A Novel Link Between REC2, a DNA Recombinase, the Retinoblastoma Protein, and Apoptosis*

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The REC2 recombinase is essential for recombinational repair following DNA damage as well as for successful meiosis and gene targeting in the corn smut Ustilago maydis. Here we report that overexpression of REC2 induced apoptotic cell death in human HuH-7, Hep G2, and Hep 3B hepatoma cells. Apoptosis was related to recombinase activity and was significantly increased by inhibition of retinoblastoma (Rb) expression with transforming growth factor-β1. REC2-induced apoptosis was associated with a significantly reduced percentage of cells in the G1 phase of the cell cycle and a significant reduction in G2/M only in the Rb(-/-) Hep 3B cells. Overexpression of REC2 resulted in increased abundance of the hyperphosphorylated form of Rb. However, by immunoprecipitation REC2 was associated primarily with hypophosphorylated Rb, suggesting that REC2 may be involved in modulating the phosphorylation state of Rb. The A and B pocket domains with the spacer amino acid sequence and the carboxyl-terminal region of Rb were required for maximal binding to REC2. Overexpression of Rb significantly inhibited REC2-induced apoptosis even in the presence of transforming growth factor-β1. Taken together, these data suggest a novel interaction of Rb with the recombinase REC2 and a role for this complex in bridging DNA recombination and apoptosis.

The retinoblastoma tumor suppressor gene product (Rb) is a nuclear phosphoprotein that is involved in regulating progression through the cell cycle (1). It is hypophosphorylated in the quiescent state and early G1 where it blocks cell cycle progression. Phosphorylation of Rb is initiated during mid to late G1, and it becomes fully phosphorylated prior to the G1-S phase transition. Moreover, the phosphorylation status of Rb is also involved in modulating its ability to associate and/or bind with other cellular and viral factors. In fact, the number of partner proteins that interact with Rb has increased dramatically in recent years and includes certain viral proteins (2–5), the transcription factors Myc and E2F, the proto-oncogene MDM2, members of the cyclin family, the nuclear matrix proteins lamin A and C, and proteins involved in nucleosome disruption (6). In addition to its central role in regulating the cell cycle, it has been shown that Rb is involved in the TGF-β1-associated apoptotic pathway in hepatocytes and hepatoma cells (7, 8). The ability of TGF-β1 to induce apoptosis in these cells was associated with decreased phosphorylation of Rb as well as reduced expression. Depletion of Rb by antisense oligonucleotides also resulted in cell death, whereas overexpression of the protein protected against TGF-β1-induced apoptosis (8).

Recently, a eukaryotic recombinase REC2 was cloned from the fungus Ustilago maydis and shown to play an essential role in facilitating homologous recombination with high target specificity as well as recombinational repair following DNA damage (9–11). Sequence analysis revealed that the REC2 gene product shares a significant region of homology with the Escherichia coli RecA protein, comprising an amino acid stretch of conserved residues that span an ATP binding domain (9). The region is essential for biological activity of RecA in recombination as well as in homologous pairing reactions performed in vitro. The requirement of functional REC2 for these biochemical pathways has facilitated the isolation of recombinase-deficient mutants, thus aiding in the characterization of the mechanisms involved in recombination and repair (9).

In higher eukaryotes, the role of DNA recombinases in cell growth and survival is largely uncharacterized. In this study we examined the effect of REC2 overexpression on the growth characteristics of several human hepatoma cell lines. Our results indicate that overexpression of REC2 is associated with an increase in apoptosis that is regulated in part by the level of recombinase activity. In addition, the response is modulated by Rb, which binds REC2 directly, thereby suggesting a role for this complex in bridging DNA repair and apoptosis.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfections, and Immunohistochemistry—Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, grown at 37 °C, and transfected as described previously (8, 12). Briefly, 5 μg of each expression plasmid pCMV.CAT, pCMV.REC2, pCMV.REC2-10, and pCMV.REC2-5 (9), and pCMV.Rb (13) under control of the cytomegalovirus promoter was transfected into the cell lines using Lipofectin® (Life Technologies, Inc.). After 36 h, 1 ng TGF-β1 was added to the cultures, which were then maintained for an additional 30 h. Transfection efficiency was monitored by chloramphenicol acetyltransferase gene expression and fluorescence labeling of the transfected gene products. Morphological evaluation of apoptosis was carried out as described (8). A minimum of 300–500 cells were counted for each culture, and the percentage of apoptotic cells was determined from at least three independent experiments.

For immunohistochemical analysis, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, at room temperature for 10 min. Following three washes with PBS, they were incubated with rabbit polyclonal antibody against U. maydis REC2 (2.5 μg/ml) and fluorescent-labeled secondary antibody. Cell death was evaluated by fluorescence microscopy.

The abbreviations used are: TGF-β1, transforming growth factor-β1; PBS, phosphate-buffered saline; GST, glutathione S-transferase; GSH, glutathione.

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REC2 Induces Apoptosis and Binds the Retinoblastoma Protein

FIG. 1. REC2 expression and morphologic changes in Hep G2 and HuH-7 human hepatoma cell lines. A, Hep G2 cells were transfected with pCMV.CAT (a–c); pCMV.REC2 (d–f); pCMV.REC2-10 (g–i); and pCMV.REC2-5 (j–l). a, d, g, and j, REC2 and Hoechst dye staining. c, f, i, and l, REC2 and Hoechst dye staining. B, HuH-7 cells were transfected with either pCMV.REC2 or pCMV.CAT. a–c, low levels of REC2 expression. d–f, high levels of REC2 expression. a and d, REC2 staining. b and e, REC2 and Hoechst dye staining. c and f, Hoechst dye staining. High level expression of REC2, REC2-10, and REC2-5 were similar (p = 0.39). High versus low level expression of REC2 was significantly different (p < 0.0001). Bar, 20 μm.

μg/ml) in PBS containing 0.3% Triton X-100 at 4 °C overnight. After blocking with 5% normal goat serum, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG in PBS for 1 h at room temperature. Cell nuclei were stained using Hoechst dye 33258 (1 μg/ml) for 5 min. The images were acquired, and the nuclear morphology was evaluated as described previously (8). Levels of REC2 expression were quantitated by gray scale image analysis as described under “Experimental Procedures.” b, e, h, and k, Hoechst dye staining; c, f, i, and j, REC2 and Hoechst dye staining.

Stable REC2 transfectants of Hep G2 and Hep 3B cells were established using the pCMV.REC2 construct containing a neomycin resistance gene. The transfected cells were selected using G418 (800 μg/ml), and the resulting clonal cells were maintained in G418 at 400 μg/ml.

FIG. 2. Western blot analysis of REC2 expression in stably transfected Hep G2 and Hep 3B cells. Whole cell lysates from stable pCMV.REC2-transfected Hep G2 and Hep 3B cells following SDS-polyacrylamide gel electrophoresis and transfer were incubated with either Rb monoclonal antibody XZ161 (A) or polyclonal anti-REC2 antibody (C) and detected using ECL chemiluminescence. Additional cell lysates were co-immunoprecipitated using either anti-REC2 (B) or Rb XZ161 (D) antibodies, and the resulting precipitates were subjected to Western blot analysis using either Rb XZ161 (B) or anti-REC2 (D) antibodies. The phosphorylation state of Rb (A and B) is indicated. pRb, hypophosphorylated Rb, 110 kDa; ppRb, hyperphosphorylated Rb, 114 kDa. +, transfected with pCMV.REC2; −, transfected with vector pCMV.

REC2 positive cells were identified by immunohistochemistry as outlined above, and the positive clonal lines from the Hep 3B or Hep G2 transfectants were pooled and expanded.

Immunoprecipitation and Western Blotting—Whole cell lysates were prepared and analyzed by Western blotting as described previously (7) using monoclonal anti-Rb XZ161 and polyclonal anti-REC2 antibodies. Co-immunoprecipitation using anti-Rb XZ161 monoclonal antibody or anti-REC2 polyclonal antibody was performed as outlined (14, 15), and the resulting precipitates were subjected to immunoblot analysis using the same two antibodies. The proteins were detected using the ECL® Chemiluminescence System (Amer sham Life Science, Inc.).

In Vitro Binding Assays—Glutathione S-transferase (GST)-Rb fusion proteins included amino acids 379–792, 379–928, 379–928 with the missense C706F mutation, 379–928 with exon 22 deletion (amino acids 733–769), 379–928 with exon 21 deletion (amino acids 701–733), 403–816, 379–792 with deletion of amino acids 573–645 (16), and amino acids 1–400 of the full-length Rb protein and were fused to the carboxy-

TABLE I

Distribution of the cell cycle phases in REC2-transfected HuH-7 and Hep 3B cells

Results are the means ± S.D. of at least three separate experiments expressed as the percentage of total cells in the designated phase of the cell cycle determined by flow cytometry as described under “Experimental Procedures.”

| Phase     | pCMV.CAT | pCMV.REC2 | pCMV.REC2 |
|-----------|----------|-----------|-----------|
| G1        | 50.9 ± 1.5 | 44.9 ± 0.8 | 49.0 ± 0.6 |
| S         | 28.7 ± 0.6 | 27.7 ± 1.1 | 24.3 ± 0.8 |
| G2/M      | 16.4 ± 3.0 | 16.9 ± 1.0 | 11.4 ± 1.1 |
| Sub-G1    | 3.9 ± 0.7 | 10.5 ± 0.8 | 6.6 ± 0.4 |

a p < 0.05 relative to controls.

b p < 0.001 relative to controls.
REC2 Overexpression Induces Apoptosis in Human Hepatoma Cells—To identify the effects of REC2 on cell growth and survival, Hep G2 human hepatoma cells were transfected with an expression construct containing the full-length REC2 gene from *U. maydis* under control of the cytomegalovirus promoter. Immunofluorescent labeling with anti-REC2 antibody revealed that overexpression of REC2 was associated with cell blebbing, nuclear fragmentation, and subsequent cell death by apoptosis (Fig. 1A, d-f). Apoptosis was also observed with overexpression of *REC2*-10 (T697A) (Fig. 1A, g-i), a recombinase-active missense mutant defective in the putative cdc2 kinase association motif (9). In contrast, similar overexpression of the recombinase-inactive missense mutant *REC2*-5 (D234A) did not induce apoptosis (Fig. 1A, j-l). The same results were observed when the wild type and the two missense *REC2* mutants were overexpressed in rat primary hepatocytes (data not shown).

Previous studies indicated that the Rb gene product protected hepatocytes and human hepatoma cells from apoptosis induced by TGF-β1 (8). We therefore investigated whether the high levels of endogenous Rb expressed in HuH-7 cells (7) could modulate REC2-induced apoptosis. We characterized cells expressing two significantly different levels of the transfected REC2 protein. HuH-7 cells with low level expression of REC2 exhibited nuclear blebbing and fragmentation similar to that observed in the transiently transfected Hep G2 cells (Fig. 1B, d-f). Cell cycle analysis was performed on pCMV-REC2-transfected HuH-7 cells as well as on transfected Hep 3B cells, a Rb−/− human hepatoma cell line (19). The results demonstrated that a “sub-G1” cell population of apoptotic cells (20, 21) increased significantly (*p* < 0.001) in both *REC2* transiently transfected cell lines (Table I). However, the percentage of Hep 3B cells in sub-G1 increased 4-fold compared with a 2.7-fold increase observed in the transfected HuH-7 cells. Both cell lines exhibited significant decreases in G1 cell populations, but the decrease was 2-fold greater in Hep 3B than HuH-7 cells. The S phase populations in both cell lines were unchanged by overexpression of REC2. Interestingly, only the Hep 3B cells showed a decrease in the G2/M phase cell population relative to control (*p* < 0.001) (Table I). These data suggested that expression of Rb may affect the ability of REC2 to promote apoptosis
as well as alter the phase of the cell cycle that exits into apoptosis.

REC2 Binds Hypophosphorylated Rb—The induction of apoptosis in HuH-7 cells has been linked to decreased Rb expression and/or inactivation (8). Thus, if overexpressed REC2 binds Rb during the cell cycle, the interaction could be a potential trigger for REC2-induced apoptosis. In fact, Western blot analyses demonstrated that stable expression of REC2 in Hep G2 cells resulted in an increase of the hyperphosphorylated inactive form of Rb from approximately 10 to 60% (Fig. 2, A and C). Meanwhile, those selected clones no longer exhibited extensive apoptosis, suggesting that compensatory changes had occurred. The up-regulation of Rb steady-state levels may, in fact, be a compensatory mechanism(s) required for survival with overexpression of the recombinase. However, by immunoprecipitation with anti-REC2 polyclonal antibody, only the hypophosphorylated Rb species was associated with REC2 (Fig. 2B). Immuno-precipitation using the anti-Rb monoclonal antibody ZX161 also established the presence of REC2 in this complex (Fig. 2D). The results suggested that REC2 binds Rb either directly or indirectly through a bridge protein and appears to modulate its phosphorylation state promoting accumulation of the functionally inactive hyperphosphorylated Rb.

In vitro binding assays were performed (22) to confirm binding between the REC2 and Rb proteins and to identify the putative REC2 binding region in Rb. Recombinant GST-Rb fusion proteins containing different regions of Rb (Fig. 3B) were bound to GSH-Sepharose and used as an affinity reagent for recombinant REC2 protein purified from E. coli (11). The results established that REC2 bound to the GST-Rb fusion proteins (Fig. 3A) but not to the GST-Sepharose nor the beads alone. Binding required the Rb A and B pocket domains as well as the spacer sequence (lanes 1 and 3–6). In fact, deletion of the spacer region abolished binding (lane 7), whereas three naturally occurring Rb loss-of-function alleles with alterations in the B pocket domain consisting of exon 22 deletion (lane 4), exon 21 deletion (lane 5), or a missense mutation (C706F) (lane 6) only decreased REC2 binding activity. The strongest binding between REC2 and Rb required the Rb carboxy-terminal region in addition to the pocket domains and spacer sequence (lane 2). Finally, REC2 did not bind to the Rb amino-terminal region (lane 8). The in vitro binding assay also confirmed that the REC2 present in the lysate from the transfected Hep 3B cells was capable of binding to the GST-Rb (379–928) fusion protein containing the A and B pocket domains, spacer sequence, and carboxyl terminus (Fig. 3C).

These results indicated that REC2 binds to the regions of Rb that have been identified for binding of the viral proteins SV40 T antigen, E1A, HPV E7, and Rep A (2–5), as well as the E2F proteins (13, 23). However, only the E2F transcription factors require both the A and B pocket domains as well as the carboxy-terminal region of Rb for high affinity binding. In addition, protein sequence alignment analysis demonstrated that neither the REC2 nor the E2F proteins contain the Rb-binding

**Fig. 4. Effect of TGF-β1 on REC2-induced apoptosis in HuH-7 cells.** The percentage of apoptotic cells was determined 66 h after transient transfection with the indicated construct or vehicle alone (Control). Incubation was with either no TGF-β1 (−) or 1 nM TGF-β1 (+) for 30 h. *, statistically significant increase (p < 0.001) relative to control and pCMV.CAT-transfected cells. ‡, statistically significant increase (p < 0.001) relative to TGF-β1-treated control and pCMV.CAT-transfected cells. §, significantly different from REC2-transfected cells (p < 0.01 REC2-10; p < 0.001 all others) and TGF-β1-treated REC2-transfected cells (p < 0.001).

**Fig. 5. Overexpression of wild type Rb inhibits REC2-induced apoptosis in transiently transfected HuH-7 cells.** A, apoptosis induced by REC2 or 1 nM TGF-β1 alone versus REC2 + 1 nM TGF-β1. B, TGF-β1-induced apoptosis in REC2-positive cells that were co-transfected with wild type Rb. −, neither transfected with the indicated construct nor incubated with TGF-β1; +, transfected with the indicated construct or incubated with 1 nM TGF-β1. *, statistically significant increase over pCMV control (p < 0.05 pCMV.Rb + TGF-β1; p < 0.001, all others) (A) or over pCMV.REC2 (p < 0.001) (B). ‡, statistically significant decrease relative to non-pCMV.Rb-transfected cells cultured under identical conditions (p < 0.001).
motif LXCXE previously identified in the viral E1A, large T, E7, and Rep A transforming protein products (2–5). Therefore, REC2 and the E2F transcription factors require similar structural domains of the Rb protein for maximal binding.

TGFB-1 Modulates Apoptosis Associated with REC2 Overexpression in HuH-7 Cells—We next determined whether the interaction between REC2 and Rb was involved in the Rb-mediated apoptotic pathway. High level overexpression of REC2 in HuH-7 cells was associated with apoptosis in 24.0% of cells compared with less than 0.4% in the chloramphenicol acetyltransferase plasmid-transfected controls (Figs. 4 and 5B). In addition, overexpression of the recombinase-active REC2-10 mutant allele was also associated with significantly increased apoptosis (p < 0.001). In contrast, the recombinase-inactive REC2-5 mutant allele did not induce apoptosis. Interestingly, it also exhibited no detectable binding to Rb in Hep G2 cells (Fig. 6) despite high level overexpression of the protein. When the REC2-overexpressing HuH-7 cells were incubated with 1 nM TGF-b1, which inhibits Rb expression and phosphorylation (8), REC2-induced apoptosis was significantly increased (p < 0.001) in both wild type and REC2-10-transfected cells (Fig. 4). However, in the wild type REC2-transfected cells the increase in apoptosis was significantly greater (~20%) than the sum of the individual inducers (Figs. 4 and 5, A and B). These data suggested that Rb expression modulates a TGF-b1/REC2 apoptotic pathway(s) and that the putative association of REC2 with CDK may be involved in the induction of apoptosis. Because loss of functional Rb results in apoptosis in these cells (8), we determined whether alterations in the Rb levels would modulate the increased apoptosis observed in TGF-b1-treated REC2-transfected cells.

Overexpression of Rb Inhibits REC2-induced Apoptosis—When co-overexpressed in HuH-7 cells, Rb inhibited TGF-b1 as well as REC2-induced apoptosis by an average of 74.0% (p < 0.001) (Fig. 5, A and B). Interestingly, an 82% decrease (p < 0.001) in the percentage of apoptosis was observed in the cells overexpressing both REC2 and Rb that were also treated with TGF-b1 (Fig. 5, A and B). These data coupled with that from the REC2-10 mutant imply that the interactive effect of TGF-b1 and REC2 is likely mediated by Rb and that the putative association of CDK with REC2 may be involved in modulating the phosphorylation state of Rb. The inability of the REC2-5 recombinase-deficient mutant to bind Rb and induce apoptosis in either HuH-7 cells or primary rat hepatocytes suggests that Rb potentially monitors the level of recombinase activity during the cell cycle. Cells with an abnormal increase in REC2 activity may then, in part, be regulated via apoptosis.

Our results support a novel link between DNA recombination and apoptosis in which Rb appears to be involved. Interestingly, it has recently been reported that the tumor suppressor p53 associates with Rad51, a human homolog of REC2, and suppresses spontaneous homologous recombination (24, 25). It remains to be elucidated how these tumor suppressor genes balance both DNA repair and apoptosis in the control of cell growth.

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