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TRANSLATIONAL AUTOREGULATION OF THYMIDYLATE SYNTHASE AND DIHYDROFOLATE REDUCTASE

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1. ABSTRACT

The folate-dependent enzymes, thymidylate synthase (TS) and dihydrofolate reductase (DHFR) are critical for providing the requisite nucleotide precursors for maintaining DNA synthesis and DNA repair. In addition to their essential roles in enzyme catalysis, these two enzymes have now been shown to function as RNA binding proteins. Using *in vitro* and *in vivo* experimental model systems, we have shown that the functional consequence of binding of TS protein to its own cognate mRNA, as well as binding of DHFR to its own DHFR mRNA, is translational repression. Herein, we review and update studies focusing on the translational autoregulatory control of TS and DHFR expression and discuss the molecular elements that are required for these specific RNA-protein interactions. Moreover, we present evidence showing that abrogation of these normal translational autoregulatory feedback mechanisms provides the molecular basis for the rapid development of cellular drug resistance.

2. INTRODUCTION

Translational regulation is based on the central concept that cellular gene expression is governed by the efficiency of translation of a given mRNA in the absence of a corresponding change in steady-state mRNA levels (1,2). This control mechanism is a relatively acute process that can alter cellular gene expression in response to genotoxic and/or cytotoxic stresses. Translational regulation ensures the precise and desired level of a given cellular protein, and in so doing, works in close concert with other regulatory events, including transcription, post-transcription, and post-translation. This process offers a precise, efficient means to regulate cellular gene expression. The significant time lag that is required for critical signaling pathways within the nucleus to exert their biological effects, including gene amplification, activation of transcription, processing, and nucleo-cytoplasmic transport of RNA, is effectively bypassed. Rapid changes in protein synthesis can, therefore, be acutely effected. As translation represents the last step in protein synthesis, the cellular metabolic requirements are minimized that then allows for proper conservation of energy. An additional advantage of this control mechanism is that it is readily reversible. Once the cellular needs have been satisfied, protein synthesis can be restored to basal levels. Thus, translational control ensures and maintains the proper balance within the cell to allow for normal growth and proliferation to occur.

Translational autoregulation is a specialized form of translational regulation which is highly conserved in evolution. It is a well-established mechanism for the regulation of expression of bacteriophage and prokaryotic systems (3-7). There is now growing evidence that eukaryotic cellular gene expression is regulated in a similar manner. Thymidylate synthase (TS) (8,9) and dihydrofolate reductase (DHFR) (10-12) represent the first two eukaryotic genes whose expression is controlled by this process of translational autoregulation. Recent studies have demonstrated that expression of another folate-dependent enzyme serine hydroxymethyltransferase (13) and the p53 tumor suppressor gene (14,15) is controlled by an identical translational autoregulatory process. Herein, we will review and update the basic studies investigating the translational autoregulation of TS and DHFR.

3. THYMIDYLATE SYNTHASE

TS is a folate-dependent enzyme that catalyzes the reductive methylation of deoxyuridylate (dUMP) by the reduced folate 5,10-methylenetetrahydrofolate (CH_2THF) to thymidylate (dTMP) and dihydrofolate (16,17) (figure 1). Once synthesized, dTMP is then metabolized intracellularly to the dTTP triphosphate form, an essential precursor for DNA biosynthesis and DNA repair. dTMP can also be formed through the salvage pathway via phosphorylation of thymidine by the thymidine kinase-mediated pathway, although this pathway appears to play only a relatively minor role in dTMP synthesis. The TS-catalyzed reaction provides for the sole intracellular *de novo* source of dTMP. Given its central role in dTMP and DNA biosynthesis and given the observation that inhibition of this reaction results in

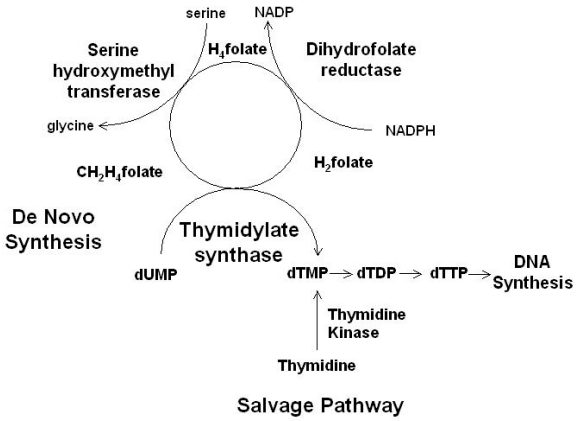

Figure 1. Enzyme reactions catalyzed by thymidylate synthase and dihydrofolate reductase.

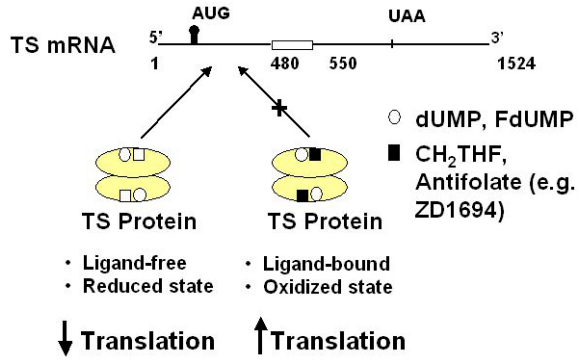

Figure 2. Model for TS translational autoregulation and for the induction of TS in response to TS inhibitor compounds.

cessation of cellular proliferation and growth, TS has served as an important target for the design and development of anticancer agents for well over 40 years (18). In fact, TS is one of the first well-established molecular targets in cancer chemotherapy.

Using various *in vitro*, *in vivo*, and clinical model systems, several investigators have described acute increases in TS enzyme levels following short-term exposure to fluoropyrimidine compounds (19-24). It was postulated that the drug-mediated induction of TS may represent a mechanism for the rapid development of resistance to 5-fluorouracil (5-FU) chemotherapy with direct biological and clinical relevance. Although the underlying mechanisms for the enhanced expression of TS in response to 5-FU and other fluoropyrimidine analogs were not well-characterized in these initial studies, several possibilities were proposed including increased transcription, enhanced stability of TS mRNA, increased efficiency of TS mRNA translation, and enhanced stability of TS protein. Significant efforts subsequently focused on elucidating the critical biochemical and molecular events that controlled the 5-FU-mediated acute induction of TS. Keyomarsi and Pardee (25) showed that treatment of human breast cancer MCF-7 cells with the quinazoline antifolate analog raltitrexed (ZD1694) resulted in an acute increase (up to 40-fold) in TS enzyme levels with no associated change in TS mRNA levels. The presence of the protein synthesis inhibitor cycloheximide blocked the elevation in TS enzyme levels following exposure to ZD1694, providing suggestive evidence that a translational regulatory event was involved.

Using the human colon cancer H630 cell line as our model system, studies from our own lab showed that the increase in both TS enzyme activity and TS protein expression in response to short-term exposures to 5-FU was not associated with a corresponding change in the level of TS mRNA expression (26,27). An approximately 50% increase in free TS protein levels was observed. Additional studies revealed that the increase in TS protein expression resulted directly from the synthesis of TS protein and not from alterations in protein stability. Moreover, we observed that the cytokine IFN-γ, at non-growth inhibitory drug concentrations, was able to abrogate the 5-FU-mediated induction of TS, and in so doing, enhance the cytotoxic effects of 5-FU by nearly 20-fold. This study provided the first piece of evidence for the role of translational regulation in an intact biological system and emphasized the potential biological relevance of this control mechanism. Welsh et al (28) recently investigated the induction of TS in several human cancer cell lines as well as in non-transformed human fibroblasts following short-term exposure to the antifolate analog ZD9331. While induction of TS protein expression, in the range of 6- to 10-fold was documented in human cancer cell lines, an even higher level of TS induction was observed in non-transformed human fibroblasts.

Based on these initial pre-clinical studies, our laboratory performed a series of experiments to more precisely investigate the regulation of TS mRNA translation. Our current working model for the translational autoregulatory control of TS and the interaction between TS protein and its own TS mRNA is presented in figure 2. As noted above, this regulatory process is well-established as an important control mechanism for the expression of various bacteriophage and prokaryotic systems. However, TS represents the first eukaryotic gene whose expression is controlled in such a manner.

Two different *cis*-acting sequences have been identified on human TS mRNA, and each interacts with high affinity (1-3 nM) to human TS (8,9). The first site is a 30-nt sequence corresponding to nt 80-109 and includes the translational start site within the loop aspect of a stable stem-loop structure. The second element is contained within a 70-nt sequence corresponding to nt 480-550 in the coding region. This second *cis*-element is sufficient to confer the property of translational regulation onto a heterologous luciferase reporter gene and requires the presence of an intact wild-type TS protein for its biological effect (29). While this sequence is able to exert translational control independent of the 5'-upstream *cis*-element *in vivo*, it appears that both elements are required for complete translational autoregulatory activity.

Significant efforts have been placed on defining the essential molecular factors that mediate the interaction between TS protein and its own target TS mRNA. Our lab

has shown that the RNA binding activity of human recombinant TS is exquisitely redox-sensitive and requires the presence of at least one free sulfhydryl group (30). There are five different cysteine amino acid residues on human TS protein. The role of these cysteine sulfhydryls on RNA binding of human TS was investigated by mutating each cysteine moiety to a corresponding alanine residue (31). Mutations at C195, C199, and C210 did not alter the RNA binding activity of the TS protein. The RNA binding activity of the C43A mutant protein was reduced by 30% when compared to wild-type TS. In contrast, the C180A mutant was completely inactive with respect to RNA binding. Further studies have shown that Cys-180 was a critical residue for mediating RNA recognition and for the *in vitro* and *in vivo* translational regulatory effects of human TS (31). At the present time, the precise mechanism by which this specific cysteine sulfhydryl mediates RNA binding remains unclear. One possibility is that the sulfhydryl group may form a direct Michael adduct with the C-6 position of a uracil ring on human TS mRNA. An alternative possibility is that occupation of this cysteine residue may alter RNA binding via a steric hindrance mechanism. The final potential mechanism is that the cysteine at residue 180 is essential in maintaining the TS protein in a certain conformation that then makes the true domain on the protein more accessible for RNA binding.

In addition to the redox state of TS, another critical determinant of RNA binding relates to the state of occupancy of the protein. When TS is ligand-free, maximal RNA binding activity is maintained, which then leads to translational repression of TS mRNA. In contrast, when TS is ligand-bound by either its physiologic nucleotide substrate dUMP or its physiologic folate CH_2THF or bound by the 5-FU nucleotide metabolite FdUMP, TS is no longer able to bind with high affinity to its target mRNA (see figure 2). In addition, incubation of TS with various antifolate analogs including raltitrexed (ZD1694), pemetrexed (LY231514) and MTA significantly reduces its ability to form an RNP complex with its cognate TS mRNA. This then leads to abrogation of the translational repressive effects of TS, thereby resulting in increased synthesis of new TS protein (32). Such a condition would exist in cancer cells exposed to inhibitor compounds of TS. This model provides the molecular basis for the acute induction of TS that arises in direct response to exposure to the class of TS inhibitor compounds. Abrogation of this normal TS translational autoregulatory process would appear then to represent a biologically relevant mechanism that helps to maintain normal cellular synthetic function in the setting of an acute cellular stress such as exposure to an anticancer agent. Moreover, it offers a novel mechanism for the development of acute drug resistance to compounds that specifically target TS.

4. DIHYDROFOLATE REDUCTASE

The enzyme dihydrofolate reductase (DHFR) catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate, a key intermediate in one-carbon transfer reactions (33,34) (figure 1). DHFR plays a critical role in folate homeostasis, and provides the one-carbon carrier units that are required for the *de novo* synthesis of purines, thymidylate, and certain amino acids. For this reason, DHFR represents an important target enzyme in cancer chemotherapy (35), and as in the case of TS, it represents one of the first molecular targets to be identified.

Several *in vitro* and *in vivo* model systems have demonstrated that exposure of malignant cells to the antifolate analog methotrexate (MTX) is accompanied by acute increases in DHFR enzyme activity and DHFR protein (36-42). Bertino et al first reported that treatment with MTX resulted in an acute increase in DHFR enzyme activity in the leukemic cells of patients and that this increase appeared to be secondary to stabilization of the DHFR enzyme by MTX (36,37). However, transcriptional, post-transcriptional, and post-translational events were also proposed as potential mechanisms mediating this induction of DHFR expression. The possibility for translational control of DHFR was initially proposed by Cheng and colleagues (39,40) following their observation that exposure of human nasopharyngeal cancer KB cells to increasing concentrations of MTX resulted in up to a five-fold increase in DHFR protein levels. Protein stability studies revealed that the half-life of DHFR remained unchanged in control and drug-treated cells. The fact that drug treatment did not alter the protein half-life effectively ruled out the possibility for a post-translational event as a causative mechanism for protein induction. In a similar series of experiments, Grem et al (42) showed that treatment of H630 human colon cancer cells with the antifolate analog trimetrexate (TMQ) gave rise to a 3.5-fold increase in levels of DHFR protein. This TMQ-mediated induction of protein was nearly completely abrogated in the presence of cycloheximide, suggesting a potential role for translational control in the synthesis of new DHFR protein. Of note, both of these initial studies were somewhat limited in scope in that neither one specifically determined the levels of DHFR mRNA following exposure to the respective antifolate analog nor were the effects of antifolate treatment on protein stability directly investigated using more precise immunoprecipitation pulse-labeling techniques.

Gollerkeri et al (43) from our group recently investigated the effect of MTX on DHFR expression using the human colon cancer RKO model system. Treatment with MTX led to a significant time- and dose-dependent induction of DHFR protein levels. DHFR protein levels were maximally induced by 10- to 12–fold after 24-hour treatment with MTX. Northern blot analysis revealed no change in levels of DHFR mRNA in control and MTX-treated cells. Immunoprecipitation pulse-labeling experiments showed no change in the half-life of DHFR protein in control and MTX-treated cells, being on the order of 22-24 hours in either case. These results, taken together, suggested that the MTX-mediated induction of DHFR protein in RKO colon cancer cells was controlled by a translational regulatory process that directly involved translation of DHFR mRNA.

To more directly investigate translation of DHFR mRNA, we used a rabbit reticulocyte lysate *in vitro* translation system to show that translation of human DHFR

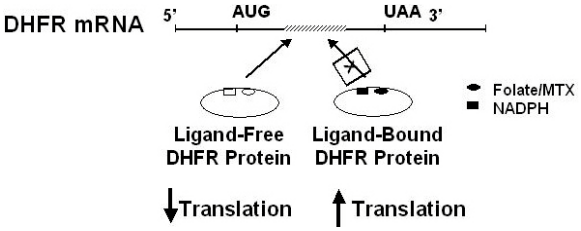

Figure 3. Model for DHFR translational autoregulation and for the induction of DHFR in response to DHFR inhibitor compounds.

mRNA was specifically inhibited in the presence of human recombinant DHFR protein (10-12). RNA binding studies confirmed a direct interaction between human recombinant DHFR protein and its own DHFR mRNA. Of note, this RNA-protein interaction did not require the presence of other cellular proteins and/or cofactors. Furthermore, dihydrofolate, the normal physiologic folate substrate for DHFR, and MTX, an antifolate inhibitor analog, each interfered with the ability of the protein to bind to its cognate DHFR mRNA.

To date, one *cis*-acting element in the protein-coding region has been identified that interacts with relatively high affinity (2-4 nM) to DHFR protein (44). Using a series of gel shift and nitrocellulose filter binding assays, our laboratory has localized this element to a 164-nt RNA sequence corresponding to nt 401-564. This specific sequence binds to DHFR protein with an affinity similar to that of the full-length DHFR mRNA. To document *in vivo* biological activity, this 164-nt sequence was cloned onto the 5' end of a luciferase reporter plasmid, and transient transfection experiments were performed using human colon cancer RKO cells. In cells transfected with the recombinant p644/DHFR:401-564 plasmid, luciferase activity was decreased by 50% when compared to cells transfected with p644 plasmid alone. In cells transfected with p644/DHFR:401-564, luciferase activity was restored to almost 100% of control when cells were treated with the antifolate analog methotrexate or with an siRNA targeting DHFR mRNA. These findings provided evidence that the DHFR:401-564 sequence was indeed responsive to alterations in DHFR protein expression and was, therefore, a true DHFR-response element.

Further studies have recently localized this element to an 82-nt sequence corresponding to nt 401-482. Preliminary footprinting analysis has recently identified a 27-nt core stem-loop structure contained within this sequence that is the actual *cis*-acting response element (personal communication). In addition, *in vitro* RNA binding studies suggest that human DHFR may also be able to interact with another sequence contained within the coding-region, corresponding to DHFR:1-200. Preliminary analysis of these two elements, however, has failed to identify a consensus nucleotide sequence and/or a consensus secondary structure. However, more precise structural studies are required to more completely characterize each of these binding sites. More recently, the Bertino lab has identified a short sequence in the 5'-untranslated region that may function as a determinant of DHFR mRNA translational efficiency (45). However, their studies suggest that a cellular protein other than DHFR may be functioning as the *trans*-acting element and forming a RNP complex with this DHFR mRNA sequence.

In addition to investigating the *cis*-acting elements on DHFR mRNA, our lab also focused on identifying the key elements on the DHFR protein, itself, that are required for RNA binding. Using a series of DHFR mutant proteins and RNA gel shift and nitrocellulose binding experiments, we have shown that the cysteine residue at the amino acid 6 position as well as Ile-7, Arg-28, and Phe-34 are critical amino acid residues for RNA binding (46). Our studies suggest that point mutations at these specific residues completely abrogate the ability to form RNP complexes with either the full-length DHFR mRNA or with the shorter DHFR:401-564 and DHFR:401-482 *cis*-acting response elements. At present, it remains unclear as to whether these amino acids form direct contact points with the DHFR mRNA and/or whether they maintain the protein in a proper conformational state that then allows for the actual binding domain on the protein to interact with its cognate mRNA. However, our studies would tend to support the notion that these residues play a more critical role in maintaining the DHFR protein in the proper structural state for RNA binding. Studies are in progress to resolve the crystal structure of the DHFR protein-DHFR mRNA complex in order to directly address this very important issue.

Together, these findings support a model of DHFR translational autoregulation (figure 3). When the metabolic needs of the cell are met or when the cell is in a quiescent state, DHFR binds with high affinity to its cognate mRNA and effectively represses translation. However, when growth requirements are increased, when substrate levels are elevated, and/or when cells are exposed to a cytotoxic and/or genotoxic stress as in the case of treatment with DHFR inhibitor compounds, the DHFR protein is no longer able to bind to its target DHFR mRNA, thereby allowing for translation and synthesis of new protein to proceed.

5. PERSPECTIVE

In this review, we have highlighted the role of translational autoregulation in controlling the expression of TS and DHFR, two folate-dependent proteins that are critically involved in the normal function of the cell cycle and in maintaining the cellular requirements for growth and proliferation. The events of the cell cycle represent a highly ordered process, and progression through the cell cycle is controlled by the expression of certain proteins that act at critical checkpoints. Translational autoregulation allows the cell a rapid and efficient means of altering cellular gene expression in response to various external stimuli and/or cytotoxic stresses.

TS and DHFR each represent two well-defined targets in cancer chemotherapy for the past 40-45 years. The ability to regulate the expression of both of these genes at the translational level represents a biologically relevant

process that serves three important functions. First, this is an important mechanism by which normal cellular synthetic function can be tightly regulated. Second, it serves a critical defensive mechanism for cancer cells to rapidly develop cellular resistance in response to exposure to inhibitor compounds that directly target TS and/or DHFR so as to maintain cellular synthetic function. Finally, translational regulation provides an efficient protective mechanism by which cells can protect themselves against the deleterious effects of cytotoxic and/or genotoxic stresses. Studies are presently on-going in our laboratory to further elucidate the key *cis*- and *trans*-acting elements that modulate these specific RNA-protein interactions. This work contributes to our growing understanding of the important role of translational control in the regulation of cellular gene expression and provides new insights into the critical molecular elements that mediate RNA-protein interactions. Moreover, these molecular-based studies should provide the rational basis for the design and development of novel strategies that can be translated into the clinic for the treatment of human cancer.

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