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c-Rel Arrests the Proliferation of HeLa Cells and Affects Critical Regulators of the G_1/S-Phase Transition

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A tetracycline-regulated system was used to characterize the effects of c-Rel on cell proliferation. The expression of c-Rel in HeLa cells led to growth arrest at the G_1/S-phase transition, which correlated with its nuclear localization and the induction of endogenous IκBα expression. These changes were accompanied by a decrease in E2F DNA binding and the accumulation of the hypophosphorylated form of Rb. In vitro kinase assays showed a reduction in Cdk2 kinase activity that correlated with elevated levels of p21^{WAF1} Cdk inhibitor and p53 tumor suppressor protein. While the steady-state levels of *WAF1* transcripts were increased, pulse-chase analysis revealed a sharp increase in p53 protein stability. Importantly, the deletion of the C-terminal transactivation domains of c-Rel abolished these effects. Together, these studies demonstrate that c-Rel can affect cell cycle control and suggest the involvement of the p21^{WAF1} and p53 cell cycle regulators.

c-Rel is a member of the Rel/NF-κB family of transcription factors, which also includes the retroviral v-Rel oncoprotein, the cellular RelA, RelB, p105 (NF-κB1), and p100 (NF-κB2) precursor proteins, *Xenopus* X-Rel1, and the *Drosophila* Dorsal and Dif factors (reviewed in references 5 and 84). Rel proteins have homologous N-terminal domains that control their nuclear localization, dimer formation, and binding to DNA. Their distinct C-termini participate in transcriptional activation or in the control of Rel activity. These proteins also have similar modes of regulation. The prototypic NF-κB factor is a heterodimer of p50 (NF-κB1) and p65 (RelA) that is retained in an inactive cytoplasmic form by one of several IκB inhibitors in unstimulated cells. In vivo activation of these complexes by a variety of stimuli results in the phosphorylation and rapid degradation of IκB through the ubiquitin-proteasome pathway (reviewed in reference 82). The nuclear entry of active NF-κB complexes is followed by their binding to κB DNA sites and the activation of gene expression (reviewed in references 4 and 79).

Several members of the Rel/NF-κB family have been implicated in the control of cell proliferation and differentiation. For instance, the temporal expression of c-Rel correlates with lymphoid cell differentiation (14, 15, 18, 40, 55, 63). However, their effects have been the object of conflicting observations. While the DNA-binding activity of NF-κB factors is increased in response to proliferative signals (6, 67), the induction of these factors was also implicated in cell growth arrest and differentiation (26, 35). Rel/NF-κB factors have also been shown to regulate cell response to a variety of stimuli ranging from stress to infection (5, 7, 36, 66, 79) and were recently implicated in the promotion or the inhibition of apoptosis (1, 8, 9, 11, 37, 52, 56, 65, 83, 85, 87, 91, 94). The rearrangement and amplification of several *rel* and *nf-κb* genes in hematopoietic and solid tumors are consistent with their participation in the control of cell growth and survival (reviewed in references 31 and 57).

In this study, we used a tetracycline-regulated system to investigate the effects of the chicken c-Rel protein on cell proliferation. We show that the expression of c-Rel arrested the proliferation of HeLa cells at the G_1/S-phase transition. We also demonstrate that this effect correlated with changes in the activity, modification, expression, and stability of critical cell cycle regulators including E2F, Rb, Cdk2, p21^{WAF1}, and p53. Interestingly, the deletion of c-Rel-activating sequences abolished these effects. Taken together, these findings indicate that the expression of c-Rel can affect cell cycle control and suggest the involvement of the p21^{WAF1} and p53 cell cycle regulators.

MATERIALS AND METHODS

Plasmids. c-*rel* was conditionally expressed in HeLa cells by using a tetracycline-regulated system (34). pUHD10-3-CCR expressed a chicken c-*rel* cDNA (17) under the control of the minimal cytomegalovirus promoter and heptamerized tetracycline operator sites of pUHD10-3 (34). pHMR272 was used to confer resistance to the drug hygromycin B (10). CCR-H is a c-*rel* mutant that contains a stop codon at the unique *Hin*cII site of c-*rel*. This mutant synthesizes a truncated c-Rel protein lacking the c-*rel* activation domains. CCR-H was cloned into a pUHD10-3 vector containing the hygromycin B resistance gene (pUHD10-3-hygro-CCR-H).

Cell culture and transfection. HtTA-1 cells, which stably expressed a fusion protein comprising the *Escherichia coli* tetracycline repressor and the activation domain of the herpes simplex virus VP16 protein (tTA), were a gift from H. Bujard (Heidelberg, Germany) (34). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 1× vitamin solution, 1× nonessential amino acids, and antibiotics (100 U of penicillin per ml, 100 μg of streptomycin per ml, and 125 μg of G418 per ml in potency units; Gibco). Cells were maintained at 37°C in an atmosphere of 5% CO_2.

Cells were conditioned to tetracycline-HCl (1 μg per ml; Sigma) for 4 days prior to transfection. HtTA-1 cells were transfected with pUHD10-3-CCR and pHMR272, or with pUHD10-3-hygro-CCR-H, by a modified calcium phosphate procedure (19). Cell clones were selected in the presence of hygromycin B (225 U per ml; Calbiochem). Drug-resistant colonies were picked and screened for the inducible expression of c-Rel or CCR-H. Cell clones were maintained in the presence of tetracycline (1 μg per ml) and refed every other day.

Immunological reagents. Polyclonal antibodies Ab1801 and Ab3, which recognize the unique carboxyl terminus of chicken c-Rel or the Rel homology region, respectively, were described previously (33, 50). Other antibodies used in this study were specific for IκBα, Cdk2, cyclin E, and p53 (sc-203, sc-163, sc-247, and sc-126, respectively; Santa Cruz Biotechnology); Rb (PharMingen), p21^{WAF1}

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(Ab-1; Oncogene Science); p53 (Ab-1 and Ab-2; Oncogene Science); or actin (Sigma).

Immunoblotting. CCR43 and CCR-H5 cells maintained in the presence of tetracycline were induced to express c-*rel* or the CCR-H mutant in medium lacking tetracycline. Cell extracts were prepared in lysis buffer (50 mM Tris HCl [pH 7.5], 150 mM sodium chloride, 1% sodium deoxycholate, 1% Triton X-100, 10 μg of leupeptin per ml, 10 μg of pepstatin per ml, 20 μg of aprotinin per ml, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.5 mM sodium orthovanadate) (70). Extracts were quantitated for total protein concentration by the method of Bradford (12). Proteins (20 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Schleicher & Schuell) in Tris-glycine–methanol buffer (16). Membranes were blocked in PBST (80 mM disodium hydrogen orthophosphate anhydrous, 20 mM sodium dihydrogen orthophosphate [pH 7.5], 100 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk. Membranes were incubated with immune serum, followed by incubation with donkey anti-rabbit or sheep anti-mouse horseradish peroxidase-linked antibodies and detection by enhanced chemiluminescence (Amersham). Immunoblotting to detect p53 was performed with antibody Ab-2 as described previously (21).

Immunocytochemistry and immunofluorescence. Cells were seeded onto coverslips in the presence of tetracycline and induced for c-Rel expression upon removal of the drug. Cells were fixed in 4% paraformaldehyde and permeabilized for 10 min in phosphate-buffered saline (PBS) containing 0.3% Triton X-100 and 5% goat serum. Cells were stained for c-Rel expression by immunocytochemistry with antibody Ab1801 and the Vectastain Elite ABC kit (Vector Laboratories). IκBα expression was analyzed by immunofluorescence with an anti-IκBα antibody and a fluorescein-conjugated goat anti-rabbit immunoglobulin G secondary antibody (Cappel). After nuclear staining with the intercalating dye Hoechst 33258, coverslips were mounted in the presence of 0.2% *para*-phenylenediamine (Sigma).

DNA-binding assay. CCR43 and HtTA-1 cells were maintained in the presence or absence of tetracycline for 120 h. Extracts were prepared and quantitated as described above for the immunoblotting procedure. Gel retardation assays were performed with cell extracts (20 μg) and ^{32}P-labeled double-stranded oligonucleotides containing a palindromic κB DNA site from interleukin 2-Rα (5′-CAACGGCAGGGGAATTCCCCTCTCCTT-3′), an E2F-1 consensus binding site (5′-ATTTAAGTTTCGCGCCCTTTCTCAA-3′), or an Oct-1 consensus binding site (5′-TGTCGAATGCAAATCACTAGAA-3′). Supershift analysis was performed with anti-Rel (Ab-3) (32), anti-p65 (KD 13/2), and anti-p50 antibodies (1263). Anti-p50 and anti-p65 antibodies were gifts from Nancy Rice (Advanced BioSciences Laboratories, National Cancer Institute, Frederick, Md.) and Rodrigo Bravo (Bristol-Myers Squibb, Princeton, N.J.), respectively. DNA-protein complexes were resolved by electrophoresis in 5% nondenaturing polyacrylamide gels.

Thymidine incorporation assays. CCR43 and HtTA-1 cells were maintained in the presence or absence of tetracycline for 72 h. Cell cultures were incubated with [^{3}H]thymidine (1 μCi; Amersham) for 4 h, fixed in methanol, exposed to NTB-2 silver grain emulsion (Kodak) for 5 days, and subjected to autoradiography.

Flow cytometry. The flow cytometry profiles of uninduced and induced CCR43 cells were determined by propidium iodide staining of nuclear DNA. Cells maintained in the presence of tetracycline were induced for c-Rel expression in the absence of tetracycline for 120 or 168 h. Cells were trypsinized and fixed in 70% ethanol at −20°C. Cells were washed in PBS–0.5% Tween 20–1% bovine serum albumin and resuspended in PBS containing propidium iodide (10 μg per ml). Cells were analyzed by flow cytometry with the Modfit program for cell cycle analysis.

In vitro kinase and immunoprecipitation assays. A glutathione *S*-transferase-Rb (GST-Rb) fusion protein was used as a substrate in in vitro kinase assays. A GST-Rb expression vector was obtained from W. Kaelin (Dana Farber Institute). GST-Rb was produced in the BL21 strain of *E. coli* and purified as described previously (47).

In vitro kinase assays were performed with extracts from HtTA-1 or CCR43 cells, uninduced or induced for 120 h in the absence of tetracycline. Extracts were prepared in EBC buffer (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40 (NP-40), 50 μg of phenylmethylsulfonyl fluoride per ml, 10 μg of aprotinin per ml, 5 μg of leupeptin per ml, 5 mM sodium orthovanadate, 100 mM sodium fluoride) and quantitated (12). Proteins (75 μg) were immunoprecipitated with anti-Cdk2 antibodies in EBC buffer. Immunoprecipitates were washed twice in NETN (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) and twice in IPK buffer (50 mM Tris [pH 7.5], 10 mM $MgCl_2$) and then incubated in IPK buffer containing 1 mM dithiothreitol, 150 ng of purified GST-Rb protein, and 10 μCi of [γ-^{32}P]ATP (Amersham) for 1 h at 30°C. Samples were boiled in protein loading buffer and resolved by SDS–10% PAGE. For coimmunoprecipitation of Cdk2-p21 complexes, extracts (150 μg) were immunoprecipitated with anti-Cdk2 antibodies and protein A-Sepharose (Pharmacia). Complexes were resolved by SDS-PAGE, followed by immunoblotting with anti-Cdk2 and anti-p21 antibodies.

Pulse-chase analysis. CCR43 cells were maintained in the presence or absence of tetracycline for 66 h and incubated for 2 h with methionine-free Dulbecco's modified Eagle's medium supplemented with 2% dialyzed fetal bovine serum, 2 mM glutamine, 1× vitamin solution, 1× nonessential amino acids, and antibiotics, in the presence or absence of tetracycline. Cells were washed with 1× Hanks buffer (Gibco) and labeled for 90 min with 400 μCi of EXPRESS ^{35}S protein-labeling mix (NEN Dupont) with or without tetracycline. Then they were washed twice with PBS and chased for the indicated periods of time with normal growth medium supplemented with 5 mM L-methionine. Cells were harvested in PBS and lysed in a mixture containing 50 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, 100 μg of bacitracin per ml, 1 μg of pepstatin A per ml, and 10 mM sodium bisulfite (41). Cell supernatants were precleared with 50 μl of protein A-Sepharose and quantitated. Cell lysates were immunoprecipitated with anti-p53 antibodies Ab-1 (amino acids [aa] 371 to 380; Oncogene Science) or sc-126 (aa 11 to 25; Santa Cruz Biotechnology) and resolved by SDS–7.5% PAGE.

Northern blotting. Total RNA was extracted with RNAzol B (TEL-TEST). RNA samples (20 μg) were analyzed in 1% agarose-formaldehyde gels and transferred onto Hybond-N membranes (Amersham). Membranes were baked for 10 min at 80°C and were UV cross-linked with a Stratalinker (Stratagene). Membranes were hybridized to ^{32}P-labeled probes specific for c-*rel*, *iκbα*, *WAF1*, *p53*, or *actin* in a mixture containing 5× SSPE (0.75 M NaCl, 50 mM sodium phosphate, 5 mM EDTA, pH 7.4), 50% formamide, 0.5% SDS, and 100 μg of sheared salmon sperm DNA per ml at 42°C overnight. Membranes were washed twice in 2× SSPE–0.1% SDS at room temperature and once in 1× SSPE–0.1% SDS at 65°C, followed by autoradiography.

RESULTS

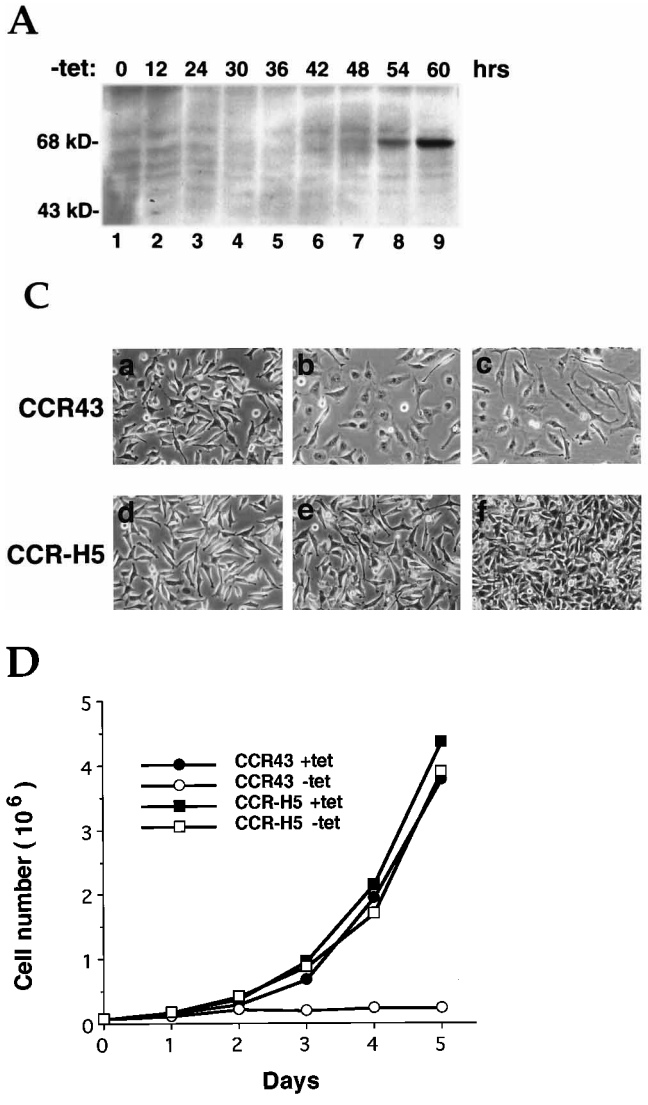
Tetracycline-regulated expression of c-Rel. A tetracycline-regulated system was used to characterize the effects of c-Rel on cell proliferation (34). The chicken c-*rel* gene was expressed under the control of the tTA fusion activator, comprising the *E. coli* tetracycline repressor and the activation domain of the VP16 protein of herpes simplex virus. The addition of tetracycline to the culture medium prevented the association of the tTA activator with the tetracycline operator sites, thereby arresting c-*rel* transcription. Stable cell clones were generated by cotransfection of the HtTA-1 cell line (34) with pUHD10-3-CCR and the pHMR272 hygromycin B resistance plasmid (10).

The inducible expression of c-Rel was analyzed in immunoblots. Whereas the protein was undetectable in cells maintained in the presence of tetracycline, removal of the drug led to the detection of c-Rel 48 to 54 h later and to its accumulation thereafter (Fig. 1A). DNA-binding assays confirmed that c-Rel produced in this system was functional for binding to the IL2-Rα NF-κB DNA site (data not shown).

High-level expression of c-Rel alters cell morphology. The induction of c-Rel led to significant morphological changes and to a marked decrease in the proliferation of HeLa cells. The morphology of the CCR43 c-Rel cell clone maintained in the presence of tetracycline was identical to that of the parental HtTA-1 cells that only expressed the tTA activator (Fig. 1B, panel a, and data not shown). In contrast, CCR43 cells adopted a flattened morphology and their growth rate decreased significantly after the removal of tetracycline (panels b and c). Prolonged induction of the protein led to the progressive elongation of the cells (panel d). Eventually, all of the cells adopted this morphology and ultimately died. The kinetics of c-Rel induction and the phenotype associated with its expression in cell clone CCR43 were representative of three independent clones analyzed.

Changes in cell morphology correlate with the nuclear localization of c-Rel and the induction of IκBα expression. Immunocytochemistry analysis showed that the morphological changes in CCR43 cells correlated with the expression and subcellular localization of c-Rel. Following the removal of tetracycline, c-Rel was predominantly found in the nuclei of giant flattened cells that stopped proliferating (Fig. 1B, panels b and c). After prolonged periods of induction, c-Rel appeared to localize to the cytoplasm of cells that showed a sharply elongated morphology (panel d).

Parallel immunofluorescence staining for endogenous IκBα expression together with nuclear DNA staining with Hoechst



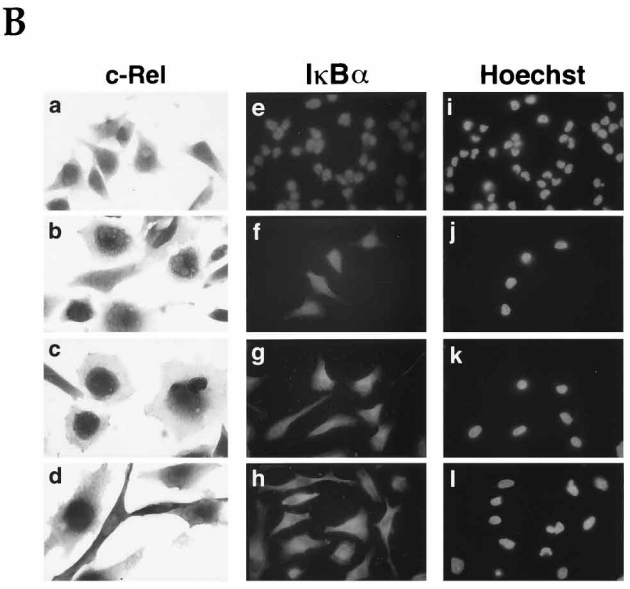


FIG. 1. The subcellular localization of c-Rel correlates with changes in cell morphology and the expression of IκBα. (A) Immunoblot analysis of c-Rel expression. Extracts (20 μg) from CCR43 cells maintained in the presence (lane 1) or absence (lanes 2 to 9) of tetracycline were resolved by SDS–7.5% PAGE and analyzed by enhanced chemiluminescence-immunoblotting with anti-c-Rel antibody Ab1801. (B) Immunocytochemistry analysis of c-Rel expression and immunofluorescence analysis of IκBα expression. Uninduced and induced CCR43 cells were analyzed by immunocytochemistry with anti-c-Rel antibody Ab1801 and a horseradish peroxidase-conjugated secondary antibody (a to d). Cells were also analyzed by immunofluorescence with an anti-IκBα antibody and a fluorescein-conjugated secondary antibody (e to h), and their nuclei were stained with Hoechst dye (i to l). CCR43 cells were maintained in the presence of tetracycline (a, e, and i) or in the absence of tetracycline for 48 (b, f, and j), 72 (c, g, and k), or 144 h (d, h, and l). (C) Morphology of cells expressing full-length c-Rel (CCR43) or only the Rel homology domain lacking the c-Rel-activating sequences (CCR-H5). CCR43 and CCR-H5 cells were maintained in the presence of tetracycline (a and d) or in the absence of the drug for 72 (b and e) or 120 h (c and f). (D) Growth curve of CCR43 and CCR-H5 cells cultured in the presence (+tet) or absence (−tet) of tetracycline.

dye showed IκBα staining in the cytoplasm of induced CCR43 cells (Fig. 1B, panels f to h and j to l). Western blot analysis confirmed the induction of IκBα expression in response to c-Rel (data not shown). Previous studies showing that c-Rel regulates IκBα gene expression and that IκB factors in turn regulate the subcellular localization of Rel proteins support our data (5, 13, 51, 62, 73).

Together, these results suggested that changes in the morphology of CCR43 cells likely resulted from the nuclear localization of c-Rel and the consequent activation of cellular gene expression. In agreement with this model, control cells expressing the CCR-H mutant of c-Rel that encodes the Rel homology domain alone, lacking the c-Rel transactivating sequences, failed to undergo morphological changes (CCR-H5; Fig. 1C, compare panels a to c to panels d to f).

c-Rel blocks cell cycle progression at the G_1/S-phase transition. The flattened morphology of CCR43 cells expressing c-Rel and their decreased proliferation suggested that overexpression of the protein may drive cells into growth arrest. Growth curves of CCR43 and CCR-H5 cells cultured in the presence or absence of tetracycline supported this hypothesis and showed that CCR-H5 cells expressing a transcription-defective mutant of c-Rel failed to undergo growth arrest (Fig. 1D). To further characterize this effect of c-Rel, the proliferative capacity of CCR43 cells was examined in thymidine incorporation assays. The parental HtTA-1 cells producing the tTA activator alone and the uninduced CCR43 cells proliferated efficiently, as evidenced by abundant dark nuclei (Fig. 2A, panels a to c). In sharp contrast, induction of c-Rel expression in CCR43 cells resulted in negligible incorporation of [^{3}H]thymidine (Fig. 2A, panel d). This result demonstrated a block in DNA synthesis and suggested the interruption of cell proliferation at the G_1/S-phase transition.

Flow cytometry was then used to further evaluate the effects of c-Rel on cell cycle progression in cells stained with propidium iodide for determining nuclear DNA content. Uninduced CCR43 cells showed a normal pattern of cell cycle distribution, similar to that of uninduced or induced parental HtTA-1 cells (Fig. 2B, left panel, and data not shown). Conversely, the induction of c-Rel decreased the number of cells that progressed from G_1 into S phase (Fig. 2B, middle and right panels). The flow cytometry profiles also suggested the inefficient progression of cells from S into G_2/M, as the cells seemed to be arrested with a DNA content between 2 and 4 N. Similarly, cells in G_2/M appeared to progress inefficiently back into G_0/G_1. Combined with the thymidine incorporation assays demonstrating the interruption of DNA synthesis (Fig. 2A),

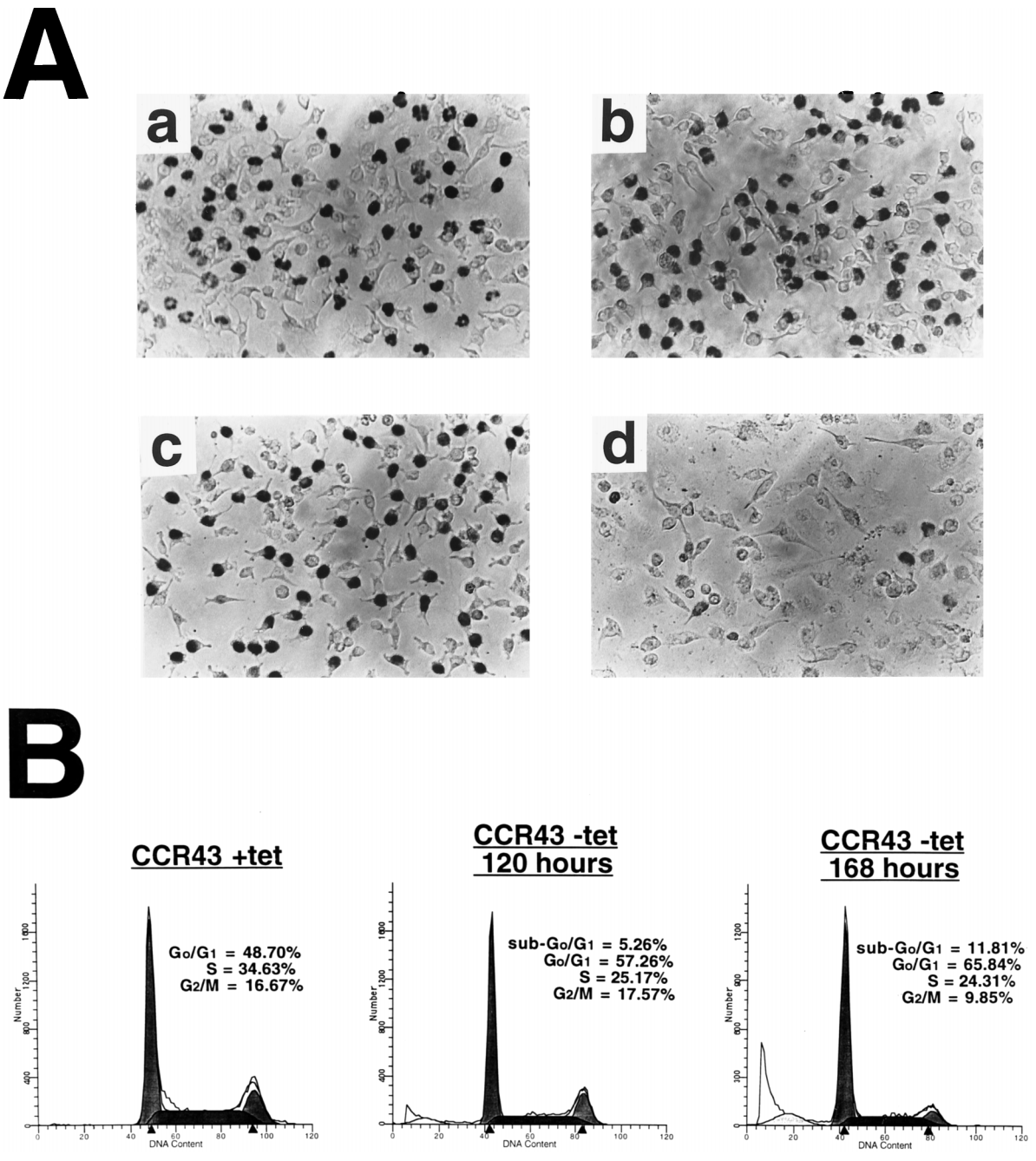


FIG. 2. c-Rel arrests cell cycle progression at the G_1/S-phase transition. (A) Analysis of c-Rel effects on cellular DNA synthesis. Assay of [^{3}H]thymidine incorporation in HtTA-1 (a and b) and CCR43 (c and d) cells maintained in the presence (a and c) or absence of tetracycline for 72 h (b and d). (B) Flow cytometry analysis of c-Rel effects on cell cycle progression. CCR43 cells were analyzed for nuclear DNA content by propidium iodide staining. Cells maintained in the presence (left) or absence of tetracycline for 120 (center) or 168 (right) h. Cell number (*y* axis) is plotted against nuclear DNA content (*x* axis). The distributions of cells in the sub-G_0/G_1, G_0/G_1, S, and G_2/M phases of the cell cycle are shown.

these results indicated that the expression of c-Rel initiated a block at the G_1/S-phase transition together with a possible block at the S-phase checkpoint or an overall slowdown of cell cycle progression.

c-Rel expression leads to a decrease in E2F DNA binding and the accumulation of hypophosphorylated Rb. The marked effects of c-Rel on cell proliferation led us to examine the mechanisms involved by characterizing its effects on the activity, modification, and expression of important regulators of the G_1/S-phase transition. Given the central role of E2F transcription factors in the control of G_1/S-phase progression (reviewed in reference 2), we examined the effects of c-Rel on the DNA-binding activity of E2F factors. Extracts from uninduced or induced CCR43 cells were analyzed for binding to a consensus E2F DNA site in gel retardation assays. The parental HtTA-1 cell clone was used as a control.

As shown in Fig. 3A, the DNA-binding activity of E2F was unaffected by the removal of tetracycline from HtTA-1 cells

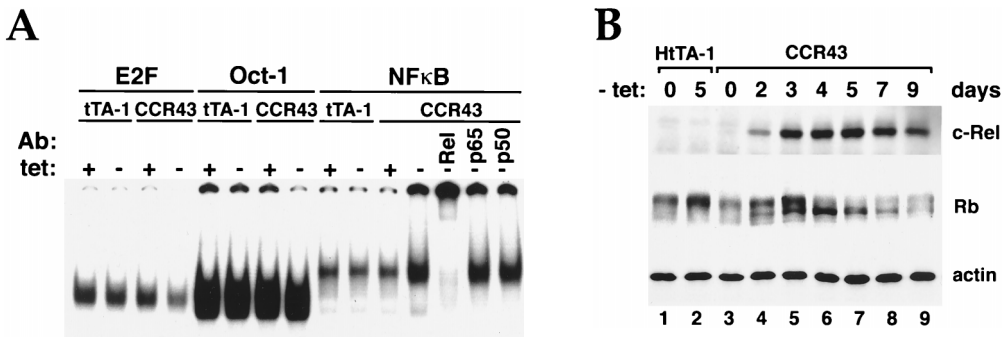


FIG. 3. c-Rel expression leads to a decrease in E2F DNA-binding activity and the accumulation of hypophosphorylated Rb. (A) Gel retardation analysis of E2F DNA-binding activity. Cell extracts (20 μg) were analyzed for binding to ^{32}P-labeled oligonucleotide probes (3×10^4 cpm) containing an E2F-1 DNA-binding site (lanes 1 to 4) or control Oct-1 (lanes 5 to 8) or NF-κB DNA sites (lanes 9 to 15). HtTA-1 (lanes 1, 2, 5, 6, 9, and 10) or CCR43 (lanes 3, 4, 7, 8, and 11 to 15) cells were maintained in the presence (lanes 1, 3, 5, 7, 9, and 11) or absence of tetracycline for 120 h (lanes 2, 4, 6, 8, 10, and 12 to 15). Supershift analysis of DNA-bound complexes was done with anti-Rel (lane 13), anti-p65 (lane 14), and anti-p50 (lane 15) antibodies. DNA-protein complexes were analyzed by electrophoresis in 5% native polyacrylamide gels. (B) Immunoblot analysis of the Rb protein. Cell extracts (20 μg) were analyzed by enhanced chemiluminescence-immunoblotting with antibodies specific for c-Rel (CCR), Rb, or actin. HtTA-1 (lanes 1 and 2) or CCR43 (lanes 3 to 9) cells were maintained in the presence (lanes 1 and 3) or absence of tetracycline for 48 (lane 4), 72 (lane 5), 96 (lane 6), 120 (lanes 2 and 7), 168 (lane 8), or 216 (lane 9) h.

(lanes 1 and 2). However, E2F DNA binding was significantly reduced upon induction of c-Rel in CCR43 cells (lanes 3 and 4). The induction of c-Rel had no significant effect on the binding to an unrelated Oct-1 DNA oligonucleotide (lanes 5 to 8). As anticipated, a control reaction showed increased κB site DNA binding in CCR43 cells expressing c-Rel (compare lane 12 to lanes 9 to 11). A supershift analysis demonstrated that this activity predominantly consisted of homodimeric c-Rel complexes. Whereas an anti-Rel antibody supershifted the bound complex, antibodies against the p65 and p50 subunits of NF-κB failed to do so (lanes 13 to 15). Together, the data suggested that the expression of c-Rel interfered with the ability of endogenous E2F factors to bind to their cognate DNA sites.

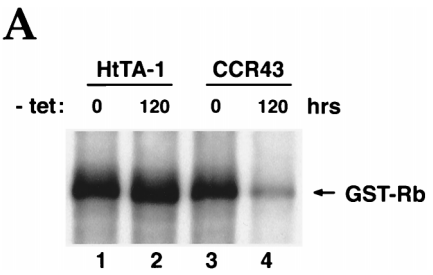
The DNA-binding activity of E2F factors is regulated by their differential association with members of the retinoblastoma (Rb) tumor suppressor family (reviewed in references 20 and 86). Hypophosphorylated Rb, found in growth-arrested and differentiated cells, associates with E2F to block its DNA-binding and transcriptional activities. The phosphorylation of Rb by cyclin-dependent kinases at the G_1/S-phase transition leads to its dissociation from E2F, allowing the cells to resume cell cycle progression. Immunoblot analysis was used to determine whether the c-Rel-induced growth arrest correlated with a change in the phosphorylation status of Rb. A comparative analysis of HtTA-1 and CCR43 cells during a time course of induction showed the specific accumulation of hypophosphorylated Rb in response to c-Rel induction in CCR43 cells (Fig. 3B; compare lanes 4 to 9 to lanes 1 to 3).

c-Rel expression is accompanied by a reduction in Cdk2 kinase activity. The phosphorylation of Rb at the G_1/S-phase transition is mediated by complexes of cyclin D-Cdk4/6 and cyclin E-Cdk2 (reviewed in references 43 and 77). To investigate the mechanism by which c-Rel interfered with the phosphorylation of Rb, we examined the kinase activities of these complexes. Extracts from uninduced or induced CCR43 cells were immunoprecipitated with antibodies specific for Cdk2 or Cdk4 and assayed for the phosphorylation of an exogenous GST-Rb substrate. The parental HtTA-1 cell clone was used as a control.

Whereas GST-Rb was efficiently phosphorylated by Cdk2 in HtTA-1 cells and in uninduced CCR43 cells, its phosphorylation was significantly decreased following c-Rel expression (Fig. 4A; compare lane 4 to lanes 1 to 3). Similar results were obtained when histone H1 was used as a substrate (data not shown). This suggested that c-Rel may decrease proliferation at the G_1/S-phase transition by interfering with Cdk2 kinase activity. Parallel experiments failed to detect endogenous Cdk4 kinase activity in either the control HtTA-1 cells or in CCR43 cells (data not shown). This observation is supported by studies showing that human papillomavirus E6 and E7 proteins, similar to those endogenous to HtTA-1 and CCR43 cells, disrupt cyclin D-Cdk4 complexes and promote the formation of inactive Cdk4-p16^{INK4A} complexes (92). Our inability to coimmunoprecipitate cyclin D with Cdk4 in HtTA-1 and CCR43 cells further supports this model (data not shown). This lack of coimmunoprecipitation is in contrast to the coimmunoprecipitation of cyclin E with Cdk2 that we observed in these cells (data not shown).

c-Rel does not affect the steady-state levels of cyclin E or Cdk2. The activities of Cdks are controlled by their transient association with cyclins, whose levels oscillate during the cell cycle and determine the phase at which the complex is active (reviewed in reference 64). The decrease in Cdk2 activity upon c-Rel expression and the important role of Cdk2-cyclin E complexes in the G_1/S-phase transition led us to look for possible changes in the expression of cyclin E and Cdk2 in CCR43 cells. Immunoblot analysis showed no effect of c-Rel on the steady-state levels of cyclin E or Cdk2 (Fig. 4B). Thus, it appears that the decreased activity of the Cdk2 kinase did not result from a reduction in the steady-state levels of Cdk2 or its cyclin E partner.

Accumulation of the p21^{WAF1} Cdk inhibitor in response to c-Rel expression. Another important mode of Cdk regulation involves the association of Cdks with inhibitors that modulate cell cycle progression. Among them, p16^{INK4A} and p15^{INK4B} control the activities of Cdk4- and Cdk6-cyclin complexes, whereas the p21 (CIP1/WAF1/CAP20/SDI1) and p27 (KIP1)



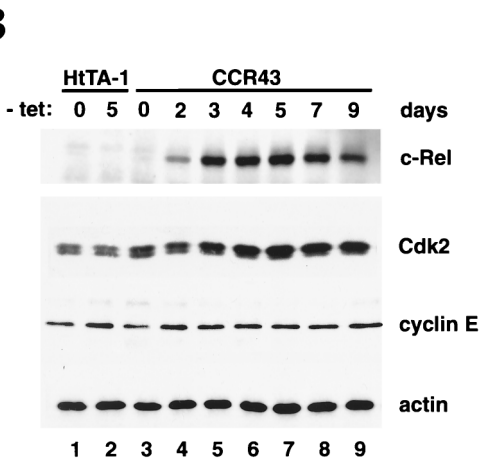


FIG. 4. c-Rel expression correlates with a decrease in Cdk2 kinase activity. (A) Effect of c-Rel on Cdk2 kinase activity. Cell extracts (75 μg) were immunoprecipitated with an anti-Cdk2 antibody and assayed for the in vitro phosphorylation of a GST-Rb substrate. HtTA-1 (lanes 1 and 2) or CCR43 (lanes 3 and 4) cells were maintained in the presence (lanes 1 and 3) or absence of tetracycline for 120 hours (lanes 2 and 4). The substrate was resolved by electrophoresis in a SDS–10% polyacrylamide gel. (B) Western blot analysis of Cdk2 and cyclin E expression. Extracts (20 μg) were analyzed by enhanced chemiluminescence-immunoblotting for the steady-state levels of cyclin E and Cdk2. HtTA-1 (lanes 1 and 2) or CCR43 (lanes 3 to 9) cells were maintained in the presence (lanes 1 and 3) or absence of tetracycline for 48 (lane 4), 72 (lane 5), 96 (lane 6), 120 (lanes 2 and 7), 168 (lane 8), or 216 (lane 9) h. An antiactin antibody was used as a control.

proteins inhibit Cdk2- and Cdk4-cyclin complexes (reviewed in reference 78). Given the strong inhibitory effect of c-Rel on the Cdk2 kinase, we looked for a possible change in the steady-state levels of $p21^{WAF1}$ in immunoblots. As anticipated, low levels of p21 were detected in uninduced CCR43 cells, levels equivalent to those found in uninduced or induced control HtTA-1 cells (Fig. 5A, lanes 1 to 3). Interestingly, a significant accumulation of $p21^{WAF1}$ was observed following the induction of c-Rel in CCR43 cells (lanes 4 to 9).

We investigated whether the decreased Cdk2 kinase activity in CCR43 cells resulted from its association with p21 in coimmunoprecipitation assays. Cell extracts were immunoprecipitated with an anti-Cdk2 antibody, followed by immunoblotting with an antibody against p21. As anticipated, very low levels of p21 were associated with Cdk2 in uninduced CCR43 cells, i.e., levels similar to those found in the control HtTA-1 cells (Fig. 5B, lanes 1 to 3). In contrast, abundant levels of p21 were found associated with Cdk2 in CCR43 cells expressing c-Rel (lane 4). This suggested that the c-Rel-dependent increase in the steady-state levels of p21 resulted in the functional inactivation of Cdk2 complexes and the consequent growth arrest.

Next, we examined whether the elevated levels of p21 protein were due to an increase in *WAF1* transcripts. Northern blot analysis during a time course of induction showed levels of endogenous *WAF1* mRNAs in uninduced CCR43 cells equivalent to those in the control HtTA-1 cells (Fig. 5C, lanes 1 to 10). In contrast, the induction of c-Rel expression led to a significant increase in *WAF1* transcripts (Fig. 5C, lanes 11 to 18). Thus, the accumulation of $p21^{WAF1}$ protein in CCR43 cells correlated with an increase in the steady-state levels of *WAF1* transcripts.

c-Rel expression correlates with increased p53 protein stability. The study of p53 knockout mice revealed two pathways for the regulation of *WAF1* expression (61). The first pathway involves transcriptional activation by p53 and is activated by DNA damage (24, 25, 27). The second is independent of p53 and is activated in response to differentiation signals (46, 81, 93). To investigate the possibility that the c-Rel-induced growth arrest that we observed could involve the activation of p21 in a p53-dependent manner, we examined the levels of the p53 protein in CCR43 cells.

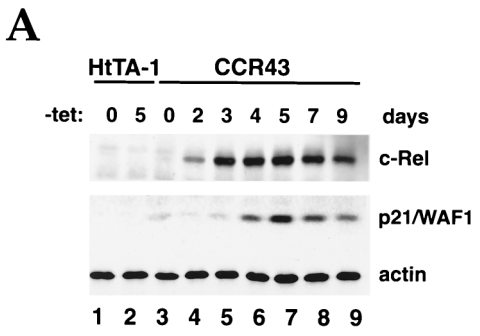
Immunoblots showed a marked increase in the steady-state levels of p53 following the induction of c-Rel, which correlated with the accumulation of $p21^{WAF1}$ (Fig. 6A, top panel; compare lanes 4 to 9 with lanes 1 to 3). Importantly, no increase was observed for cells expressing a c-Rel protein with its transactivation domains deleted (CCR-H5; Fig. 6A, bottom panel). This was consistent with the failure of these cells to undergo growth arrest (Fig. 1C and D) and suggested a role for the transcription activation domain of c-Rel in this process.

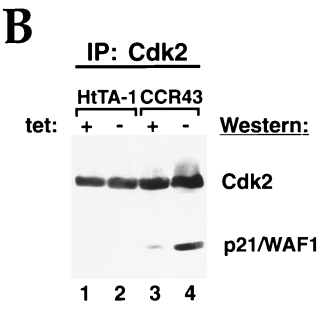
The presence of an NF-κB site in the p53 promoter (90) led us to investigate whether the increased levels of p53 in cells expressing c-Rel resulted from regulation at the transcriptional level. Northern blots failed to show any change in the levels of p53 transcripts during a time course of c-Rel induction (Fig. 6B, lanes 10 to 18). Rehybridization to a probe for *iκbα* confirmed that c-Rel expressed in CCR43 cells was competent for transactivation, as endogenous *iκbα* transcripts were induced with kinetics that paralleled that for c-*rel* mRNAs (Fig. 6B, lanes 10 to 18).

Our results raised the possibility that c-Rel may affect the stability of the p53 protein. Wild-type p53 has a half-life of less than 30 min and undergoes degradation through the ubiquitin-proteasome pathway (22, 58). We examined whether the expression of c-Rel led to the stabilization of p53 in a pulse-chase analysis. The human 293 cell line expressing the adenovirus E1B 55,000-molecular-weight (55K) protein associated with p53 was used as a control for the mobility of p53 (Fig. 6C, lane 1) (72). As expected, the half-life of p53 was less than 30 min in uninduced CCR43 cells (Fig. 6C, lanes 2 to 7). In contrast, its half-life was extended to about 5 h following the induction of c-Rel (lanes 8 to 13). The fact that antibodies directed against different epitopes of p53 recognized the same band confirmed the identity of p53 in this assay (lanes 14 and 15). Combined, these experiments showed that the expression of c-Rel led to a significant increase in p53 stability and suggested that this effect may play an important role in the G_1/S-phase arrest observed following the expression of c-Rel.

DISCUSSION

This report shows that the expression of c-Rel in HeLa cells led to G_1/S-phase arrest and altered cell morphology in a way that correlated with its nuclear localization. An investigation of the mechanisms involved revealed the accumulation of Rb in its hypophosphorylated form and a marked decrease in E2F DNA binding. The inhibition of Cdk2 kinase activity correlated





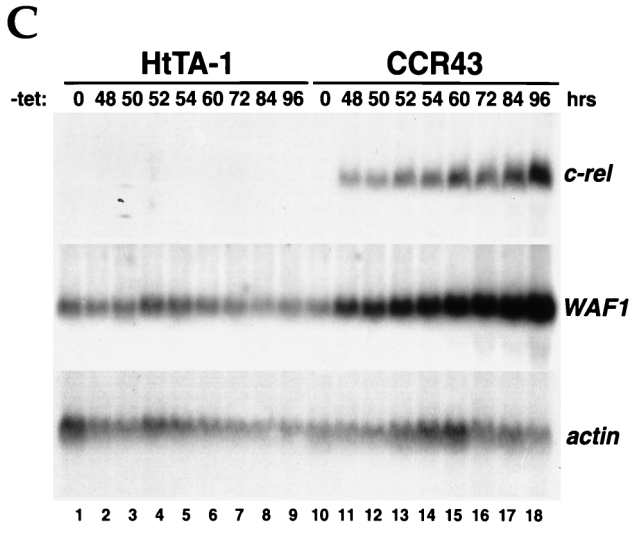


FIG. 5. The expression of c-Rel correlates with the accumulation of the $p21^{WAF1}$ Cdk inhibitor. (A) Immunoblot analysis of $p21^{WAF1}$ expression. Extracts (20 μg) were analyzed by enhanced chemiluminescence-immunoblotting with antibodies specific for c-Rel (CCR), $p21^{WAF1}$, or actin. HtTA-1 (lanes 1 and 2) or CCR43 (lanes 3 to 9) cells were maintained in the presence (lanes 1 and 3) or absence of tetracycline for 48 (lane 4), 72 (lane 5), 96 (lane 6), 120 (lanes 2 and 7), 168 (lane 8), or 216 (lane 9) h. (B) Coimmunoprecipitation assay of Cdk2 with $p21^{WAF1}$. Cell extracts (150 μg) were immunoprecipitated with an anti-Cdk2 antibody and resolved by SDS–15% PAGE. Immunoprecipitated complexes were analyzed in Western blots with anti-Cdk2 (top) or anti-$p21^{WAF1}$ (bottom) antibodies. HtTA-1 (lanes 1 and 2) or CCR43 (lanes 3 and 4) cells were maintained in the presence (lanes 1 and 3) or absence of tetracycline for 120 h (lanes 2 and 4). (C) Northern blot analysis of *WAF1* gene expression. Total RNA (20 μg) was resolved in a 1% formaldehyde-agarose gel, transferred onto a nylon membrane, and hybridized to ^{32}P-labeled probes specific for chicken c-*rel* (top) or *WAF1* (center). An *actin* probe was used as a control (bottom). HtTA-1 (lanes 1 to 9) or CCR43 (lanes 10 to 18) cells were maintained in the presence (lanes 1 and 10) or absence of tetracycline for 48, 50, 52, 54, 60, 72, 84, or 96 h (lanes 2 to 9 and 11 to 18).

with increased production of the $p21^{WAF1}$ Cdk inhibitor. c-Rel expression also led to the stabilization of p53, suggesting the p53-dependent regulation of *WAF1* gene expression. Importantly, a c-Rel mutant defective for transcriptional activation failed to increase the stability of p53 and to induce growth arrest. Combined, these results indicate that c-Rel can affect cell cycle regulation and suggest a role for its C-terminal transcriptional activation domain in this process.

c-Rel and cell cycle control. The expression of c-Rel in CCR43 cells led to the inhibition of E2F DNA binding and the accumulation of hypophosphorylated Rb. Given the role of E2F in the activation of genes important for DNA synthesis (reviewed in reference 2), it is likely that the down-modulation of E2F activity contributed to the G_1/S block that we observed. In turn, the inhibition of Cdk2 kinase and the accumulation of hypophosphorylated Rb are conceivably responsible for the inhibition of E2F DNA binding. Previous studies showed that $p21^{WAF1}$ regulates the activity of Cdk2, inhibits DNA replication, and promotes cell growth arrest and terminal differentiation (reviewed in references 29 and 78). The accumulation of the p21 transcript and protein suggests a role for this factor in the pathway leading to c-Rel-induced growth arrest in CCR43 cells (Fig. 5A and C). Future studies will help to clarify whether the expression of p21 alone is sufficient for the G_1 arrest that we observed.

The important role of p53 in p21 gene regulation (28) and its reported activation by NF-κB complexes (90) led us to investigate the effects of c-Rel on p53. Our analysis of CCR43 cells showed the posttranscriptional accumulation of p53 in response to c-Rel induction. This correlated with a sharp increase in the p53 half-life, from less than 30 min in uninduced cells to more than 5 h in c-Rel-expressing cells. Importantly, no such increase was observed in cells expressing a mutant c-Rel with its transactivation domain deleted, suggesting a possible role for the transcriptional activity of c-Rel in this process. Alternatively, the pathway leading to increased p53 stability in CCR43 cells may require specific protein interactions with the C-terminal domain of c-Rel.

Wild-type p53 undergoes rapid degradation through the ubiquitin-proteasome pathway (22, 58). This process is accelerated by its association with the E6 protein of human papillomavirus in combination with the cellular E6AP protein (74). Given that p53 is wild type in HeLa cells (75), it is unlikely that its half-life would be extended to greater than 5 h if c-Rel acted simply by inhibiting the activity of endogenous E6 in CCR43 cells. Moreover, the induction of c-Rel was also accompanied by a sharp increase in the steady-state levels of endogenous IκBα and $p27^{Kip1}$ Cdk inhibitor (data not shown). Like p53 and IκBα, $p27^{Kip1}$ is targeted for degradation by the ubiquitin-proteasome pathway (68). It is thus intriguing to hypothesize that c-Rel may exert its effects on cell proliferation by affecting the stability of critical cell cycle regulators. The ability of NF-κB to regulate the expression of proteasome subunit LMP2 is consistent with this model (89). It will therefore be interesting to examine the possible effects of c-Rel on the ubiquitin-proteasome pathway.

Interestingly, a transient increase in the overall amount of Rb was also observed in CCR43 cells expressing c-Rel. This effect is similar to that seen upon the inhibition of cell proliferation by interferon treatment of human lymphoid cells and in response to bovine papillomavirus E2 protein expression in HeLa cells, which led to the stabilization of Rb (44, 71). In CCR43 cells, this effect was followed by a decrease in Rb and p53 protein amounts that correlated with the progressive relocalization of c-Rel to the cytoplasm. Together with our find-

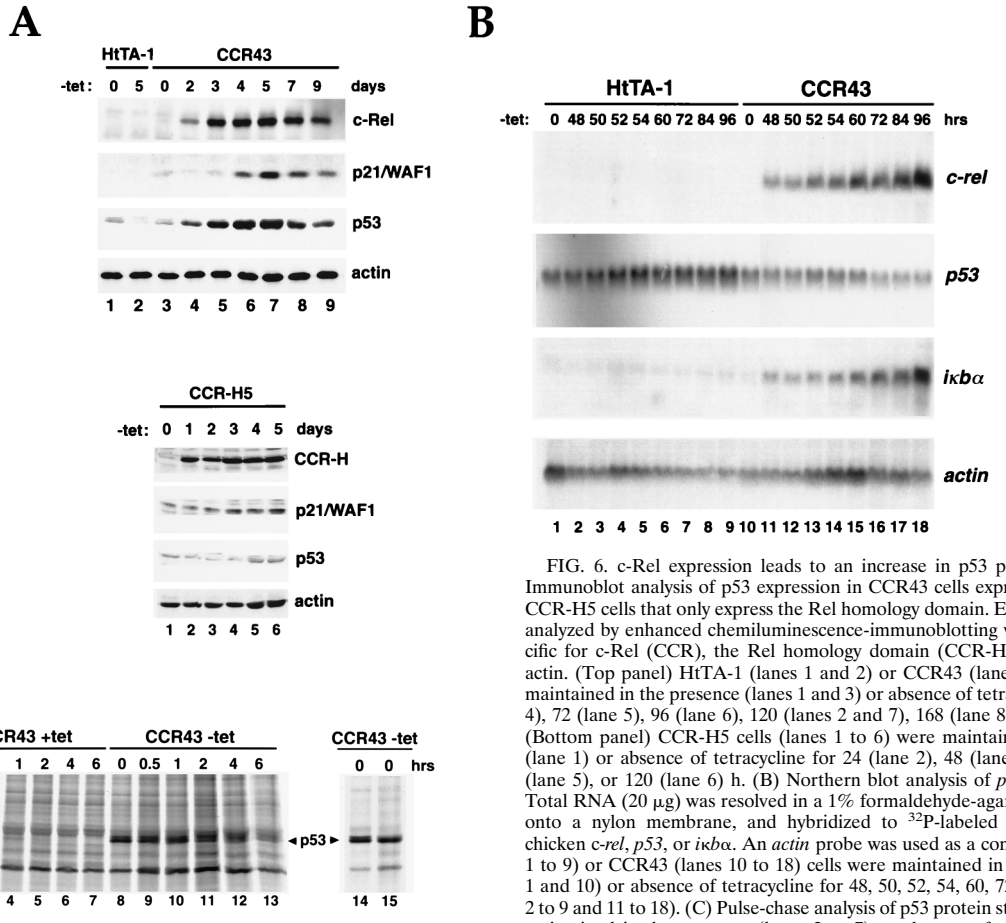


FIG. 6. c-Rel expression leads to an increase in p53 protein stability. (A) Immunoblot analysis of p53 expression in CCR43 cells expressing c-Rel and in CCR-H5 cells that only express the Rel homology domain. Extracts (20 μg) were analyzed by enhanced chemiluminescence-immunoblotting with antibodies specific for c-Rel (CCR), the Rel homology domain (CCR-H), p21^{WAF1}, p53, or actin. (Top panel) HtTA-1 (lanes 1 and 2) or CCR43 (lanes 3 to 9) cells were maintained in the presence (lanes 1 and 3) or absence of tetracycline for 48 (lane 4), 72 (lane 5), 96 (lane 6), 120 (lanes 2 and 7), 168 (lane 8), or 216 (lane 9) h. (Bottom panel) CCR-H5 cells (lanes 1 to 6) were maintained in the presence (lane 1) or absence of tetracycline for 24 (lane 2), 48 (lane 3), 72 (lane 4), 96 (lane 5), or 120 (lane 6) h. (B) Northern blot analysis of *p53* gene expression. Total RNA (20 μg) was resolved in a 1% formaldehyde-agarose gel, transferred onto a nylon membrane, and hybridized to ^{32}P-labeled probes specific for chicken c-*rel*, *p53*, or *ikbα*. An *actin* probe was used as a control. HtTA-1 (lanes 1 to 9) or CCR43 (lanes 10 to 18) cells were maintained in the presence (lanes 1 and 10) or absence of tetracycline for 48, 50, 52, 54, 60, 72, 84, or 96 h (lanes 2 to 9 and 11 to 18). (C) Pulse-chase analysis of p53 protein stability. CCR43 cells maintained in the presence (lanes 2 to 7) or absence of tetracycline for 66 h (lanes 8 to 15) were pulse-labeled for 90 min and chased for 0 (lanes 2, 8, 14, and 15), 0.5 (lanes 3 and 9), 1 (lanes 4 and 10), 2 (lanes 5 and 11), 4 (lanes 6 and 12), or 6 (lanes 7 and 13) h. Human 293 cells were labeled for 6 h and used as a control for p53 protein mobility (lane 1). The positions of p53 (black arrowhead) and the associated adenovirus E1B 55K protein (gray arrowhead) in 293 cells are shown (lane 1). The exposure time for lane 1 was 1/10 of that for lanes 2 to 15. p53 was immunoprecipitated with anti-p53 antibodies Ab-1 (lanes 1 to 14) and sc-126 (lane 15). The p53 doublet seen in CCR43 cells was previously observed by others in HeLa cells (59, 60).

ings suggesting a correlation between the C-terminal activation domain of c-Rel and the stabilization of p53, these observations raise the possibility that c-Rel might also affect the synthesis or stability of Rb.

Growth arrest and apoptosis. In addition to revealing a block at the G_1/S-phase transition, our flow cytometry analysis revealed a sub-G_0/G_1 peak indicative of cell death that was observed after the prolonged induction of c-Rel (Fig. 2B, middle and right panels) (23). Staining with the intercalating dye Hoechst 33258 revealed the presence of some fragmented nuclei in CCR43 cells induced to express c-Rel for 168 h, suggestive of death by apoptosis (data not shown) (30). A terminal deoxynucleotidyltransferase-mediated dVTP nick end-labeling (TUNEL) assay confirmed that the long-term growth arrest associated with the expression of c-Rel eventually led to some programmed cell death (data not shown). However, it is important to note that the kinetics and the efficiency with which c-Rel arrested the proliferation of CCR43 cells differed markedly from those for which apoptosis was observed. Whereas growth arrest was seen within less than 72 h after the removal of tetracycline, only 12% of the cells had undergone apoptosis at 168 h after removal of the drug. This argues against a direct role for c-Rel in the induction of programmed cell death in CCR43 cells and rather suggests that apoptosis perhaps occurred as a result of prolonged cell cycle arrest (reviewed in references 42 and 53). In agreement with the inability of c-Rel to potently activate expression of the c-*myc* gene implicated in the apoptotic pathway (52), the expression of c-Rel in CCR43 cells failed to increase that of c-Myc (data not shown).

The phenotype of c-Rel in CCR43 cells is strikingly similar to that observed upon the tetracycline-regulated expression of p21^{WAF1} in DLD1 colorectal carcinoma cells (76). Similar to c-Rel expression, p21 expression led to growth arrest and giant cell formation. While p21 did not induce massive cell killing in DLD1 cells, a small proportion of the cells ultimately underwent apoptosis after 5 to 8 days of induction (76). The effects of c-Rel in CCR43 cells are also similar to those that accompanied its expression in undifferentiated P19 mouse embryonal carcinoma cells (45). P19 cells expressing c-Rel were enlarged, flattened, and growth inhibited, and they expressed several differentiation-specific antigens. It is noteworthy that P19 cells induced to differentiate by retinoic acid also showed reduced Cdk2 kinase activity, hypophosphorylation of Rb, decreased E2F DNA binding, and G_1 arrest (49). Our inability to isolate stable clones of NIH 3T3 cells constitutively expressing the mouse c-Rel protein is also consistent with the c-Rel-induced growth arrest that we observed in CCR43 cells (data not shown).

In light of these observations, it is conceivable that p21 is

responsible for initiating the growth arrest and limited apoptosis that we observed. Alternatively, the delayed and limited apoptotic response that we saw could be due to an imperfect growth arrest resulting in some DNA damage. This would be consistent with a role for c-Rel in growth arrest and with the induction of p53 in response to DNA damage (reviewed in references 48 and 88). Since the p53 and Rb pathways cooperate to determine whether cells experience growth arrest or apoptosis (39), the observed shift between growth arrest and cell death may also reflect a change in the ratio of hypophosphorylated Rb and p53 after prolonged induction of c-Rel. The results of our immunoblot analyses would be consistent with this model.

Biological relevance. Accumulating evidence supports a role for the Rel and NF-κB factors in the inhibition of apoptosis (8, 9, 11, 52, 56, 65, 83, 85, 87, 91, 93a, 94). However, the mechanism by which Rel functions in this process remains to be determined. In light of our present findings, it is tempting to speculate that the ability of c-Rel to arrest cell growth could help to divert the apoptotic response toward cell cycle arrest. The fact that c-Rel also enabled CCR43 cells to escape tumor necrosis factor alpha (TNFα)-induced cell death agrees with this hypothesis (93a). Interestingly, an intact c-Rel transcription activation domain was necessary to induce growth arrest in CCR43 cells and to inhibit TNFα-induced apoptosis (Fig. 1C and D) (93a). According to this model, the transient induction of c-Rel expression and/or nuclear translocation in response to external stimuli could contribute to the survival of cells receiving apoptotic signals by promoting a transient growth arrest. Future studies will help to evaluate whether the effects of c-Rel on cell proliferation play a role in the cell decision between life and death and whether p21 contributes to its antiapoptotic activity.

Our finding of a correlation between c-Rel and the activity of cell cycle regulators is supported by the recent demonstration of a functional interaction between the C-terminal transactivation domain of p65 (RelA) and $p21^{WAF1}$ and Cdk2 through the p300 and CBP coactivators (69). Importantly, experimental evidence also supports the functional interaction of p300 and CBP with p53 to induce cell cycle arrest (3, 38, 54, 80). Together with our studies, these results point to a link between transcription factors of the Rel family and cell cycle regulation. Given the correlation between the activation of NF-κB in cells subjected to ionizing radiation and genotoxic agents and its ability to rescue them from apoptosis (8, 56, 83, 85, 91), our findings raise the possibility that the ability of Rel factors to affect cell cycle control may be a part of their antiapoptotic function. Further studies will help to address these issues.

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