Structural Determinants for the Intracellular Degradation of Human Thymidylate Synthase†

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ABSTRACT: Thymidylate synthase (EC 2.1.1.45) (TS) catalyzes the conversion of dUMP to dTMP and is therefore indispensable for DNA replication in actively dividing cells. The enzyme is a critical target at which chemotherapeutic agents such as fluoropyrimidines (e.g., 5-fluorouracil and 5-fluoro-2′-deoxyuridine) and folic acid analogues (e.g., raltitrexed, LY231514, ZD9331, and BW1843U89) are directed. These agents exert their effects through the generation of metabolites that bind the active site of TS and inhibit catalytic activity. The binding of ligands to the TS molecule leads to dramatic changes in the conformation of the enzyme, particularly within the C-terminal domain. Stabilization of the enzyme and an increase in its intracellular level are associated with ligand binding and may be important in cellular response to TS-directed drugs. In the present study, we have examined molecular features of the TS molecule that control its degradation. We find that the C-terminal conformational shift is not required for ligand-mediated stabilization of the enzyme. In addition, we demonstrate that the N-terminus of the TS polypeptide, which is extended in the mammalian enzyme and is disordered in crystal structures, is a primary determinant of the enzyme's half-life. Finally, we show that TS turnover is carried out by the 26S proteasome in a ubiquitin-independent manner. These findings provide the basis for a mechanistic understanding of TS degradation and its regulation by antimetabolites.

Thymidylate synthase (EC 2.1.1.45) (TS)[1] catalyzes the reductive methylation of dUMP by 5,10-methylene-5,6,7,8-tetrahydrofolic acid (CH_2H_4PteGlu) to generate dTMP and dihydrofolic acid (*1*, *2*). Since this reaction provides the sole de novo source of dTMP for DNA replication in actively dividing cells, the enzyme has been a critical target for chemotherapeutic agents that are widely used in the treatment of solid tumors (*1*). The fluoropyrimidines 5-fluorouracil and 5-fluoro-2′-deoxyuridine (FdUrd) exert their toxicity through the generation of the nucleotide analogue 5-fluoro-2′-deoxyuridylic acid (FdUMP), which inhibits TS via the formation of a stable ternary complex containing the enzyme, the nucleotide analogue, and the cosubstrate CH_2H_4PteGlu (*3*−*6*). Data from X-ray crystal structures of natural TS substrates bound within the enzyme's active site cleft have led to the development of the folate-based TS inhibitors (*2*, *6*−*8*). Several folate analogues such as raltitrexed (RTX), LY231514, ZD9331, and BW1843U89 have advanced to clinical trials and have demonstrated significant activity against a variety of cancers (*5*, *7*−*9*).

A number of studies with a variety of human tumor cell lines, tumor cells, and clinical specimens have shown that TS levels increase by about 2−4-fold following exposure to various TS-directed inhibitors (*10*−*15*). Since elevated TS levels have been associated with reduced response to these inhibitors, the phenomenon of TS induction may have important consequences to the clinical efficacy of these agents (*9*, *16*−*18*). Ligand-mediated induction of TS occurs at the posttranscriptional level, but the exact mechanism has been controversial. Work by Chu and colleagues has led to a model, termed autoregulatory translation, which proposes that the efficiency of TS mRNA translation is repressed by the binding of the enzyme to its mRNA; induction of TS in response to ligands occurs through breakdown of the enzyme−mRNA complex and relief of translational repression (*19*−*22*). Our laboratory has shown that the polysome profiles for TS mRNA are not altered in cells treated with fluoropyrimidines, making it unlikely that induction occurs at the level of translation (*23*). Rather, we observed that the ligand-bound enzyme is more stable than the ligand-free enzyme, suggesting that induction results from reduced turnover of the TS polypeptide (*23*). A similar conclusion was reached by Washtien a number of years ago following exposure of human gastrointestinal tumor cells lines to 5-fluorouracil (*11*).

X-ray analyses have shown that, upon ligand binding, TS undergoes a conformational change in which the C-terminal residues translocate and close over the active site cavity (*24*−*29*). The movement from the ligand-free "open" conformation to the ligand-bound "closed" conformation is induced by binding of the folate cosubstrate and is necessary to achieve the correct orientation of substrates for product formation (*29*, *30*). A hydrogen bond network involving the C-terminal valine residue (Val-313 in the human enzyme) stabilizes the closed conformation (*27*, *30*). With few exceptions, all TS molecules examined to date adopt the

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[1] Abbreviations: TS, thymidylate synthase; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridylic acid; CH_2H_4PteGlu, 5,10-methylene-5,6,7,8-tetrahydrofolic acid; RTX, raltitrexed.

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closed conformation upon formation of the ternary complex (*31*−*33*). We have suggested that closure of the C-terminus over the active site is responsible for the stabilization of TS upon binding of inhibitors, resulting in increased intracellular levels of the protein (*18*, *23*).

In this report, we have identified structural features of the TS polypeptide that determine its stability and its induction by ligands. We show that the C-terminal conformational shift is not required for ligand-mediated stabilization of TS. In addition, we have identified the N-terminal region of the TS polypeptide, which is extended in the mammalian enzyme and exhibits a disordered conformation (*33*, *34*), as a primary determinant of the enzyme's intracellular half-life. Finally, we show that TS turnover is carried out by the 26S proteasome in a ubiquitin-independent manner. The implications of these findings to regulation of TS levels within the cell are discussed.

MATERIALS AND METHODS

Cells and Cell Culture. All cells were cultured in a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Cellgro) containing 4.5 g/L glucose and supplemented with 10% heat-inactivated fetal bovine serum (Cellgro). COS-7 cells were obtained from the American Type Culture Collection. Cell line RJK88.13, which is a TS-deficient derivative of V79 Chinese hamster lung cells, was obtained from Dr. Robert Nussbaum (University of Pennsylvania) and maintained in medium supplemented with 10 mM thymidine. Chinese hamster lung cell line E36 and its E1-defective derivative ts20 (*35*) were provided by Dr. William Dunn (University of Florida Health Science Center) and were maintained at 32 °C.

Cells were treated with 50 nM FdUrd (with 10 μM folinic acid), 50 nM RTX (AstraZeneca), or 100 nM BW1843U89 (AstraZeneca) for 24 h prior to harvesting. Where appropriate, pretreatment with the proteasome inhibitors lactacystin or MG132 (Sigma-Aldrich) was for 4 h.

Plasmid Constructs and Transfection. In vitro mutagenesis was carried out by sequential polymerase chain reaction, as described previously (*18*). Introduction of an ochre termination codon in place of Val-313 was done by mutating codon 313 from GTT to TAA within plasmid pTF487, which contains a full-length cDNA copy of the P303L form of human TS mRNA between nucleotides 25 and 1354 (*18*); the resulting mutant was termed V313Oc. The final PCR product was cloned into vector pCRII-TOPO (Invitrogen) and sequenced to verify the presence of the correct mutation-(s). A *Bgl*II/*Hpa*I fragment containing the mutant codon was excised and cloned in place of the corresponding fragment in expression vector pJZ205, which contains the wild-type TS cDNA under the control of the SV40 promoter. The final construct, containing both the P303L and the V313Oc mutations, was denoted pZR560.

The D218N substitution was also generated from pTF487 by mutating codon 218 from GAC to AAC. The PCR product was cloned into pCRII-TOPO and sequenced to verify the presence of the correct mutations. A *Bgl*II/*Hpa*I fragment containing the mutant codon was excised and cloned in place of the corresponding fragment in expression vector pJZ205. The final construct, containing both the P303L and the D218N mutations, was denoted pZR564.

The E30M substitution was produced by introducing a GAG → ATG change at codon 30 of plasmid pKB169, which contains a full-length cDNA copy of the wild-type TS mRNA between nucleotides 25 and 1354. The final PCR product was digested with *Hin*dIII/*Pst*I, cloned in place of the corresponding fragment of pKB169, and sequenced to verify the mutation. A *Hin*dIII/*Bgl*II fragment containing the mutant codon was excised and cloned into the expression construct pJZ205. This final construct, containing the E30M mutation, was denoted pTF590.

Deletion mutants lacking various lengths of the N-terminal end of the TS polypeptide were generated by inactivating the normal ATG initiator codon by mutagenesis and introducing new ATG codons at the appropriate downstream positions. The normal ATG of pKB169 was converted to CAG using PCR mutagenesis; the final product of the mutagenesis reaction was gel purified, digested with *Hin*dIII/*Pst*I, cloned into the parental plasmid pKB169 in place of the corresponding fragment, and sequenced to verify the presence of the proper mutations. The mutation-containing *Hin*dIII/*Bgl*II fragment was cloned in place of the corresponding fragment of expression vector pJZ205, resulting in plasmid pTF573. ATG initiator codons were introduced into pTF573 at various positions. The del6 mutation was generated by a GAG → ATG change at codon 7, the del12 mutation by a TTG → ATG change at codon 13, the del22 mutation by a GAG → ATG change at codon 23, and the del29 mutation by a GAG → ATG change at codon 30. In each case, the final PCR product was gel-purified, digested with *Hin*dIII/*Pst*I, cloned in place of the corresponding fragment within pKB169, and sequenced to verify the mutation; the *Hin*dIII/*Bgl*II fragment of the resulting plasmid was extracted and cloned into the expression construct pJZ205, as described above. Plasmids pTF577, pTF584, pTF578, and pTF568 correspond to the del6, del12, del22, and del29 mutants, respectively.

A plasmid encoding a His_6-tagged derivative of TS was generated by PCR amplification of the amino-terminal half of pKB169 using a 5′-primer that included six histidine codons inserted between codons 2 and 3. The resulting 624 bp fragment was gel-purified, cloned into the pGEM-T Easy vector (Promega), and sequenced. A *Hin*dIII/*Bgl*II fragment from the resulting plasmid, which contains the His_6-tagged region, was cloned in place of the corresponding fragments in expression plasmid pJZ205, which encodes wild-type TS; the final construct was denoted His_6-TS.

Plasmids expressing hemagglutinin epitope-tagged ubiquitin (HA-Ub) and His_6-tagged c-Jun (c-Jun-His_6) were kindly provided by Dr. Dirk Bohmann (University of Rochester Medical Center) and have been described elsewhere (*36*).

Transient transfection into COS-7 cells was performed using FuGENE-6 (Roche, Inc.) according to the manufacturer's instructions. Transfection into RJK88.13 cells was done by calcium phosphate−DNA coprecipitation in media containing 10 μM thymidine, as described previously (*18*); stable transfectants were selected in thymidine-free medium, pooled, and maintained in mass culture.

Measurement of TS Levels. Extracts from logarithmically growing cells were prepared by sonicating the cells in 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 10 mM dithiothreitol, and 0.05% NP-40, followed by centrifugation

at 100000*g* for 1 h. The concentration of active TS in the extracts was determined by the FdUMP binding assay (*18*). Measurement of TS levels by Western blotting was carried out with an anti-human TS monoclonal antibody as probe (the antibody was provided by Dr. Sondra Berger, University of South Carolina); the antigen−antibody complex was detected using the ECL chemiluminescence kit (Amersham).

k_{cat} values were estimated in crude cell extracts based upon the ratio of catalytic enzyme activity (as determined by the tritium release assay; see ref *37*) to the concentration of enzyme dimers (as determined by the FdUMP binding assay; see above). Values are expressed as s^{-1}.

Northern Blotting. Aliquots of total cellular RNA (10 μg) were subjected to electrophoresis through 1.5% agarose gels containing 2.2 M formaldehyde, transferred to nylon membranes, and hybridized to a radiolabeled 1.3 kilobase *Acc*I/*Hpa*I fragment of the TS cDNA from plasmid p*K*B148 (*18*). Equivalence of RNA loading was determined by ethidium bromide staining.

Analysis of Protein Ubiquitination. COS-7 cells were transiently transfected with a plasmid encoding His_6-TS with or without cotransfection with the HA-Ub plasmid (*36*). Twenty-four hours after transfection, cells were treated with 25 μM MG132 for 6 h to allow accumulation of ubiquitinated proteins. TS (including any ubiquitin-bound isoforms) was isolated using Ni-NTA−agarose according to the procedure of Trier et al. (*36*). Briefly, cells were lysed in 0.1 M NaH_2PO_4/NaH_2PO_4 (pH 8.0) containing 6 M guanidinium chloride and 5 mM imidazole, and the lysate was sonicated (4 × 30 s) using a VirSonic50 sonicator (Virtis Co., New York). The lysate was incubated overnight at 4 °C on a rotator in the presence of 0.25 mL of Ni^{2+}-NTA−agarose beads (Quiagen). The slurry was transferred to 1.5 mL Eppendorf tubes and centrifuged at 3000 rpm for 3 min. To remove nonspecifically bound proteins, the beads were successively washed using previously described buffers (36), and the protein conjugates were eluted with 0.2 mL of 200 mM imidazole containing 50 mM Na_2HPO_4/NaH_2PO_4 (pH 8.0), 100 mM KCl, 20% glycerol, and 0.2% NP-40. The eluate was quantitated using the Bio-Rad protein assay (Bio-Rad) and analyzed by Western blotting.

As a positive control, COS-7 cells were transiently transfected with a plasmid encoding c-Jun-His_6 (*36*) with or without HA-Ub. Transfected cells were processed and analyzed as described above.

Blots were probed using antibodies against HA (Santa Cruz Biotechnology), human TS, or c-Jun (Santa Cruz Biotechnology).

Determination of the Role of the Ubiquitin Conjugation Pathway in TS Degradation. Plasmid pTF489, encoding the P303L form of TS, was transiently transfected into both E36 and ts20 cells in 100 mm plates at 32 °C using FuGene-6 transfection reagent (Roche, Inc.). Twenty-four hours post-transfection, cells were trypsinized, plated in 60 mm dishes, and incubated for 12 h at 32 °C and then overnight at 39 °C. Cycloheximide (50 μg/mL) was added, and cells were harvested at the indicated time points. The cells were lysed by sonication (3 × 10 s) in 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 0.05% NP-40, 10 mM DTT, 2 mM β-mercaptoethanol, 5 mM PMSF, 200 μg/mL aprotinin, 100 μg/mL pepstatin, and 50 μg/mL leupeptin. The lysates were centrifuged at 11000 rpm for 1 h at 4 °C, and protein was quantitated using the Bio-Rad protein assay reagent.

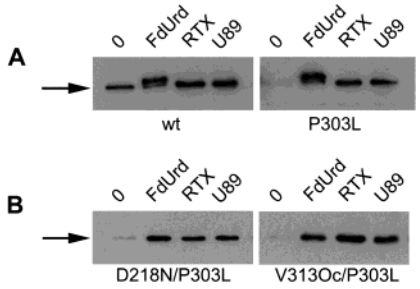

FIGURE 1: Effect of TS inhibitors on enzyme levels in transiently transfected cells. COS-7 cells were transfected with constructs encoding wild-type TS, the P303L mutant, the D218N/P303L double mutant, or the V313Oc/P303L double mutant. Two days after transfection, FdUrd (50 nM)/folinic acid (10 μM), RTX (50 nM), or BW1843U89 (100 nM) was added to the medium, and TS levels were measured 24 h later by Western blotting. The arrows indicate the position of ligand-free TS.

RESULTS

The C-Terminal Conformational Shift Is Dispensable for Ligand-Mediated Induction of TS. Previously, we suggested that ligand-mediated closure of the C-terminus over the active site cavity of TS renders the enzyme resistant to proteolysis and is responsible for enzyme stabilization in response to drug binding (*23*). To test this hypothesis, we examined mutant TS molecules that are incapable of undergoing the ligand-mediated conformational shift. Functional studies of the *Lactobacillus casei* enzyme have shown that deletion of the C-terminal residue (Val-316) or substitution of asparagine in place of aspartic acid at position 221 (D221N) results in a catalytically inactive enzyme that does not form the covalent ternary complex in the presence of ligands and remains in an open conformation (*2*, *26*, *27*, *30*, *38*). We asked if ligand-mediated induction of the enzyme is maintained in analogous mutants of the human enzyme (i.e., at residues Val-313 and Asp-218).

Site-directed mutagenesis was used to generate constructs encoding the V313Oc and the D218N mutants of human TS. To facilitate the detection of TS induction, each construct also contained the P303L substitution, which causes the enzyme to undergo a 10−15-fold induction in the presence of ligands, as opposed to the modest 2−3-fold increase that is characteristic of wild-type TS (*18*). The various constructs were transiently transfected into COS-7 cells, and enzyme induction in response to fluoropyrimidines (i.e., FdUrd) or folate analogues (i.e., RTX and BW1843U89) was assessed by Western blotting. As shown in Figure 1, similar to the P303L form, induction of both the V313Oc/P303L and the D218N/P303L enzymes occurred in response to the drugs, indicating that neither the V313Oc nor the D218N substitution abrogates ligand-mediated induction. The slower migrating band exhibited by the wild-type and P303L enzymes in FdUrd-treated cells is characteristic of the covalent, ternary complex containing the enzyme, FdUMP, and CH_2H_4PteGlu; as expected, this complex does not occur with either the V313Oc or the D218N mutant. We conclude, on the basis of these results, that the C-terminal conformational shift is dispensable for stabilization of TS by ligands.

The N-Terminal Domain of TS Modulates the Enzyme's Stability. Comparisons among TS polypeptides from numer-

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          1        10        20        30
Wildtype  MPVAGSELPRRPLPPAAQERDAEPRPPHGELQYGQIQHIL…

del29                                 MLQYGQIQHIL…

del6            MLPRRPLPPAAQERDAEPRPPHGELQYGQIQHIL…

del12                 MPPAAQERDAEPRPPHGELQYGQIQHIL…

del22                                 MPRPPHGELQYGQIQHIL…
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FIGURE 2: Amino acid sequence of the N-terminal region of human TS. The first 40 residues of the wild-type enzyme and deletion mutants del29, del6, del12, and del22 are aligned.

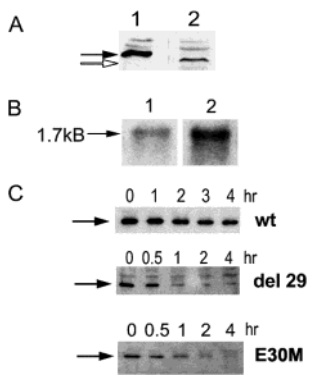

FIGURE 3: Expression and half-life of wild-type TS and the del29 mutant. (A) TS levels were measured by Western blotting in extracts prepared from wild-type cells (lane 1) or the del29 mutant (lane 2). The positions of the wild-type and the del29 enzymes are indicated by closed and open arrows, respectively. (B) TS mRNA levels were determined by Northern blotting of total RNA prepared from wild-type (lane 1) or del29 (lane 2) cells. The arrow indicates the position of TS mRNA. (C) The half-lives of the wild-type, del29, and E30M forms of TS were estimated by Western blotting at various times following addition of 50 μg/mL cycloheximide to the medium. The time after cycloheximide addition is indicated; arrows mark the position of ligand-free TS.

ous species have shown that the N-terminus of the mammalian enzyme is extended relative to that in other species (*2*, *33*). Most of the amino acid sequence differences that occur in the human, rat, and mouse enzymes are located within this region, suggesting that it is evolving at an accelerated rate relative to the remainder of the molecule (*39*). X-ray crystallographic analyses have indicated that the N-terminal domain, which tolerates large deletions without effects on catalytic activity, is disordered and makes no significant contacts with the body of the TS molecule (*31*, *38*). No function has been assigned to this region of the TS polypeptide.

To gain insight into the role, if any, of the N-terminal domain in TS degradation, we examined a mutant that lacks the first 29 residues (Figure 2). A construct encoding this mutant, termed del29, was stably transfected into the TS-deficient Chinese hamster lung cell line RJK88.13, and TS expression was measured in cell-free extracts by Western blotting. As shown in Figure 3A, steady-state levels of the mutant enzyme were ~10% of that in cells transfected with the wild-type enzyme. This was verified by FdUMP binding assays, which measure the extent of analogue binding into a stable, ternary complex (data not shown).

Northern blotting (Figure 3B) indicated that TS mRNA expression in the del29 transfectant was approximately 2−5-fold higher than that in a wild-type transfectant. The discordance between enzyme and mRNA expression could be due the del29 enzyme being unstable. This was tested by comparing the rates of decay of the wild-type and del29 forms in cycloheximide-treated cells. The half-life of the del29 enzyme was found to be about 0.5−1 h, which is considerably shorter than that for the wild-type enzyme (Figure 3C). Thus, the discordance between enzyme and mRNA expression exhibited by the del29 transfectant is due to the unstable nature of the mutant enzyme. This suggests that the N-terminal domain stabilizes the enzyme and therefore plays a role in TS turnover.

A caveat to this conclusion relates to the function of residue Asp-30, which was converted to Met during generation of the del29 construct (see Materials and Methods and Figure 2). Asp-30 is analogous to Met-1 of *Escherichia coli* TS. In the *E. coli* enzyme, Met-1 is carbamylated and forms hydrogen bonds with conserved residues Thr-48 and Thr-49, generating a network that has been postulated to preserve the structural integrity of the enzyme during the conformational change accompanying ligand binding (*2*, *40*, *41*). The acidic side chain of Asp-30 in human TS forms hydrogen bonds with the analogous threonine residues (i.e., Thr-75 and Thr-76) (*32*). It is therefore possible that the instability of the del29 mutant does not reflect a role for the N-terminal extension in enzyme turnover; rather, it may be due to loss of the hydrogen bond network between Asp-30 and Thr-75/76. Indeed, an E30M mutant that retains residues 1−29 exhibits a degradation rate that is nearly indistinguishable from that for the del29 mutant (Figure 3C).

To address this ambiguity and to determine if the N-terminal domain does, in fact, play a role in TS stability, we analyzed additional deletion mutants within the region. Constructs encoding TS molecules lacking the first 6, 12, or 22 amino-terminal residues (mutants del6, del12, and del22, respectively; see Figure 2) were introduced into RJK88.13 cells, and stable transfectants were selected in thymidine-free medium and analyzed for enzyme expression by FdUMP binding assays. The del12 mutant was expressed at very low levels relative to the wild-type enzyme (i.e., 0.3 vs 2.5 pmol of FdUMP bound/mg of extract protein), while the del22 enzyme was expressed at levels similar to the wild type (i.e., about 1.7−2.5 pmol of FdUMP bound/mg of protein) and the del6 mutant was expressed at strikingly high levels (i.e., 19 pmol of FdUMP bound/mg of protein). The differences in FdUMP binding activities for the various mutants reflect altered concentrations of enzyme, rather than changes in FdUMP affinities, as demonstrated by Western blotting (data not shown) and by the fact that doubling the concentration of the nucleotide analogue in the assays did not increase the amount of bound FdUMP (data not shown).

The k_{cat} values for the various mutants were estimated in cell-free extracts and were found to be quite similar to that of the wild-type enzyme. Values were 1.8 s^{-1} for wild-type TS, 1.7 s^{-1} for the del6 mutant, 0.74 s^{-1} for the del12 mutant, 1.0 s^{-1} for the del22 mutant, and 1.0 s^{-1} for the del29 mutant.

Since TS mRNA levels were similar in all of the transfected cells and did not parallel enzyme levels (data not shown), we surmised that the differences in enzyme expression among the mutants reflected variations in protein stability. Therefore, the half-lives of the del6, del12, and del22 mutants were determined in cycloheximide-treated cells (Figure 4). The del6 mutant was extremely stable, showing no detectable decay up to 48 h after initiation of cyclohex-

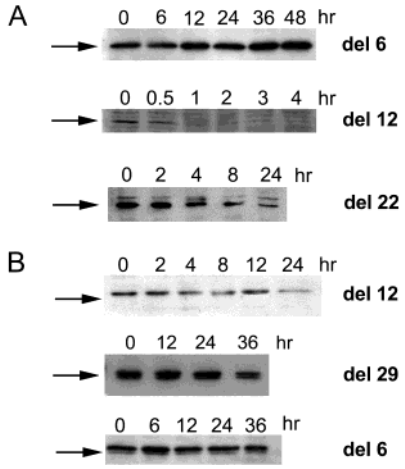

FIGURE 4: Effects of the N-terminal region on the half-life of the TS polypeptide. (A) Half-lives were estimated by addition of cycloheximide (50 μg/mL) to the medium of RJK88.13 cells stably expressing the indicated deletion mutants; at the indicated times, enzyme levels were assessed by Western blotting. The time after cycloheximide addition is indicated; arrows mark the position of ligand-free TS. (B) RJK88.13 cells stably expressing the indicated deletion mutants were pretreated for 24 h with 50 nM FdUrd/10 μM folinic acid, and enzyme half-lives were analyzed as a (A).

imide treatment (Figure 4A). In fact, in some experiments, the relative concentration of the del6 enzyme underwent a slight increase after 36−48 h, probably as a consequence of enrichment resulting from decay of other cellular proteins. The del12 and del22 mutants were markedly more unstable, with half-lives of about 1 and 4 h, respectively (Figure 4A). In general, the half-lives of the mutant polypeptides paralleled enzyme expression. Thus, the stable del6 enzyme was expressed at very high levels, while the unstable del12 enzyme was expressed at low levels; the del22 and wild-type enzymes were intermediate in both stability and expression. Taken as a whole, the results indicate that various deletions within the N-terminal domain of human TS lead to marked alterations in its half-life. Thus, this region of the polypeptide appears to play a significant role in regulating TS stability.

The N-Terminal Domain Affects Stabilization of TS by Ligands. In previous work, we showed that induction of TS concentrations in response to inhibitors is associated with a lengthening of the enzyme's half-life, i.e., from about 7−10 h to 20−24 h (*23*). Since the del6, del12, and del29 mutants were considerably different with regard to stability, we assessed the effects of ligand binding on their half-lives. In the presence of FdUrd, the del12 and del29 enzymes, which were very unstable in the absence of drug (see Figure 4A), exhibited half-lives of about 24−36 h (Figure 4B), which is similar to that for the wild-type enzyme. Thus, FdUrd treatment resulted in a striking (i.e., 20−50-fold) stabilization of both mutant polypeptides, indicating that their relative instability is "rescued" by ligands. This is similar to what was previously observed with the P303L form, which is unstable relative to the wild-type enzyme in the absence of ligands but is as stable as the wild-type enzyme in the presence of ligands (*23*).

The long half-life of the del6 enzyme was maintained in the presence of FdUrd (Figure 4B), precluding determination of whether this mutant is stabilized by drug.

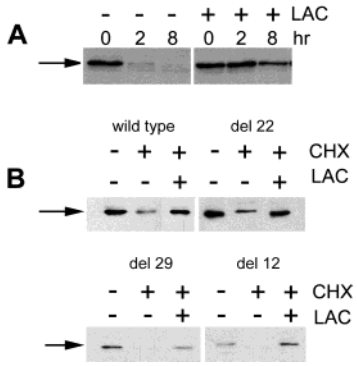

FIGURE 5: Stabilization of the TS polypeptide by lactacystin. (A) RJK88.13 cells stably expressing the P303L mutant were treated with 50 μg/mL cycloheximide (CHX) for the indicated times in the absence (−) or presence (+) of 7.5 μM lactacystin (LAC); TS levels were assessed by Western blotting. The arrow indicates the position of TS. (B) RJK88.13 cells stably expressing wild-type TS or various N-terminal deletion mutants of TS were treated with 50 μg/mL cycloheximide (CHX) in the absence (−) or presence (+) of 7.5 μM lactacystin (LAC), as indicated. After a period of time equal to 3−4 half-lives (based upon determinations similar to those in Figure 4A), TS levels were assessed by Western blotting. The arrow indicates the position of TS.

TS Degradation Is Carried Out by the 26S Proteasome. To begin to identify the cellular machinery responsible for TS turnover, we determined if enzyme degradation is mediated by the 26S proteasome. We tested the effect of lactacystin, a highly specific inhibitor of the proteasome, on the half-life of TS (*42*). Transfected cells expressing the P303L mutant were pretreated with lactacystin for 4 h, and the rate of TS decay was measured following addition of cycloheximide to the medium. As shown in Figure 5A, the enzyme was stabilized by pretreatment with lactacystin, suggesting that the 26S proteasome mediates its degradation. Similarly, the wild-type, del29, del22, and del12 mutants were also stabilized by lactacystin (Figure 5B). Comparable results were obtained using MG132, another proteasome inhibitor (data not shown). These data indicate that TS degradation is carried out by the 26S proteasome.

The TS Polypeptide Is Not Ubiquitinated. Most proteins that are degraded by the 26S proteasome are covalently linked to ubiquitin, a small, evolutionarily conserved protein of length 76 amino acids (*43*). The conjugation of one or more polyubiquitin chains to a target protein is required for efficient recognition and degradation of that protein by the proteasome (*43*). To determine if ubiquitination might be involved in proteasomal turnover of TS, we assayed for the presence of ubiquitin-conjugated TS isoforms in isolated preparations of the enzyme. COS-7 cells were transiently transfected with a plasmid encoding His_6-tagged human TS (His_6-TS), either with or without cotransfection of a plasmid encoding hemagglutinin-tagged ubiquitin (HA-Ub) (*36*). His_6-TS and any ubiquitin conjugates were isolated from extracts of the transfected cells using Ni-NTA−agarose and subjected to Western blotting. As a control, we analyzed cells transfected with His_6-tagged c-Jun (c-Jun-His_6), a protein known to be ubiquitinated (*36*). Probing the blot with an antibody to HA (Figure 6A) resulted in a heterogeneous "smear" of bands only in cells transfected with both c-Jun-His_6 and HA-Ub (lane 5); such a smear is characteristic of ubiquitin-conjugated proteins. No signal was detected without cotransfected HA-Ub (Figure 6A, lane 4). Importantly, no

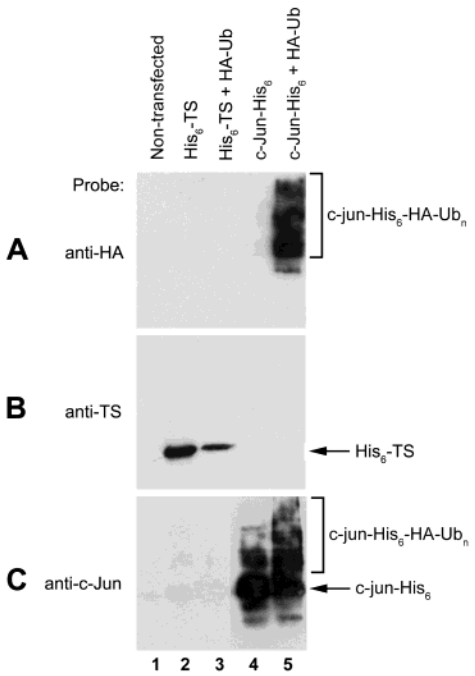

FIGURE 6: Measurement of TS ubiquitination. COS-7 cells were transiently transfected with the following constructs: His_6-TS (lane 2), His_6-TS and HA-Ub (lane 3), c-Jun-His_6 (lane 4), or c-Jun-His_6 and HA-Ub (lane 5); mock-transfected controls (lane 1) were included in the analysis. Twenty-four hours posttransfection, cells were treated with 25 μM MG132 for 6 h, and His_6-TS or c-Jun-His_6 (and their ubiquitinated isoforms) was isolated using Ni-NTA−agarose beads. The isolated proteins were analyzed by Western blotting using antibodies against HA (A), human TS (B), or c-Jun (C) as probes. Ubiquitinated forms of c-Jun-His_6 are indicated with brackets; the position of His_6-TS and c-Jun-His_6 are indicated.

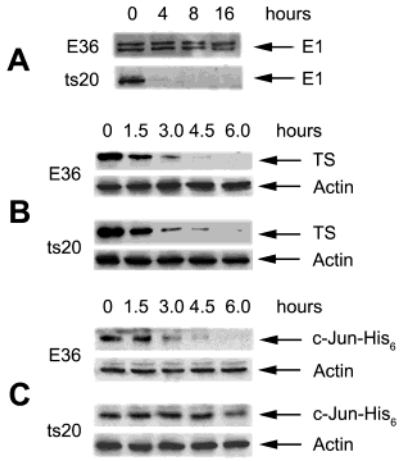

FIGURE 7: TS degradation in cells lacking the ubiquitin conjugation pathway. (A) E36 and ts20 cells cultured at 32 °C were shifted to the nonpermissive temperature (39 °C) for various times. E1 levels were assessed at various times after the temperature shift by Western blotting using antibodies against E1a/E1b (Calbiochem) as a probe. The number of hours at 39 °C is shown at the top of the figure. The E1 band is indicated. (B) E36 and ts20 cells were transiently transfected with a construct encoding the P303L form of TS at 32 °C. Twenty-four hours posttransfection, cells were split and incubated at 32 °C for 12 h and at 39 °C overnight. While the cells were maintained at 39 °C, the half-life of the TS polypeptide was determined after the addition of cycloheximide to the medium. The time (hours) after addition of cycloheximide is shown at the top of the figure. To control for sample loading, blots were stripped and probed with a monoclonal antibody against actin (Sigma). Bands corresponding to TS and actin are indicated. (C) E36 and ts20 cells were transiently transfected with a construct encoding c-Jun-His_6, and the cells were processed and analyzed exactly as in (B). The bands corresponding to c-Jun-His_6 and actin are indicated.

signal was observed in cells transfected with His_6-TS, even in the presence of cotransfected HA-Ub (Figure 6A, lanes 2 and 3). Probing the same blot with an antibody against TS showed the expected band at 36 kDa in His_6-TS-transfected cells, both with or without cotransfected HA-Ub (Figure 6B, lanes 2 and 3); no high molecular mass bands indicative of ubiquitin-conjugated TS isoforms were observed, even after prolonged exposure of the films. Finally, probing with antibody against c-Jun revealed the expected band at 39 kDa along with a smear of ubiquitinated species in cells transfected with c-Jun-His_6 both with and without cotransfected HA-Ub (Figure 6C, lanes 4 and 5). Thus, under conditions where ubiquitin conjugates of c-Jun-His_6 are readily detected, no such conjugates of His_6-TS are observed. These results suggest that TS is not ubiquitinated to any appreciable extent and that the enzyme does not require ubiquitination to be targeted to the 26S proteasome.

TS Degradation Does Not Require the Ubiquitin Conjugation Pathway. As a further test of the role of protein ubiquitination in TS degradation, we utilized a cell line that lacks the ubiquitin conjugation pathway. Cell line ts20, which is a derivative of Chinese hamster lung cell line E36, bears a temperature-sensitive mutation in the ubiquitin-activating enzyme E1 (*35*), which catalyzes the first step in attachment of ubiquitin to target proteins. At the restrictive temperature of 39 °C, E1 protein in ts20 cells is inactivated, resulting in the disruption of protein ubiquitination and stabilization of proteins that are degraded in a ubiquitin-dependent manner (*35*). We used the E36 and ts20 cell lines to assess the effect of disrupting the ubiquitin conjugation pathway on TS stability.

As an initial experiment, we assayed E1 levels in ts20 cells at several time points after the temperature shift. As expected, shifting ts20 cells from 32 to 39 °C resulted in a profound decrease in E1 levels, while no change was observed in parental E36 cells (Figure 7A). A plasmid encoding the P303L form of TS or c-Jun-His_6 was transiently transfected into E36 and ts20 cells at 32 °C; the transfected cells were shifted to 39 °C for 16 h, and the half-lives of the exogenously introduced proteins were determined after addition of cycloheximide to the medium (see Materials and Methods for details). TS was rapidly degraded with a half-life of ~1 h in both E36 and ts20 cells (Figure 7B); in contrast, c-Jun-His_6 was rapidly degraded in E36 cells, yet was relatively stable in ts20 cells (Figure 7C). TS is stabilized by proteasome inhibitors and by FdUrd at the restrictive temperature (data not shown), indicating that targeting of the enzyme to the 26S proteasome and ligand-mediated stabilization still occur under conditions where the ubiquitin conjugation pathway is inactive.

These results indicate that disruption of the ubiquitin conjugation pathway does not affect degradation of the TS polypeptide. Thus, TS turnover by the 26S proteasome does not require ubiquitination, implying that the enzyme is degraded in a ubiquitin-independent manner.

DISCUSSION

In this study, we have made several important observations. First, conformational closure of the C-terminus over

the active site cavity is not required for ligand-mediated stabilization of TS. This conclusion is based on the properties of two mutant enzymes (V313Oc and D218N) that bind ligands noncovalently and that remain in the open conformation (*2*, *26*, *27*, *30*, *38*). The observation that binding of ligands to the mutant enzymes results in the induction of TS levels (Figure 1) indicates that the C-terminal conformational shift is dispensable to stabilization of the polypeptide. Thus, noncovalent ligand binding, including that in aberrant modes, can promote escape from the cellular degradation machinery, resulting in enzyme stabilization and increased enzyme levels.

These conclusions rest on the assumption that the V313Oc and D218N substitutions within human TS mimic the well-studied effects of analogous substitutions (i.e., V316Am and D221N) in the *L. casei* enzyme. Although this assumption has not been formally tested, there are several reasons why it is very likely to be valid. The amino acid sequence and the three-dimensional structure of the TS polypeptide are highly conserved across a wide range of organisms, and many contacts among critical, conserved residues are maintained (*2*). Indeed, both Val-313 (Val316 in the *L. casei* enzyme) and Asp-218 (Asp221 in *L. casei*) play important and fundamental roles in TS function. Val-313, which occurs in most TS polypeptides and undergoes only conservative replacements, makes critical hydrogen bond contacts with other amino acid residues and with the folate cosubstrate, all of which mediate conformational closure of the active site cavity (*27*). Asp-218 is invariant among species and is intimately involved in binding of the folate cosubstrate and in catalysis (*30*, *38*). It is therefore quite likely that analogous amino acid substitutions within the TS polypeptides from different species exert identical effects on enzyme function. In addition, the slower migrating band in FdUrd-treated cells expressing the wild-type or P303L enzyme, which corresponds to the covalent ternary complex, does not occur with the V313Oc and D218N mutants (Figure 1). This is consistent with the notion that the latter two mutants, like the V316Am and D221N mutants of *L. casei* TS, undergo only noncovalent interactions with ligands.

The second major observation made in the current study is that the N-terminal region of the TS polypeptide, which is extended by about 30 amino acids in the mammalian as compared to the prokaryotic enzyme (*2*, *39*), governs TS stability and its response to ligands. This was a rather surprising finding, since the region exhibits few features indicative of a critical function. It has a disordered structure, makes no known contacts with the body of the polypeptide, tolerates mutations without loss of catalytic activity, and is the location of most amino acid differences that have evolved among the human, rat, and mouse enzymes (*31*, *38*, *39*). Interestingly, progressive deletion of N-terminal amino acid residues has variable effects on polypeptide stability. Removal of the first 6 amino acids results in an enzyme that is very stable relative to the wild-type enzyme and that is expressed at high levels within the cell. Deletion of the first 12 amino acids results in an enzyme that is markedly unstable and expressed at very low levels. Removal of 22 amino acids changes the degradation rate only marginally from the wild-type enzyme. Finally, deletion of 29 amino acids creates a very unstable enzyme that is a consequence, at least in part, of the loss of the hydrogen-bonding network between residue Glu-30 and conserved residues Thr-75/76. The half-lives of the del12 and del29 mutants increase to about 24−36 h in the presence of FdUrd, which is typical of the wild-type enzyme and reflects a large (i.e., 20−50-fold) stabilization. Thus, high rates of degradation resulting from deletion of the first 12 or 29 residues are reversed by ligand binding. Clearly, the exact mode of action of this region remains a topic for further study.

The third important finding is that degradation of the TS molecule is carried out by the 26S proteasome and is ubiquitin-independent. Very few proteins have been shown to be targeted to the proteasome without a requirement for ubiquitination (*44*, *45*). Those that have been identified as degraded in a ubiquitin-independent manner include ornithine decarboxylase (ODC; *46*, *47*), $p21^{WAF1/CIP1}$ (*48*, *49*), and Rb (*50*). Interestingly, several aspects of the degradation of ODC and TS are shared between the two proteins. ODC degradation is governed by the enzyme's C-terminal region, which, like the N-terminal domain of TS, is disordered in crystal structures (*51*). Detailed biochemical analyses have indicated that the C-terminus of ODC recognizes the same site on the proteasome as does the polyubiquitin chains of proteins that are degraded in a ubiquitin-dependent manner (*47*). Indeed, deletion of as few as six amino acids from the C-terminal end of ODC results in reduced binding to the proteasome and resistance to intracellular degradation (*47*, *52*). The present results indicate that the N-terminus of TS is likewise a major determinant of proteasome-mediated degradation of the protein. Furthermore, just like removal of the last six residues of ODC, deletion of the first six amino acids of TS stabilizes the polypeptide (see Figure 4). It is likely, therefore, that the disordered termini of TS and ODC, both of which mediate proteasomal degradation in a ubiquitin-independent fashion, function via similar mechanisms. Current studies are directed at understanding the mechanism by which the N-terminal domain of TS mediates recognition by the 26S proteasome. In this regard, it will be of particular interest to determine how the ligand-bound enzyme escapes the proteasomal machinery and becomes stabilized.

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REFERENCES

1. Danenberg, P. V. (1977) *Biochim. Biophys. Acta 473*, 73−92.
2. Carreras, C. W., and Santi, D. V. (1995) *Annu. Rev. Biochem. 64*, 721−762.
3. Berger, S. H., and Berger, F. G. (1988) *Mol. Pharmacol. 34*, 474−479.
4. Berger, S. H., Davis, S. T., Barbour, K. W., and Berger, F. G. (1988) *Adv. Exp. Med. Biol. 244*, 59−69.
5. Bertino, J. R. (1997) *Semin. Oncol. 24*, S18-52−S18-56.
6. Santi, D. V., McHenry, C. S., Raines, R. T., and Ivanetich, K. M. (1987) *Biochemistry 26*, 8606−8613.
7. Takemura, Y., and Jackman, A. L. (1997) *Anticancer Drugs 8*, 3−16.
8. Danenberg, P. V., Malli, H., and Swenson, S. (1999) *Semin. Oncol. 26*, 621−631.

9. Bertino, J. R. (1997) *Semin. Oncol. 24*, S18-3−S18-7.
10. Gorlick, R., Metzger, R., Danenberg, K. D., Salonga, D., Miles, J. S., Longo, G. S., Fu, J., Banerjee, D., Klimstra, D., Jhanwar, S., Danenberg, P. V., Kemeny, N., and Bertino, J. R. (1998) *J. Clin. Oncol. 16*, 1465−1469.
11. Washtien, W. L. (1984) *Mol. Pharmacol. 25*, 171−177.
12. Van der Wilt, C. L., Pinedo, H. M., Smid, K., and Peters, G. J. (1992) *Cancer Res. 52*, 4922−4928.
13. Spears, C. P., Shahinian, A. H., Moran, R. G., Heidelberger, C., and Corbett, T. H. (1982) *Cancer Res. 42*, 450−456.
14. Peters, G. J., Backus, H. H., Freemantle, S., van Triest, B., Codacci-Pisanelli, G., van der Wilt, C. L., Smid, K., Lunec, J., Calvert, A. H., Marsh, S., McLeod, H. L., Bloemena, E., Meijer, S., Jansen, G., van Groeningen, C. J., and Pinedo, H. M. (2002) *Biochim. Biophys. Acta 1587*, 194−205.
15. Welsh, S. J., Titley, J., Brunton, L., Valenti, M., Monaghan, P., Jackman, A. L., and Aherne, G. W. (2000) *Clin. Cancer Res. 6*, 2538−2546.
16. Berger, S. H., Barbour, K. W., and Berger, F. G. (1988) *Mol. Pharmacol. 34*, 480−484.
17. Gorlick, R., and Bertino, J. R. (1999) *Semin. Oncol. 26*, 606−611.
18. Kitchens, M. E., Forsthoefel, A. M., Barbour, K. W., Spencer, H. T., and Berger, F. G. (1999) *Mol. Pharmacol. 56*, 1063−1070.
19. Chu, E., and Allegra, C. J. (1996) *Cancer Treat. Res. 87*, 175−195.
20. Chu, E., and Allegra, C. J. (1996) *Adv. Enzyme Regul. 36*, 143−163.
21. Chu, E., Koeller, D. M., Casey, J. L., Drake, J. C., Chabner, B. A., Elwood, P. C., Zinn, S., and Allegra, C. J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8977−8981.
22. Chu, E., and Allegra, C. J. (1993) *Adv. Exp. Med. Biol. 339*, 143−151; discussion 165−167.
23. Kitchens, M. E., Forsthoefel, A. M., Rafique, Z., Spencer, H. T., and Berger, F. G. (1999) *J. Biol. Chem. 274*, 12544−12547.
24. Matthews, D. A., Appelt, K., Oatley, S. J., and Xuong, N. H. (1990) *J. Mol. Biol. 214*, 923−936.
25. Montfort, W. R., Perry, K. M., Fauman, E. B., Finer-Moore, J. S., Maley, G. F., Hardy, L., Maley, F., and Stroud, R. M. (1990) *Biochemistry 29*, 6964−6977.
26. Carreras, C. W., Climie, S. C., and Santi, D. V. (1992) *Biochemistry 31*, 6038−6044.
27. Perry, K. M., Carreras, C. W., Chang, L. C., Santi, D. V., and Stroud, R. M. (1993) *Biochemistry 32*, 7116−7125.
28. Phan, J., Koli, S., Minor, W., Dunlap, R. B., Berger, S. H., and Lebioda, L. (2001) *Biochemistry 40*, 1897−1902.
29. Kamb, A., Finer-Moore, J. S., and Stroud, R. M. (1992) *Biochemistry 31*, 12876−12884.
30. Chiericatti, G., and Santi, D. V. (1998) *Biochemistry 37*, 9038−9042.
31. Almog, R., Waddling, C. A., Maley, F., Maley, G. F., and Van Roey, P. (2001) *Protein Sci. 10*, 988−996.
32. Sotelo-Mundo, R. R., Ciesla, J., Dzik, J. M., Rode, W., Maley, F., Maley, G. F., Hardy, L. W., and Montfort, W. R. (1999) *Biochemistry 38*, 1087−1094.
33. Phan, J., Steadman, D. J., Koli, S., Ding, W. C., Minor, W., Dunlap, R. B., Berger, S. H., and Lebioda, L. (2001) *J. Biol. Chem. 276*, 14170−14177.
34. Schiffer, C. A., Clifton, I. J., Davisson, V. J., Santi, D. V., and Stroud, R. M. (1995) *Biochemistry 34*, 16279−16287.
35. Kulka, R. G., Raboy, B., Schuster, R., Parag, H. A., Diamond, G., Ciechanover, A., and Marcus, M. (1988) *J. Biol. Chem. 263*, 15726−15731.
36. Treier, M., Staszewski, L. M., and Bohmann, D. (1994) *Cell 78*, 787−798.
37. Boles, J. O., Cisneros, R. J., Weir, M. S., Odom, J. D., Villafranca, J. E., and Dunlap, R. B. (1991) *Biochemistry 30*, 11073−11080.
38. Sage, C. R., Michelitsch, M. D., Stout, T. J., Biermann, D., Nissen, R., Finer-Moore, J., and Stroud, R. M. (1998) *Biochemistry 37*, 13893−13901.
39. Ciesla, J., Weiner, K. X., Weiner, R. S., Reston, J. T., Maley, G. F., and Maley, F. (1995) *Biochim. Biophys. Acta 1261*, 233−242.
40. Fauman, E. B., Rutenber, E. E., Maley, G. F., Maley, F., and Stroud, R. M. (1994) *Biochemistry 33*, 1502−1511.
41. Finer-Moore, J. S., Maley, G. F., Maley, F., Montfort, W. R., and Stroud, R. M. (1994) *Biochemistry 33*, 15459−15468.
42. Lee, D. H., and Goldberg, A. L. (1998) *Trends Cell Biol. 8*, 397−403.
43. Ciechanover, A., Orian, A., and Schwartz, A. L. (2000) *J. Cell. Biochem., Suppl. 34*, 40−51.
44. Verma, R., and Deshaies, R. J. (2000) *Cell 101*, 341−344.
45. Orlowski, M., and Wilk, S. (2003) *Arch. Biochem. Biophys. 415*, 1−5.
46. Murakami, Y., Matsufuji, S., Hayashi, S. I., Tanahashi, N., and Tanaka, K. (1999) *Mol. Cell. Biol. 19*, 7216−7227.
47. Zhang, M., Pickart, C. M., and Coffino, P. (2003) *EMBO J. 22*, 1488−1496.
48. Sheaff, R. J., Singer, J. D., Swanger, J., Smitherman, M., Roberts, J. M., and Clurman, B. E. (2000) *Mol. Cell 5*, 403−410.
49. Touitou, R., Richardson, J., Bose, S., Nakanishi, M., Rivett, J., and Allday, M. J. (2001) *EMBO J. 20*, 2367−2375.
50. Kalejta, R. F., and Shenk, T. (2003) *Proc. Natl. Acad. Sci. U.S.A. 100*, 3263−3268.
51. Almrud, J. J., Oliveira, M. A., Kern, A. D., Grishin, N. V., Phillips, M. A., and Hackert, M. L. (2000) *J. Mol. Biol. 295*, 7−16.
52. Li, X., and Coffino, P. (1993) *Mol. Cell. Biol. 13*, 2377−2383.

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