoration of the MTA-to-adenine nucleotide salvage pathway in MTAP-deficient malignant cells might decrease their purine *de novo* synthesis rates. To test this, we studied the rates of purine *de novo* synthesis in the MTAP-transfected versus wild-type or sham-transfected pancreatic carcinoma cell lines, using an assay system that measures the incorporation of [(U)^{14}C]glycine into the purine nucleotide precursor FGAR during a blockade of phosphoribosylformylglycineamidine synthetase by azaserine. As shown in Fig. 3, the two MiaPaCa-2 cell lines in which the MTA-to-adenine nucleotide salvage pathway has been restored, MiaPaCa-2/*MTAP*-AzG and MiaPaCa-2/*MTAP*-G, displayed statistically significant decreases in their rates of *de novo* purine synthesis (34–47%) relative to the corresponding MTAP-deficient lines. The rates of incorporation of [(U)^{14}C]glycine into FGAR in the wild-type or sham-transfected PANC-1 cell types were lower than that of the comparable MiaPaCa-2-derived cell lines, presumably due to their longer doubling times. The *MTAP*-transfected PANC-1 lines had *de novo* purine synthesis rates that were 18–36% lower than the corresponding MTAP-deficient PANC-1 lines, although this difference was statistically significant only when compared with the values for the PANC-1 wild-type cells.

Role of the MTAP-dependent salvage pathways in determining sensitivity to purine *de novo* synthesis inhibitors. Part of the mechanism of action of MTX involves the inhibition of several folate-requiring steps of purine *de novo* synthesis (e.g., GARFT) by polyglutamylated forms of MTX and/or dihydrofolate, the latter accumulating as results of inhibition by MTX of dihydrofolate reductase (30, 31). Recently, we showed that the antipurine actions of MTX are more efficacious in $MTAP^-$ pancreatic carcinoma cell lines, including PANC-1 and MiaPaCa-2, than $MTAP^+$ normal epithelial cells or pancreatic carcinoma lines; it was postulated that in cells in which the MTAP-dependent adenine salvage pathway is operational, the efficient recycling of purine moieties might render these cells less sensitive to the purine *de novo* synthesis inhibitory effects of MTX (7). If so, reintroduction of MTAP activity via transfection should decrease the sensitivity of such originally MTAP-deficient cells to the antipurine effects of MTX and other agents that inhibit *de novo* purine synthesis.

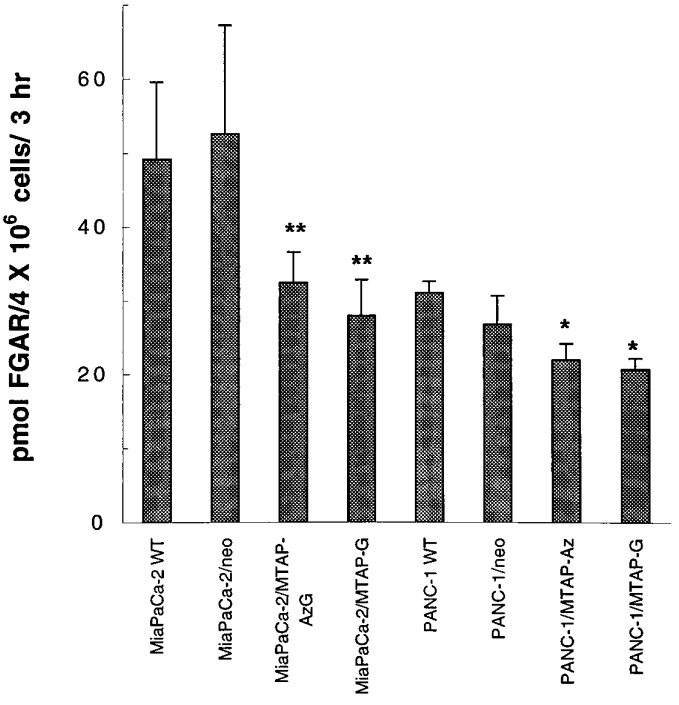

Fig. 3. Effect of restoration of the MTAP-dependent adenine salvage pathway in MTAP-deficient cells on the rate purine *de novo* synthesis. Purine *de novo* synthesis was determined by measuring the cellular rate of incorporation of [(U)-^{14}C]glycine into the purine nucleotide precursor, FGAR, in the presence of azaserine, as described in the text. Values are mean ± standard deviation of three to six independent determinations. **, Significantly different from the corresponding wild-type (*WT*) or sham-transfected cell lines, as determined by a two-tailed paired *t* test ($p < 0.05$). *, Significantly different ($p < 0.05$) only from those of the corresponding wild-type cell lines, as determined by a two-tailed paired *t*-test.

We compared the dose-response curves for the antipurine-associated growth-inhibitory actions of MTX and azaserine in the MTAP-transfected cell lines (in particular, the MiaPaCa-2/*MTAP*-G and PANC-1/*MTAP*-G cell lines) with that of the corresponding sham-transfected cell lines (i.e., those transfected only with *neo*-containing vectors). These cell lines were appropriate for this study because none of them had received prior exposure to purine *de novo* inhibitors (unlike MiPaCa-2/*MTAP*-AzG or PANC-1/*MTAP*-Az). In experiments involving MTX, exogenous thymidine (20 μM) was added to the medium to abrogate the inhibition of thymidine nucleotide synthesis effected by this antifolate, thereby isolating its antipurine actions. Under these conditions, MTX was highly efficacious against the MTAP-deficient MiaPaCa-2 or PANC-1 *neo*-transfected cell lines, effecting a nearly complete growth-inhibitory action in the high ($>10^{-6}$ M) concentration range (Fig. 4, A and B, ○). These dose-response curves were characterized by a steep slope in the 10^{-9} to 10^{-7} M range (ID_{50} values for the antipurine-related growth inhibitory action of MTX were 1.0×10^{-8} M for the MiaPaCa-2/*neo* cell line and 3.6×10^{-8} M for the PANC-1/*neo* cell line). In contrast, the *MTAP*-transfected counterpart cell lines MiaPaCa-2/*MTAP*-G and PANC-1/*MTAP*-G, in which the MTAP-dependent adenine salvage pathway is operational, displayed a markedly different dose-response curve, with decreased potency and incomplete efficacy at the higher MTX doses (Fig. 4, A and B, ●). The ID_{50} values for MTX were 6.7×10^{-8} M for the MiaPaCa-2/*MTAP*-G line and 7.7×10^{-7} M for the PANC-1/*MTAP*-G line, or ~6.7- and ~21.4-fold higher, respectively, than their counterpart MTAP-deficient lines.

A similar trend was observed when dose-response curves were obtained for these cell lines in response to the specific inhibitor of purine *de novo* synthesis azaserine. The azaserine dose-response curves for both the MTAP-expressing MiaPaCa-2/*MTAP*-G and PANC-1/*MTAP*-G cell lines were shifted rightward by a 3-fold factor relative to their respective MTAP-deficient counterparts (Fig. 4, C and D). The efficacy of azaserine for mediating an inhibition of cell growth was decreased in the MTAP-expressing cell lines relative to the MTAP-deficient lines, although this decrease is not as pronounced as that observed in the case of MTX. Thus, MTAP-transfected cell types are less sensitive to the antipurine actions of MTX or azaserine than their corresponding MTAP-deficient cell types.

Discussion

In previous work, it was demonstrated that the antipurine-related growth-inhibitory action of the antifolate agent MTX was more pronounced in subsets of pancreatic carcinoma cell lines that were *p16*$^-$, *MTAP*$^-$ than in pancreatic carcinomas or normal keratinocyte epithelial cells that were *p16*$^+$, *MTAP*$^+$ (7). Furthermore, it was shown that the coaddition of an inhibitor of MTAP enhanced both the potency and efficacy of the antipurine-related growth-inhibitory actions of MTX in *MTAP*$^+$ but not *MTAP*$^-$ cell lines (7). These data were consistent with the hypothesis that operation of the adenine salvage pathway in MTAP-containing cells, including normal cells, decreases their dependence on *de novo* purine synthesis and permits the maintenance, to some degree, of purine pools even in the face of a pharmacological blockade of purine *de novo* synthesis. Thus, *MTAP*$^+$ cells are less sensitive to the inhibitory effects that antifolates such as MTX have on purine *de novo* synthesis. In contrast, MTAP-deficient malignant cells, which are unable to recycle the purine moiety of MTA, are more dependent on purine *de novo* biosynthesis and are more sensitive than MTAP-containing cells to the antipurine actions of antifolates. In the current study, we demonstrated that reexpression of MTAP in *p16*$^-$, *MTAP*$^-$ malignant cells, via transfection, (i) restores their MTAP-dependent salvage pathways, (ii) decreases their rates of purine *de novo* synthesis, and (iii) decreases their sensitivity to the antipurine actions of MTX and azaserine, all of which are predicted by the original hypothesis. These concepts are supported by a recent study by Hori *et al.* (8), who found that transfection of MTAP cDNA into the *MTAP*$^-$ non-small cell lung carcinoma cell line A549 renders these cells less responsive to antifolates such as 5,10-dideaza-5,6,7,8-tetrahydrofolate, which acts as a specific inhibitor of GARFT of the purine *de novo* synthetic pathway (8).

Previous studies on MTX have demonstrated that there are a number of biochemical factors that can affect the responsiveness of a cell to this agent. The activities of two distinct carrier-mediated active transport systems, the reduced folate carrier and the high affinity folate binding protein (human folate receptor), are important factors in determining the intracellular levels of MTX within a given tissue; in addition, both the activity of FPGS, which traps MTX in

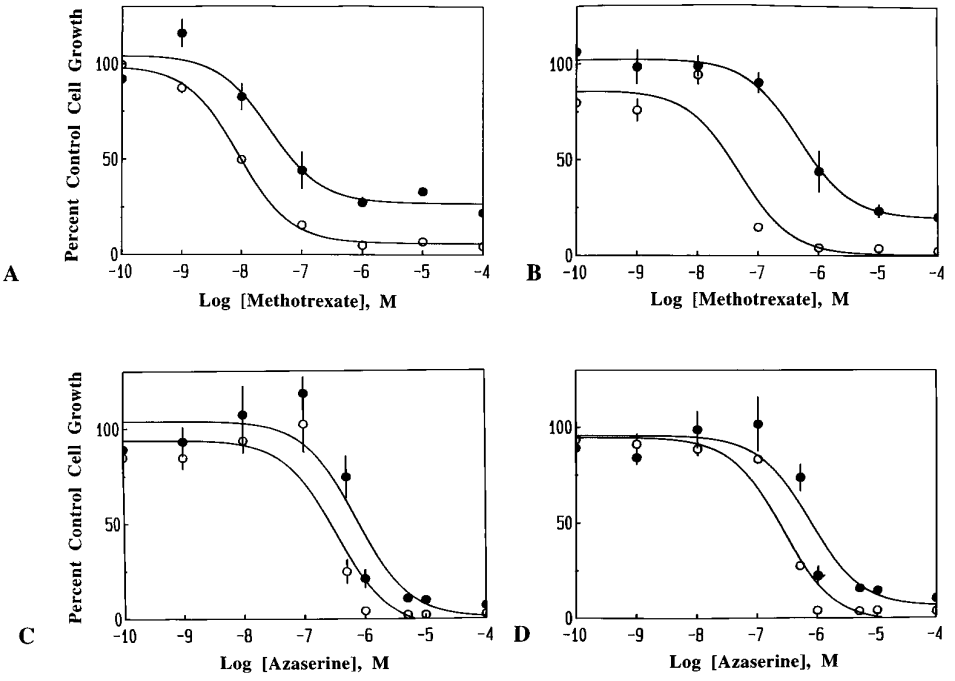

Fig. 4. Dose-response curves for the antipurine-related growth-inhibitory effects of MTX (A and B) or azaserine (C and D) in *MTAP*-transfected versus control PANC-1- and MiaPaCa-2-derived cell lines. Experiments were carried out in DMEM containing 10% horse serum in 12-well dishes (20,000 cells/well). For experiments involving MTX, the medium was supplemented with 20 μM thymidine to eliminate the thymidine nucleotide-depleting actions of this antifolate. Cells were incubated at 37° in a humidified incubator with a 95% air/5% CO_2 atmosphere for 4 days (MiaPaCa-2-derived cells) or 7 days (PANC-1-derived cells), and cell numbers were assessed using the MTT-based assay. A, Dose-response curves for the antipurine-related growth-inhibitory actions of MTX on MiaPaCa-2/*neo* cells (○, control cell doublings, 4.65 ± 0.06) and MiaPaCa/*MTAP*-G cells (●, control cell doublings, 2.47 ± 0.20). B, Dose-response curves for the antipurine-related growth-inhibitory actions of MTX on PANC-1/*neo* cells (○, control cell doublings, 4.93 ± 0.02) and PANC-1/*MTAP*-G cells (●, control cell doublings, 3.73 ± 0.14). C, Dose-response curves for the growth-inhibitory actions of azaserine on MiaPaCa-2/*neo* cells (○, control cell doublings, 4.63 ± 0.25) and MiaPaCa-2/*MTAP*-G cells (●, control cell doublings, 3.00 ± 0.21). D, Dose-response curves for the growth-inhibitory actions of azaserine on PANC-1/*neo* cells (○, control cell doublings, 5.13 ± 0.05) and PANC-1/*MTAP*-G cells (●, control cell doublings, 3.81 ± 0.17). Values are mean ± standard deviation of three independent determinations. Curve fitting by nonlinear regression was carried out using InPlot (GraphPAD, San Diego, CA).

the cell by catalyzing its polyglutamation, and DHFR, which is one of the initial targets of MTX and its polyglutamated forms, are also determinants of MTX efficacy (32). Interestingly, in patients with acute lymphoblastic leukemias, p53 mutations have been associated with DHFR amplification and consequent MTX resistance (33). The current study, which used syngeneic cell model systems in which the above-mentioned determinants of MTX action (e.g., folate carrier, FPGS and DHFR activities, and p53 status) are presumably equivalent, have demonstrated that MTAP status and operation of its attendant salvage pathways can also be determinants of MTX efficacy, at least under controlled conditions. The finding that a purine salvage pathway can influence the action of antifolates is not unprecedented: Sano *et al.* (34) showed that cultures of HL-60 promyelocytic leukemia cells that lack the purine salvage enzyme hypoxanthine-guanine phosphoribosyltransferase are 3-fold more sensitive to the antiproliferative actions of MTX than are wild-type cells. These findings support the postulate that loss of a purine recycling mechanism renders cells more dependent on purine *de novo* synthesis during proliferation and hence more vulnerable to inhibitors of the *de novo* pathway. Operation of the MTA-to-methionine pathway may also be important in conferring a decreased responsiveness to the actions of MTX. MTX, at relatively high concentrations ($>10^{-6}$ M), has been shown to block the cellular uptake of methionine (35); thus, MTAP-expressing cells, which have the ability to convert endogenously generated MTA to methionine, might be less prone to the growth-inhibitory consequences of this blockade.

There are a number of potential implications of these findings for clinical cancer chemotherapy. If normal cells, which are uniformly MTAP positive (5), are intrinsically less sensitive to the antipurine actions of antifolates such as MTX, then this might in part be the basis for the relatively selective action these agents have against certain malignancies. If the corollary is correct that MTAP-deficient malignant cells are especially sensitive to certain antifolates that act in whole or in part by inhibiting purine *de novo* synthesis, one would theoretically be able to obtain an improved therapeutic index by identifying the subset of patients with MTAP-deficient malignancies and treating them with such agents. From this point of view, it is not surprising that the more MTX-responsive malignancies, such as T cell acute lymphocytic leukemias, happen to have high incidences of 9p21 deletions, including the *MTAP* locus (36). The development of methodologies for detecting MTAP in clinical samples (e.g., those based on *in situ* hybridization or immunohistochemistry) is a priority.

These considerations may also influence the design of clinical trials of some of the new antifolates currently under development. Of particular interest are third-generation antifolates such as LY309887; this thienyl derivative of 5,10-

dideaza-5,6,7,8-tetrahydrofolate acts as a potent and selective GARFT inhibitor and requires relatively low polyglutamation for activation (37). One would predict that this compound, which exclusively targets purine *de novo* synthesis, might be especially efficacious against MTAP-deficient malignancies, regardless of their FPGS activity: indeed, among the xenografted tumors that were reported to be highly responsive to LY309887 are the *MTAP*$^{-}$ pancreatic carcinomas PANC-1 and BxPC-3 (37). This hypothesis is being investigated. In any case, the fact that MTAP deficiency in malignant cells can alter their sensitivity to particular antineoplastic agents may have had an impact on past drug development efforts. Two of the early model systems used for screening the activity of anticancer drugs, the L1210 and P388 murine leukemias, are MTAP deficient (10); this may explain in part their high degree of responsiveness to antifolates such as methotrexate. One can only speculate whether the use of these MTAP^{-} model systems might have favored the development, for example, of particular antifolates with strong antipurine activity. Even among the current cell models for drug screening of the National Cancer Institute (includes 60 cell lines), 47% have been found to bear homozygous deletions of *p16* (38); presumably a high percentage of this subset also bear homozygous deletions of *MTAP*. Having both *MTAP*$^{+}$ and *MTAP*$^{-}$ cell lines within a drug screening system is not disadvantageous and can in fact be advantageous with awareness of this genotypic/phenotypic difference so data are appropriately interpreted.

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