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Ionizing Radiation and Teniposide Increase p21$^{waf1/cip1}$ and Promote Rb Dephosphorylation but Fail to Suppress E2F Activity in MCF-7 Breast Tumor Cells

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SUMMARY

Ionizing radiation and the topoisomerase II inhibitor, teniposide (VM-26) both increase levels of the cyclin dependent kinase inhibitor, p21$^{waf1/cip1}$ and promote dephosphorylation of the retinoblastoma tumor suppressor protein, Rb, in MCF-7 breast tumor cells, perturbations associated with suppression of the activity of the transcription factor, E2F. However, studies using an E2F binding site-luciferase reporter plasmid transfected into MCF-7 cells failed to demonstrate a reduction in E2F activity in response to VM-26 or to ionizing radiation. In contrast, E2F activity (both basal and E1A stimulated) could be suppressed by transfection with a plasmid expressing Rb, indicating that the capacity of E2F to bind to Rb and to be inactivated by Rb is functionally intact in MCF-7 cells. These findings in MCF-7 breast tumor cells suggest that E2F activity may not be directly susceptible to modulation by endogenous p21$^{waf1/cip1}$ and Rb.

Cellular progression through the restriction point (the G_1 to S transition) is stringently controlled through the successive activation of cyclin-dependent kinases (1) that modulate the activity of the Rb family of tumor suppressor proteins, including Rb, p107, p130, and p300 (1–3). In turn, binding of the Rb proteins to a family of E2F transcription factors (4) influences the activity of E2F (2, 5) and the transition to S phase (6). Initiation of the S phase is thought to be a consequence of E2F transactivation of genes such as dihydrofolate reductase, DNA polymerase α, thymidylate synthase, thymidine kinase, c-*myc*, and c-*myb* (7). Recent studies have determined that, in addition to the Rb family of proteins, E2F activity may be influenced directly by p53 (8), by the mdm2 protein that is downstream of p53 (9), by the cyclin-dependent kinase inhibitory protein p21$^{waf1/cip1}$ (10), and through alterations in E2F phosphorylation by the cyclin E/cdk2 and cyclin A/cdk2 complexes (11).

In addition to promoting the G_1 to S transition, there is indirect evidence suggesting that E2F could be involved in arresting cell growth in response to DNA damage. Alterations in elements putatively upstream of E2F in response to DNA damage include an increase in the levels of the p53 tumor suppressor (12–14) and the p21$^{waf1/cip1}$ proteins (15, 16), as well as dephosphorylation of Rb (14, 17). However, other findings argue (again indirectly) against a role for E2F in growth arrest. Although there is evidence for a growth-suppressive action of Rb (18), presumably through inactivation of E2F, transfection of Rb-negative cells with vectors constitutively expressing Rb does not uniformly result in abrogation of cell growth (19, 20). Furthermore, although DNA damage generally results in the up-regulation of p53 and p21$^{waf1/cip1}$ and the induction of Rb dephosphorylation, events that should lead to G_1 arrest through suppression of E2F activity, tumor cells in which DNA is damaged generally arrest in G_2 (21–23). However, to our knowledge, no studies have been reported assessing the response to DNA damage at the level of E2F activity in the intact cell.

The studies in this report describe the effects of ionizing radiation and teniposide on E2F activity in MCF-7 breast tumor cells. Ionizing radiation has been reported to induce p53, to increase p21$^{waf1/cip1}$ levels (14, 15), and to promote dephosphorylation of Rb in MCF-7 cells (15). We have substantiated the influence of ionizing radiation on p21$^{waf1/cip1}$ levels and Rb dephosphorylation and demonstrated similar effects by the topoisomerase II inhibitor, VM-26. To further extend these findings, we evaluated the influence of VM-26 and ionizing radiation on the activity of E2F by assessing the

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ABBREVIATIONS: PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

expression of an E2F binding site-luciferase reporter plasmid transfected into MCF-7 cells. The studies described below indicate that an increase in endogenous $p21^{waf1/cip1}$ protein levels and/or the dephosphorylation of endogenous Rb in MCF-7 cells may not be sufficient to suppress E2F activity. Consequently, it remains to be determined what role is played by E2F in the signal transduction pathway that responds to DNA damage through p53, $p21^{waf1/cip1}$, and Rb.

Experimental Procedures

Materials. Dulbecco's modified Eagle's medium (56-439) was obtained from Hazelton Research Products (Denver, PA); L-glutamine, penicillin/streptomycin (10,000 units penicillin/ml and 10 mg/ml streptomycin), and fetal bovine serum were obtained from Whittaker Bioproducts (Walkersville, MD); defined bovine calf serum was obtained from Hyclone Laboratories (Logan, UT). Trypsin-EDTA (10×; 0.5% trypsin, 5.3 mM EDTA) was obtained from GIBCO/BRL (Grand Island, NY) and maintained as a frozen stock. VM-26 (teniposide) was generously provided by Bristol-Myers. (Wallingford, CT). VM-26 was dissolved in dimethylsulfoxide (Aldrich Biochemicals, Milwaukee, WI) and maintained as a frozen stock solution for a maximum period of 2–3 weeks. Drug was diluted in incubation medium on the day of the experiment. [α-^{32}P]dCTP (3000 Ci/mmol) was obtained from DuPont NEN Research Products (Boston, MA). The restriction endonucleases were obtained from New England Biolabs, Beverly, MA, and the nick-translation kit was obtained from GIBCO/BRL. Anti-Cip1 (p21) was obtained from Transduction Laboratories (Lexington, KY). Anti-Rb was obtained from Pharmingen (San Diego, CA).

Probes and plasmids. A plasmid containing functional E2F consensus binding sites linked to a luciferase reporter (PGL2-E2F/Luc) and a plasmid containing mutated E2F binding sites linked to a luciferase reporter (PGL2-E2Fmut/Luc) were generously provided to our laboratory by Dr. Srikumar Chellappan (Columbia University College of Physicians and Surgeons, New York, NY). The wild-type pGL2 plasmid contains four E2F consensus sites (—TTTCGCGC—), whereas the mutant plasmid contains four E2F sites mutated at 3 base pairs (—TTTCTATC—) blocking the binding of the E2F protein. The normal and the mutated E2F consensus sequences were linked to a (—GATCTGTCCGAGTTATAA—) sequence containing a TATAA box, which was then attached to a luciferase reporter gene. The pCEP4 vector containing the β-galactosidase gene under the control of the CMV promoter was obtained from Dr. Eric Westin's laboratory at the Medical College of Virginia (Richmond, VA). The pSVE-Rb plasmid was generously provided by Dr. Lila Adnane at the Molecular Genetics Department, University of Pittsburgh. The pSVE-Rb plasmid contains the 4.7-kb human retinoblastoma gene placed in the *Bam*HI site of the pSVE vector. This plasmid overexpresses Rb through the SV-40 promoter that is upstream from the *Bam*HI site. The pGE1A plasmid, which expresses E1A through the SV-40 promoter, was a gift from Dr. Joseph Nevins (Duke University Medical Center, Durham, NC).

Cell line. The MCF-7 breast tumor cell line was kindly provided by the laboratory of Dr. Kenneth Cowan at the National Cancer Institute (Bethesda, MD). Cells were maintained as monolayers in Dulbecco's modified Eagle's medium supplemented with glutamine (0.292 mg/ml), penicillin/streptomycin (0.5 ml/100 ml of medium), 5% fetal bovine serum, and 5% defined bovine serum. All cells were cultured at 37° in an atmosphere of 5% CO_2 and 100% humidity. For the studies described in this report, cells were subcultured at densities in which the cells were maintained in logarithmic growth during the assay procedure.

Cell cycle analysis. MCF-7 breast tumor cells were grown in 75-cm^2 flasks to approximately 30–40% confluence. Cells were irradiated with the indicated dose of ionizing radiation or incubated with VM-26 for 3 hr at 37°. Cells were washed twice with fresh medium and permitted to grow in fresh medium. Samples were isolated at 0-, 24-, 48-, and 72-hr intervals postexposure. At appropriate times, the cells were washed once with 1 × PBS and detached from the flasks by trypsinization. Cells were centrifuged for 5 min at 1500 rpm and resuspended in ice cold 1 × PBS. The cell number was determined, and 5×10^6 cells/ml were resuspended in 500 μl of 1 × PBS and 500 μl of ethanol from a 100% stock. Cells were either incubated for 15 min on ice or stored at 2–8°C until the day of use. The samples that had been fixed in ethanol were centrifuged for 15 min at 1200 rpm, and the supernatant was discarded. The pellet was resuspended in 1 ml of filtered 1 × PBS (0.2-μm filter) and transferred to a specimen collection vial. Using a 3-ml syringe and a 26-gauge needle, the samples were passed through the syringe six times. One milliliter of solution A (3.4 mM trisodium citrate dihydrate, 1% (v/v) Nonidet P-40, 1.5 mM spermine tetrahydrochloride, and 500 μM Tris, pH 7.6) was added to the cells, and the cell suspension was incubated for 10 min at room temperature with gentle agitation. One milliliter of solution B (0.5 mg/ml trypsin inhibitor, 0.1 mg/ml ribonuclease A, 3.3 mM spermine tetrahydrochloride, pH 7.6) was added, and the cell suspension was incubated for an additional 10 min at room temperature with gentle agitation. Finally, solution C (748 μM propidium iodide, 3.33. mM spermine tetrahydrochloride, pH 7.6) was added to the samples and incubated for 10 min at room temperature with gentle agitation. The samples were then centrifuged for 5 min at 1200 rpm. The supernatant was removed, and the pellets were resuspended in 1 ml of filtered PBS and transferred to 12 × 75-mm tubes. The samples were maintained on ice or at 2–8°C and in the dark for up to 4 days until analysis by flow cytometry. For flow cytometric analysis, an argon laser emitting at 488 or 514 nm was used to determine DNA content, and the Verity Mod Fit LT Software was used to analyze the results obtained from the Becton Dickinson FACScan, model FC.

Western blot analyses. Cells were grown in T-75 flasks to approximately 60% confluence before irradiation or treatment with VM-26. Cells were then washed with ice-cold PBS, and crude cellular protein was extracted using hot lysis buffer (50 mM Tris·HCl, pH 6.8, containing 0.1% sodium dodecyl sulfate) at 0, 3, 6, 9, and 24 hr. Samples were denatured by boiling for 10 min followed by centrifugation. Total protein was quantified using the Pierce Coomassie Protein Assay Reagent (Pierce, Rockford, IL). Proteins (25 μg per lane) were electrophoresed on sodium dodecyl sulfate polyacrylamide gels (6% for Rb and 15% for p21) and transferred to nitrocellulose membranes. The blots were blocked with TBS containing 5% nonfat dry milk and 0.1% Tween 20 for at least 4 hr at room temperature. The blocking buffer was then decanted, and the blots were probed with the respective primary antibody diluted in TBS-Tween (containing 2% nonfat dry milk) for 1 hr at room temperature. p21 and Rb proteins were detected using 0.5 μg/ml of the respective antibodies. After washing in TBS-Tween, the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (0.2 μg/ml; Kirkegaard & Perry Laboratories, Gaithersburg, MD), then washed again in TBS-Tween. Antibody binding was detected using a chemiluminescence kit (Pierce). The blots were exposed to X-ray film and quantified using NIH Image software.

Luciferase reporter assay for E2f activity. Approximately 1.5×10^5 cells/ml were plated in each cylinder of a 6-well plate containing 2 ml of growth medium, and the cells were allowed to proliferate overnight. The following day, the various plasmids were diluted in 100 μl of Opti-MEM (GIBCO BRL); to keep the total concentration of DNA transfected in each condition constant, Bluescript (KSP$^+$) plasmid containing nonspecific sequences was added as needed. The LipofectAMINE transfection procedure by GIBCO BRL was performed as described by the manufacturer. After either irradiation or drug treatment (VM-26 or the vehicle, DMSO), the cells were washed twice with 2 ml of Ca^{+2}/Mg^{+2}-free PBS, and then lysed using 235 μl/well reporter lysis buffer (Promega, Madison, WI) containing 125 mM Tris, pH 7.8, with H_3PO4, 10 mM EDTA, 10 mM dithiothreitol, 50% glycerol, and 5% Triton X-100 (diluted 1:4) for 15

min at room temperature. The cell lysate was scraped using a rubber policeman, collected in 1.5-ml microcentrifuge tubes, and centrifuged at 10,000 rpm for 2 min at 4°. The supernatant was transferred to a 1.5-ml Eppendorf tube and stored at −70° until the extract was used for the determination of luciferase and β-galactosidase activities.

The luciferase activity of the cellular extract was determined by mixing 20 μl of cell extract with 100 μl of Promega luciferase reagent containing 270 μM coenzyme A (lithium salt), 470 μM luciferin, 530 μM ATP, 20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, and 33.3 mM dithiothreitol, pH 7.8, at room temperature and the relative light units measured for 20 sec in a Berthold LB 9501 luminometer. Transfection efficiency was monitored by the co-transfection of a plasmid expressing β-galactosidase from a cytomegalovirus promoter. β-Galactosidase activity was determined as described in Sambrook *et al.* (24). Briefly, 5–20 μl of cell extract were brought up to a final volume of 30 μl by the addition of lysis buffer (Promega). A substrate mixture (270 μl) containing *O*-nitrophenyl-β-D-galactopyranoside, 0.1 M $MgCl_2$, 4.5 M β-mercaptoethanol, and 0.1 M sodium phosphate (pH 7.5) was added to 30 μl of the cell extract. The solution was then incubated for 30 min at 37°. The reaction was stopped by the addition of 500 μl of 1 M Na_2CO_3. The product of β-galactosidase activity, *O*-nitrophenol, was measured spectrophotometrically at 420 nm.

Results

Effects of VM-26 and ionizing radiation on cell cycle progression. A primary goal of the present work was to determine whether suppression of E2F activity is a component of the pathway leading to growth arrest in MCF-7 breast tumor cells. This first series of experiments was designed to assess the nature of growth arrest in MCF-7 breast tumor cells exposed to either ionizing radiation or to VM-26.

Drugs that inhibit topoisomerase II, such as VM-26, have generally been reported to produce arrest in the G_2 phase of the cell cycle (22–23). Asynchronously growing MCF-7 breast tumor cells exposed to 10 μM VM-26 for 3 hr were analyzed for cell cycle distribution at 24-hr intervals subsequent to the drug exposure. Table 1 indicates that within 24 hr the percentage of cells in the G_2M phase of the cell cycle more than doubled, whereas the proportion of cells in the G_0-G_1 phase was reduced. By 72 hr, 35.7 ± 8.7% of the cell population was in the G_2M phase compared with 13.2 ± 1.3% of cells in G_2M before treatment. There was a modest but significant decline in the proportion of cells in the G_0-G_1 phases of the cell cycle, whereas the S phase fraction remained essentially unchanged.[2]

Ionizing radiation has been reported to produce a combined G_1 and G_2 arrest in MCF-7 cells (25). This finding was verified in our own studies. As indicated in Table 2, exposure of MCF-7 cells to 6 Gy of ionizing radiation resulted in an accumulation of cells in both the G_0-G_1 and the G_2M phases of the cell cycle with a marked decline in the S phase population. The accumulation of cells in G_2M was more pronounced; the percentage of cells in G_2M increased from 13.2 ± 1.3% to 19.5 ± 0.5% by 72 hr, whereas the G_0-G_1 fraction increased from 58.5 ± 4.7% to 71 ± 1.0%.

[2] In separate studies, in which cell number was assessed by trypan blue exclusion over a period of 3 days, we independently verified that both VM-26 and ionizing radiation interfere with the growth of MCF-7 breast tumor cells.

TABLE 1

Influence of VM-26 on cell cycle progression

MCF-7 breast tumor cells were exposed to 10 μM VM-26 for 3 hr. Cell cycle distribution was determined in treated cells at intervals of 24, 48, and 72 hr. The values represent means ± SE for three independent experiments (VM-26) and four independent experiments (medium control).

Condition	G_0–G_1	S	G_2 + M
	%	%	%
Medium, 0 hr	58.5 ± 4.7	28.3 ± 5.1	13.2 ± 1.3
VM-26, 24 hr	50.7 ± 5.8	21.7 ± 4.7	27.6 ± 7.5
VM-26, 48 hr	42.0 ± 6.0	23.0 ± 6.0	35.0 ± 11
VM-26, 72 hr	41.3 ± 8.0	23.0 ± 4.1	35.7 ± 8.7

TABLE 2

Influence of ionizing radiation on cell cycle progression

MCF-7 breast tumor cells were irradiated (6 Gy) and cell cycle distribution was determined in irradiated cells at intervals of 24, 48 and 72 hr. The values represent means ± SE for two independent experiments (irradiated) and four independent experiments (medium control).

Condition	G_0–G_1	S	G_2 + M
	%	%	%
Medium, 0 hr	58.5 ± 4.7	28.3 ± 5.1	13.2 ± 1.3
Irradiated, 24 hr	79.0 ± 4.0	4.5 ± 1.5	16.5 ± 2.5
Irradiated, 48 hr	72.5 ± 1.5	6.0 ± 1.0	21.5 ± 1.5
Irradiated, 72 hr	71.0 ± 1.0	9.5 ± 1.5	19.5 ± 0.5

Influence of VM-26 on the levels of $p21^{waf1/cip1}$ and on the phosphorylation state of Rb. The induction of $p21^{waf1/cip1}$ in response to DNA damage has been demonstrated to occur in cells exposed to ionizing radiation as well as to other modalities that induce DNA damage (12–14, 16). The influence of 10 μM VM-26 on the levels of the $p21^{waf1/cip1}$ protein was assessed over a time frame of 24 hr by Western analysis. Fig. 1A indicates that there was an approximately 2-fold increase in $p21^{waf1/cip1}$ levels by 3 hr, and a 3-fold elevation within 6 hr that was sustained over 24 hr. This represents a significant, though far from imposing, increase in $p21^{waf1/cip1}$ levels.

The $p21^{waf1/cip1}$ protein acts as a generalized inhibitor of the cyclin dependent kinases (26, 27). One consequence of this inhibition is conversion of the phosphorylated form of the Rb tumor suppressor protein to the hypophosphorylated form (28), which is thought to activate Rb (28) and to facilitate its binding to the transcription factor E2F (29). The phosphorylation state of Rb in response to 10 μM VM-26 was determined over the same time frame as the levels of the $p21^{waf1/cip1}$ protein. Results of the Western analysis, pre-

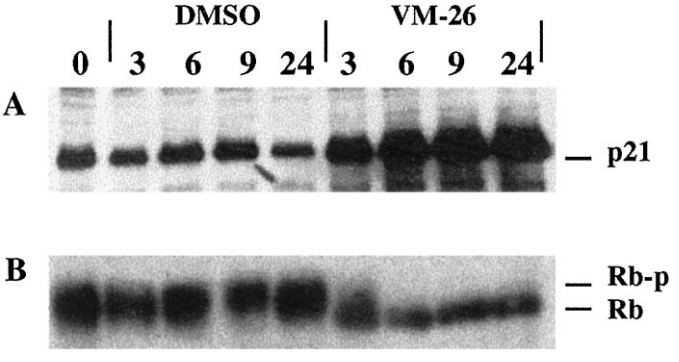

Fig. 1. Influence of VM-26 on $p21^{waf1/cip1}$ levels and Rb dephosphorylation in MCF-7 cells. MCF-7 cells were exposed to 10 μM VM-26. $p21^{waf1/cip1}$ levels and Rb phosphoprylation/dephosphorylation were monitored as a function of time by immunoblotting. $p21^{waf1/cip1}$ levels and Rb phosphorylation/dephosphorylation were also monitored in cells treated with volume equivalents of DMSO. A, $p21^{waf1/cip1}$ levels. B, Rb. *Rb-p*, phosphorylated Rb; *Rb*, dephosphorylated Rb. The time after initiation of exposure to VM-26 or the vehicle control (*DMSO*, dimethylsulfoxide) is indicated above each lane.

sented in Fig. 1B, are similar to what has been reported by other investigators assessing the status of Rb in proliferating MCF-7 breast tumor cells (15, 30, 31); that is, in control cells (the first five lanes) we observe a broad band indicative of the multiple phosphorylated states of the Rb protein. In cells exposed to VM-26, there was a clearly discernible conversion to the dephosphorylated form of the protein that corresponded closely with the increase in levels of the $p21^{waf1/cip1}$ protein; i.e., the change was evident at 3 hr, more pronounced at 6 hr, and maintained over a period of at least 24 hr.

Effects of ionizing radiation on the levels of $p21^{waf1/cip1}$ and on the phosphorylation of Rb. Exposure of MCF-7 cells to 10 Gy of ionizing radiation resulted in a time-dependent increase in $p21^{waf1/cip1}$ levels (Fig. 2A) that was similar to that induced by VM-26. As was the case with VM-26, the increase in p21 was roughly 2-fold after 3 hr; a 3-fold increase, which was evident by 6 hr, was maintained over a period of 24 hr. These observations are similar to the findings of other investigators using a dose of 5 Gy in MCF-7 cells (14, 15).

The phosphorylation state of Rb in response to ionizing radiation was determined over the same time frame as the levels of the $p21^{waf1/cip1}$ protein. Fig. 2B indicates that the dephosphorylated form of Rb was discernible after 3 hr, and was further visible throughout the 24-hr interval subsequent to irradiation. However, in these studies with ionizing radiation, the phosphorylated form of Rb was still evident even at 24 hr after irradiation (*last lane*). Furthermore, there was an indication of a decline in the overall levels of the Rb proteins at 24 hr. It should be noted that this decline is not a result of DNA fragmentation associated with apoptotic cell death (31a).

Effects of VM-26 and ionizing radiation on E2F activity. The drug and radiation induced increases in $p21^{waf1/cip1}$ levels and the dephosphorylation of Rb should result in a concomitant inactivation of the transcription factor E2F (10, 29). However, this possibility has, to our knowledge, not been assessed directly in cells responding to DNA damage. To assess the effects of ionizing radiation and teniposide on E2F activity, an E2F-luciferase reporter plasmid (PGL2-E2F/luc; see Experimental Procedures) that permits assessment of the activity of the multiple forms of E2F in the cell (4) was transfected into MCF-7 cells. Fig. 3A presents values for E2F activity in MCF-7 cells exposed to VM-26 for various time periods as well as E2F activity in control cells exposed to volume equivalents of DMSO (the vehicle for VM-26). Even continuous exposure of MCF-7 cells to VM-26 for up to 12 hr failed to reduce the transactivation activity of

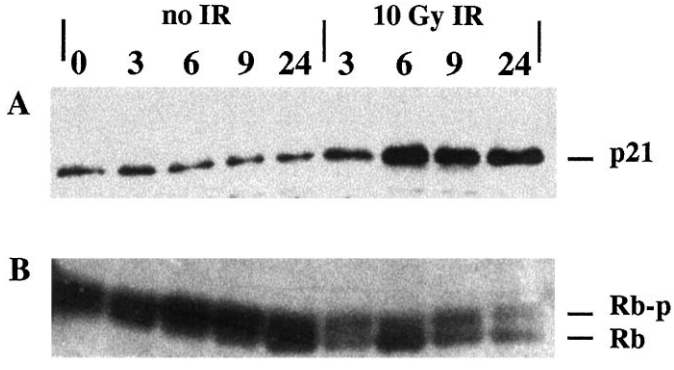

Fig. 2. Influence of ionizing radiation on $p21^{waf1/cip1}$ levels and Rb phosphorylation in MCF-7 cells. MCF-7 cells were exposed to 10 Gy of ionizing radiation. $p21^{waf1/cip1}$ levels and Rb levels were monitored as a function of time by immunoblotting. $p21^{waf1/cip1}$ levels and Rb phosphorylation/dephosphorylation were also monitored in untreated controls. A, $p21^{waf1/cip1}$ levels. B, Rb. Rb-p, phosphorylated Rb; Rb, dephosphorylated Rb. The time after initiation of irradiation (*IR*) or mock irradiation (*no IR*) is indicated above each lane.

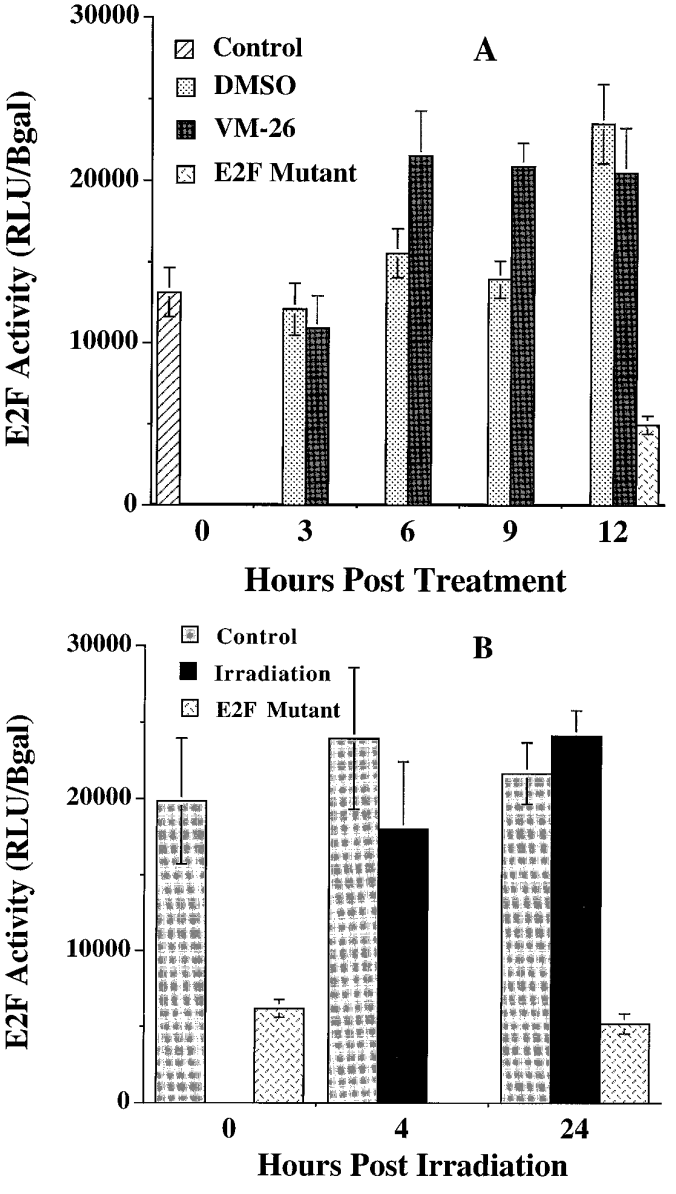

Fig. 3. Effects of VM-26 and ionizing radiation on E2F activity in MCF-7 Cells. MCF-7 breast tumor cells were transfected (see Experimental Procedures) with 1 μg of the pGL2-E2F/luc plasmid and 1 μg of pCEP4/β-galactosidase. The cotransfected cells were irradiated (6 Gy) or treated chronically with either the vehicle control (*DMSO*, dimethylsulfoxide) or VM-26 for 3, 6, 9, and 12 hr. A set of control cells was also transfected with 1 μg of the E2F mutant plasmid pGL2-E2F-mut/luc and 1 μg of pCEP4/β-galactosidase, and the cells were incubated in medium. In each independent transfection, the transfection efficiency was normalized by dividing the luciferase activity by the β-galactosidase activity. The values are the mean (relative luciferase units (*RLU*)/β-galactosidase (*Bgal*) expression) and mean $\pm$ standard error of between 6 and 12 independent transfections. A, VM-26. B, ionizing radiation.

E2F.[3] In fact, at 6 and 9 hr, there was a small but significant increase in E2F activity (when compared with cells exposed to DMSO). Fig. 3 indicates that a reporter plasmid containing mutations in the E2F binding site (pGL2-E2F-mut/luc; see Experimental Procedures) that was utilized as a negative control at 12 hr had relatively low expression of luciferase as compared with the wild-type plasmid containing intact E2F binding sites.

The influence of ionizing radiation on E2F activity was assessed at intervals of 4 and 24 hr after irradiation with 6 Gy. Fig. 3B indicates that there was no discernible diminution of E2F activity by ionizing radiation. The reporter plasmid containing mutations in the E2F binding site was again utilized as a negative control at 0 and 24 hr.

Determination of the functional interaction between Rb and E2F in the MCF-7 cell line

The absence of suppression of E2F activity by either VM-26 or ionizing radiation in cells with elevated levels of the $p21^{waf1/cip1}$ protein and dephosphorylation of Rb might be ascribed to problems with the reporter assay or to defects in the association between Rb and E2F. These issues were addressed by transfecting cells with vectors constitutively expressing exogenous Rb or the adenoviral protein, E1A, either alone or in combination. Transfection with Rb was designed to demonstrate that endogenous E2F activity could be inhibited by its binding to the Rb tumor suppressor protein. Transfection with E1A, which dissociates E2F complexed with the Rb family of proteins (32, 33) was designed to further validate the utility of the reporter assay in detecting alterations in E2F activity. As expected in cells with functional E2F, the Rb expressing vector produced a marked reduction in E2F activity (Fig. 4A). The average reduction in E2F activity was 67.7 ± 16.5%. Conversely, as shown in Fig. 4B, transfection with E1A stimulated E2F activity, indicating the dissociation of endogenous complexes involving E2F and the Rb family of proteins (i.e., p130, p107, and Rb) in MCF-7 cells. Transfection with Rb was able to reverse the stimulation of E2F activity induced by E1A, a finding that is consistent with the capacity of E2F to be bound to and inhibited by Rb. In both sets of experiments, the activity of the pGL2-E2F-mut/luc plasmid was included as a negative control.

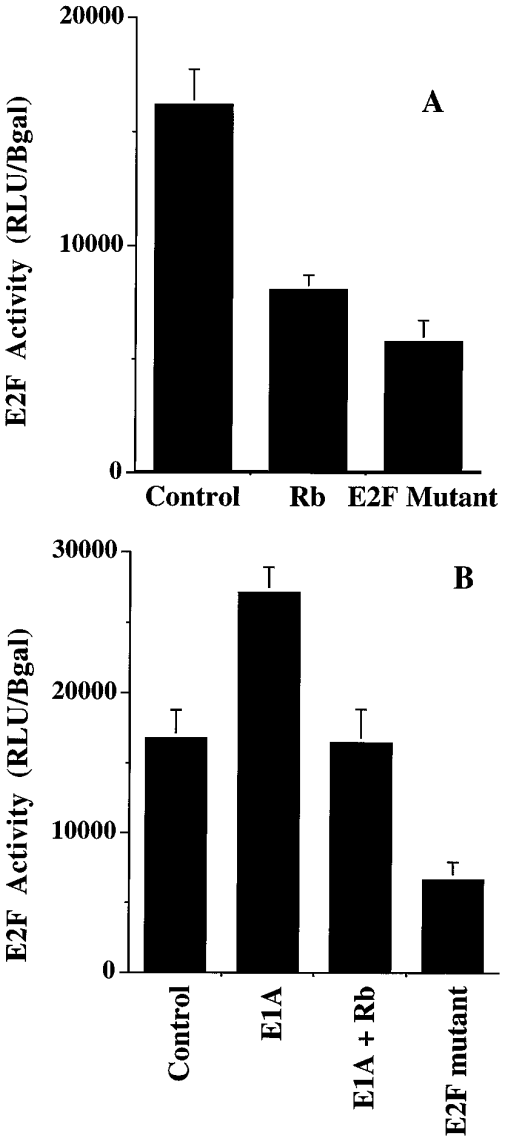

Fig. 4. Effects of transfection with exogenous plasmids expressing Rb or E1A on E2F activity in MCF-7 cells. MCF-7 breast tumor cells were transfected with 1 μg of the pGL2-E2F/luc plasmid, 1 μg of pCEp4/β-galactosidase, and 1 μg of pSVE-RB (A) and/or 0.25 μg of pGE1A (B), each under the control of the SV-40 promoter. A set of (negative) control cells was also transfected with the E2F mutant plasmid, pGL2-E2F-mut/luc. In each independent transfection, the transfection efficiency was normalized by dividing the luciferase activity by the β-galactosidase activity. The values are the mean relative luciferase units (*RLU*)/β-galactosidase (*Bgal*) ± standard error of between 8 and 12 independent transfections.

Discussion

It is generally accepted that DNA damage, such as that induced by ionizing radiation, increases levels of the p53 tumor suppressor protein (12–15), up-regulates the cyclin-dependent kinase inhibitory protein, $p21^{waf1/cip1}$ (13–16) and promotes dephosphorylation of Rb (14, 15, 17). However, although these perturbations are thought to suppress the activity of the transcription factor E2F (2, 5, 10, 28, 29) the influence of DNA damage on E2F activity has not previously been assessed in the intact cell. Consequently, a primary focus of the studies described in this report was to determine whether exposure of MCF-7 breast tumor cells to ionizing radiation or to VM-26 would alter the transactivational activity of E2F in concert with their effects on $p21^{waf1/cip1}$ levels and on the phosphorylation state of Rb.

Gudas *et al.* (14) and Wosikowski *et al.* (15) have reported that ionizing radiation increases levels of p53 and of $p21^{waf1/cip1}$ and promotes the dephosphorylation of Rb in the MCF-7 breast tumor cell line. We have substantiated the effects of ionizing radiation on $p21^{waf1/cip1}$ levels and Rb dephosphorylation and further demonstrated that the topoisomerase II inhibitor VM-26 produces similar (and in the case of Rb, more pronounced) alterations in these growth-regulatory proteins in the MCF-7 breast tumor cell line. However, despite the 2–3-fold increase in $p21^{waf1/cip1}$ levels and the dephosphorylation of Rb, neither VM-26 nor ionizing radiation suppressed E2F activity. Thus, it appears that in

[3] In a separate study using four sample replicates, we found no decrease in E2F activity by VM-26 even after 24 hr.

MCF-7 cells, the damage responses involving up-regulation of $p21^{waf1/cip1}$ and dephosphorylation of Rb occur as expected, but that these effects, either alone or in combination, are not sufficient to suppress E2F activity. The lack of effect on E2F activity might explain, in part, the absence of G_1 arrest in MCF-7 cells exposed to VM-26; however, this observation is not consistent with the combined G_1 and G_2 arrest induced by ionizing radiation.

Dbaibo *et al.* (34), using gel-shift analysis, demonstrated that treatment of the MOLT-4 human lymphoblastic leukemia cell line with sphingosine, a breakdown product of membrane sphingolipids, promoted the loss of association between E2F and its specific DNA sequence elements; this effect was associated with sphingosine-induced dephosphorylation of Rb (35). These observations suggest that the relationship between Rb and E2F may be intact in other tumor cell lines under different experimental conditions, although transcriptional repression by the Rb-E2F complex is generally thought to require occupation by E2F of its binding site on the promoter.

Our studies raise the question of why $p21^{waf1/cip1}$ and the dephosphorylated form of Rb fail to suppress E2F activity in MCF-7 cells. We considered the possibility that the existence of a mutation in the E2F family of proteins in MCF-7 cells might prevent their association with Rb. This possibility appears to have been ruled out by the studies in which E2F activity was suppressed by transfection with a plasmid expressing Rb; these studies indicate that the capacity of endogenous forms of E2F to bind to Rb is intact in MCF-7 cells. Although this approach does not identify the forms of E2F bound to Rb, these should include E2F-1, E2F-2, E2F-3 (36), as well as E2F-4 (37).

One explanation that might account for the failure of VM-26 and ionizing radiation to inactivate E2F activity in the MCF-7 breast tumor cell is that endogenous Rb levels are too low to suppress E2F. An and Dou (38) have reported proteolytic cleavage of Rb in HL-60 and U937 leukemic cells exposed to VP-16, whereas we observe what appears to be an overall reduction in Rb protein levels in irradiated MCF-7 cells after 24 hr. Although the MCF-7 cells contain a wild-type Rb protein, we must consider the possibility of defects in the capacity of the endogenous Rb protein to either bind to E2F or, once bound, to inactivate E2F. Consequently, it is possible that in MCF-7 cells exposed to VM-26 or ionizing radiation, where Rb (and possibly p107 and/or p130) are dephosphorylated, inactivation of E2F does not occur because Rb is functionally inactive. This might occur if the dephosphorylation is incomplete or occurs at sites that fail to confer activity on the Rb protein.

The current studies are the first to suggest that dephosphorylation of Rb as well as a 3-fold increase in $p21^{waf1/cip1}$ levels and an increase in p53 in concert with dephosphorylation of Rb (14, 15) are not sufficient to predict a concomitant suppression of E2F activity in the MCF-7 breast tumor cell. Although we cannot yet predict the relevance of the current findings to other experimental tumor cell lines or to the clinical situation, these findings suggest that the putative signaling pathway linking p53, $p21^{waf1/cip1}$, and Rb to E2F could be dysfunctional or dysregulated at the level of the Rb-E2F complex, at least in the MCF-7 cell line. Although mutations in p53 represent one well established mechanism for loss of restriction point control (39, 40), the failure of signals downstream of p53 (e.g., increased $p21^{waf1/cip1}$ and Rb dephosphorylation) to abrogate E2F function (7) could contribute to loss of restriction point control in cells with functional p53.

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