Translation inhibition during cell cycle arrest and apoptosis: Mcl-1 is a novel target for RNA binding protein CUGBP2

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Subramaniam D, Natarajan G, Ramalingam S, Ramachandran I, May R, Queimado L, Houchen CW, Anant S. Translation inhibition during cell cycle arrest and apoptosis: Mcl-1 is a novel target for RNA binding protein CUGBP2. Am J Physiol Gastrointest Liver Physiol 294: G1025–G1032, 2008. First published February 21, 2008; doi:10.1152/ajpgi.00602.2007.—CUGBP2, a translation inhibitor, induces colon cancer cells to undergo apoptosis. Mcl-1, an antiapoptotic Bcl-2 family protein, interferes with mitochondrial activation to inhibit apoptosis. Here, we have determined the effect of CUGBP2 on Mcl-1 expression. We developed a HCUG2 cell line by stably expressing CUGBP2 in the HCT-116 colon cancer cells. HCUG2 cells demonstrate decreased levels of proliferation and increased apoptosis, compared with HCT-116 cells. Flow cytometry analysis demonstrated higher levels of cells in the G2-M phase. Western blot analyses demonstrated that there was decreased Bcl-2 and Mcl-1 protein but increased expression of Bax, cyclin B1, and Cdc2. Immunocytochemistry also demonstrated increased levels of cyclin B1 and Cdc2 in the nucleus of HCUG2 cells. However, there was colocalization of phosphorylated histone H3 with transferase-mediated dUTP nick-end labeling (TUNEL). Furthermore, immunostaining for α-tubulin demonstrated that there was disorganization of microtubules. These data suggest that CUGBP2 expression in HCUG2 cells induces the cells to undergo apoptosis during the G2-M phase of the cell cycle. We next determined the mechanism of CUGBP2-mediated reduction in Mcl-1 expression. Mcl-1 protein, but not Mcl-1 mRNA, was lower in HCUG2 cells, suggesting translation inhibition. CUGBP2 binds to Mcl-1 3′-untranslated region (3′-UTR) both in vitro and in HCUG2 cells. Furthermore, CUGBP2 increased the stability of both endogenous Mcl-1 and luciferase mRNA containing the Mcl-1 3′-UTR. However, luciferase protein expression from the luciferase-Mcl-1 3′-UTR mRNA was suppressed. Taken together, these data demonstrate that CUGBP2 inhibits Mcl-1 expression by inhibiting Mcl-1 mRNA translation, resulting in driving the cells to apoptosis during the G2 phase of the cell cycle.

Bcl-2 family member; G2-M arrest; checkpoint kinases; RNA stability

Both proapoptotic and antiapoptotic proteins are included in the Bcl-2 family, and the choice between survival and death for a cell is in part dependent on these proteins (2, 9). Mcl-1 is a unique member of the family that was originally identified in the ML-1 human myeloid leukemia cell line when the cells underwent phorbol ester-induced differentiation (24, 27). Mcl-1 functions to inhibit cell death. Although Mcl-1 expression is required to maintain cell viability and promote ML-1 cell differentiation, downregulation of the protein triggers the cell to undergo apoptosis (49). Mcl-1 expression has also been shown to be essential for embryonic development because knockout of the gene resulted in the lack of preimplantation development and implantation (37). On the other hand, transgenic overexpression of Mcl-1 results in B-cell lymphomas and resistance to chemotherapeutic agents (28, 38, 40).

Mcl-1 is also unique in that it has a short half-life and quickly decreases in response to various cell death-related signals, including growth factor withdrawal, radiation treatment, and viral infection (19, 48). Both growth factor withdrawal and radiation have been shown to reduce Mcl-1 gene expression by inhibiting the translation of Mcl-1 mRNA. Recently, inhibition of Mcl-1 mRNA translation in response to UV radiation was determined to occur through phosphorylation of the α-subunit of eukaryotic translation initiation factor 2 (eIF2) at serine-51, leading to decreased formation of the ternary complex required for the binding of Met-tRNA^{Met} to the 40S ribosomal subunit (11, 18). Furthermore, the double-stranded RNA-activated protein kinase (PKR), which is known to phosphorylate eIF2 at serine-51, is known to bind to the 3′-untranslated regions (3′-UTRs) of the cytoskeletal mRNAs tropomyosin, troponin, and cardiac actin and induce muscle cell differentiation (8, 34).

The CELF (CUGBP- and ETR-3-like) family of RNA binding proteins is composed of six members and is involved in various cellular processes including mRNA splicing, editing, stability, and translation (3). The proteins encoded by this family contain three RNA binding domains of the RNA recognition motif (RRM) type (17). RRMs are 80-amino acid domains with conserved sequences that are present in many RNA binding proteins to regulate various posttranscriptional functions. In the CELF proteins, the first two RRMs are located in tandem near the NH2 terminus and are separated from the third RRM by an intervening bridge segment rich in basic amino acid residues (21, 39). The bridge region is highly divergent between the proteins. The proteins have been shown to actively regulate splicing in muscle cells. Transcripts that are regulated by CELF proteins include cardiac troponin T (cTNT) exon 5, insulin receptor (IR) exon 11, chloride channel 1 (CIC1) intron 2, N-methyl-D-aspartic acid (NMDA) receptor-1 (NMDAR-1) exons 5 and 21, and the α-actinin muscle-specific exon (7, 12, 22, 25, 42). CUGBP2, a member of the CELF family, is expressed ubiquitously, albeit at higher levels in muscle cells. However, the physiological function of CUGBP2 expression in the epithelial cells is currently unknown. In previous studies, we have demonstrated that CUGBP2 expression is upregulated in HT-29 colon cancer cells when the cells
were exposed to UV or γ-irradiation (31). Furthermore, CUGBP2 translocated to the cytoplasm and bound to U-rich sequences in the 3′-untranslated region (3′-UTR) of cyclooxygenase-2 (COX-2) mRNA, resulting in increased stability of COX-2 mRNA while inhibiting its translation (31). COX-2 is the rate-limiting enzyme in the prostaglandin synthesis pathway that is overexpressed in inflammation and cancers (5, 30).

The facts that Mcl-1 is overexpressed in cancers and that Mcl-1 protein expression is rapidly lost following radiation exposure but its mRNA remains unaffected suggest that Mcl-1 might be a target for CUGBP2 function. To determine whether CUGBP2 regulates Mcl-1 expression, we generated stable colon cancer cells expressing CUGBP2. Cells overexpressing CUGBP2 had a lower growth rate, eventually underwent apoptosis, and did not survive very long. Furthermore, Mcl-1 protein levels are lower in the CUGBP2-overexpressing cells compared with control, vector-transfected cells. We also determined that CUGBP2 interacts with the Mcl-1 3′-UTR to inhibit the translation. These data demonstrate that Mcl-1 mRNA is a novel target during CUGBP2-mediated cell death during prolonged G2 phase transition.

**MATERIALS AND METHODS**

Cells. HCT-116 human colon adenocarcinoma cell line (American Type Culture Collection, ATCC) was grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and standard antibiotic-antimycotic agents in a humidified chamber at 37°C with 5% CO2. For CUGBP2 expression, HCT-116 cells were transfected with plasmid pCMV-Tag2B encoding CUGBP2, and stable transfectants (named HCU2G) were isolated as individual colonies following incubation in 800 μg/ml G418.

Cloning and plasmids. The full-length coding region of human CUGBP2 was amplified by RT-PCR from HCT-116 cells and cloned in pGEM-T EZ. After sequence, the product was cloned into pCMV-Tag2B at the HindIII and XhoI restriction sites and expressed as amino-terminal FLAG epitope-tagged proteins. The 2.8-kb full-length region of the Mcl-1 3′-UTR was amplified from HCT-116 cells by RT-PCR using the primers 5′-GCTCTAGAGTAGGAGCTGGTT-3′ and 5′-GCTCTAGACAGAAAGTTAGGGAAA-3′, which were underlined in both primers. The PCR product was first cloned into pGEM-T EZ. After sequence, the fragment was isolated and cloned into plasmid pGL3-control at the XhoI site downstream of the firefly (Photinus pyralis) luciferase gene. The cloning was verified with DNA sequencing using BigDye Terminator chemistry on both strands.

**Cell proliferation and apoptosis.** To assess proliferation, HCT-116 and HCU2G cells were plated onto 96-well plates at a density of 1 × 10⁵ cells/well, allowed to adhere, and grown overnight in 10% heat-inactivated fetal bovine serum containing DMEM. Cell proliferation was assessed for up to 72 h at 24-h intervals by the hexosaminidase assay (23). For apoptosis, the cells were grown in 96-well black plates. After 24 h, caspase 3/7 activity was measured by use of the Apo-one Homogeneous Caspase-3/7 Assay kit (Promega, Madison, WI) according to the manufacturer’s protocol.

**Cell cycle analyses.** Cells were plated at a density of 5 × 10⁵ cells/well on six-well plates. After 48 h, the cells were trypsinized and suspended in PBS. Single-cell suspensions were fixed using 70% ethanol for 2 h and subsequently permeabilized with PBS containing 1 mg/ml propidium iodide (Sigma-Aldrich St. Louis, MO), 0.1% Triton X-100 (Sigma-Aldrich), and 2 μg DNase-free RNase (Sigma-Aldrich) at room temperature. Flow cytometry was measured by use of a FACSCalibur analyzer (Becton Dickinson, Mountain View, CA), capturing 50,000 events for each sample. Results were analyzed with ModFit LT software (Verity Software House, Topsham, ME).

**Immunocytochemistry and immunofluorescence.** Cells were plated onto coverslips in six-well dishes and allowed to grow for 24 h. The cells were fixed with 10% buffered formalin for 10 min and subsequently washed thrice with PBS. For immunocytochemistry, the cells were then permeabilized with PBS containing 0.5% Triton X-100 for 10 min at room temperature. The coverslips were incubated with rabbit anti-cyclin B1 (Santa Cruz Biotechnologies, Santa Cruz, CA) and rabbit anti-Cdc2 antibodies (Abcam, Cambridge, MA), followed by biotinylated anti-rabbit IgG. The slides were further processed by using a Vectastatin ABC kit (Vector Laboratories, Burlingame, CA) followed by DAB staining. For immunofluorescence, the cells were incubated with anti-FLAG antibody (Affinity Bioreagents, Golden, CO) followed by FITC-conjugated anti-rabbit IgG. The nucleus was counterstained with 4′,6-diamidino-2-phenylindole. The slides were mounted and examined with a Zeiss Axioskop 2 MOT plus microscope (Carl Zeiss, Thornwood, NY).

**Real-time RT-PCR.** Total RNA was isolated from cells by use of TRIzol reagent and reverse transcribed with Superscript II reverse transcriptase in the presence of random hexanucleotide primers (all from Invitrogen, Carlsbad, CA). Complementary DNA was then used for real-time RT-PCR using Jumpstart Tag DNA polymerase (Sigma Chemical) and SYBRgreen nucleic acid stain (Molecular Probes, Eugene, OR). Crossing threshold values for individual genes were normalized to β-actin. Changes in mRNA expression were expressed as fold change relative to control. Primers used in this study were as follows: β-actin, 5′-GCTGATCCACATCTGCTGGAA-3′ and 5′-ATCATTGCCTCCTCAGGACG3′; Mcl-1, 5′-TCTCAAGGCG-3′ and 5′-TCTGTAATGGTGTCGACG3′; Bcl-2, 5′-CGCCCTGTCGATGACTGAGTA-3′ and 5′-CCCATGGTCCGTATTCCTG-3′.

**Western blot analysis.** Cell lysates were subjected to SDS-PAGE and blotted onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). Antibodies were purchased from Cell Signaling Technology (Beverly, MA), Santa Cruz Biotechnology, and Abcam. Specific proteins were detected by the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ).

**Immunoprecipitation-coupled RT-PCR analyses.** Cells were subjected to treatment with 1% formaldehyde for 10 min, and cell lysates were prepared by sonication. Cell lysates were immunoprecipitated with an anti-CUGBP2 antibody. Total RNA was isolated from the precipitate and supernatant and subjected to RT-PCR analyses for Mcl-1 and β-actin as mentioned above.

**Recombinant protein.** Recombinant CUGBP2 was expressed as His6-terminal glutathione S-transferase (GST) fusion proteins from the plasmid pGEX-4T3 (Amersham-Pharmacia) following the cloning of the CUGBP2 full-length PCR products as previously described (41). Purity of protein following affinity purification on a GST-Sepharose column was determined by Coomassie brilliant blue staining. Protein was assessed to be >95% pure (data not shown).

**In vitro transcription and EMSA.** The pGEM-T EZ plasmid containing the full-length Mcl-1 3′-UTR was linearized with NsiI and used as template for in vitro transcription with T7 RNA polymerase in the presence of [32P]UTP. For EMSA, 32P-labeled cRNA template (50,000 cpm) was incubated with increasing concentrations of GST-CUGBP2 in a binding buffer containing 20 mM HEPES (pH 7.9), 100 mM KCl, and 1 mM DTT for 20 min at room temperature and then treated with RNase T1 (1 U/reaction). The mixture was immediately analyzed by electrophoresis in a 5% native PAGE using 0.5% Trisborate-EDTA buffer.

**RNA stability assay.** HCT-116 and HCU2G cells were plated and allowed to grow overnight. Actinomycin D (10 μg/ml final concentration), a potent inhibitor of mRNA synthesis, was added to the cells and total mRNA was extracted from 0 to 8 h. RNA was subjected to real-time RT-PCR as described above. To determine the effect of CUGBP2 on the stability of the luciferase mRNA encoding the full-length Mcl-1 3′-UTR, the cells were transfected with either pGL3 control or pGL3-Mcl-1 3′-UTR plasmid overnight, following which
actinomycin D was added. Again, total mRNA was extracted at 0–8 h and treated with DNase to remove any residual plasmid DNA and subsequently subjected to real-time RT-PCR. Data is presented as relative to the presence of RNA in the control cells, at the time of addition of actinomycin D. Assays were performed in triplicate wells and experiments were repeated three times.

Luciferase assay. HCT-116 and HCUG2 cells were transiently transfected with the construct encoding control luciferase (Luc) or the luciferase-Mcl-1 3′-UTR, along with plasmid pRL-TK that encodes Renilla luciferase under the control of the RSV thymidine kinase promoter (Promega). The plasmids were transfected with FuGENE 6 (Roche). Luciferase levels were determined as per manufacturer’s instructions (Dual-Luciferase Reporter Assay System, Promega) by using a BioTek Synergy HT reader. Luciferase activity was normalized to Renilla luciferase activity and presented as luciferase units relative to control. Assays were performed in triplicate wells and experiments were repeated three times.

Statistical analysis. The values are expressed as means ± SE. Data were analyzed by an unpaired Student’s t-test. We considered a P value of less than 0.05 to be statistically significant.

RESULTS

CUGBP2 overexpression induces a mitotic crisis. In previous studies, we have demonstrated that CUGBP2 overexpression induced colon cancer cells to undergo apoptosis (31, 32). To confirm that CUGBP2 affects cell growth, we first aimed to develop a cell line that stably expressed CUGBP2. Since COX-2 and CUGBP2 have opposing functions, we opined that cells expressing high levels of COX-2 might not be favorable for stable CUGBP2 expression. We chose HCT-116 cells since they have been shown to express low levels of COX-2. Isolation of stable CUGBP2 expressing cells was difficult with a
majority of the cells dying. However, a few colonies did eventually develop, especially when a graded incubation with G418 from 200 μg/ml to 800 μg/ml was performed. Five independent clones were selected for further study. It was immediately apparent that the cells, which we named HCUG2, grew at a slower rate compared with the parent HCT-116 cells. To determine the effect of CUGBP2 overexpression on cell cycle progression, cells were plated and grown for 48 h and subjected to flow cytometry analyses following propidium iodide (PI) staining. There was a significant increase in cells in the G2-M phase of the cell cycle compared with wild type HCT-116 cells (30.47 and 11.64% in HCUG2 and HCT-116 cells, respectively) (Fig. 1A). This suggests that the cells have a prolonged G2-M transit when CUGBP2 is overexpressed. We next determined the effect of CUGBP2 overexpression on cell proliferation. HCT-116 and HCUG2 cells were tested for hexosaminidase activity for up to 72 h after plating. There was a steady decrease in proliferation of HCUG2 cells compared with control HCT-116 cells (Fig. 1B). Since CUGBP2 has been demonstrated to induce cells to undergo apoptosis, we determined the levels of activated effector caspase proteins, caspase 3 and caspase 7. There was a 2.5-fold increase in activated caspase 3/7 in HCUG2 cells compared with HCT-116 WT cells (Fig. 1C). To confirm that the cells were undergoing apoptosis, we performed transferase-mediated dUTP nick-end labeling (TUNEL) immunostaining. HCT-116 cells did not demonstrate any TUNEL-positive cells (Fig. 1D). How- ever, significant number of HCUG2 cells were positive for TUNEL staining, confirming that the cells were undergoing apoptosis (Fig. 1D). We also coimmunostained the cells for phosphorylated histone H3, a marker of mitotic cells. All the TUNEL-positive HCUG2 cells were also positive for phosphorylated histone H3 (Fig. 1D). To determine whether the reason for the higher levels of cells in G2-M is due to an arrest at G2 resulting in a lack in progression to mitosis, we next determined the expression and localization of cyclin B1 and the serine/threonine kinase Cdc2. To induce mitosis, cyclin B1 and Cdc2 heterodimerize and localize into the nucleus, which results in activating enzymes that regulate chromatin condensation, nuclear membrane breakdown, and mitosis-specific microtubule reorganization (6, 35, 43). Western blot analysis demonstrated increased expression of both cyclin B1 and Cdc2 in HCUG2 cells (Fig. 1E). Furthermore, immunocytochemistry analyses demonstrated that there were significantly higher nuclear levels of both proteins in HCUG2 cells compared with HCT-116 cells (Fig. 1F). These data suggest that cells undergoing apoptosis are also in mitosis, suggesting mitotic catastrophe. A hallmark of cells undergoing mitotic catastrophe is the lack of tubulin polymerization. To confirm that the HCUG2 cells were undergoing mitotic catastrophe, we immunostained the cells for α-tubulin. Although the HCT-116 cells demonstrated active tubulin organization and spindle formation, tubulin was disorganized and there was no appearance of spindle formation in HCUG2 cells, confirming that HCUG2 were undergoing mitotic catastrophe (Fig. 1G). Together, these data suggest that overexpression of CUGBP2 results in a crisis resulting in cells undergoing apoptosis during the G2-M phase of the cell cycle.

**Overexpression of CUGBP2 affects Mcl-1 expression.** Mcl-1 contributes to the control of mitochondrial integrity, which is...
critical for maintaining cell viability. Furthermore, depletion of Mcl-1 can trigger apoptosis during prolonged mitotic arrest. Therefore, we next determined the expression of Mcl-1 in HCUG2 cells. Real-time RT-PCR analyses demonstrated a twofold increase in Mcl-1 mRNA levels compared with control HCT-116 cells (Fig. 2A). Similarly, Bcl-2 mRNA expression is upregulated by twofold in HCUG2 cells compared with HCT-116 cells (Fig. 2B). To further demonstrate that Mcl-1 and Bcl-2 are overexpressed in the cells, a Western blot analysis was performed with extracts from the two cell lines. However, in contrast to the mRNA expression, there was a reduction in both Mcl-1 and Bcl-2 proteins in the HCUG2 cells. In contrast, there was an increase in the proapoptotic Bax protein (Fig. 2C). Furthermore, there was a fivefold reduction in Bcl-2-to-Bax ratio, suggesting that the cells overexpressing CUGBP2 are prone to apoptosis (Fig. 2D). These data suggest that CUGBP2 inhibits the expression of antiapoptotic proteins Mcl-1 and Bcl-2 at the protein but not the mRNA level.

CUGBP2 is a Mcl-1 3′-UTR interacting protein. In previous studies, CUGBP2 has been identified to interact with alanine- and uridine-rich (AU-rich) sequences with a high affinity (41). In addition, it can interact with CUG triplet repeat sequence and with UGUU motifs (10). Scanning the Mcl-1 3′-UTR demonstrated the presence of multiple AU-rich and UGUU sequences (data not shown). Therefore, we determined whether CUGBP2 binds Mcl-1 3′-UTR. For this, we first amplified the full-length Mcl-1 3′-UTR sequence using sequence-specific primers and cloned it into plasmid pGEM-T EZ. Using T7 RNA polymerase, we generated the 3′-UTR cRNA in the presence of [32P]UTP. Recombinant GST-CUGBP2 containing the GST moiety at the NH2 terminus was generated and affinity purified to >95% purity. In the EMSA assay, increasing amounts of the recombinant GST-CUGBP2 were allowed to interact with the radiolabeled Mcl-1 3′-UTR cRNA and subsequently visualized following non-denaturing gel electrophoresis. As shown in Fig. 3A, there was a low-level binding with 50 ng of recombinant protein, but significantly higher binding at 100 ng of the recombinant protein. Further study, however, is required to determine the affinity of CUGBP2 binding. To confirm that CUGBP2 binds Mcl-1 mRNA, immunoprecipitation of RNA-protein complexes was performed followed by RT-PCR analysis for Mcl-1 and β-actin mRNAs with specific primers. Although β-actin mRNA was not observed in the immunoprecipitate, Mcl-1 mRNA was observed, demonstrating that CUGBP2 binds to Mcl-1 mRNA in the cells (Fig. 3B). Furthermore, there were higher levels of Mcl-1 mRNA in the precipitate of HCUG2 cells compared with HCT-116 cells. These data suggest that CUGBP2 binds Mcl-1 3′-UTR.

CUGBP2 increases the stability of Mcl-1 mRNA following binding to Mcl-1 3′-UTR. We have previously shown that CUGBP2 binding to COX-2 3′-UTR increases the stability of COX-2 mRNA (31, 41). Since CUGBP2 also binds to Mcl-1 mRNA, it is therefore likely that CUGBP2 could affect the rate of Mcl-1 mRNA degradation. To evaluate this, we examined the half-life of Mcl-1 mRNA in HCT-116 and HCUG2 cells following addition of the de novo transcription inhibitor actinomycin D. RNA was harvested at various time points for up to 8 h and then Mcl-1 mRNA levels were determined by real-time RT-PCR. Although Mcl-1 mRNA decayed with a half-life of 30 min in HCT-116 cells, it increased to 3 h in HCUG2 cells (Fig. 4A). These data indicate that Mcl-1 mRNA stability is regulated by CUGBP2. To further determine whether the increased stability occurs through the 3′-UTR, we generated a firefly luciferase reporter construct containing the Mcl-1 3′-UTR (Fig. 4B). The half-life of the Luc-Mcl-1 mRNA was 30 min whereas that of Luc mRNA was 45 min (Fig. 4C, compare left and right). This provide evidence that the Mcl-1 3′-UTR contains cis-acting sequence elements that confer basal mRNA destabilization and probably contribute to the regulation of Mcl-1 mRNA stability. We next determined whether CUGBP2 affects the stability of Luc-Mcl-1 mRNA. For this, we transiently transfected plasmid vectors expressing Luc and Luc-Mcl-1 mRNAs in HCUG2 cells. Although the Luc control mRNA stability does not change between HCT-116 and HCUG2 cells, that of Luc-Mcl-1 mRNA increases from 30 min in HCT-116 cells to 3 h in HCUG2 cells (Fig. 4C). These data suggest that CUGBP2 regulated Mcl-1 mRNA stability through the 3′-UTR.
DISCUSSION

This is the first demonstration that Mcl-1 mRNA is regulated at the posttranslational levels of mRNA stability and translation by the 3'-UTR. A scan of the sequence suggests the presence of multiple sites in the 3'-UTR that might bind to CUGBP2. These include AUUUA and UGUU sequences. Interestingly, these sequence elements are distributed throughout the 3'-UTR, which is over 2.8 kb in length. Careful analysis is necessary to identify the sequence motif(s) that mediate CUGBP2 function. This will be a focus of our future studies. Another point of interest is that CUGBP2 also downregulated Bcl-2 levels in the cells. Bcl-2 also contains a long 3'-UTR and is loaded with AU-rich and UGUU sequences. Further studies, also currently ongoing in the laboratory, are aimed at determining whether CUGBP2 also regulates Bcl-2 through 3'-UTR mediated translation inhibition.

Previous studies have demonstrated that Mcl-1 expression is regulated at the transcriptional and posttranslational levels (13). At the transcriptional level, Mcl-1 promoter is regulated by transcription factors SRF/ETS, STAT3, cAMP-responsive element-binding protein, and PU.1 in various cell types. Post-translational control of Mcl-1 protein occurs through the ubiquitin-proteasome degradation pathway (4, 44–46). However, loss of Mcl-1 protein synthesis also occurs during times of crisis. For example exposure of HeLa cells to UV irradiation resulted in a rapid decline in protein expression but no change in Mcl-1 mRNA levels, suggesting that translation inhibition occurs (33, 36). Earlier studies from our group have demonstrated rapid induction of CUGBP2 following exposure of cells to UV and γ-irradiation (31). Now, in this manuscript, we have demonstrated that CUGBP2 binds to the Mcl-1 3'-UTR to stabilize Mcl-1 mRNA but at the same time inhibits its translation. This is similar to what was observed with COX-2. Although CUGBP2 increased COX-2 mRNA stability, it inhibited its translation (31, 41). These data together suggest that, under conditions of high levels of expression, CUGBP2 binds to the 3'-UTR of transcripts that encode antiapoptotic proteins and inhibit their expression. We recognize, however, that the

CUGBP2 inhibits Mcl-1 mRNA translation through the 3'-UTR. We next examined the effect of Mcl-1 3'-UTR on luciferase activity in HCT-116 and HCUG2 cells. Plasmids encoding the control Luc and Luc-Mcl-1 mRNA were transfected overnight in both HCT-116 and HCUG2 cells, and luciferase activity was determined. There was no difference in luciferase activity from the control luciferase mRNA between HCT-116 and HCUG2 cells (Fig. 5, compare left and right). Furthermore, presence of the Mcl-1 3'-UTR resulted in a 40% reduction in luciferase activity in HCT-116 cells. However, overexpression of CUGBP2 resulted in a further 40% reduction in luciferase activity, resulting in ~10% luciferase levels compared with the Luc control (Fig. 5). These data suggest that CUGBP2 inhibits Mcl-1 mRNA translation through the 3'-UTR.
context of its interactions with the 3′-UTR might play a role in regulating CUGBP2 activity. For example, CUGBP2 interacts with the RNA stabilizing protein HuR and affects HuR-induced COX-2 mRNA translation (41).

It is not clear what role HuR plays in regulating Mcl-1 expression. Recent studies have suggested that HuR can interact with both Bcl-2 and Mcl-1 mRNA and that knockdown of HuR reduces steady-state levels of the two mRNAs (1). However, the context of this interaction might also be important, especially since radiation has been shown to induce HuR expression while at the same time inhibiting Mcl-1 (11, 29, 33). In this regard, it should be noted that HuR was shown to synergize with the translational silencer TIA-1 to reduce the translation of TNF-α mRNA (15). Similar results were observed with translation of cytochrome c mRNA (16). Under normal conditions, HuR bound to the 3′-UTR of cytochrome c mRNA and the mRNA was actively translated. However, under conditions of endoplasmic reticulum stress, HuR and TIA-1 coordinate to inhibit cytochrome c mRNA translation (16). Given that CUGBP2 also interacts with HuR, and the two proteins coordinate to inhibit COX-2 mRNA translation in intestinal epithelial cells, the possibility exists that HuR might contribute to CUGBP2-mediated inhibition of Mcl-1 when cells are undergoing mitotic catastrophe (41). Additional studies are therefore required to understand the mechanism by which CUGBP2 increases stability but inhibits translation following binding to the Mcl-1 3′-UTR.

The balance between pro- and antiapoptotic Bcl-2 family members determines the mitochondrial response to apoptotic stimuli (20, 26). Increased expression of proapoptotic members along with suppression of antiapoptotic members would lead to the formation of pores in the mitochondria and the release of cytochrome c and other proapoptotic molecules, which results in the formation of the apoptosome and the activation of the caspase cascade (20, 26). In this study, we have demonstrated that overexpression of CUGBP2 results in increased apoptosis of HCT-116 colon cancer cells, at a time when the cells are in the G2-M phase. Our studies also demonstrate the presence of Cdc2 kinase and cyclin B1 in the nucleus of HCUG2 cells. Presence of these two proteins is a hallmark of progression through mitosis (35). Hence, further analyses are required to determine whether the overexpression of CUGBP2 in the cancer cells results in the induction of spindle checkpoint, which would delay sister chromatid separation and lead to mitotic spindle and chromosome segregation abnormalities. Hence, at this time, the data suggest that CUGBP2 induces cell death during prolonged G2 phase transition.

We have also demonstrated that, when CUGBP2 is overexpressed, Bcl-2 and Mcl-1 levels are reduced whereas Bax is upregulated, resulting in a Bcl-2-to-Bax ratio that favors an apoptotic response. One interesting point to note is that Mcl-1 protein has a short half-life. Phosphorylation at threonine-163 results in rapid ubiquitination-dependent proteosomal degradation (14). In addition, phosphorylation at serine-159 may hinder its interaction with proapoptotic Bcl-2 including Bim and Noxa and allow these proteins to act on the mitochondria (18). On the one hand, Mcl-1 phosphorylation at serine-64 is increased during the G2–M phase of the cell cycle and is more pronounced when cells are exposed to microtubule-disrupting agents (18). Furthermore, this increased phosphorylation contributes to the antiapoptotic activity of Mcl-1, suggesting the need for Mcl-1 during mitosis. This would also therefore explain why CUGBP2-mediated inhibition of Mcl-1 expression results in the prolonged G2 transition, ultimately leading to apoptosis, a profound disastrous effect on the cells.

It remains to be seen whether ectopic overexpression of Mcl-1 can overcome CUGBP2-mediated induction of prolonged G2 transition-coupled apoptosis. In this regard, it should be noted that Mcl-1 protein is rapidly cleaved by caspase-3 during tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis (47). We have shown that overexpression of CUGBP2 resulted in activation of caspase-3. Although the mechanism by which CUGBP2 induces caspase-3 activation is not known, this suggests that CUGBP2 affects Mcl-1 protein levels by two methods, a direct and an indirect mechanism. The direct mechanism involves inhibition of Mcl-1 mRNA translation by binding to the Mcl-1 3′-UTR and the indirect mechanism involves caspase-mediated degradation of Mcl-1 protein.

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REFERENCES

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