CUGBP2 downregulation by prostaglandin E2 protects colon cancer cells from radiation-induced mitotic catastrophe

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Natarajan G, Ramalingam S, Ramachandran I, May R, Queimado L, Houchen CW, Anant S. CUGBP2 downregulation by prostaglandin E2 protects colon cancer cells from radiation-induced mitotic catastrophe. Am J Physiol Gastrointest Liver Physiol 294: G1235–G1244, 2008. First published March 6, 2008; doi:10.1152/ajpgi.00037.2008.—Prostaglandin E2 (PGE2) is a potent inhibitor of ionizing radiation (IR)-induced cell death. Exposure of colon cancer cells to IR leads to increased CUGBP2 expression. Therefore, we tested the hypothesis that PGE2 radioprotects colon cancer cells by inhibiting CUGBP2 expression. Exposure of HCT-116 cells to γ-IR (0–12 Gy) resulted in a dose-dependent reduction in cell growth and an increase in the G2-M phase of the cell cycle. Western blot analyses demonstrated increased levels of activated caspase 9 and caspase 3. In addition, whereas Bax expression is increased, that of Bcl-2 and Bcl-xL was reduced. Further analyses demonstrated increased activation of Chk1 and Chk2 kinases, coupled with higher levels of nuclear cyclin B1 and Cdc2. Pretreatment with PGE2 suppressed the activation of caspase 3 and caspase 7 and inhibited Bax expression. In addition, PGE2 treatment restored growth and colony formation to control levels. IR significantly upregulated the expression of CUGBP2 in the cells, which was suppressed when cells were pretreated with PGE2. Ectopic overexpression of CUGBP2 also induced apoptosis. Furthermore, it reversed the PGE2-mediated protection from IR-induced mitotic catastrophe. Furthermore, there was an increase in nuclear localization of cyclin B1 and Cdc2 coupled with increased phosphorylation of p53, Chk1, Chk2, and Cdc25c proteins. Cell cycle analysis also demonstrated increased G2-M transition. In contrast, siRNA-mediated suppression of CUGBP2 expression restored normal cell cycle progression and decreased IR-induced apoptosis. Taken together, these data demonstrate that PGE2 protects colon cancer cells from IR-induced mitotic catastrophe in part through suppression of CUGBP2 expression.

Another set of proteins that have structural homology to the ELAV proteins is the CUGBP-ETR3-like factors (CELF), which have been implicated in the regulation of mRNA splicing, editing, and translation (19, 32).

CUGBP2 (also known as ETR3, NAPOR2, and BRUNOL2) is a member of the CELF family that was originally identified to bind to CUG triplet repeats in a protein kinase mRNA-regulating myotonic dystrophy (19, 32). In muscle, CUGBP2 regulates alternative splicing of several transcripts including cardiac troponin T, insulin receptor, chloride channel 1, N-methyl-D-aspartic acid receptor-1, and α-actinin (2). However, subsequently CUGBP2 was identified to be ubiquitously expressed.

Previously, CUGBP2 was identified as a transcript that is highly expressed in neuroblastoma cells undergoing colchicine-induced apoptosis (9, 10). Similarly, we have demonstrated that CUGBP2 is a nuclear protein in intestinal epithelial cells and that its expression is induced when the cells are exposed to γ-irradiation (IR), at a time when the cells are undergoing apoptosis (22, 23). Furthermore, upon induction, CUGBP2 translocates to the cytoplasm, where it binds AU-rich sequences in the 3′-untranslated region of cyclooxygenase-2 (COX-2) mRNA to inhibit its translation (22). COX-2 is the rate-limiting enzyme in the prostaglandin synthesis pathway that is overexpressed in inflammation and cancers (35). One such product, prostaglandin E2 (PGE2), protects the intestinal epithelium from IR by increasing stem cell survival and diminishing apoptosis (4, 15, 28).

Recent studies have demonstrated that when cells are treated with IR they undergo mitotic catastrophe (6, 30). However, the mechanisms of PGE2 protection are not well characterized. Given that IR induces whereas PGE2 inhibits CUGBP2 expression, we explored the possibility that CUGBP2 and PGE2 may have opposing functions in regulating IR-mediated mitotic catastrophe and that PGE2-mediated protection occurs in part through suppression of CUGBP2 expression. To demonstrate this, intestinal epithelial cells in culture were first exposed with increasing doses of IR to demonstrate that the cells undergo mitotic catastrophe. However, pretreatment with PGE2 inhibited IR-mediated mitotic catastrophe. Furthermore, IR induced CUGBP2 expression, and silencer RNA (siRNA)-mediated knockdown of CUGBP2 decreased IR-mediated mitotic catastrophe. Finally, we demonstrate that IR induces the transcription of CUGBP2 gene, which is suppressed by PGE2. These results demonstrate that colon cancer cells
exposed to IR undergo mitotic catastrophe in a CUGBP2-dependent manner.

MATERIALS AND METHODS

Cell culture. HCT-116 human colon adenocarcinoma cell line was obtained from the American Type Culture Collection and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 U/ml of penicillin-streptomycin in a humidified chamber at 37°C with 5% CO2. For stable expression, HCT-116 cells were stably transfected and colonies were isolated following incubation in 800 μg/ml geneticin.

Plasmids and siRNA. Sequence targeting the CUGBP2 mRNA 5’-GCAAACCCUACUGAUCCUA-3’ and a scrambled control siRNA not matching any of the human genes were obtained from Ambion and transfected with Transfectol (Ambion). The full-length coding region of human CUGBP2 was amplified by RT-PCR from HCT-116 cells and expressed as amino-terminal FLAG epitope-tagged proteins in plasmid pCMV-Tag2B (a cytomegalovirus immediate early promoter driven expression vector) after cloning at the HindIII and Xhol restriction sites.

Cell proliferation. HCT-116 cells were seeded on a 96 well plates at a density of 1 × 10^3 cells/well and allowed to adhere. The cells were subjected to 6-Gy γ-irradiation in a Gammacell 40-cesium irradiator at 0.90 Gy/min. Where indicated, cells were transfected with pCMV-Tag2B-CUGBP2 or silencer RNA for CUGBP2. Also, in some cases, cells were pretreated with 1 μM PGE2. Following 48 h incubation, cell proliferation was determined by hexosaminidase enzyme assay. Results were further confirmed by manual cell counts.

Apoptosis. HCT-116 cells were grown in 96-well black plates. As mentioned above, the cells were exposed to increasing doses of IR (0–12 Gy). After 24 h, caspase 3/7 activity was measured by using the Apo-one Homogeneous Caspase-3/7 Assay kit per the manufacturer’s instructions (Promega, Madison, WI). Where indicated, cells were pretreated with 1 μM PGE2 2 h before IR treatment. In some studies, cells were transfected with 10 nM of CUGBP2-specific or scrambled siRNA 24 h before IR treatment.

Cell cycle analyses. HCT-116 cells treated with PGE2 or IR and/or transfected with CUGBP2-specific or scrambled siRNA were harvested by trypsinization and suspended in phosphate-buffered saline (PBS). The single-cell suspensions were fixed using 70% ethanol for 2 h and subsequently permeabilized with PBS containing 1 mg/ml propidium iodide (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich), and 2 μg DNase-free RNase (Sigma-Aldrich) at room temperature. Flow cytometry was done with a FACScalibur analyzer 3 color (Becton Dickinson, Mountain View, CA), capturing 50,000 events for each sample; results were analyzed with ModFit LT software (Verity Software House, Topsham, ME).

Colony formation assay. Six-well dishes were seeded with 1,000 viable cells and simultaneously treated with PGE2 or IR and/or transfected with CUGBP2-specific or scrambled siRNA on complete medium. After 48 h, the cells were washed in PBS and incubated for an additional 14 days in complete medium. Each treatment was done in triplicate. The colonies obtained were washed with PBS, fixed in 10% formalin for 10 min at room temperature, and subsequently washed with PBS followed by staining with hematoxylin. The colonies were counted and compared with untreated cells.

RT-PCR analysis. Total RNA was isolated from the cells using Trizol. Complementary DNA was prepared by using random hexamer oligonucleotides and used for real-time RT-PCR analyses using Jumpstart Tag DNA polymerase (Sigma Chemical). Primers used in this study were as follows: β-actin, 5’-GAATGCTGTCCTCCATGTTTAGT-3’ and 5’-CTTAAATGTTCCAAGCCCTTC-3’; CUGBP2, 5’-AGCTCGATTTCCTTTCCCCAG-3’ and 5’-CCGGAGGATCCGGCATCTC-3’.

Western blot analyses. HCT-116 cells after the various treatments were allowed to grow for 48 h. Cell lysates were prepared and subjected to 10% SDS-polyacrylamide gel electrophoresis and blotted onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). Antibodies for CUGBP2 were generated against the peptide sequence NPLSTTSSALGALT located at amino acid 280–294 of human CUGBP2 (Sigma-Aldrich). All other antibodies were purchased from either Santa Cruz Biotechnology (Santa Cruz, CA) or Cell Signaling (Bedford, MA). Specific proteins were detected by the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunocytochemistry and immunofluorescence. Cells were transfected with a NH2-terminal FLAG-tagged CUGBP2 plasmid or plasmid vector alone as control and allowed to grow for 48 h. Where indicated, cells were also subjected to 6-Gy IR and/or 1 μM PGE2. The cells were fixed with 10% buffered formalin and then permeabilized with PBS containing 0.5% Triton X-100. For immunocytochemistry analyses of cyclin B1 and Cdc2, the cells 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 then processed by using Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) followed by DAB staining. For immunofluorescence, the cells were incubated with rabbit anti-FLAG antibody (Affinity Bioreagents) followed by FITC-conjugated anti-rabbit IgG. The slides were mounted and examined with a Zeiss Axioskop 2 MOT plus microscope (Carl Zeiss).

Statistical analysis. The values are expressed as means ± SE. The mean results for each group were compared by ANOVA followed by Mann-Whitney test for multiple comparisons. We considered P < 0.05 to be statistically significant.

RESULTS

Prostaglandins protect from IR-induced cell death. IR is a potent inducer of cell death. To determine the mechanism by which PGE2 protects from radiation-induced cell death, we initially measured the dose necessary for colon cancer cells to undergo IR-induced cell death. HCT-116 cells were subjected to increasing doses of IR and the cells were allowed to grow for 2 wk, at which point the number of colonies was determined. There was a dose-dependent decrease in the number of colonies that developed (Fig. 1, A and B). A dose of 6-Gy was sufficient to decrease the number of colonies by 50% compared with control, untreated cells. Similarly, 6-Gy IR treatment reduced colony formation of two other colon cancer cell lines, SW480 and HT-29, by 48 and 42%, respectively (data not shown). To determine whether radiation affects cell cycle progression, cells were collected at 24 h following IR and subjected to flow cytometry analyses. There was a dose-dependent increase in cells in the G2-M phase of the cell cycle, with almost a threefold increase observed at 6-Gy (Fig. 1C). At the low dose of 2-Gy, there was a decrease in cells in the S phase, whereas at the higher doses, there was a decrease in cells in G0/G1 phase. These data suggest that there is a rapid response of the cells to exposure to IR, resulting in prolonged G2-M transition.

We next determined whether the reduction in growth of cells following IR is due to an extended G2-M state. We and others have previously demonstrated that PGE2 radioprotecets cells (11, 15, 22). Therefore, we determined whether PGE2 treatment affects cell growth and restores colony formation ability. HCT-116 cells treated with 6-Gy IR demonstrated lower levels of proliferation and higher levels of apoptosis (Fig. 2, A and B). However, pretreatment of cells with 1 μM PGE2 suppressed the IR-induced inhibition of cell proliferation (Fig. 2A) More-
However, pretreatment with PGE2 partially suppresses the phase (22.48% compared with 6.32% in controls, Fig. 2). G2-M arrest, resulting in only 13.15% in this phase of the cell cycle (Fig. 2C). These data suggest that PGE2 blocked IR-induced suppression of cell growth and restored cell cycle progression. To determine the effects of PGE2 on long-term growth of the cells, the cells were allowed to grow for 10 days following PGE2 and IR treatments. At low doses of PGE2 (≤0.5 μM) there was no effect on IR-mediated reduction in colony formation (data not shown). However, 1 μM PGE2 pretreatment significantly protected the cells from IR-induced cell death, resulting in numbers of colonies that are comparable to those of control, untreated cells (Fig. 2D).

**PGE2 protects from IR-induced mitotic catastrophe.** The data suggest that the number of cells at the G2-M transition of the cell cycle is higher, whereas at the same time there is increased apoptosis. To confirm that the cells are undergoing apoptosis, Western blot analyses were performed. Radiation is known to induce the intrinsic caspase pathway that involves caspase 9 (17). Western blot analysis for caspase 9 and the effector caspase 3 demonstrated increased amount of the cleaved (activated) product for both proteins following 6-Gy IR (Fig. 3A). However, expression of both caspase 9 and caspase 3 was similar to that observed in control levels when cells were pretreated with 1 μM PGE2. We also examined the expression of Bcl2, Bcl-xL, and Bax proteins and calculated the Bax-to-Bcl2 ratio, a critical determinant of apoptosis. Whereas Bax expression was induced following IR, expression of Bcl2 and Bcl-xL proteins were reduced (Fig. 3A). Furthermore, expression of all three proteins was similar to baseline levels in the PGE2-treated cells, again suggesting that PGE2 protected the cells from IR-induced apoptosis.

Cyclin B1 and its interacting partner, the Cdc2 serine/threonine kinase heterodimerize, localize in the nucleus and induce mitosis by phosphorylating and activating enzymes regulating chromatin condensation, nuclear membrane breakdown, and mitosis-specific microtubule reorganization (1, 6). Previous studies have also demonstrated that cyclin B1 protein levels rapidly increase during IR-induced apoptosis (25, 27). Given that we observed increased G2-M transition following IR, we next determined the expression and localization of these two proteins. Both cyclin B1 and Cdc2 were upregulated following 6-Gy IR (Fig. 3B). In addition, cyclin B1, but not Cdc2, expression was also upregulated by 1 μM PGE2 treatment. This was further confirmed by immunocytochemistry, in which increased levels of cyclin B1 were found in the nucleus following IR (Fig. 3C). PGE2 treatment also induced the expression of the proteins, especially cyclin B1. However, the protein was distributed throughout the cells (Fig. 3C).

Cdc2 needs to be rapidly dephosphorylated at Thr14 and Tyr15 at the end of the G2 phase of the cell cycle and before entering mitosis (6). Dephosphorylation of Cdc2 occurs by simultaneous inactivation of the Wee-1/Myt-1 kinases and the activation of the dual-specificity Cdc25c phosphatase. Checkpoint kinases Chk1 and Chk2, inactivate Cdc25c by phosphorylation on the Ser216 residue (6, 34). Phosphorylation of Cdc25c at this residue creates a binding site for 14-3-3 proteins, which then sequester Cdc25c in the cytoplasm, thereby preventing Cdc25c from interacting with Cdc2 in the nucleus (7, 24). To determine the effect of IR on Cdc25c, Western blot analysis was performed with an antibody that recognizes the phosphorylated protein at Ser-216. Treatment with 6-Gy IR resulted in increased levels of phosphorylated Cdc25c protein (Fig. 3B). PGE2 treatment did not affect Cdc25c protein phos-
Fig. 2. PGE₂ protects from IR-induced cell death. 

A: cell proliferation. HCT-116 cells treated with IR or PGE₂ or both were analyzed for proliferation based on hexosaminidase enzyme activity. PGE₂ increased whereas IR decreased cell proliferation. Moreover, pretreatment with PGE₂ protected the cells from IR-mediated inhibition of proliferation. Statistically significant differences: *P < 0.05 compared with control and **P < 0.05 compared with radiation alone.

B: apoptosis. Following the indicated treatments, cells were analyzed for caspase 3/7 activity. PGE₂ suppressed IR-induced caspase 3/7 activity. Statistically significant differences: *P < 0.05 compared with control and **P < 0.05 compared with radiation alone.

C: cell cycle. Cells were treated as above and cell cycle profiles were analyzed by flow cytometry. Whereas the percentage of cells in the G2-M phase following IR was increased compared with control cells, PGE₂ reduced this effect. 

D: colony formation. Cells were allowed to grow for 2 wk after IR and/or PGE₂ treatment. PGE₂ alone increased whereas IR decreased the number of colonies. Furthermore, addition of PGE₂ reversed the IR-mediated suppression and increased colony numbers. Statistically significant differences: a, P < 0.05 compared with control; b, P < 0.05 compared with radiation alone.
Fig. 3. PGE2 protects against IR-induced mitotic catastrophe. A: lysates from HCT-116 cells following IR and/or PGE2 treatment were subjected to Western blot analysis using specific antibodies for caspase 3, caspase 9, Bax, Bcl2, and Bcl-xL. IR exposure resulted in cleavage and therefore activation of caspase 3 and caspase 9 proteins. This was suppressed when cells were pretreated with PGE2. In addition, the proapoptotic Bax protein was upregulated whereas antiapoptotic proteins Bcl2 and Bcl-xL were downregulated after IR. Again, pretreatment with PGE2 resulted in the expression of these proteins comparable to control levels. Actin was used as internal control for loading. NS, nonspecific band. B: Western blot analysis using specific antibodies for cyclin B1, Cdc2, phospho-Chk1 (Ser345), phospho-Chk2 (Thr68), and phospho-Cdc25c (Ser216). IR increased cyclin B1, Cdc2, and phosphorylation of Chk2. In addition, IR induced phosphorylation of Cdc25c. Pretreatment with PGE2 before IR also results in increased cyclin B1 and Cdc2 and phosphorylation of Chk1, Chk2, and Cdc25c protein. C: cells treated with IR and/or PGE2 were immunostained for cyclin B1 and Cdc2. The images show increased levels of both proteins in the nucleus only following IR treatment, but not when cells were also treated with PGE2. D: cells were immunostained for apoptosis with terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL; green) and for active mitosis by phospho-histone H3 (red). The merged images show that exposure to IR had resulted in higher levels of cells undergoing apoptosis and mitosis simultaneously compared with controls or PGE2-treated cells. DAPI (4’-6-diamidino-2-phenylindole) was used to stain the nucleus.
phorylation. Moreover, PGE2 did not affect IR-induced Cdc25c phosphorylation. Since Cdc25c is phosphorylated, we next determined whether Chk1 and/or Chk2 is also activated. Whereas exposure to IR resulted in increased phosphorylation of Chk2 kinase, it did not affect Chk1 phosphorylation (Fig. 3B). In contrast, PGE2 treatment resulted in increased phosphorylation of both Chk1 and Chk2, although there was a higher level of Chk1 phosphorylation compared with Chk2. These data suggest that the cells are undergoing mitosis following IR treatment. However, as mentioned above, there was also increased apoptosis following IR treatment. To determine whether the cells undergoing apoptosis were also in mitosis, immunocytochemistry was performed. Levels of phosphorylated histone H3, a marker of mitotic cells, were determined along with terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) staining for cells undergoing apoptosis. Control cells did not demonstrate any TUNEL-positive labeling, but it was prevalent in IR-treated cells (Fig. 3D). Moreover, there were higher levels of phosphorylated histone H3-positive cells following IR treatment. In addition, many cells demonstrated positive staining for both phosphorylated histone H3 and TUNEL. In contrast, pretreatment of cells with PGE2 inhibited apoptosis of the cells as evidenced by the lower levels of TUNEL-positive cells (Fig. 3D). This suggests that IR-treated cells undergo apoptosis when they are transiting through mitosis and that pretreatment with PGE2 prevents the apoptosis and suppresses the increased mitosis, thereby protecting the cells from mitotic catastrophe.

**IR treatment induces CUGBP2, which is suppressed by PGE2.** In previous studies, we have demonstrated that treatment of intestinal epithelial cells with 12-Gy IR results in the induction of RNA binding protein CUGBP2, and cells undergo apoptosis (22, 23). However, the effect of lower doses of IR on CUGBP2 expression is not known. The fact that HCT-116 cells that were exposed to 6-Gy IR underwent apoptosis suggested that CUGBP2 expression might be induced. To determine whether CUGBP2 expression is affected by low-dose IR, total RNA from HCT-116 cells was isolated 24 h following exposure to 6-Gy IR and subjected to RT-PCR analyses. As shown in Fig. 4A, CUGBP2 mRNA levels were increased by over twofold in cells exposed to 6-Gy IR compared with untreated cells. Furthermore, pretreatment with 1 μM PGE2 suppressed both baseline and IR-induced levels of CUGBP2 mRNA expression (Fig. 4A). Western blot analyses were performed to confirm the CUGBP2 protein expression. CUGBP2 expression was significantly induced in cells following 6-Gy IR, but not in cells also treated with PGE2 (Fig. 4B).

**CUGBP2 overexpression induces cells to undergo mitotic catastrophe.** The significant induction of CUGBP2 following 6-Gy IR suggested a potential role for CUGBP2 in cells undergoing mitotic catastrophe. To determine this, CUGBP2 was stably overexpressed in HCT-116 cells and its effects on HCT-116 cell growth were determined. First, we observed that the majority of the cells did not grow and only a few cells grew and developed colonies in petri dishes (Fig. 5A). To determine the mechanism by which CUGBP2 inhibits HCT-116 cell growth, cell cycle analysis was performed. There was a significant increase in cells transiting the G2-M phase, compared with untreated controls (Fig. 5B). Furthermore, a comparison of the CUGBP2 expressing cells with those of IR-treated cells suggest that CUGBP2 overexpression affects cell cycle progression of colon cancer cells in a manner similar to cells exposed to 2-Gy IR (compare Fig. 5A with Fig. 1A).

We next determined the effect of CUGBP2 expression on the markers of mitotic catastrophe. First, we determined the effect of CUGBP2 on the activation of caspase 9 and caspase 3, the intrinsic pathway and the effector caspase proteins, respectively. Western blot analysis demonstrated higher levels of cleaved caspase 9 and caspase 3 proteins, suggesting that apoptotic pathways are activated in the cells (Fig. 5C). Next, Western blot analyses were performed to determine whether G2-M checkpoint proteins are affected. Higher levels of Chk1 (Ser345) and Chk2 (Thr68) and its target Cdc25c (Ser216) protein were observed (Fig. 5C). In addition, higher levels of p53 phosphorylation at Ser15 were observed in the CUGBP2-expressing cells (Fig. 5C). Western blot and immunocytochemistry analyses also demonstrated higher levels of cyclin B1 and Cdc2 and increased nuclear localization of both proteins (Fig. 5D). Taken together, these data suggest that CUGBP2 overexpression results in cells undergoing mitotic catastrophe.

Since we observed that PGE2 inhibits IR-mediated cell death, we next determined whether ectopic CUGBP2 expression affects PGE2-mediated protection from IR-induced mitotic catastrophe. CUGBP2 was transiently expressed in HCT-116 cells, following which the cells were sequentially treated with 1 μM PGE2 and 6-Gy IR. Subsequently, the cells were immunostained for phosphorylated histone H3 (mitosis) and TUNEL (apoptosis). CUGBP2 expression before PGE2 and IR treatment resulted in a significant number of cells that were positive for both TUNEL and phosphorylated histone H3, similar to that seen when CUGBP2 was expressed in the cells but no further treatments were given (Fig. 5, E and F). These data suggest that PGE2-mediated protective effect occur in part due to inhibition of CUGBP2 expression.
CUGBP2 is essential for IR-induced mitotic catastrophe. Next, we determined whether IR-mediated mitotic catastrophe occurs in part because of induction of CUGBP2 expression. For this, we developed a siRNA specific for CUGBP2 and a scrambled siRNA. Western blot analyses confirmed that the CUGBP2-specific siRNA inhibited the expression of CUGBP2 protein, compared with the scrambled siRNA (Fig. 6A). To determine the effect of decreasing CUGBP2 expression on IR-mediated apoptosis, cells were transfected with either CUGBP2-specific or the scrambled siRNA and subsequently exposed to 6-Gy IR. The cells that were subjected to only 6-Gy IR or transfected with the scrambled siRNA before IR exposure demonstrated a 3.5-fold increase in caspase 3/7 activity, suggesting that the cells were undergoing apoptosis (Fig. 6B). In contrast, when the cells were transfected with CUGBP2-specific siRNA, there was a significant reduction in IR-induced caspase 3/7 activity compared with cells that did not receive any siRNA, suggesting that CUGBP2 plays a major role in IR-mediated apoptosis.

Given that overexpression of CUGBP2 resulted in a G2-M transition similar to that observed with IR exposure, we also determined the effect of CUGBP2 downregulation on HCT-116 cell cycle progression following 6-Gy IR. There was a 2.5-fold increase in the G2-M transition in IR-treated cells, which was not affected when cells were also transfected with the scrambled siRNA (Fig. 6C). However, when the cells were...
transfected with CUGBP2-specific siRNA, there was a ~40% reduction in the number of cells in the G2-M phase (13.1% in IR/CUGBP2 siRNA compared with 22.4% in IR alone, Fig. 6C). Taken together, these data suggest that IR-induced mitotic catastrophe was significantly suppressed in CUGBP2-downregulated colon cancer cells.

Given the requirement for CUGBP2 in IR-induced mitotic catastrophe, we next determined the effect of CUGBP2 knockdown in IR and PGE2-mediated cell proliferation and apoptosis. Western blot analysis showed that, whereas IR induced CUGBP2 expression, this was significantly reduced when cells were transfected with a CUGBP2-specific siRNA (Fig. 7A). To determine the effect of exposing cells to PGE2 in the setting of CUGBP2 knockdown, cells were first transfected with the CUGBP2 siRNA followed by treatment with PGE2 and IR. Treatment with IR alone resulted in a 30% reduction in cell proliferation (Fig. 7B). Both PGE2 and CUGBP2 siRNA suppressed the IR-mediated inhibition of cell proliferation. Furthermore, when the two were administered to the same cells in a sequential manner (CUGBP2 siRNA transfection first followed by PGE2), cell proliferation was similar to that observed in control, untreated cells (Fig. 7B). Similarly, the combination of CUGBP2 siRNA and PGE2 resulted in reducing activated caspase 3 and caspase 7 activity to baseline levels (Fig. 7C). These data suggest that PGE2 treatment coupled with CUGBP2
downregulation is additive on reversing IR-mediated effects on apoptosis and cell proliferation compared with CUGBP2 siRNA + IR or PGE2 + IR.

**DISCUSSION**

RNA-binding proteins have various cellular functions in the regulation of gene expression. The data presented here demonstrate that CUGBP2 is a critical player in IR-induced mitotic catastrophe. More important, the novelty of the findings is that PGE2 inhibits the expression of CUGBP2, a mechanism by which PGE2 protects the cells from IR-mediated mitotic catastrophe. However, we suggest that this is not the only mechanism by which PGE2 mediates its protective function, but rather that the inhibition of CUGBP2 expression could be a major player in PGE2 activity.

In previous studies, we have demonstrated that CUGBP2 is a potent inducer of cell death (22, 23). Here, we have further determined that the apoptotic effect occurs specifically during mitosis, resulting in mitotic catastrophe. Overexpression of CUGBP2, either ectopically or following IR, resulted in increased number of cells in the G2-M phase of the cell cycle. We have further demonstrated that siRNA-mediated knockdown of CUGBP2 partially rescues the cells from the mitotic catastrophe phenotype following IR. However, the mitotic catastrophe that was observed did not occur when the cells were arrested in the G2 phase of the cell cycle. The cells were actively transiting through mitosis based on the increased nuclear localization of cyclin B1 and Cdc2 and enhanced phosphorylation of checkpoint kinases Chk1/2 and its target protein Cdc25c. This suggests a novel mechanism of mitotic catastrophe wherein the cells are not arrested in any stage but are active in the process of cell division. Given that chemotherapeutic drugs require actively dividing cells and not quiescent cells for their activity, the possibility exists that a greater level of efficacy for the drugs would be possible if the cells are induced to also express CUGBP2. If this were the case, then lower levels of the drug might be required, thereby decreasing the bystander toxic effects on neighboring cells. In this regard, it should be noted that we have observed that natural chemopreventive products such as curcumin also induce CUGBP2 (S. Ramalingam and S. Anant, unpublished observations). This might explain why these natural compounds might be highly efficacious in reducing cancer growth while having lower toxicity.

We and others have previously shown that administration of bacterial LPS prior to IR radioprotects the intestine in a COX-2-dependent manner (23, 29). Administration of LPS induced COX-2-mediated PGE2 production, and administration of COX-2 inhibitors was able to override the protection from IR. Here, we have demonstrated that PGE2 inhibits CUGBP2 expression and that suppressing CUGBP2 expression protects the cells from IR-mediated cell death. The fact that PGE2 inhibits CUGBP2 expression suggests that LPS-mediated radioprotection also occurs through inhibiting CUGBP2 expression. In previous studies, we have demonstrated that CUGBP2 binds to AU-rich sequences in the 3' untranslated region of COX-2 mRNA and inhibits COX-2 mRNA translation (22). The consequence of this is that lack of COX-2 expression results in decreased PGE2 production. As shown here, PGE2, a major product of COX-2 activity in colon cancers, inhibits CUGBP2 expression. Hence this suggests that a closed negative regulatory loop occurs wherein expression of CUGBP2 inhibits COX-2 and therefore PGE2 expression, whereas PGE2 inhibits CUGBP2 expression, resulting in normal cell growth. However, additional studies are necessary to determine the mechanism by which PGE2 inhibits CUGBP2 expression. One possibility could be through effects on transcription factor activity. PGE2 has been shown to inhibit IL-12 p40 gene transcription in macrophages, and this is dependent on the presence of a functional activator protein-1 (AP1) (21). In addition, PGE2 treatment of T cells has been shown to induce the expression of ICER, a potent transcriptional inhibitor that modulates CREB transcriptional factor activity (3, 13). In silico analysis of the promoter region for CUGBP2 identified binding sites for AP1 and CREB transcription factors −250 bp upstream from the transcription factor binding site (data not shown). These suggest that PGE2 might inhibit CUGBP2 expression through one or both of these transcription factors.

So how does PGE2 downregulate CUGBP2 expression? PGE2 can act via any one of four EP receptors termed EP1 to EP4. HCT-116 cells are known to express EP1, EP2, and EP4 (31, 33). While signaling through EP1 results in activation of phospholipase C and inositol triphosphate, that from EP2 and EP4 results in increased intracellular Ca2+. In previous studies, we have demonstrated that PGE2 suppresses IR-induced apoptosis of HCT-116 cells through a mechanism involving Akt activation and Bax translocation, most likely by signaling through the EP2 receptor (33). In addition, EP4 has been shown to preferentially use the phosphatidylinositol 3-kinase-dependent-Akt pathway, which also results in ERK activation (14, 16). PGE2 has also been implicated in the activation of the epidermal growth factor receptor, which subsequently can also activate the Akt and ERK pathways (5, 26). Hence, the possibility exists that PGE2-mediated protection from IR-induced mitotic catastrophe could involve one or many of these pathways to suppress CUGBP2 expression.

In summary, our study provides mechanistic evidence that PGE2-mediated radioresistance of the intestinal epithelial cell occurs through inhibition of CUGBP2 expression. Since PGE2 is overexpressed in many malignancies, these findings further imply that CUGBP2 might be an effective strategy for cancer chemotheraphy, for which in vivo studies are warranted.

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**REFERENCES**