Thymidylate synthase as an oncogene: A novel role for an essential DNA synthesis enzyme

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Summary

Thymidylate synthase (TS) is an E2F1-regulated enzyme that is essential for DNA synthesis and repair. TS protein and mRNA levels are elevated in many human cancers, and high TS levels have been correlated with poor prognosis in patients with colorectal, breast, cervical, bladder, kidney, and non-small cell lung cancers. In this study, we show that ectopic expression of catalytically active TS is sufficient to induce a transformed phenotype in mammalian cells as manifested by foci formation, anchorage independent growth, and tumor formation in nude mice. In contrast, comparable levels of two TS mutants carrying single point mutations within the catalytic domain had no transforming activity. In addition, we show that overexpression of TS results in apoptotic cell death following serum removal. These data demonstrate that TS exhibits oncogene-like activity and suggest a link between TS-regulated DNA synthesis and the induction of a neoplastic phenotype.

Introduction

Thymidylate synthase (TS) plays a central role in the biosynthesis of thymidylate, an essential precursor for DNA synthesis. TS catalyzes the reductive methylation of 2′-deoxyuridine 5-monophosphate (dUMP) by transfer of a methylene group from a cofactor, CH_2H_4 folate, to generate deoxythymidine-5′-monophoshate (dTMP). dTMP is further phosphorylated to the triphosphate state (dTTP), which is a direct precursor for DNA synthesis. Since the TS catalyzed reaction is the sole intracellular de novo source of dTMP, the inhibition of TS results in the cessation of cellular proliferation and growth (Friedkin and Kornberg, 1957; Navalgund et al., 1980).

Several clinical studies have shown that TS protein and mRNA levels are higher in cervical, breast, kidney, bladder, lung, and gastrointestinal tumor tissues than in their normal counterparts and that high TS levels have been associated with poor clinical outcome in these cancers (Johnston et al., 1995; Leichman, 2001; Mizutani et al., 2003; Nomura et al., 2002; Pestalozzi et al., 1997; Shintani et al., 2003; Suzuki et al., 1999). Tumors with elevated TS levels are thought to undergo more active cellular proliferation, which in turn is associated with tumor invasiveness and metastasis (Edler et al., 2000; Mizutani et al., 2003; Nomura et al., 2002). Whether TS overexpression is a consequence of aberrant cellular proliferation or whether overexpression of TS participates directly in stimulating the increase in cell growth and proliferation associated with the transformed phenotype remains to be established.

TS transcription is regulated by several protein factors, including members of the E2F gene family (DeGregori et al., 1995; Dong et al., 2000; Dyson, 1998; Trimarchi and Lees, 2002). The importance of E2F function has been demonstrated by studies which show that inhibition of E2F-1 prevents entry into S phase (Wu et al., 1996) and that ectopic expression of E2F-1 in quiescent cells is sufficient for progression into DNA synthesis (Johnson et al., 1993). Overexpression of E2F-1 also results in cellular transformation of rodent cells (Singh et al., 1994; Xu et al., 1995). In addition, it has been demonstrated that overexpression of E2F-1 in both human fibrosarcoma cells and quiescent rat fibroblast cells results in an increase in TS mRNA and protein levels (Banerjee et al., 1998; DeGregori et al., 1995), suggesting that E2F-1 might play an important role in the regulation of TS gene expression in vivo. E2F-1 plays a central role in regulating the transition from the G_1 to S phase during cell cycle progression (Wu et al., 1996) by serving as a bifunctional transcription factor which can repress gene transcription when bound to the

SIGNIFICANCE

Elevated levels of thymidylate synthase (TS) activity have been identified as an important prognostic biomarker for colorectal cancer and for many other common human malignancies. A common interpretation for this observation has been that elevated levels of TS protein arise simply as epiphenomena which are secondary to aberrant tumor cell proliferation. In this report, we have demonstrated that ectopic expression of human TS can transform murine cells in vitro and in vivo, which instead infers a causal role for TS in mammalian tumorigenesis. This data also suggests that regulating TS activity in vivo may be important for the development of both cancer prevention and therapeutic strategies.

retinoblastoma (RB) protein and other corepressor molecules in nonmitotic cells (Cam and Dynlacht, 2003). In response to external and internal growth signals, however, RB undergoes conformational changes due to cyclin-mediated phosphorylation and is released from E2F-1 binding. Free E2F-1 binds to DNA where it exhibits a transcriptional activation function that appears to be critical for proper entry into S phase. Loss of RB function, therefore, results in the constitutive induction of a set of E2F-1 regulated genes that encode several DNA synthesis enzymes, including TS, dihydrofolate reductase (DHFR), DNA polymerase α, and thymidine kinase (TK) (Helin, 1998). Overexpression of E2F-1 in rat embryo fibroblast, however, resulted in a greater increase in TS as compared to DHFR and TK (Banerjee et al., 1998), suggesting that the TS promoter activity exhibits enhanced sensitivity to levels of E2F-1 activity and that increased levels of TS may not be due solely to a general increase in DNA synthesis enzymes.

Since E2F-1 overexpression leads to both cellular transformation and the induction of TS expression (Singh et al., 1994), and since TS levels are elevated in malignant tissues (Gorlick et al., 1998; Mizutani et al., 2003; Nomura et al., 2002; Shintani et al., 2003; Suzuki et al., 1999), we tested whether TS might act as a downstream effector of E2F-1 to participate in the oncogenic pathway. We found that overexpression of TS can result in neoplastic transformation and in programmed cell death upon removal of survival factors.

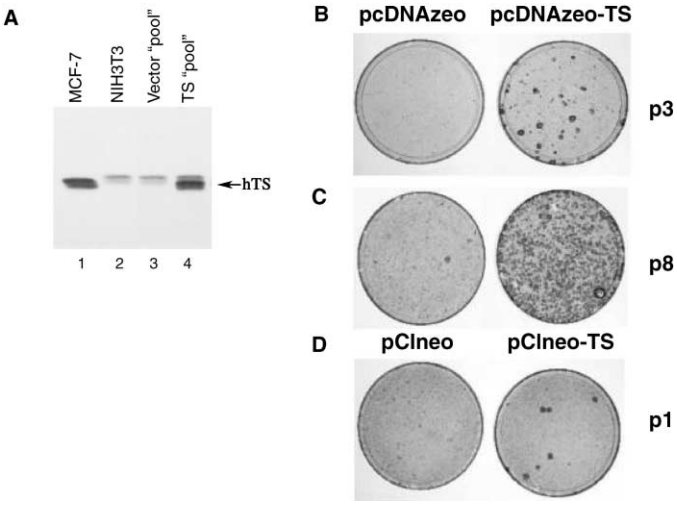

Figure 1. Thymidylate synthase (TS) induced foci in NIH/3T3 cells

A: Immunoblot analysis of cell extracts from stably transfected TS and control cell pools (lanes 3 and 4). The MCF-7 and NIH/3T3 cells represent positive and negative controls for the expression of human TS, respectively (lanes 1 and 2). The arrow depicts ectopic expression of human TS (hTS) detected with TS106 monoclonal antibody.

B–D: Focus assays. NIH/3T3 cell pools transfected with the indicated plasmids and tested at different passage numbers (p1, p3, and p8) for foci formation.

Results

Overexpression of thymidylate synthase induces transformed foci in NIH/3T3 cells

To determine whether overexpression of human thymidylate synthase could lead to cellular transformation, immortalized NIH/3T3 mouse fibroblast cells were transfected with either a TS expression plasmid (pcDNA3.1zeo-TS) or the empty control vector (pcDNA3.1zeo). Stable zeocin-resistant clones were pooled and protein extracts from both the TS and vector-transfected cell pools were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) using anti-TS antibody (Behan et al., 1998). The TS-transfected cell pool contained high levels of human TS that could be resolved from the endogenous mouse TS (Figure 1A). The difference in the electrophoretic mobility of mouse and human TS was verified by the comparison of the migration pattern of TS in protein extracts obtained from human MCF-7 breast cancer cells and mouse NIH/3T3 cells. The migration pattern displayed by the human TS that was ectopically expressed in transfected rodent NIH/3T3 cells was similar to the migration pattern exhibited by the endogenous human TS in MCF-7 cells (Figure 1A, lanes 1 and 4), while the empty vector-transfected NIH/3T3 cells exhibited only low levels of the endogenous mouse TS.

To determine whether overexpression of TS results in a transformed phenotype of the NIH/3T3 cells, we tested the ability of TS-transfected cells to form foci in monolayer cultures. Cells were selected in zeocin-containing medium to enrich for the transfected cells, and the foci assay was performed using the third passage of pooled clones from either control or TS transfected cells. We found that cells overexpressing human TS gave rise to foci 4–6 weeks after inoculation, while no foci were observed at 6 weeks in the cells transfected with the vector control (Figure 1B). Microscopic examination of the TS-induced foci revealed multilayered growth and crisscross morphology characteristic of transformed cells (data not shown). The number of foci in the TS-transfected cell pool was approximately 10-fold higher when tested at passage 8, demonstrating the selective advantage of transformed cells (Figure 1C). To confirm this observation, NIH/3T3 cells were transfected with a different TS expression vector, pCIneo-TS, containing the neomycin resistance gene or with the empty control vector, pCIneo. G418-resistant cell clones were pooled and tested for foci formation immediately without passage. We again found that overexpression of TS gave rise to foci formation in NIH/3T3 cells, while transfection of the pCIneo vector had no effect (Figure 1D). This data suggests that overexpression of TS in mammalian cells is sufficient to induce foci with a dysplastic morphology in NIH/3T3 cells in vitro and that additional passaging of these cells was not required for the acquisition of a transformed phenotype.

Neoplastic transformation induced by thymidylate synthase

To establish whether overexpression of TS may result in a neoplastic phenotype, single cell clones were isolated from NIH/3T3 cells transfected with either TS or the empty control vector. Protein extracts isolated from each clone were tested for TS expression by immunoblot analysis. Of the eleven TS-transfected clones examined, seven expressed detectable exogenous human TS protein (Figure 2A, lanes 3–9), while the human TS was absent in the empty control vector (Figure 2A, lane 2) and in parental NIH/3T3 cells (Figure 2A, lanes 1 and 10).

To determine whether the ectopically expressed human TS retained catalytic activity in the NIH/3T3 mouse cells, we measured the conversion of dUMP to dTMP by a tritium release assay (Allegra et al., 1985). We found that exogenously expressed human TS retained catalytic activity in mouse NIH/3T3

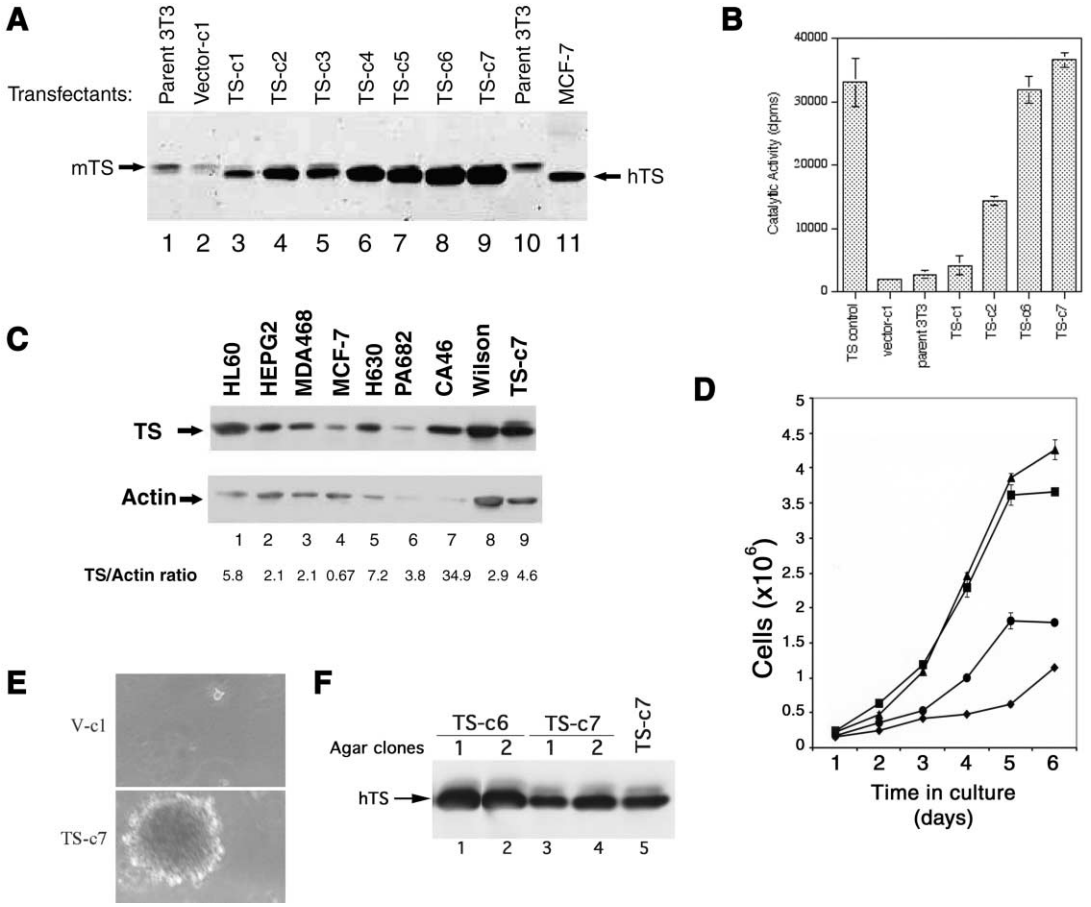

Figure 2. In vitro transformation by thymidylate synthase (TS)

A: Expression of ectopic human thymidylate synthase (TS) in single cell clones derived from NIH/3T3 cells. Immunoblot analysis with anti-TS detects mouse (mTS) and human (hTS) as indicated by the arrows in TS-expressing cell clones (lanes 3–9), vector alone (lane 2), parental cells (lanes 1 and 10), and the control human MCF-1 cells (lane 11).

B: TS catalytic activity was measured in the indicated single cell clones. The TS protein control was purified from L. caseii. Results shown are mean ± standard deviation (SD) of three to four separate experiments.

C: Comparison of ectopic TS levels in NIH/3T3 cells to TS levels in human tumor cell lines. Immunoblot analysis detects TS and actin as indicated by the arrows. TS and actin levels were measured using NIH image program.

D: Growth analysis of TS overexpressing cells. Growth curves of TS transfected cell lines (TS-c5, squares; TS-c7, triangles) compared to vector alone cells (V-c1, circles; V-c2, diamonds). Results shown represent the average from triplicate plates.

E: Growth of thymidylate synthase-transformed cells in soft agar. Photomicrograph of representative agar clones from TS-overexpressing cells (TS-c7) and vector control cells (V-c1).

F: Immunoblot analysis with anti-TS detects human TS in protein extracts isolated from agar clones. Single agar clones were isolated from two independent TS-transfected clones, TS-c6 and TS-c7 (lanes 1–4). Protein extract from TSc-7 were used as a positive control for detection of human TS (hTS).

cells with up to a 20-fold increase in TS catalytic activity in cells transfected with human TS as compared to the empty vector transfectants or to the parental NIH/3T3 cells (Figure 2B). In addition, TS enzymatic activity directly correlated with the amount of steady-state TS detected by immunoblot analysis (Figures 2A and 2B). Thus, the ectopically expressed human TS maintained high steady-state levels of biological activity in mouse NIH/3T3 cells.

Since previous studies using immunoblotting or quantitative PCR showed that TS protein content and RNA levels may vary up to 60-fold in primary human colorectal cancer biopsies or cancer cell lines (Grem et al., 2001; Leichman, 2001), we performed immunoblot analysis to compare the amount of TS protein relative to endogenous actin levels in the transfected clone TS-c7 with protein extracts from different types of tumor cell lines (Figure 2C). We found that TS protein levels varied up to 50-fold between the different tumor lines, and that the levels of ectopic human TS in the mouse cell clone TS-c7 were intermediate within this range (7.5-fold lower than the cell line with the highest steady-state TS/actin expression (CA46) and 6.5 fold higher than the cell line with the lowest TS/actin expression [MCF-7]) (Figure 2C).

Morphological examination of the TS-overexpressing clones under light microscopy revealed a characteristic transformed cell morphology. The TS transformants were small, refractile, and spindle shaped, and displayed multilayered growth. In contrast, the empty vector-transfected cells grew in a monolayer and morphologically resembled normal NIH/3T3 cells (data not shown). TS-overexpressing cells lost contact inhibition, resulting in piling up and crisscrossing with a 2- to 3-fold increase

Table 1. Colony growth in soft agar

Cell line	Average number of clones*		Total number of clones	% efficiency in agar
	Small	Large		
V-c1	16	0	16	0.016
V-c3	14	0	14	0.014
TS-c5	554	65	619	0.619
TS-c6	417	11	428	0.428
TS-c7	714	52	766	0.766

1×10^5 cells were plated in 0.3% agar, 0.2% peptone, and DMEM supplemented with 1% Glutamine, 1% PenStrep, 15% FBS, 4 mM HEPES, and 400 μg zeocin per ml. Numbers refer to mean number of colonies per 60 mm dishes (in triplicate) appearing in 14 days.
*Average of two to three separate experiments.

Table 2. Tumor formation in Balb/c nude mice

Cell line	# of mice with tumors/# of mice injected				%	Latency (weeks)[a]
	Exp 1	Exp 2	Exp 3	Total		
V-c1	0/7	0/7	0/7	0/21	0	
V-c3	–[b]	–	0/6	0/6	0	
V-c4	–	–	0/4	0/4	0	
TS-c5	7/7	–	7/7	14/14	100	6.5–9
TS-c6	4/7	2/2	1/7	7/16	44	9–15
TS-c7	4/7	5/6	3/7	12/20	60	6.7–17

[a] All animals were sacrificed at 23–24 weeks.
[b] Not determined.

in saturation density as compared to the vector control culture (data not shown). In addition, comparison of the growth rates of the TS-overexpressing clones, TS-c5 and TS-c7, to the empty control vectors, V-c1 and V-c2, demonstrated faster cell growth for TS-overexpressing cells (Figure 2D). Three TS-overexpressing clones and two empty control vector clones were also tested for the acquisition of colony formation in 0.3% soft agar, an in vitro parameter for the transformed phenotype. We found that all three ectopic TS-expressing cell lines, TS-c5, TS-c6, and TS-c7, acquired an anchorage-independent phenotype and could grow in 0.3% agar. Clones TS-c5, TS-c6, and TS-c7 grew in soft agar with a 25- to 50-fold increased efficiency as compared with the control cell lines V-c1 and V-c3 (Table 1). Agar clones derived from the TS-overexpressing cell lines designated as "small" were approximately double the size of "small" clones in the control culture, V-c1 and V-c3, and grew progressively larger after two weeks of incubation. Agar clones from the empty control vector line V-c1, however, were uniformly small and did not increase in size when left in culture longer than two weeks (Figure 2E). Immunoblot analysis of extracts isolated from agar clones established from TS-overexpressing cells reveal high levels of TS protein (Figure 2F), demonstrating that cells that grew in agar retained ectopic expression of human TS. These results from both foci and soft agar assays suggest that overexpression of TS is associated with cellular transformation in vitro.

To determine whether overexpression of TS resulted in neoplastic transformation in vivo, we tested the ability of TS-overexpressing cells to form tumors in nude mice. We injected 5×10^6 cells from each of the TS-overexpressing cell lines, TS-c5, TS-c6, and TS-c7 subcutaneously into the back of five-week-old athymic Balb/c mice and monitored for tumor growth. We observed that injection of the TS-overexpressing cell lines resulted in tumor formation, whereas xenograft tumor growth was not observed with control cells. The latency period for tumor formation varied; however, tumors were first noticed at the injection site at 6 weeks for the TS-c5 and TS-c7 clones and at 9 weeks for the TS-c6 clone (Table 2). 100% of the TS-c5 injected mice developed tumors by 9 weeks, while 44% of the TS-c6 injected mice developed tumors by 15 weeks and 60% of TS-c7 injected animals developed tumors at 17 weeks following inoculation. In contrast, no tumors were observed at 24 weeks in mice injected with three independently derived vector control clones, V-c1, V-c3, and V-c4 (Table 2). Protein extracts of resected xenograft tumors derived from TS-overexpressing cells continued to demonstrate high levels of ectopic human TS as determined by immunoblot analysis (data not shown). These data suggest that TS overexpression renders NIH/3T3 cells tumorigenic in vivo.

Catalytic activity of thymidylate synthase is required for the establishment of neoplastic phenotype

Since TS is an essential S phase enzyme required for DNA synthesis, we hypothesized that the catalytic activity of the enzyme may be necessary for its transforming ability. To test our hypothesis, we overexpressed TS mutants carrying point mutations that result in the loss of catalytic activity of the enzyme. We used two distinct point mutants both located in the catalytically important and highly conserved Arg^{50}-loop of TS (Schiffer et al., 1995). Overexpression of these two TS mutants, Arg50Cys and Thr51Ala, could not rescue growth of TS-negative cells in selective medium lacking thymidine as compared to the wild-type enzyme (Tong et al., 1998). We subcloned TS-Arg50Cys and TS-Thr51Ala into the same expression vector (pcDNA3.1 zeo) that we used to express wild-type TS in our earlier transformation assays. Mutant and wild-type vectors were transfected into NIH/3T3 cells and single cell clones were isolated following zeocin selection. Protein extracts from each clone were tested for TS expression by immunoblot analysis. Both mutant and wild-type TS-transfected cells expressed exogenous human TS (Figure 3A, lanes 3 to 11), while the enzyme was absent in the empty vector control (Figure 3A, lane 1). Protein extract from clone TS-c7 was used as a positive control (Figure 3A, lane 2). To determine the catalytic activity of these mutants when expressed in NIH/3T3 cells, we selected cell clones that expressed similar steady-state protein levels as shown by immunoblot analysis (Figure 3A) and measured the conversion of dUMP to dTMP by TS using an in vitro catalytic activity assay (Figure 3B, lanes 2 to 7). In addition, a protein sample isolated from a clone transfected with empty vector was used as a control to measure the catalytic activity of endogenous mouse TS (Figure 3B, lane 1). We found that the exogenously expressed mutants TS-Arg50Cys (cell lines mTS-c3 and mTS-c5) and mutant TS-Thr51Ala (cell lines mTS-c6 and mTS-c7), with similar levels of TS expression (Figure 3A), showed reduced catalytic activity as compared to the wild-type TS (Figure 3B). Thus, ectopically expressed human TS that contain a point mutation in the UTP-folate binding site maintains high steady-state protein levels that lacks biological activity in NIH/3T3 cells.

To establish whether high levels of exogenously expressed, catalytically inactive TS are capable of inducing transformation,

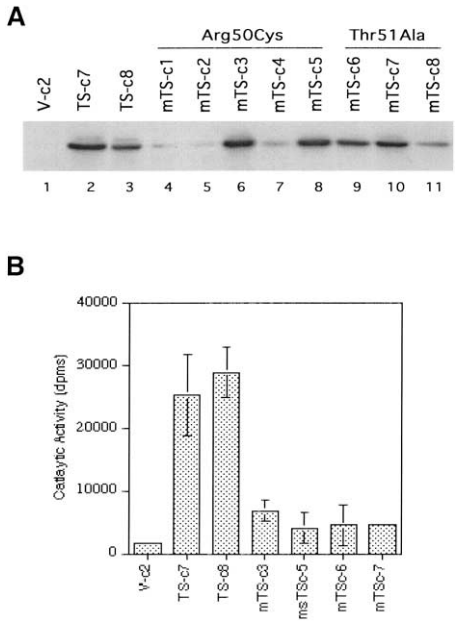

Figure 3. Characterization of overexpressed mutants of human thymidylate synthase (TS) lacking catalytic activity of the enzyme

A: Expression of mutated human TS in single cell clones derived from NIH/3T3 cells. Immunoblot analysis with anti-TS detects wild-type clones (TS-c7 and TS-c8) and mutant TS clones (mTS-c1 to mTS-c8) in TS-expressing cells (lanes 2–11). Vector control cell clone V-c2 is shown in lane 1. Mutations from arginine to cysteine and from threonine to alanine at positions 50 (Arg50Cys) and 51 (Thr51Ala), respectively, are indicated.
B: Catalytic activity of mutated human thymidylate synthase (TS). TS catalytic activity was measured in the indicated single cell clones. Results shown are mean ± SD of three experiments.

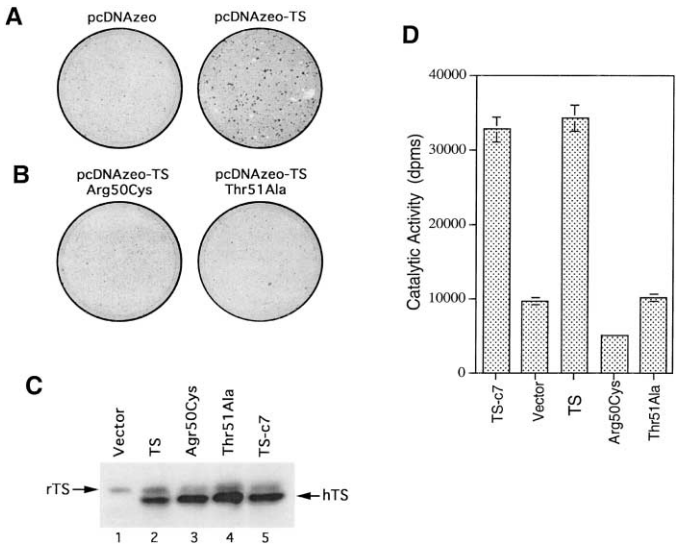

Figure 4. In vitro transformation of rat kidney (RK3E) cells by thymidylate synthase

A and B: Focus assays. RK3E cell pools transfected with the indicated plasmids were tested at passage 2 for foci formation.
C: Immunoblot analysis of cell extracts from pools of stably transfected cells with empty vector (lane 1), wild-type TS (lane 2), and mutants TS, TS(Arg50Cys), and TS(Thr51Ala) (lanes 3 and 4). The TS-c7 cells represent a positive control for the expression of human TS (lane 5), and the arrows depicts ectopic expression of human TS (hTS) or rat TS (rTS).
D: TS catalytic activity was measured from the same extracts derived from cell pools as described in **C**. Results shown are mean ± SD of three experiments.

we tested cell clones with overexpressed mutant TS for the acquisition of in vitro and in vivo parameters of transformed phenotype, including anchorage independent growth in soft agar and tumorigenicity in nude mice. We found that none of the four cell lines expressing ectopic TS-Arg50C or TS-Thr51A acquired anchorage independence for growth as compared to cell lines that overexpressed wild-type TS which could grow in soft agar (Table 3). In addition, injection of 5×10^6 cells that overexpressed mutant TS did not give rise to tumors in Balb/c nude mice by 24 weeks as compared to cells that overexpress wild-type TS which developed tumors between 8 to 18 weeks in injected animals (Table 3). These data demonstrate that a single point mutation that abolishes the catalytic activity of TS is sufficient to prevent neoplastic transformation induced by biologically active TS in vivo.

Table 3. Mutation in the catalytic site of TS abolish oncogenic activity of the enzyme

Cell line	Mutation	% efficiency in soft agar	Tumorgenicity (animals with tumors/animals injected)[a]
TS-c7	Wild-type	0.5	7/10
mTS-c3	Arg-50Cys	0	0/7
mTS-c5	Arg-50Cys	0	0/7
mTS-c6	Thr-51Ala	0	0/7
mTS-c7	Thr-51Ala	0	0/5

[a] All animals were sacrificed at 24 weeks

Overexpression of catalytically active thymidylate synthase induces transformation in RK3E cells

To test if thymidylate synthase can act as an oncogene and transform other mammalian cells in addition to NIH/3T3, we transfected the TS expression plasmid (pcDNA3.1zeo-TS) or control vector (pcDNAzeo) into E1A immortalized rat kidney cells (RK3E). Stable clones were selected in zeocin-containing medium, and foci assay was performed using second passage of pooled clones. We found that cells expressing human TS gave rise to foci three weeks after inoculation, while no foci were observed in the cells transfected with the control vector (Figure 4A). In addition, we transfected RK3E cells with the mutant TS expression plasmids (Arg50Cys and Thr51Ala). In contrast to wild-type TS, foci formation was not observed in RK3E cells transfected with the mutant enzymes (Figure 4B). To demonstrate that the inability of these cells to form foci was linked to the absence of TS catalytic activity and not the absence of TS protein, protein extracts were obtained from the wild-type, mutant, and control vector-transfected RK3E cells and analyzed by SDS-PAGE using anti-TS antibody. The TS(Arg50-Cys) and TS(Thr51Ala)-transfected cell pools contained high levels of human TS that could be resolved from the endogenous rat TS (Figure 4C). To confirm that TS(Arg50Cys) and TS(Thr51Ala) mutants lacked catalytic activity in RKE3 cells, we measured the conversion of dUMP to dTMP by a tritium release

assay (Allegra et al., 1985). We found that exogenously expressed TS mutants were catalytically inactive in mouse RKE3 cells (Figure 4D). These results demonstrate that wild-type TS can induce transformation in two distinct mammalian cell lines and confirm that catalytic activity of the enzyme is required for the induction of oncogenic activity of TS.

Induction of programmed cell death by overexpression of thymidylate synthase

Overexpression of certain oncogenes has been shown to induce programmed cell death following the removal of growth factors (Evan et al., 1992; Shan and Lee, 1994). Since we have now shown that TS can behave as an oncogene, we asked whether TS overexpression could also promote programmed cell death upon serum deprivation. TS-overexpressing cell clones TS- c6, TS-c7, and the control vector clone, V-c1, were grown in media containing 0.1% or 10% serum and examined after 24 and 48 hr for morphological changes associated with apoptosis. In both TS-overexpressing cell lines, the majority of cells were either detached from the plates or were rounded after 24 hr in 0.1% serum, displaying cytoplasmic blebs characteristic of cells undergoing apoptosis (Figure 5A). In contrast, the control cells grown in 0.1% serum (Figure 5A) and both the TS-expressing and control cells grown in 10% serum (data not shown) displayed normal fibroblast morphology. Thus, the morphological changes observed between the TS-overexpressing and the control cells grown in 0.1% serum (Figure 5A) suggest the induction of programmed cell death in the TS-overexpressing cells. To confirm whether TS-overexpressing cells underwent programmed cell death, we performed an in situ TUNEL assay using TS-overexpressing or vector control cells that were grown for 24 hr in 0.1%, 1%, and 10% serum containing medium. We demonstrate that the TS-overexpressing cells grown in 0.1% serum-containing medium exhibited 6- to 10-fold induction of apoptosis as compared to the empty vector control cells (Figure 5B). In contrast, no apoptosis was detected in the TS-overexpressing cells or the control cells grown in 10% serum (Figure 5C). DAPI staining showed that a similar number of TS-c6 and V-c1 control cells were examined in the indicated visual fields (Figure 5B, lower panel). In addition, TUNEL assays performed on cells grown in 1% serum containing medium did not show a significant amount of apoptosis in either the control or TS-overexpressing cells (Figure 5C). We have shown that removal of serum from growth medium resulted in apoptosis with DNA fragmentation in 51% of TS-c6 and 35% of TS-c7 as compared to 6% in the control cultures as measured by the TUNEL assay (Figure 5C).

To further confirm that the observed apoptotic phenotype in the TS-overexpressing cell lines was a consequence of caspase-mediated programmed cell death and not due to a nonspecific event, we studied caspase activation in TS-induced cell death. The proteolytic cleavage of the 116 kDa poly (ADP-ribose) polymerase to an 85 kDa species is a hallmark of caspase 3-mediated apoptosis (Lazebnik et al., 1994). We observed that the 116 kDa PARP was predominant in each of the TS-overexpressing clones until 8 hr following serum deprivation (Figure 5D, lanes 5–12), which was approximately 16 hr before morphological changes were detected by light microscopy. By 24 hr, most of the protein was cleaved in the TS-c6 clone, and $>$50% of protein was cleaved in the TS-c7 cells. The cleavage was dependent on TS overexpression, as serum-starved control cells, V-c1, did not show appreciable proteolysis of PARP (Figure 5D, lanes 1–4).

Mechanisms described previously for oncogene-induced apoptosis include activation of a mitochondrial pathway (which can be inhibited by Bcl-2 or Bcl-XL) and autocrine activation of death receptors, such as Fas receptor (Hueber et al., 1997; Klefstrom et al., 2002; Phillips et al., 1999). To test if activation of the mitochondrial pathway is involved in TS-induced apoptosis, TS-c7 cells were transiently transfected with Bcl-2 or a control vector, incubated in low serum, and analyzed for apoptosis using an ELISA assay which detects fragmented DNA. Incubation of the TS-c7 cells in low serum caused a 20- to 25-fold increase in apoptosis compared to cells grown in 10% serum, and this was abrogated by transfection of Bcl-2 (Figure 5E). Thus, like c-Myc, (Hueber et al., 1997) TS induces apoptosis via a Bcl-2-dependent mitochondrial pathway.

We also demonstrated that NIH/3T3 cells express both Fas and Fas ligand and that the expression of these genes was not affected by either TS levels or the percent serum used in the media (data not shown). Unlike the apoptosis induced by c-Myc, however, inhibition of Fas-Fas ligand interactions with a soluble Fas receptor did not inhibit apoptosis induced by serum deprivation in the TS-c7 cells (data not shown).

Reduction of TS levels in TS-transformed cells correlates with anchorage-independent phenotype

To examine whether downmodulation of TS may play a role in regulating the proliferation of transformed cells, we used an RNAi strategy to decrease TS expression in TS-transformed cells. Duplex 21-mer synthetic RNA oligonucleotides directed against a portion of the TS gene were used to modulate TS levels, while RNA oligonucleotide directed against GAPDH was used as control. Each of these annealed RNA oligonucleotides was transfected into the TS-transformed cell line TS-c7, and their effects on TS protein levels were compared with mock-transfected cells by immunoblot analysis at 72 hr posttransfection. We found that RNAi directed against TS reduced the levels of TS protein in TS-c7 cells but had no effect on actin levels (Figure 6A). In addition, siRNA directed against GAPDH and the mock transfection did not alter TS expression (Figure 6A).

To determine whether the siRNA-mediated reduction in TS levels was associated with a decrease in the ability of TS-transformed cells to form colonies in soft agar, we transfected TS-c7 cells with duplex synthetic siRNA directed against TS along with the following controls: (1) GAPDH control (GAPDH-C), (2) unrelated sequences from human Mastermine ortholog (hMAM), and (3) mock transfection. At 24 hr posttransfection, the cells were placed in soft agar, and colony formations were scored 11 days after inoculation. We observed that siRNA directed against TS resulted in a significant decrease in colony formation in TS-c7 cells (Figure 6B). There was at least a 4-fold reduction in colony formation seen in TS-c7 cells transfected with siRNA directed against TS as compared to the control siRNas.

Since the siRNA results suggested that a reduction in TS levels may decrease the efficiency of transformed cells to form colonies in soft agar, we examined whether TS-c1, a TS-transfected line that expressed 7-fold less TS than TS-c7 (Figure 2, lanes 3 and 9), would also show a reduction of anchorage-independent growth. We compared growth in soft agar between these two cell lines and found a 6-fold decrease in colony formation in soft agar in TS-c1 cells as compared to TS-c7 cells

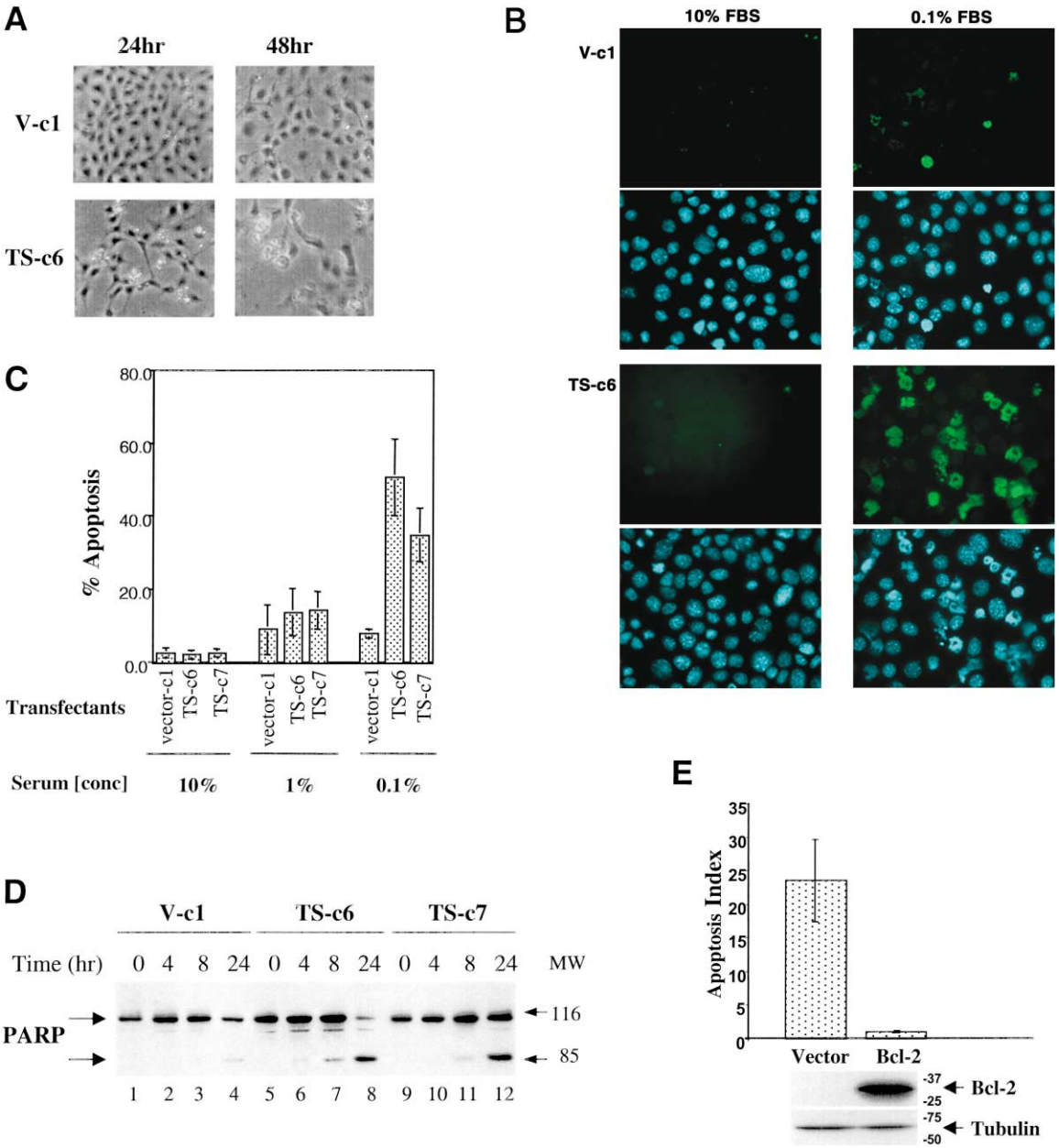

Figure 5. Programmed cell death in thymidylate synthase-transformed cells

A: TS-overexpressing clone (TS-c6) and vector alone transfected cells (V-c1) grown in 0.1% serum.

B: TUNEL assay of TS overexpressing cells and control vector alone cells grown in 10% serum or 0.1% serum for 24 hr. FITC-labeled DNA (upper panel) and DAPI staining (lower panel) for each cell line.

C: Quantification of apoptotic cells in TS-overexpressing clones (TS-c6 and TS-c7) grown for 24 hr in 10%, 1%, or 0.1% FBS. Results shown are mean ± SD of 3 to 6 experiments.

D: Time course of proteolytic cleavage by the poly (ADP-ribose) polymerase (PARP) in TS-c6 and TS-c7 cell grown in 0.1% serum as compared to vector alone clone V-c1. Arrows depict the 116 kDa PARP and the cleaved 85 kDa PARP protein detected with anti-PARP.

E: TS-c7 cells were transiently transfected with Bcl-2 or a control vector, and the cells were cultured in 10% or 0.1% serum. Apoptosis was quantitated using an Elisa assay that measured fragmented DNA. The results are presented as the increase in apoptosis seen in low serum (apoptosis index = apoptosis in 0.1% serum/apoptosis in 10% serum). The results represent the mean increase in apoptosis for three experiments ± SE. The immunoblot on the bottom panel shows expression of Bcl-2 for a representative experiment. Tubulin is shown as a loading control.

(Figure 6C). In addition, we established a tetrocyclin-inducible overexpression system for TS and asked whether repressing TS expression would affect the anchorage-independent phenotype. NIH/3T3 cells were transfected with a TS expression vector under the control of the Tet-inducible promoter, and a TS inducible clone was isolated and designated as TS/Tet-c1. We found that TS/Tet-c1 cells grew in agar with high efficiency (4.8% efficiency) when TS expression was induced, while the addition

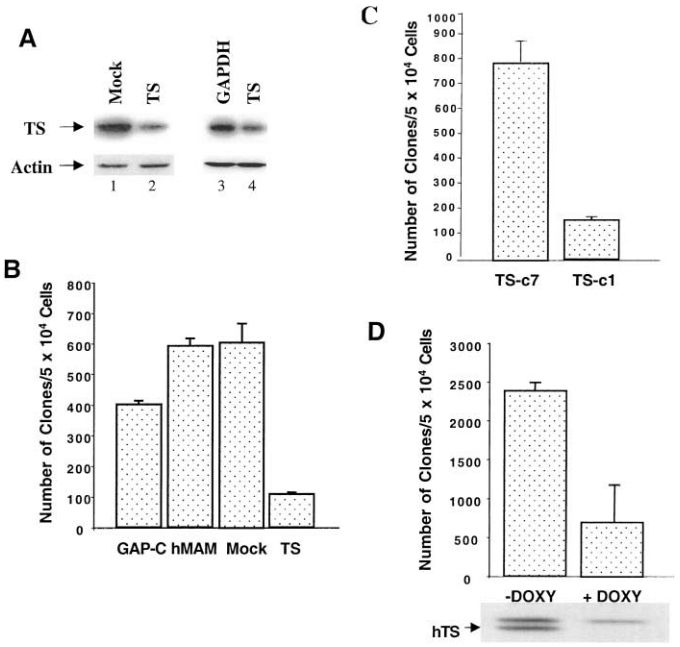

Figure 6. Decrease of TS expression results in the inhibition of anchorage-independent phenotype

A: Immunoblot analysis of TS and actin expression in TS-c7 cells 72 hr following transfection with siRNA directed against TS, GAPDH, or mock transfection.
B: siRNA directed against TS results in reduction of anchorage-independent growth. TS-c7 cells transfected with siRNA directed to TS, GAPDH control (GAP-C), human Mastermind ortholog (hMAM), or mock transfection were plated in soft agar 24 hr posttransfection and scored for colony growth at 11 days. The results represent the mean of clonal growth in soft agar from 3 to 6 dishes inoculated in two independent experiments.
C: Comparison of soft agar growth between high (TS-c7) and low (TS-c1) TS-expressing cell lines. The results represent the mean of clonal colony growth in soft agar from 6 dishes inoculated in two independent experiments.
D: Tet-inducible induction of TS expression in TS/Tet-c1 cells results in an increase of anchorage-independent growth. The immunoblot on the bottom panel shows TS expression in the absence of dexocyclin (−DOXY) and TS repression in presence of the drug (+DOXY). The results represent the mean of clonal colony growth in soft agar from 6 dishes inoculated in two independent experiments.

of doxycyclin that repressed gene expression resulted in a decrease of agar growth (1.4% efficiency) (Figure 6D). Taken together, these results suggest that decreasing TS expression by three distinct methods results in a reduction of anchorage-independent cell growth.

Discussion

TS has long been identified as a potential target for cancer chemotherapeutic agents because of the essential role it plays in the de novo synthesis of thymidylate. In addition, the level of TS protein expression has been shown to predict for the overall survival of patients with colon cancer as well as for possible resistance to chemotherapeutic agents, such as TS inhibitors (Chu and Allegra, 1996; Johnston et al., 1995; Peters et al., 1995). High levels of TS have also been correlated with poor prognosis in patients with ovarian cancer, rectal cancer, and non-small cell lung carcinoma (Edler et al., 2000; Mizutani et al., 2003; Nomura et al., 2002; Pestalozzi et al., 1997; Shintani et al., 2003; Suzuki et al., 1999). Over the past decade, several studies have also suggested a possible link of TS gene expression with cell cycle regulation and cell proliferation. These reports have associated RB growth suppressor function with its ability to repress the transcriptional activation of E2F target genes (Harbour and Dean, 2000), which include a wide variety of metabolic enzymes such as TS, DHFR, and TK (DeGregori et al., 1995; Dyson, 1998; Helin, 1998). Consistent with the model that E2F can regulate the expression of these enzymes, it has been shown that ectopic expression of E2F-1 can stimulate the expression of TS in quiescent cells (Johnson et al., 1993) and that Rb(−/−) MEF cells overproduce TS protein (Angus et al., 2002). This suggests that a specific role for RB might be to inhibit DNA synthesis (Angus et al., 2002) and that, conversely, overproduction of TS by deregulated RB could participate in aberrant stimulation of DNA synthesis that might facilitate progression to a full neoplastic phenotype. We have now tested if ectopic overexpression of TS could directly act as a transforming agent. In this report, we have demonstrated that cells that overexpress TS acquire a transformed phenotype scored by foci formation, growth in soft agar, and the ability to form tumors in nude mice. While the quantitative efficiency of these parameters for tumorigenicity is less pronounced than that measured for activated protooncogenes such as H-ras, c-*myc*, or v-src, our results nevertheless show that catalytically active TS exhibits oncogenic potential, and its transformation efficiencies are comparable to those previously reported for other transforming genes such as STAT3 or Notch 2 (Bromberg et al., 1999; Capobianco et al., 1997). In addition, we have demonstrated a direct association of elevated TS levels with anchorage-independent phenotype by (1) using a Tet-inducible system, (2) siRNA directed against TS, and (3) comparison of transfected clones with high and low TS expression.

To establish whether the delay in tumor formation was consistent with the requirement for additional somatic mutational events, we have analyzed the p53 and RB suppressor genes by protein immunoblot analysis and found low-level, full-length wild-type p53 signal and a wild-type RB migration pattern in extracts from TS-overexpressing xenograft tumors. While these results are consistent with prior studies that have failed to detect a relationship between p53 and TS levels in colon cancer biopsies (Johnston et al., 2003), additional defects in oncogene and tumor suppressor pathways remain to be defined in these tumor cells.

To confirm a link between TS activity and transformation, we overexpressed two plasmids, each carrying a different single point mutation located within the highly conserved and catalytically essential Arg^{50}-loop of TS (Schiffer et al., 1995). This loop forms a bridge between the dUMP substrate and the CH_2CH_4-folate cofactor and the C-terminal domain of the enzyme. Mutations between residues 47 to 52 disrupt the hydrogen bonding network within the Arg50 loop that mediates dUMP binding and also affects the ability of TS subunits to homodimerize (Voeller et al., 2002). We showed that the Arg50Cys and Thr51Ala mutant TS plasmids express a stable protein species that results in loss of catalytic activity. We also showed that in contrast to wild-type TS, the inactive TS mutants could not transform cells in culture and that overexpression of Arg50Cys and Thr51Ala TS mutants did not result in (1) foci formation, (2) growth in semisolid medium, or (3) tumor formation in nude mice. These data demonstrate that the TS induction of a neoplastic pheno-

type requires the catalytic activity of the enzyme. While it is not surprising that the regulation of DNA synthesis might play a role in cellular transformation, the present work demonstrates a direct link between the DNA synthetic enzyme apparatus and the establishment of neoplastic phenotype.

Interestingly, it was previously shown that overexpression of an enzyme that is also essential for DNA synthesis and repair, ribonucleotide reductase, also predisposes cells to the acquisition of a malignant phenotype. Altered expression of the R2 component of ribonucleotide reductase cooperates with ras and other oncogenes in the mechanism of neoplastic progression (Fan et al., 1996, 1998). In contrast, it was shown that the R1 subunit of ribonucleotide reductase has malignancy suppressing activity when overexpressed in ras-transformed fibroblast (Fan et al., 1997). These results suggest that ribonucleotide reductase, in addition to DNA synthesis and repair, can participate in other critical cellular functions, and that the alteration in the balance of R1 and R2 levels may affect transformation, tumorigenicity, and metastatic potential.

The TS-dependent conversion of dUMP to TMP is an essential step for providing the necessary dTTP components required for DNA replication and for maintaining a proper nucleotide balance within the cells. RB-mediated cell cycle arrest, which results in a decrease of TS expression, was shown to be associated with dTTP and dATP depletion, resulting in a higher percentage of dCTP in the total nucleotide pool (Angus et al., 2002). This data suggested that one mechanism by which the RB tumor suppressor pathway may regulate DNA replication is by controlling the levels and ratios of intracellular nucleotide pools (Angus et al., 2002). Thus, overexpression of TS might also be predicted to generate an imbalance of dNTP pools that may have multifactorial effects on cell homeostasis, including an increase in the mutational rates or an impairment of DNA repair mechanisms (Bradley and Sharkey, 1978; Davidson and Kaufman, 1978; Meuth, 1981, 1989).

As described for other oncogenes (Hueber et al., 1997), we have also shown that TS-transformed cells undergo apoptotic cell death following removal of serum from their growth medium. Serum deprivation, however, not only removes growth factors, but also removes the supply of thymidine that is required to maintain the nucleotide pool balance and DNA synthesis. Since removal of thymidine has been previously shown to result in apoptosis, referred to as "thymineless death" (Houghton et al., 1994), it was possible that overexpression of TS might protect these cells from undergoing apoptotic cell death. In contrast, however, we observed that TS overexpression resulted in the induction of apoptosis, as measured by PARP cleavage, which occurred as early as 8 hr after serum removal, and by TUNEL assay that was measured 24 hr after serum deprivation. Thus, high levels of TS may lead to an accumulation of genetic alterations in the genome due to direct or indirect dNTP imbalance that can result in transformed phenotype, and in enhanced susceptibility to apoptotic cell death upon serum removal. The observation that Bcl-2 can inhibit TS-induced apoptosis in low serum suggests a role for activation of mitochondrial pathway in these cells. The mechanism that underlines the ability of ectopic TS overexpression to induce apoptotic cell death and how TS activates the mitochondrial apoptosis pathway and the neoplastic transformation remains to be defined. In addition, the observations that ectopic TS could participate in tumorigenicity and that TS-overexpressing cells could be susceptible to an apoptotic cell death pathway support the use of TS as a potential biomarker (Chu et al., 2003) in cancer, and may provide further rationale for the development of effective antitumor agents.

Experimental procedures

Plasmids

Plasmid pCIneo-TS was constructed by PCR amplification of the plasmid pcEHTS (ref) using the following primers: sense 5′TTT TCT AGA CCG CCC GCC GCG CCA TGC CTG TGG CCG GCT CGG 3′ and antisense 5′TTT TCT AGA ACC CTA AAC AGC CAT TTC CAT3′, which amplified the region from −146 bp from the ATG site to 944 bp. PCR fragments were subcloned into the Xba I site of the pCIneo vector (Invitrogen) generating the pCIneo-TS expression plasmid. For construction of pcDNA3.1zeo-TS, the 958 bp Xba I DNA fragment excised from pCIneo-TS was subcloned into the mammalian expression vector, pcDNA3.1 zeo (+) (Invitrogen). The mutant plasmids (gift from D. Banerjee and J. Bertino) were subcloned into the HindIII/Xho site of the pcDNA3.1 zeo (+) vector. Nucleotide sequences were obtained to confirm the presence of Arg50Cys and Th51Ala mutations. Plasmid Tet-TS was constructed by subcloning a blunt-ended 948 bp HindIII/PmeI DNA fragment of TS into the blunt-ended BamHI site of the tetracycline-inducible vector, Ec1514A (Li et al., 1996). Bcl-2 expression plasmid was cloned in pcDNA 3 (Invitrogen) and was a gift from M. Blagosklonny.

Cell culture, transfections, foci assays, and growth analysis

NIH/3T3 and RK3E cells were maintained in Dulbelcco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1% glutamine, and 1% penicillin/streptomycin. NIH/3T3 cells were transfected with 10 μg of plasmids by calcium phosphate precipitation as previously described (Reinhold et al., 1995). Cells from each transfection were split 1:3 and grown for 14 days in 800 μg/ml of zeocin or 600 μg/ml of G418 selection medium. Foci assays were performed at passage 3 or passage 8 by plating 5×10^5 cells per 100 mm petri dishes in zeocin selection, and at passage 1 when plated in G418-containing medium. Cells were incubated for 5–6 weeks, fixed with 10% methanol, 10% acidic acid solution, and stained with 20% ethanol, 0.4% crystal violet for 5 min. For generating growth curves, NIH/3T3 cells were plated at 2×10^5 cells per 100 mm dish and counted in triplicate using a Beckman Coulter Counter. RK3E cells were transfected with 15 μg of plasmids by Fugene 6 as described by the manufacturer (Roche Molecular). Cells from each transfection were split by plating 1.4×10^6 cells per 100 mm dish and grown for 10 days in 100 μg/ml of zeocin. Foci assays were performed at passage 2 by plating 1.5×10^6 cells per 100 mm petri dishes in zeocin selection. Cells were incubated for 3 weeks and fixed as described above. For isolation of single cell clones containing Tet-inducible TS gene, NIH/3T3 cells were transfected with 15 μg of Tet-TS plasmid using Fugene 6 and single cell clones were selected in 600 μg/ml of G418. Cells were expanded in medium with serum containing trace amounts of tetrocyclin and then switched to a medium supplemented with tetracycline-free fetal calf serum (BD Biosciences, Palo Alto, CA). For soft agar analysis, cells were grown for 48 hr in the presence or absence of 2 μg/ml doxycyline in medium containing a tetracycline-free fetal calf serum.

Cellular extract preparation, antibodies, and Western blot analysis

Whole cell extracts were prepared as previously described (Niklinski et al., 2000). Cells were lysed in EBC buffer (50 mM Tris [pH 7.5], 200 mM NaCl, 50 mM NaF, 5 mM EDTA, 0.1% Nonidet P-40 5 mM Mg2Cl) supplemented with 0.5 mM dithiothreitol, 1 mM phenyl-methylsulfonyl fluoride, and leupeptin and aprotenin (10 μg/ml each). One hundred micrograms of protein extract was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose filters, and immunoblotted with anti-TS antibody (clone TS-106) in a 1:100 dilution as previously described (Alexandrova et al., 1995; Behan et al., 1998). For analysis of apoptosis by PARP cleavage, cells were grown in 0.1% serum, and both attached and detached cells were used for cell extract preparation. The anti-PARP antibody (H-250) and the Bcl-2 antibody (100) were obtained from Santa Cruz Biotechnology and both used in a 1:200 dilution. The α-Tubulin antibody (clone DM1A) was obtained from Sigma and used in 1:500 dilution.

Transfection of RNA oligonucleotides and RNAi sequences

For TS gene expression by immunoblot analysis, 3×10^5 cells were plated per 100 mm dish and transfected with 4 μg duplex siRNA and 60 μl of Effectene (Qiagen, Valencia, CA) as recommended by the manufacturer. Cells were harvested at 72 hr and lysed in EBC buffer as described above. For soft agar growth analysis, 1×10^5 cells were plated per 6-well plates and transfected for 24 hr with 1 μg of duplex siRNA and 10 μl of Effectene. Cells were seeded in soft agar medium and colonies were scored 11 days after inoculation. The sequences of the sense strand of the TS RNA oligonucleotides used are as follows: TS: 5′GGAACUAGGUCAAAAAUC-UTT-3′. Small annealed duplex RNAs for TS, GAPDH, and GAPDH-C were purchased from Ambion (Austin, TX), and duplex RNA for hMAM was obtained from Qiagen.

TS catalytic activity

TS catalytic activity was determined using 100 μg of cell lysate, 10^{-5} M [5-^{3}H] dUMP, 200 pmol dUMP, 200 pmol methelyne tetrahydrofolate, and 20 μM β-mercaptoethanol in a 200 μl final volume as previously described (Allegra et al., 1985). Samples were incubated at 37°C for 30 min, and the reaction was terminated by the addition of 100 μl 20% TCA. Unmetabolized [5-^{3}H] dUMP was removed by centrifugation following the addition of 200 μl of an albumin-coated activated charcoal solution. The amount of [^{3}H] H_2O released was measured by counting the supernatant using a Beckman liquid scintillation counter.

Anchorage-independent growth and tumorigenicity assays

Anchorage independent growth was quantitated by colony formation in semisolid medium as previously described (Zajac-Kaye and Ts'o, 1984). 5×10^4 or 1×10^5 cells were seeded in triplicates per 60 mm dishes in medium supplemented with 15% serum and 400 μg/ml zeocin. Colonies were scored 11 to 14 days after inoculation. Tumorigenicity was assayed by subcutaneous injection of 5×10^6 cells into the backs of 5-week-old Balb/CAN/Ncr-nu mice (Frederick Research Center, Maryland). Cells were expanded for two passages without selection and injected in a total volume of 150 μl in DMEM supplemented with 5% serum.

Apoptosis assays

Cells were grown in 0.1%, 1%, and 10% serum containing medium for 24 or 48 hr. For the in situ detection of nuclear DNA fragmentation by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay, attached and detached cells were combined and collected on slides using cytospin centrifugation. Cells were fixed in Histochoice fixative solution (Amresco, USA) for one hour at 4°C, washed with cold PBS, and permeabilized for 2 min with 0.1% Triton X-100 in 0.1% Sodium Citrate solution at 4°C. Apoptosis was measured using fluorescein in situ cell death detection kit (Roche Molecular) as described by the manufacturer. The slides were mounted using Vectashield (Vector Laboratories) and sealed with clear nail polish. The percentage of TUNEL positive cells was determined by counting cells in a representative visual field under fluorescent microscope. For transfection of Bcl-2, 3×10^5 cells were plated per 100 mm dish and transfected with 2 μg of plasmid DNA and 20 μl of Effectene (Qiagen) as described by the manufacturer. Following 24 hr recovery after removal of transfection medium, cells were incubated for 24 hr either in 0.1% or 10% serum-containing medium. Both attached and detached cells were harvested for cell extract preparation. Quantitative determination of DNA fragmentation was performed using the Cell Death Elisa^{plus} kit (Roche Applied Science) as indicated in the instruction manual.

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