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Mechanisms of Acquired Resistance to Thymidylate Synthase Inhibitors: The Role of Enzyme Stability

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ABSTRACT

Inhibitors of the enzyme thymidylate synthase (TS), such as the fluoropyrimidines 5-fluorouracil and 5′-fluoro-2′-deoxyuridine (FdUrd) or the antifolates AG337, ZD1694, and BW1843U89, are widely used in the chemotherapy of cancer, particularly cancer of the colon and rectum. Numerous studies have shown that TS gene amplification, leading to mRNA and enzyme overproduction, is a major mechanism of resistance to these inhibitors. In the present work, we have isolated and characterized FdUrd-resistant derivatives of several human colon tumor cell lines. Although gene amplification was commonly observed, the increases in mRNA and enzyme were strikingly discordant. In one drug-resistant line, a deficiency of enzyme relative to mRNA was shown to be caused by expression of a metaboli-cally unstable TS molecule. The reduced half-life of TS in this line was caused by a Pro-to-Leu substitution at residue 303 of the TS polypeptide. The mutant enzyme conferred resistance to FdUrd as well as antifolates in transfected cells. In another FdUrd-resistant line, which had an excess of enzyme relative to mRNA, the TS molecule was more stable than in the parent line. However, no amino acid substitutions were detected in the TS polypeptide from this line, which suggests that the stabilization must be caused by changes in one or more cellular factors that regulate TS degradation. The results indicate that changes in the stability of the TS polypeptide accompany, and even contribute to, acquired resistance to TS inhibitors in colon tumor cells.

Thymidylate synthase (TS; EC 2.1.1.45) is an S-phase enzyme that catalyzes the reductive methylation of dUMP by N^5,N^{10}-methylene-5,6,7,8-tetrahydrofolic acid ($CH_2H_4PteGlu$), generating dTMP and dihydrofolate (for a review, see Carreras and Santi, 1995). The enzyme is indispensable in the de novo synthesis of dTMP and therefore plays an important role in DNA replication in actively dividing cells. The critical role of TS in dTMP synthesis has made it an attractive target at which chemotherapeutic agents are directed. Fluoropyrimidines [e.g., 5-fluorouracil (FUra) and 5′-fluoro-2′-deoxyuridine (FdUrd)] and, more recently, antifolates (e.g., AG337, ZD1694, BW1843U89) have been useful in the clinical management of tumors of the breast, colon, stomach, and head and neck (Heidelberger et al., 1982; Takemura and Jackman, 1997). In growing cells, fluoropyrimidines are metabolized to 5-fluoro-2′-deoxyuridylic acid (FdUMP), which inhibits TS via formation of a covalent complex containing the nucleotide analog $CH_2H_4PteGlu$ and TS (Carreras and Santi, 1995). This complex, which is termed the inhibitory ternary complex (ITC), is

quite stable, resulting in prolonged inhibition of the enzyme, depletion of dTMP pools, and thymineless death.

Extensive research has defined molecular mechanisms by which cells become resistant to fluoropyrimidines. Overproduction of TS via amplification of its structural gene is one such mechanism (Berger et al., 1985; Jenh et al., 1985). Changes in TS structure are also important, as indicated by the existence of amino acid substitutions in the TS polypeptide that alter binding of inhibitory ligands and modify drug sensitivity (Barbour et al., 1990; Climie et al., 1990). A number of clinical studies have indicated that high concentrations of TS in tumor biopsy specimens are associated with reduced clinical response to fluoropyrimidines (Suzuki et al., 1994; Johnston et al., 1995, 1997).

We have been interested in resistance to TS-directed inhibitors that occurs as a consequence of changes in enzyme structure. Because these inhibitors have been widely used against tumors of the gastrointestinal tract, we have focused our attention on human colonic tumor cell lines (Berger and Berger, 1988; Berger et al., 1988). Previous studies revealed that one cell line (HCT116) is relatively resistant to FdUrd because of a naturally occurring Tyr-to-His substitution at

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ABBREVIATIONS: TS, thymidylate synthase; $CH_2H_4PteGlu$, N^5,N^{10}-methylene-5,6,7,8-tetrahydrofolic acid; FUra, 5-fluorouracil; FdUrd, 5′-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridylic acid; ITC, inhibitory ternary complex; RT, reverse transcriptase or transcription; PCR, polymerase chain reaction; kb, kilobase; bp, base pair; RER, replication error.

residue 33 of the TS polypeptide (Barbour et al., 1990, 1992; Hughey et al., 1993; Reilly et al., 1995, 1997). The mutant enzyme confers fluoropyrimidine resistance as a consequence of its reduced affinity for FdUMP (Hughey et al., 1993; Reilly et al., 1997). In the present article, we have characterized fluoropyrimidine-resistant derivatives of several human colon tumor cell lines and show that changes in the stability of the TS polypeptide are associated with, and contribute to, the observed drug resistance. In one of the lines, the alteration in enzyme stability is caused by a Pro303-to-Leu substitution within the TS polypeptide. In the accompanying article, the kinetic properties of homologous substitutions within the *Escherichia coli* enzyme (i.e., at Pro254) are presented.

Experimental Procedures

Cell Lines. Human colon tumor cell lines HCT116, CBS, and C were obtained from Dr. M. Brattain, Medical College of Ohio (Toledo, Ohio) (●). All other cell lines were purchased from the American Type Tissue Collection (Rockville, MD). Cells were typically grown at 37°C in RPMI 1640 medium containing 10% fetal bovine serum (Gibco Inc., Grand Island, NY) in a humidified 5% CO_2 atmosphere.

Adaptation to FdUrd was carried out by stepwise increases in the inhibitor concentration in medium containing 10 μM folinic acid. The specific FdUrd concentrations in which cells were grown were based upon the parental line's dose-response phenotype; drug concentrations represented the concentration of drug required for 10% inhibition of cell growth (ID_{10}), and for ID_{20}, ID_{50}, ID_{100}, $2 \times ID_{100}$, and $4 \times ID_{100}$. Cell lines SW480 and SW620, which have ID_{50} values of 1.3 nM and 1.8 nM, respectively, were eventually adapted to a maximal FdUrd concentration of 40 nM; cell line HCT15, which has an ID_{50} value of 5.7 nM, was adapted to 200 nM. At each step in the selection, a vigorously growing population of cells was obtained before increasing the drug level. The adapted lines were maintained in FdUrd; they were cultured in drug-free medium for 2 weeks before analysis.

Response to TS inhibitors was measured by plating cells in various concentrations of drug in 25-cm^2 flasks (39–40,000 cells/flask). After 5 to 7 days, the number of surviving cells was assessed by staining with trypan blue and counting with a hemocytometer; the ID_{50} value was calculated. At least three flasks were used for each concentration.

Measurement of TS Levels. Extracts of logarithmically growing cells were prepared by sonication, followed by centrifugation at 100,000g for 1 h. The concentration of TS in the extracts was determined by the FdUMP binding assay (Cisneros and Dunlap, 1990), as described previously (Reilly et al., 1995). Reaction mixtures (500 μl) contained Morrison buffer [120 mM Tris, 60 mM 2-(*N*-morpholino)ethanesulfonic acid, 60 mM acetic acid, pH 7.2], 100 nM [6-^{3}H]F-dUMP (16.6 Ci/mmol; Moravek Biochemicals, Brea, CA), 65 μM CH_2H_4PteGlu, 300 μg/ml bovine serum albumin, and 50 to 100 μg of extract protein. After a 1-h incubation at 25°C, enzyme-bound radioactivity was precipitated by addition of 125 μl of 50% trichloroacetic acid, and the pellet was washed, resuspended in 50% ethanol/0.2 N NaOH, and counted. Assays were repeated using 200 nM [6-^{3}H]F-dUMP to ensure enzyme saturation. Concentrations of TS are expressed as picomoles of FdUMP bound per milligram of total protein.

Western blot analysis of TS was carried out as described previously (Reilly et al., 1997) using a monoclonal antibody against human TS as probe. The antibody-antigen complex was detected by chemiluminescence (ECL-PLUS kit; Amersham, Arlington Heights, IL); signals were quantitated with the use of a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Northern Blotting. Samples of total cellular RNA (10 μg) were subjected to electrophoresis through agarose gels containing 2.2 M formaldehyde, transferred to nylon membranes, and hybridized to radiolabeled TS-specific probes. Equivalent loading of RNA samples

was assured by ethidium bromide staining of the gel before and after transfer. The probe was a 1.3-kilobase (kb) *Acc*I/*Hpa*I fragment of plasmid pKB148, which represents a full-length human TS cDNA (Barbour et al., 1992). The blots were washed thoroughly, visualized by autoradiography, and quantitated with the use of a PhosphorImager.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Sequence Analysis of the Coding Region of TS mRNAs. RT was carried out in 20 μl of reaction mixtures containing 50 mM Tris-Cl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM dithiothreitol, 125 mM each of dCTP, dATP, dGTP, and dTTP, 5 μg/ml oligo(dT)$_{15}$, 10 μg total RNA, 20 U of RNasin (Promega, Madison WI), and 200 U of Moloney murine leukemia virus RT (Promega). After a 30-min incubation at 42°C, a 1-μl aliquot was removed and added to a PCR reaction mixture containing, in a total volume of 100 μl, the following: 10 mM Tris-Cl, pH 9.0, 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 0.1 μM each primer, 0.4 mM each of dATP, dCTP, dGTP, and dTTP, and 5 U of *Taq* polymerase (Perkin-Elmer). The forward primer (5′-ATGCCTGTGGCCGGCTCGGA-3′) corresponds to the region between nucleotides +1/+20 of the TS mRNA (+1 corresponds to the first nucleotide of the translation initiation codon); the reverse primer (5′-CACGGACAGATTTTT-GACCT-3′) is complementary to the region between nucleotides +1040/+1059 within the 3′-untranslated region of the mRNA. Amplification was carried out for 30 cycles (1 min at 95°C, 1 min at 55°C, and 1 min at 72°C), followed by an additional 7 min at 72°C. The 1.1-kb product was concentrated on a Centricon-100 filter, purified by gel electrophoresis through 1% agarose, and subcloned into plasmid pGEM-T (Promega). Sequence analysis of the entire coding region was accomplished on an automated DNA sequencer from LiCor (Lincoln, NE).

Detection of the P303L Substitution by RT-PCR. An *Aci*I polymorphism at nucleotide 908 was used to diagnose the presence of P303L substitution (see text for details). The region of the TS mRNA between nucleotides 838 and 1059 was amplified by RT-PCR; the forward primer (5′-AGGATTCTTCGAAAAGTTGA-3′) corresponded to nucleotides 838 to 857, whereas the reverse primer (5′-CACGGA-CAGATTTTTGACCT-3′) was complementary to nucleotides 1040 to 1059. The 222-base-pair (bp) amplification product was digested with *Aci*I, and analyzed by agarose gel electrophoresis. *Aci*I cleaves the RT-PCR product of the wild-type mRNA into fragments of 170 bp and 52 bp, but does not cleave the product of the mutant mRNA.

Determination of the Half-Life of TS. Cells at low density were placed into medium containing 90 μg/ml cycloheximide. At various times after addition of cycloheximide, cells were collected, extracts were prepared, and TS levels were assayed by either the FdUMP binding assay or by Western blotting (see above).

Site-Directed Mutagenesis. Leucine and aspartic acid were introduced in place of proline at residue 303 within the human TS polypeptide by PCR mutagenesis. The template for mutagenesis was plasmid pKB82, which contains a cDNA copy of the TS mRNA between nucleotides 206 and 1431. The mutagenic primers corresponded to nucleotides 899 to 918 within the human TS cDNA. The forward mutagenic primer for generation of the P303L substitution was 5′-GGTACAAT<u>CTG</u>CATCCAACT-3′ (the leucine codon is underlined), whereas the reverse mutagenic primer was 5′-AGTTGGAT-GC<u>AG</u>ATTGTACC-3′); the forward mutagenic primer for generation of the P303D substitution was 5′-GGTACAAT<u>CTG</u>CATCCAACT-3′ (the aspartic acid codon is underlined), whereas the reverse mutagenic primer was 5′-AGTTGGATGC<u>AG</u>ATTGTACC-3′). In one PCR reaction, the reverse mutagenic primer and an upstream flanking primer (5′-TATTCAGGACAGGGAGTTGA-3′) corresponding to the region between nucleotides 457 and 476 were used; in a second reaction, the forward mutagenic oligonucleotide and a T7 primer (5′-GTAATACGACTCACTATAGGGC-3′) corresponding to vector sequences located beyond the 3′ end of the TS cDNA, were used. Conditions for PCR were as described above. The PCR products from these reactions were combined and coamplified using the upstream

and the T7 primers. DNA sequencing was used to verify the presence of the mutation and to assure that no other base substitutions were present.

DNA Transfection. Plasmid pJZ205, which contains a wild-type human TS cDNA under the control of an SV40 promoter, was constructed by blunt-end ligation of the *Acc*I/*Hpa*I fragment of pKB169 (Barbour et al., 1992) into *Hind*III/*Hpa*I-digested pSV2-CAT. Plasmids containing the P303L and P303D mutant cDNAs under control of the SV40 promoter were generated by replacing the *Bgl*II/*Hpa*I fragment of pKB169 with the corresponding region from an appropriately mutagenized construct produced above. The *Acc*I/*Hpa*I fragment of the resulting plasmid was subcloned by blunt-end ligation into pSV2-CAT.

Plasmids were transfected into the TS-deficient cell line RJK88.13 (Nussbaum et al., 1985) by calcium phosphate-DNA coprecipitation (Graham and van der Erb, 1973) in medium containing 20 μg/ml thymidine; 16 to 25 μg of plasmid DNA were added per culture. After 48 h, the cells were placed in selective medium lacking thymidine, and colonies were pooled. Cells transfected with cDNA expressing wild-type TS were denoted RJK88.13/Pro303, and cells transfected with cDNA expressing the P303L and P303D mutants were denoted RJK88.13/Leu303 and RJK88.13/Asp303, respectively. The growth rates of the transfected lines were nearly identical, with doubling times of about 16 to 18 h.

Results

FdUrd Response and TS Expression in Human Colon Tumor Cell Lines. In preliminary studies, we characterized the phenotype of each of 15 human colon tumor cell lines with regard to both FdUrd sensitivity and TS expression. In drug response experiments, folinic acid was added to the growth media to maintain high $CH_2H_4PteGlu$ levels and maximize the formation and stability of the ITC (Carreras and Santi, 1995). As shown in Fig. 1, the ID_{50} values ranged from 0.1 nM in CBS to nearly 8 nM in HCT116. Interestingly, there was an association between drug sensitivity and the replication error (RER) phenotype. Cells defined as RER+, which are prone to replication errors because of mutations in any of several mismatch repair genes (Umar and Kunkel, 1996), exhibited higher ID_{50} values than cells defined as RER−, which are normal with regard to mismatch repair (Fig. 1). The statistical significance of the difference between RER+ and RER− lines (3.6 ± 2.4 nM versus 1.1 ± 1.1 nM, respectively) was $p < .02$ by a standard t test, and $p < .01$ by a Wilcoxon two-sample nonparametric test. Thus, mismatch repair-defective cell lines seem to be more resistant to FdUrd compared with mismatch repair-proficient lines.

TS enzyme concentrations were determined in cell extracts by measuring the covalent binding of radiolabeled FdUMP into an inhibitory ternary complex, an assay that measures the level of active TS (Cisneros and Dunlap, 1990). Enzyme concentrations ranged from ~0.1 pmol TS/mg protein in SW1116 to ~5 pmol TS/mg in cell line C (Fig. 2). As with FdUrd response, there was an indication that the RER status is associated with TS expression. Enzyme levels in the RER+ lines were higher than those in RER− lines; the average difference between the two groups (2.5 ± 1.2 pmol/mg versus 0.59 ± 0.35 pmol/mg, respectively) was statistically significant by both a t test ($p < .001$) and a Wilcoxon test ($p < .005$). Thus, RER+ colon tumor cell lines express higher TS concentrations than RER− lines. Western blotting verified that differences in TS expression among the cell lines reflect total TS polypeptide concentrations per se, rather than alterations

in pools of inactive enzyme (data not shown). As observed in an earlier study (Berger and Berger, 1988), ID_{50} values and TS levels were only weakly correlated among the cell lines, which indicates that the concentration of enzyme only partially explains the variations in drug response.

Characterizaton of FdUrd-Adapted Cell Lines. Fluoropyrimidine-resistant derivatives of cell lines SW480, SW620, and HCT15 were isolated by stepwise selection in progressively increasing FdUrd concentrations. The particular drug concentrations used for each line were based upon the dose-response phenotype of the parental line, and corresponded to ID_{10}, ID_{20}, ID_{50}, ID_{100}, $2 \times ID_{100}$, and $4 \times ID_{100}$. Cell lines SW480 and SW620 were adapted to a maximal FdUrd concentration of 40 nM, resulting in lines SW480/40 and SW620/40; HCT15 was adapted to a maximal concentration of 200 nM FdUrd, resulting in cell line HCT15/200. Drug response and TS enzyme levels for the resistant cells are shown in Table 1. As expected, the selected cells were resistant to high FdUrd levels compared with their drug-sensitive parents. SW480/40 and SW620/40 overproduced TS by about 60- to 100-fold, whereas HCT15/200 overproduced the enzyme by only 3- to 5-fold (Table 1). These results were verified by Western blotting (Fig. 3), which indicated 4-fold overproduction of TS in HCT15/200 and 60-fold overproduction in SW480/40. Thus, increases in TS concentrations were commonly observed during selection for FdUrd resistance; however, the extent of such increases differed among the lines.

Northern blot analysis of TS mRNA levels in the FdUrd-adapted lines revealed striking discordances between mRNA and enzyme overproduction (Fig. 4). The 60- to 100-fold overproduction of enzyme in SW480/40 and SW620/40 was asso-
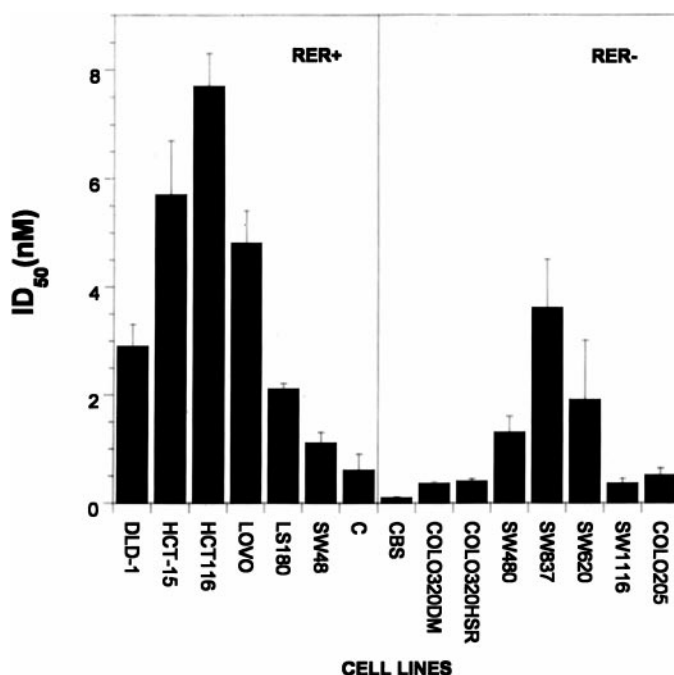

Fig. 1. FdUrd response of colon tumor cell lines. The ID_{50} value for each cell line was determined as described under *Experimental Procedures*; vertical bars indicate standard deviations. The lines are grouped according to RER status. The RER assignments were based upon microsatellite instabilities, biochemical assays of various steps in the mismatch repair pathway, or direct analysis of mutations in MMR genes (Bhattacharyya et al., 1994, 1995; Papadopoulos et al., 1994; Umar et al., 1994; Liu et al., 1995; Markowitz et al., 1995; Lengauer et al., 1997).

ciated with only a 10- to 15-fold increase in mRNA. Such discordance may derive from either an increase in the translational efficiency of TS mRNA or a stabilization of TS protein; both processes would lead to further increases in the TS level and would contribute to acquisition of drug resistance. For HCT15/200, the modest (i.e., 3–5-fold), overproduction of TS was associated with a 30- to 40-fold increase in the mRNA level, indicating that either the translational efficiency of TS mRNA is decreased or the rate of enzyme degradation is increased. It is difficult to imagine how a reduction in translational efficiency or an increase in enzyme degradation would, on its own, confer drug resistance during adaptation to FdUrd. It is probable that one or more mutations that cause amino acid substitutions within the TS polypeptide and that affect the enzyme's ability to enter into an inhibitory ternary complex have occurred; reduced translation and/or enzyme destabilization may be secondary consequences of such mutations.

Polysome analyses showed no changes in the extent of ribosome binding to TS mRNA in any of the resistant lines compared with their drug-sensitive parents (M. Kitchens,

unpublished observations). Thus, it is unlikely that differential overproduction of TS protein and mRNA is caused by alterations at the translational level. Changes in enzyme stability are more probable. Experiments presented below indicate that this is indeed the case.

Southern blotting showed that in all cases, increases in mRNA levels reflected the extent of TS gene amplification (data not shown). Thus, acquired resistance to FdUrd involves gene amplification along with mRNA and enzyme overproduction. However, the striking discordances between increases in mRNA and enzyme indicated that alterations in post-transcriptional processes (e.g., mRNA translation, protein degradation) have occurred.

Identification of an Amino Acid Substitution in the TS Polypeptide from Cell Line HCT15/200. The FdUrd-adapted cell lines were analyzed to determine if any contained mutant TS. RT-PCR was used to amplify the amino acid coding region of TS mRNA, which was subcloned and sequenced by standard methods (see *Experimental Procedures*). In initial experiments, we determined the sequence of the coding region from each of several parental cell lines, including SW480, SW620, LoVo, C, DLD1, HCT15, HCT116, and LS180. In all but one, only wild-type amino acid sequences were found. The single exception was cell line HCT116, which contains the previously identified Tyr33-to-His substitution and has been studied in some detail (Barbour et al., 1990, 1992; Hughey et al., 1993; Reilly et al., 1995, 1997).

Although the coding regions of both SW480/40 and SW620/40 were wild-type in sequence, that in HCT15/200 contained a C-to-T transition at nucleotide 908. This mutation, which destroys an *Aci*I restriction site at nucleotides 907 to 910, changes codon 303 from proline (C$\underline{C}$G) to leucine (C$\underline{U}$G). Further analyses were aimed at assessing the role, if any, of the P303L substitution in drug resistance.

To determine if the mutation occurred simultaneously with mRNA overproduction during selection of HCT15/200, cells
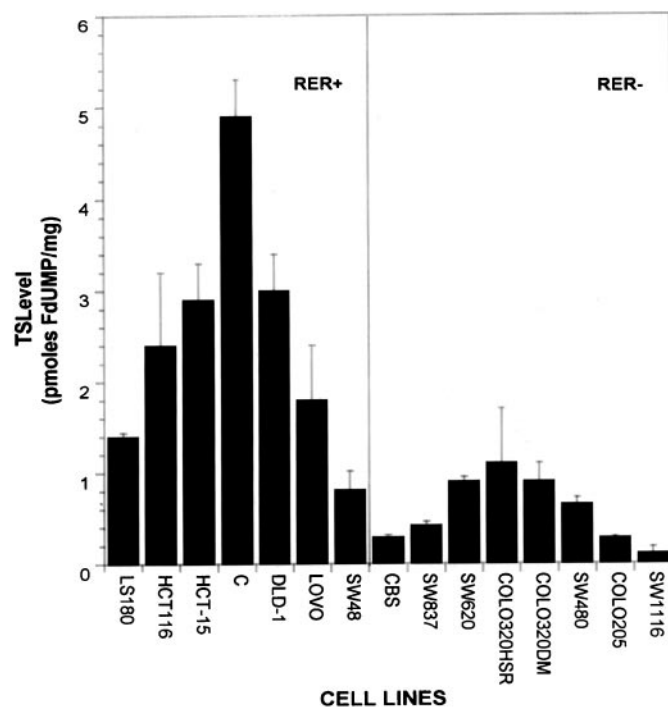

Fig. 2. TS levels in colon tumor cell lines. Enzyme was measured in extracts of each cell line by the FdUMP binding assay, as described under *Experimental Procedures*; vertical bars indicate standard deviations. The lines are grouped according to RER status. The lines are grouped according to RER status, as in Fig. 1.
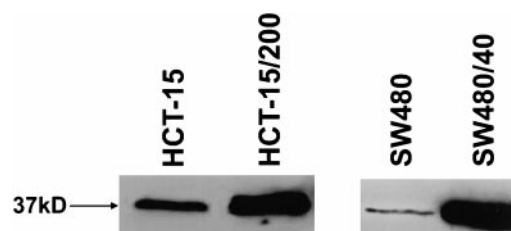

Fig. 3. Western blot analysis of TS protein concentrations. TS protein levels were assayed in cell line extracts by Western analysis, as described under *Experimental Procedures*. The molecular mass of the TS polypeptide (37 kDa) is indicated to the left.
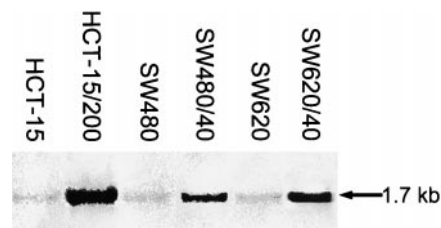
TABLE 1
Properties of FdUrd-adapted cell lines
Values represent the averages ± S.D. of at least three experiments.

Cell line	TS level	ID$_{50}$
	pmol enzyme/mg of protein	*nM*
SW480	0.65 ± 0.07	1.3 ± 0.2
SW480/40	85 ± 18	41 ± 5.1
SW620	0.90 ± 0.05	1.9 ± 0.7
SW620/40	91 ± 7.9	63 ± 5.8
HCT15	2.9 ± 0.4	5.7 ± 0.6
HCT15/200	13 ± 3.7	867 ± 58

Fig. 4. TS mRNA levels in FdUrd-adapted cell lines. Total cellular RNA from each of the indicated lines, as well as their drug-resistant derivatives, was analyzed by Northern blotting (see *Experimental Procedures*). The 1.7-kb TS mRNA is indicated.

representing each of the successive steps in the selection process were analyzed. The presence of the mutation was diagnosed by *Aci*I digestion of the 222-bp fragment generated by RT-PCR of the wild-type TS mRNA between nucleotides 838 and 1059; products of lengths 152 bp and 70 bp result from the wild-type mRNA, whereas no digestion occurs with the mutant mRNA. Figure 5 shows that parental HCT15 cells, as well as cells adapted to the ID_{10} and ID_{20} concentrations of FdUrd, expressed only the wild-type TS mRNA. The mutant mRNA was first detectable in cells adapted to the ID_{50} level of FdUrd and became more predominant during adaptation to higher drug concentrations. Cells resistant to the ID_{100} concentration expressed approximately equal amounts of both the mutant and the wild-type mRNAs. This pattern was found in each of 20 individual clones from the population (data not shown), indicating that the presence of both mRNAs represents genetic heterozygosity rather than cellular heterogeneity. Only the mutant mRNA was detected in cells adapted to a drug concentration representing $2 \times ID_{100}$; such was found in the HCT15/200 line as well (Fig. 5). Similar results were obtained by PCR analysis of genomic DNA (data not shown). Thus, expression of the mRNA reflects the presence of the corresponding gene in the various cell populations.

Northern blot analysis of TS mRNA concentrations indicated that overproduction of TS mRNA did not occur until the cells had become adapted to an FdUrd level representing $2 \times ID_{100}$ (Fig. 5). This is significantly later than the appearance of the C-to-T mutation. Thus, mRNA overproduction and the P303L substitution appeared at different times during the selection.

The P303L Substitution Is Responsible for Discordant mRNA and Protein Expression in HCT15/200 cells. To determine whether the P303L substitution causes the deficiency in TS-enzyme overproduction in cell line HCT15/200, we examined cells stably transfected with constructs encoding the wild-type and P303L enzymes. Plasmids containing the appropriate cDNAs were introduced into the TS-deficient cell line RJK88.13, and stable transfectants were selected on the basis of their ability to grow in the absence of exogenous thymidine. One population of cells
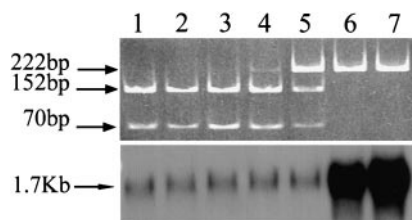

Fig. 5. Analysis of the appearance of the Pro303-to-Leu substitution and of TS mRNA overproduction during selection of HCT15/200 cells. RNA was isolated from cells at various steps during selection for FdUrd resistance. Top, the RNA was subjected to RT-PCR to amplify the region containing the C-to-U mutation at nucleotide 908 (see *Experimental Procedures*); the DNA product was digested with *Aci*I and examined by acrylamide gel electrophoresis. The sizes of the various DNA fragments are indicated to the left. Results with RNA from parental HCT15 cells are shown in lane 1, whereas those with RNAs from cells adapted to the ID_{10}, ID_{20}, ID_{50}, ID_{100}, and $2 \times ID_{100}$ concentrations of FdUrd are shown in lanes 2 to 7, respectively. Bottom, total TS mRNA concentrations were quantified by Northern blot analysis. The size of TS mRNA is indicated to the left. Results with RNA from parental HCT15 cells are shown in lane 1, and those with RNAs from cells adapted to the ID_{10}, ID_{20}, ID_{50}, ID_{100}, $2 \times ID_{100}$, and $4 \times ID_{100}$ concentrations of FdUrd are shown in lanes 2 to 7, respectively.

(RJK88.13/Pro303) expressed the wild-type TS cDNA, whereas a second population (RJK88.13/Leu303) expressed a cDNA corresponding to the P303L mutant. Enzyme and mRNA concentrations were determined in the transfected lines and compared with those in cell lines HCT15 and HCT15/200. As depicted in Fig. 6 and Table 2, the transfected lines expressed very similar levels of TS; however, RJK88.13/ Leu303 contained approximately 10-fold higher levels of mRNA than RJK88.13/Pro303. The altered mRNA/protein ratios between the two transfected lines parallel that between HCT15 and HCT15/200 (Table 2), indicating that the P303L substitution is responsible for discordant mRNA and enzyme expression.

The copy numbers for transfected genes were nearly identical in RJK88.13/Pro303 and RJK88.13/Leu303 (Fig. 6), indicating that high TS mRNA concentrations in the latter are caused by increased transcription rates per gene rather than by gene amplification. This is in contrast with cell line HCT15/200, where high TS mRNA overexpression is derived from an increase in TS gene copy number (see above).

A second mutant, P303D, was generated and analyzed as above. RJK88.13/Asp303 cells, which were transfected with the P303D construct, express mRNA and protein levels that are similar to those in cells transfected with the wild-type construct (Table 2). Thus, discordant expression of TS mRNA and protein is not a general property of all Pro303 substitutions.

The P303L Enzyme Has a Reduced Half-Life. Deficient expression of the P303L mutant may derive from a more rapid turnover rate. To test this, we compared the half-lives of the TS polypeptides in HCT15 and HCT15/200 cells. The rate of disappearance of enzyme was assayed by Western blotting at various times after the addition of cycloheximide to the growth medium. The half-life was about 7 h for the wild-type enzyme (i.e., that in HCT15) and about 1 h for the mutant (i.e., that in HCT15/200) (Fig. 7). Identical results were obtained when the FdUMP binding assay was used to monitor TS concentrations (data not shown). Thus, relative to the wild-type TS polypeptide, the P303L mutant is unstable in vivo. This instability explains the discordance between enzyme and mRNA expression in HCT15/200 and in RJK88.13/Leu303 (Fig. 6; Table 2).

The half-lives of the wild-type and P303L enzymes showed similar differences in transfected RJK88.13 cells (Z. Rafique, unpublished observations). Thus, a more rapid intracellular turnover rate is an intrinsic property of the mutant enzyme.
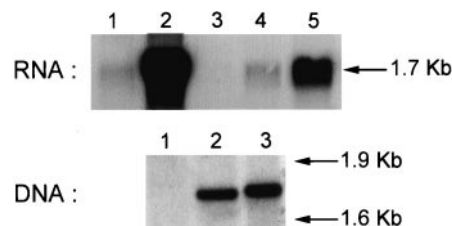

Fig. 6. TS mRNA expression in transfected cells. Top, Northern blot analysis of TS mRNA levels was carried with total RNA from cell lines HCT15 (lane 1), HCT15/200 (lane 2), RJK88.13 (lane 3), RJK88.13/ Pro303 (lane 4), and RJK88.13/Leu303 (lane 5). The size of the wild-type TS mRNA is indicated to the right. Bottom, Southern blot analysis of TS DNA was conducted with genomic DNA from cell lines RJK88.13 (lane 1), RJK88.13/Pro303 (lane 2), and RJK88.13/Leu303 (lane 3). DNA size markers are indicated to the right.

Expression of the P303L Enzyme Generates Resistance to TS Inhibitors in Transfected Cells. To assess directly whether or not the P303L mutant confers drug resistance, we examined the drug sensitivities of the transfected cell lines. Compared with transfectants expressing the wild-type enzyme, the P303L transfectants were 24-fold more resistant to FdUrd, 2.5-fold more resistant to BW1843U89, 3.7-fold more resistant to ZD1694, and >25-fold more resistant to AG337 (Table 3). Because the TS concentrations in the two lines were nearly identical (Table 2), these differences in drug sensitivity must be related to enzyme structure rather than enzyme level. Thus, the P303L mutant confers relative resistance to both fluoropyrimidine and folate inhibitors of TS. The mutant enzyme in cell line HCT15/200 must, therefore, contribute to this line's FdUrd resistance.

Compared with transfectants expressing wild-type TS, transfectants expressing the P303D enzyme were 20-fold resistant to FdUrd, but exhibited little (i.e., <2-fold) resistance to either ZD1694 or BW1843U89 (Table 3). Thus, the P303L and P303D substitutions differ with regard to their ability to confer resistance to TS inhibitors.

The Half-Life of the TS Molecule Is Increased in SW480/40. To determine whether excess TS expression in SW480/40 cells is caused by alterations in enzyme stability, we compared the half-life of the TS polypeptide in this line and in its parent (SW480). As seen in Fig. 8, TS exhibited a half-life of about 6 h in the parental line and 22 h in the drug-adapted derivative. Thus, in addition to exhibiting mRNA overproduction (Fig. 4), SW480/40 produces a TS molecule that has an increased intracellular stability. The stabilization of TS accounts for the excess enzyme overproduction, relative to mRNA, in SW480/40 cells.

Discussion

Most studies of mechanisms of drug resistance, including those involving TS inhibitors, have used model cell lines that represent a broad variety of histologic origins. We have narrowed our focus to cells of colonic origin to gain some understanding of the extent of heterogeneity in resistance mechanisms that operate in a single tumor type. Such a focus is relevant for TS inhibitors, because these agents are so widely used in the treatment of colonic neoplasms (Heidelberger et al., 1982; Takemura and Jackman, 1997). Fluoropyrimidine-based inhibitors (FUra, FdUrd) have been available for quite some time, and there is extensive information on their modes of action and the cellular mechanisms of resistance to them (Heidelberger et al., 1982). With the more recent development of a TS-targeted antifolates (e.g., AG337, ZD1694, BW1843U89), there is renewed interest in the enzyme and its role in drug response (Takemura and Jackman, 1997).

Among the 15 colon tumor cell lines that were examined in the present investigation, when comparing the lowest and highest values, TS concentrations and FdUrd sensitivities were found to vary over 50- and 80-fold ranges, respectively. During the course of the studies, we observed that mismatch repair-deficient RER+ lines express higher TS levels and are relatively resistant to FdUrd compared with repair-proficient RER− lines (Figs. 1 and 2). Thus, the RER status may be a determinant of cellular sensitivity to TS inhibitors. Mismatch repair genes have been shown to underlie the emergence of a significant number of hereditable forms of cancer and cause resistance to a variety of chemotherapeutic drugs, including cisplatin and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Umar and Kunkel, 1996). Mutations in any of five genes (*hMLH1*, *hMSH2*, *hMSH6*, *hPMS1*, and *hPMS2*) are of most importance in generating the RER+ phenotype. The nature of the link between mismatch repair and TS expression is not known. The fact that introduction of a wild-type *hMLH1* gene into HCT116 cells failed to alter TS concentrations (M. Kitchens, unpublished observations) suggests that mismatch repair genes modulate TS levels in an indirect manner. It may be that the high mutation rates in RER+ cells lead to TS regulatory mutations that result in higher enzyme levels and a selective growth advantage, allowing the cells to eventually predominate within a tumor cell population. Clearly, further studies will be necessary to ascertain the mechanisms underlying effects of the RER phenotype on TS expression.

Analysis of FdUrd-adapted cell lines indicated that acquired resistance to the drug occurs by a variety of mechanisms, affecting both the structure and the intracellular concentration of TS. Increases in enzyme levels, from ∼3- to 5-fold in HCT15/200 to ∼100-fold in SW480/40 and SW620/40 compared with parental lines, were commonly observed. These increases were associated with gene amplification and mRNA overproduction. Interestingly, the extent of mRNA overproduction, which was 30- to 40-fold in HCT15/200 and 10- to 15-fold in SW480/40 and SW620/40, did not reflect the increases in TS levels. There was a deficiency of enzyme relative to mRNA in HCT15/200 and an excess of

TABLE 2

Analysis of TS mRNA and protein expression in stably transfected RJK88.13 cells

Enzyme levels were determined by the FdUMP binding assay. RNA concentrations, in arbitrary units, were determined by Northern blotting and with the use of a PhosphorImager.

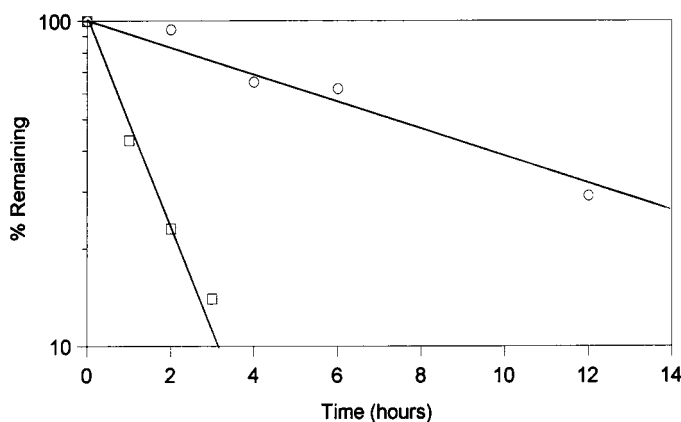| Cell line	TS level	RNA concentration	Ratio
	pmol/mg of protein		
RJK88.13/Pro303	2.6	12	4.6
RJK88.13/Leu303	2.7	118	44
RJK88.13/Asp303	3.1	18	5.8
HCT15	4.0	13	3.3
HCT15/200	12.6	769	61

Fig. 7. Stability of TS in cell lines HCT15 and HCT15/200. Cycloheximide was added to the media of exponentially growing cells, and the concentration of TS was determined by Western blotting at various times thereafter. ○, HCT15; □, HCT15/200.

enzyme in SW480/40 and SW620/40. Thus, acquired changes in enzyme levels are only partially accounted for by increases in mRNA concentrations and gene copy number, which suggests that translational and/or post-translational events are important in acquisition of FdUrd resistance.

Further analysis indicated that the discordances between enzyme and mRNA overproduction are caused by changes in the intracellular half-life of the TS polypeptide. In cell line SW480/40, TS was stabilized by a factor of 3- to 4-fold relative to that in the parental cell line (Fig. 8). No amino acid substitutions were detected in the TS polypeptide from these cells, which makes it unlikely that structural changes in the enzyme itself are responsible for the high stability phenotype. It is probable that one or more cellular factors that regulate TS turnover are involved. Perhaps, a change in the structure or function of such a factor has been selected during adaptation of SW480 cells to FdUrd. Alternatively, increased enzyme levels resulting from the 10- to 15-fold overproduction of mRNA in SW48/40 cells may exceed the concentration of one or more rate-limiting factors involved in TS degradation. Further studies will be required to sort out these possibilities.

The TS mRNA in cell line HCT15/200 contains a C-to-T mutation at nucleotide 908 within the amino acid coding region, resulting in a Pro-to-Leu substitution at residue 303. In transfected Chinese hamster cells, expression of the P303L enzyme is deficient relative to its mRNA (Fig. 6, Table 2), similar to what was observed in the HCT15/200 cells. Thus, discordant expression of mRNA and enzyme in these cells is due to the Pro303→Leu substitution. Enzyme stability measurements showed that the P303L enzyme has a more rapid turnover rate than the wild-type enzyme (Fig. 7), so that higher concentrations of mRNA are required to maintain adequate levels of TS. For *E. coli* TS, a Pro-to-Leu substitution at residue 254, which is homologous to residue 303 in human TS, causes the enzyme to be packaged into inclusion bodies (Fantz et al., 1999). This is in contrast to the wild-type enzyme and suggests that the mutant polypeptide has an aberrant structure. The fact that the P303D mutant is not discordantly expressed relative to its mRNA in transfected cells (Table 2) indicates that structural aberrations leading to enzyme instability are not conferred by all substitutions at Pro303.

Both the P303L and P303D enzymes confer resistance to FdUrd in transfected mammalian cells (Table 3). Therefore, the presence of the P303L mutant in HCT15/200 cells is a contributing factor in this line's adaptation to drug. Analysis of cells from different stages in the selection process indicated that the P303L substitution appeared earlier than gene amplification/ mRNA overproduction (Fig. 5). It is likely that the mutant enzyme is responsible for resistance to low levels of FdUrd during the initial stages of selection (i.e., at concentrations corresponding to the ID_{100}). Amplification of the mutant gene

and overproduction of its encoded mRNA occurred as the cells adapted to the more stringent selection at higher drug concentrations. The fact that two mechanisms (altered TS structure and enzyme overproduction) contribute to the resistance phenotype of HCT15/200 cells probably explains the rather large (i.e., 150-fold) increase in ID_{50}, as opposed to the 30-fold increase in SW480/40 and SW620/40 (Table 1).

In addition to conferring resistance to FdUrd, the P303L enzyme also generates reduced response to ZD1694, BW1843U89, and AG337 (Table 3). Among TS mutants that have been studied thus far, few confer resistance to antifolates. Recently, Tong et al. (1998) used site-directed mutagenesis to generate two mutant enzymes (I108A and F225W) that confer resistance to several folate analogs, including AG337, ZD1694, and BW1843U89. In another study, random oligonucleotide-mediated mutagenesis was used to isolate FdUrd-resistant mutants of TS containing amino acid substitutions within the enzyme's active site (Landis and Loeb, 1998); it is not known whether or not any of these affects sensitivity to antifolates. The P303L enzyme identified in the present report represents one of the few mutants TS mutants identified to date that directs resistance to both ZD1694 and BW1843U89.

Pro303 is highly conserved among TS molecules from a number of prokaryotic and eukaryotic species. Of 29 TS polypeptides that have been sequenced, 24 have Pro at this site, two have His, two have Ser, and one has Ala (Carreras and Santi, 1995). X-ray crystallographic studies have shown that residue 303 (as well as the homologous residue 254 of the *E. coli* enzyme) is positioned at a bend located 11 amino acids from the carboxyl terminus (Matthews et al., 1990; Schiffer et al., 1995). It is well documented that upon formation of either the catalytic or the inhibitory ternary complex, TS undergoes a conformational shift that involves a "closing" of the carboxyl terminus over the enzyme's active site cleft (Carreras and Santi, 1995). A hydrogen-bonding network involving the folate cosubstrate stabilizes the closed form. Several studies have shown that disruption of the capacity to form this network results in a catalytically inactive enzyme (Carreras and Santi, 1995). The FdUrd-resistance of the selected HCT15/200 cells may, therefore, be a consequence of destabilization of the ITC caused by perturbation of the conformational dynamics that occur as a consequence of ligand binding. Indeed, kinetic studies have shown that substitu-
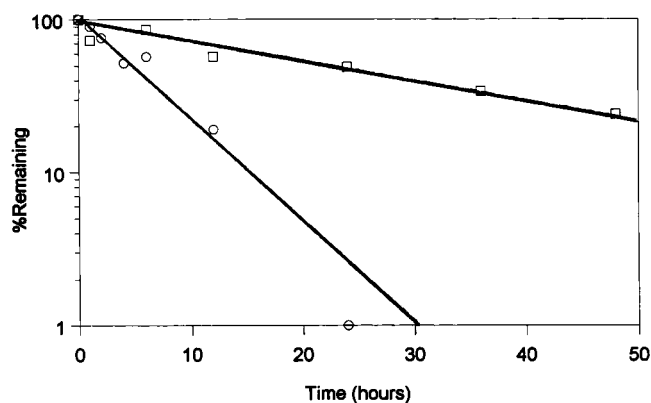
TABLE 3
Sensitivity of transfected cell lines to TS inhibitors

Cell line	ID_{50}			
	FdUrd	ZD1694	BW1843U89	AG337[a]
	nM	*nM*	*nM*	*μM*
RJK88.13/Pro303	1.8 ± 0.6	22 ± 0.4	73 ± 4.2	7.5 ± 0.5
RJK88.13/Leu303	43 ± 5	81 ± 8	195 ± 7.0	>200
RJK88.13/Asp303	36 ± 5.4	35 ± 1.7	102 ± 3.0	ND[d]

[a] Insoluble above 200 μM.
ND, not determined.

Fig. 8. Stability of TS in cell lines SW480 and SW480/40. Cycloheximide was added to the media of exponentially growing cells, and the concentration of TS was determined by Western blotting at various times thereafter. ○, SW480; □, SW480/40.

tions at Pro254 of the *E. coli* enzyme result in altered binding of $CH_2H_4PteGlu$ and various antifolates (Fantz et al., 1999).

Most chemotherapeutic agents used in cancer treatment are toxic to normal cells. For many of these agents, including TS inhibitors, myelosuppression is commonly observed, which severely limits the clinical effectiveness of therapy. A number of investigators have reduced hematopoietic toxicity in mice through retrovirus-mediated introduction of genes into bone marrow cells (Podda et al., 1992; Zhao et al., 1994; Maze et al., 1996). The success of these efforts has led to optimism with regard to the utility of gene-modified bone marrow in ameliorating myelosuppressive effects of cancer chemotherapy (Koç et al., 1996). Mutant TS molecules, such as the P303L enzyme, that have the ability to generate resistance to both fluoropyrimidines and antifolates may be specifically useful in reducing the hematopoietic toxicity induced by TS-directed inhibitors.

In summary, the results of the present study indicate that changes in the intracellular half-life of the TS polypeptide accompany, and even contribute to, resistance to TS inhibitors in colon tumor cells. This has important implications to the use of TS as a predictor of chemotherapeutic response to TS-directed agents (Suzuki et al., 1994; Johnston et al., 1995, 1997). Accurate predictions may require information on the levels of *both* TS protein and mRNA. Some tumors may overproduce the mRNA to a greater extent than the enzyme (as does cell line HCT15/200). Others may overproduce the enzyme to a greater extent than the mRNA (as in cell line SW480/40). In both cases, the tumors may be clinically refractory to FUra therapy, a result that might not be predicted on the basis of enzyme or mRNA measurements alone.

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