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Perturbation of the p53 Response by Human Papillomavirus Type 16 E7

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The p53 tumor suppressor protein can induce both cell cycle arrest and apoptosis in DNA-damaged cells. In human carcinoma cell lines expressing wild-type p53, expression of E7 allowed the continuation of full cell cycle progression following DNA damage, indicating that E7 can overcome both G_1 and G_2 blocks imposed by p53. E7 does not interfere with the initial steps of the p53 response, however, and E7 expressing cells showed enhanced expression of $p21^{waf1/cip1}$ and reductions in cyclin E- and A-associated kinase activities following DNA damage. One function of cyclin-dependent kinases is to phosphorylate pRB and activate E2F, thus allowing entry into DNA synthesis. Although E7 may substitute for this activity during cell division by directly targeting pRB, continued cell cycle progression in E7-expressing cells was associated with phosphorylation of pRB, suggesting that E7 permits the retention of some cyclin-dependent kinase activity. One source of this activity may be the E7-associated kinase, which was not inhibited following DNA damage. Despite allowing cell cycle progression, E7 was unable to protect cells from p53-induced apoptosis, and the elevated apoptotic response seen in these cells correlated with the reduction of cyclin A-associated kinase activity. It is possible that inefficient cyclin A-dependent inactivation of E2F at the end of DNA synthesis contributes to the enhanced apoptosis displayed by E7-expressing cells.

Many studies have demonstrated that the tumor suppressor protein p53 plays a major role in protecting cells from malignant progression, and p53 function is lost in the majority of human cancers (35). In normal cells, p53 is subject to high turnover but becomes stabilized following stress such as DNA damage or hypoxia (24, 28, 30, 38, 49). Activation of p53 leads ultimately to the suppression of cell growth, either through activation of a cell cycle arrest, which can be long term or temporary, or by initiation of programmed cell death or apoptosis (3). Cells showing loss of p53 function are not subject to this negative growth regulation and have been shown to accumulate genetic instability and mutations which might contribute to oncogenesis (1, 29, 46, 47, 67). In many human tumors, loss of p53 function occurs through mutations within the p53 gene itself (35), although there are alternative mechanisms by which p53 function can be circumvented. One example is found in cervical carcinomas, which are strongly associated with infection by a group of genital human papillomavirus (HPV) types (21, 27). p53 mutations are rarely found in these tumors (11, 12, 64), but the wild-type p53 protein expressed in these cells is targeted for rapid proteolytic degradation by the virally encoded E6 protein (65, 76). Cells which express E6 fail to undergo G_1 arrest or apoptosis following DNA damage (39, 68), indicating that E6 can effectively render these cells null for p53.

The mechanisms by which p53 leads to cell cycle arrest and apoptosis are not yet fully understood, although there is compelling evidence that the ability of p53 to function as a sequence-specific transcriptional activator (22, 25) contributes to both functions (10, 60, 63). A number of cellular p53 target genes have been identified, some of which have been implicated as downstream mediators of p53 function. Notably, the gene encoding the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$ has been shown to be regulated, at least in part, through a p53 response element (23, 31, 77) and is transcriptionally activated following DNA damage (19). The $p21^{waf1/cip1}$ inhibitor downregulates the activities of several cyclin-dependent kinases (cyclin E/CDK2, cyclin A/CDK2, and cyclin D/CDK4) which are essential for cell cycle progression (2, 26, 56–58, 62, 70) and thereby contributes to the DNA damage-induced G_1 phase arrest. The importance of $p21^{waf1/cip1}$ as a mediator of p53 function is supported by the observation that cells deficient in $p21^{waf1/cip1}$ show a clear defect in their cell cycle arrest response to DNA damage (6, 18, 73).

Several potential substrates for the cyclin-dependent kinases have been identified (54), including the retinoblastoma gene product (pRB) and other members of the pRB protein family, such as p107 (4, 44, 45). pRB is also a tumor suppressor protein which negatively regulates cell growth, in part, through binding to and inactivating certain transcription factors such as members of the E2F family (34). Cell cycle progression is associated with the phosphorylation of pRB, which renders the protein inactive and releases the transcription factors, thus initiating expression of genes necessary for progression into S phase (7, 9, 15). It has therefore been suggested that the induction of $p21^{waf1/cip1}$ by p53 could lead to the accumulation of hypophosphorylated pRB and that this may be at least partially responsible for the G_1 arrest (20). As with p53, pRB function is lost in most tumor cells through several mechanisms, including mutation within the pRB gene (5, 42, 43, 71, 78), overexpression of cyclin D (33, 36), or loss of the specific cyclin D kinase inhibitor p16 (37, 55). The E7 protein encoded by the genital HPVs found in cervical tumors, like simian virus 40 large T antigen and adenovirus E1A, forms a direct complex with the hypophosphorylated, active form of pRB, resulting in the release of transcriptionally active E2F (8, 52). Previous studies showed that expression of E7 was able to overcome a p53-induced G_1 phase cell cycle arrest (17, 32, 66), consistent with the notion that pRB is a target of p53 function. In this study we

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have investigated the mechanism by which E7 can overcome the p53 signal for cell cycle arrest and the consequences of this activity to the cell.

MATERIALS AND METHODS

Cell culture. RKO and MCF-7 cells were grown in Dulbecco's modified Eagle medium (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL). For Western analysis, kinase assays, and fluorescence-activated cell sorter (FACS) analysis, cells were treated with actinomycin D (Sigma) and harvested for analysis as described below. RKO, RKO+E6, and RKO+E7 cells have been described previously (39, 66), as have MCF-7 and MCF-7+E7 cells (32).

FACS analysis. Cells prepared as outlined above were harvested by trypsinization, taking care to include detached and adherent cells. The cells were collected by centrifugation, resuspended in phosphate-buffered saline (PBS), and made up to 90% methanol with cold (−20°C) methanol. Cells were fixed at −20°C overnight and then rehydrated with PBS. The cells were treated with 40 μg of RNase A per ml, stained with 20 μg of propidium iodide per ml, and analyzed by FACS.

BrdU pulse analysis. After 24 h of actinomycin D treatment, cells were labeled for 1 h with 10 μM bromodeoxyuridine (BrdU) before being washed and returned to label-free medium. At 0, 2, 4, 8, and 24 h after being labeled, the cells were harvested into methanol as described above. The fixed cells were stained with fluorescein isothiocyanate-conjugated anti-BrdU antibody (PharMingen) and propidium iodide as previously described (32).

Western blot analysis. Equal amounts of cell lysates were run on 8, 10, or 15% polyacrylamide gels, and proteins were transferred overnight onto nitrocellulose membranes. The membranes were probed with antibodies against p53 (DO-1), p21$^{waf1/cip1}$ (Oncogene Science Inc.), cyclin A (monoclonal antibody E23, a gift of J. Gannon and T. Hunt), or cyclin E (HE12; Pharmingen). The blots were processed by one- or two-step antibody detection and electrochemiluminescence fluorography (Amersham).

Purification of bacterially expressed protein. Logarithmically growing 500-ml cultures of *Escherichia coli* JG-1 transformed with glutathione *S*-transferase (GST)–RB plasmids (51) were grown for 4 h in the presence of 100 μg of isopropyl-β-D-thiogalactopyranoside (IPTG) per ml. The cells were spun down and resuspended in 20 ml of cold MTPBS (150 mM NaCl, 16 mM Na_2HPO_4, and 4 mM NaH_2PO_4, adjusted to pH 7.3) (52) and then sonicated at 4°C. Cell debris was removed by centrifugation, and the GST fusion protein was collected on glutathione-Sepharose beads (Pharmacia) at 4°C for 1 h. The beads were washed twice in MTPBS and then three times in wash buffer (100 mM Tris [pH 8.0], 120 mM NaCl). The protein was eluted from the beads in elution buffer (100 mM Tris [pH 8.0], 25 mM glutathione [adjusted to pH 8.0]) at room temperature for 1 h. The yield was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Kinase assays. Cells were washed in PBS and lysed on ice in 1 ml of the appropriate lysis buffer. For cyclin A-, cyclin E-, and E7-associated kinase activities, NP40M buffer (150 mM NaCl, 50 mM Tris [pH 8.0], 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM NaF, 0.1 mM NaV_3O_4) was used. E7-associated kinase activity was also tested in ELB buffer (250 mM NaCl, 0.1% Nonidet P-40, 50 mM HEPES [pH 7.0], 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 0.1 mM NaF, 0.1 mM NaV_3O_4). Immunoprecipitations were carried out by using a polyclonal rabbit antibody raised against bacterially expressed E7 (52); JG39, an anti-cyclin A polyclonal rabbit serum (a gift of J. Gannon and T. Hunt); and HE67 (PharMingen), a monoclonal antibody against cyclin E. Kinase reactions were carried out in 50 mM Tris–10 mM $MgCl_2$–250 μM cold ATP with 20 μg of histone H1 (Boehringer Mannheim) or 1 μg of bacterially expressed GST-RB substrate and 5 μCi of [γ-^{32}P]ATP (Amersham) per reaction. The reactions were stopped after 20 min by addition of sample buffer, and the reaction products were analyzed by autoradiography following sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

RESULTS

HPV type 16 E6 and E7 each overcome a DNA damage-induced cell cycle arrest. DNA damage responses were investigated in RKO and MCF-7 cells, human colon and breast carcinoma cell lines which are wild type for both p53 and pRB, and in E6- and E7-expressing clones of these lines (32, 39, 66). Treatment with 1 nM actinomycin D induced a dramatic reduction in the S phase population in control RKO cells, to around 3% of that seen in the untreated population, consistent with the observation that this drug can elicit a p53-induced cell cycle arrest (53). The E6-expressing lines, in contrast, demonstrated resistance to cell cycle arrest, presumably due to the lack of functional p53 in these cells (39), and expression of the E7 protein conferred at least a partial abrogation of the p53-induced cell cycle arrest. Typically, E7-expressing lines retained approximately 60% of their original S phase population during 120 h of actinomycin D treatment (data not shown). The resistance of both E6- and E7-expressing RKO and MCF-7 cells to DNA damage-induced cell cycle arrest has been described previously (17, 32, 66). These results demonstrate that E7 can overcome a checkpoint activated by p53 which prevents cells from entering DNA synthesis, but they do not indicate whether E7 can also abrogate p53-dependent S or G_2 phase checkpoints, which might allow DNA synthesis but prevent cell division (1, 13, 74). In order to address this question, RKO cells expressing E6 or E7 were incubated for 1 h with BrdU to label newly synthesized DNA, and then the progression of these labeled cells through the cell cycle was monitored over 24 h (Fig. 1). Untreated cells were seen in G_2 within 4 h and had returned to G_1 by 8 h. Cells grown in the presence of 1 nM actinomycin D moved into G_2 at a somewhat lower rate but had clearly returned to the G_1 phase by 24 h. Interestingly, E6- and E7-expressing cells reentered G_1 at a similar rate, indicating that both viral oncogenes can facilitate full cell cycle progression. Full cell cycle progression was also seen with E7-expressing cells in 5 nM actinomycin D, although the profiles were complicated by the extremely high rates of cell death seen under these culture conditions (see below).

The p53 response pathway remains intact in E7-expressing cells. A model in which E7 overcomes the p53-induced cell cycle arrest by directly inactivating pRB, a target of the p53-induced cyclin-dependent kinase inhibitor p21$^{waf1/cip1}$ (17, 32, 66), has been proposed. This model predicts that the initial steps of the p53 response would remain intact in E7-expressing cells, and in order to ascertain whether this was indeed the case, we examined some of the components of this pathway in these lines (Fig. 2). Consistent with previous studies, p53 was shown to accumulate upon actinomycin D treatment in the control cells, and the expected increase in p21$^{waf1/cip1}$ levels is also observed, indicating that p53 is transcriptionally active. The cells expressing E6 contain little p53 and fail to induce either p53 or p21$^{waf1/cip1}$ in response to treatment. E7-expressing cells were indistinguishable from the control cells, with similar elevations of both p53 and p21$^{waf1/cip1}$. As we have shown previously, E7-expressing MCF-7 cells expressed somewhat higher levels of p53 than control cells (32). However, there was no evidence that this resulted in an increase in p53 transcriptional activity, with no elevation of p21$^{waf1/cip1}$ expression. Following DNA damage, both p53 and p21$^{waf1/cip1}$ levels increased similarly in both E7-expressing and control MCF-7 cells, indicating that these cells also maintained a normal p53 response pathway (data not shown).

Cyclin-dependent kinase activity is reduced after p53 induction in E7-expressing cells. One of the principal functions of p21$^{waf1/cip1}$ is the inhibition of cyclin-dependent kinase activity, which plays an important role in mediating cell cycle progression. We therefore examined cyclin-dependent kinase activity in control and E7-expressing cells. Steady-state levels of cyclin E protein were elevated following actinomycin D treatment in MCF-7 and RKO cells (Fig. 3A and B), consistent with the observation that cyclin E protein levels are not reduced in rat cells following activation of wild-type p53 function (41). A similar increase in cyclin E levels was also observed in the E7-expressing cells, indicating that the accumulation is not dependent on cell cycle arrest. We saw no evidence that E7 expression resulted in an increase in cyclin E expression; in several repeats of this experiment, the total cyclin E protein levels in untreated E7-expressing cells were either the same as or slightly lower than those in control cells. Cyclin D1 levels remained constant in all of the lines under all of the conditions tested here (data not shown). Despite accumulation of protein

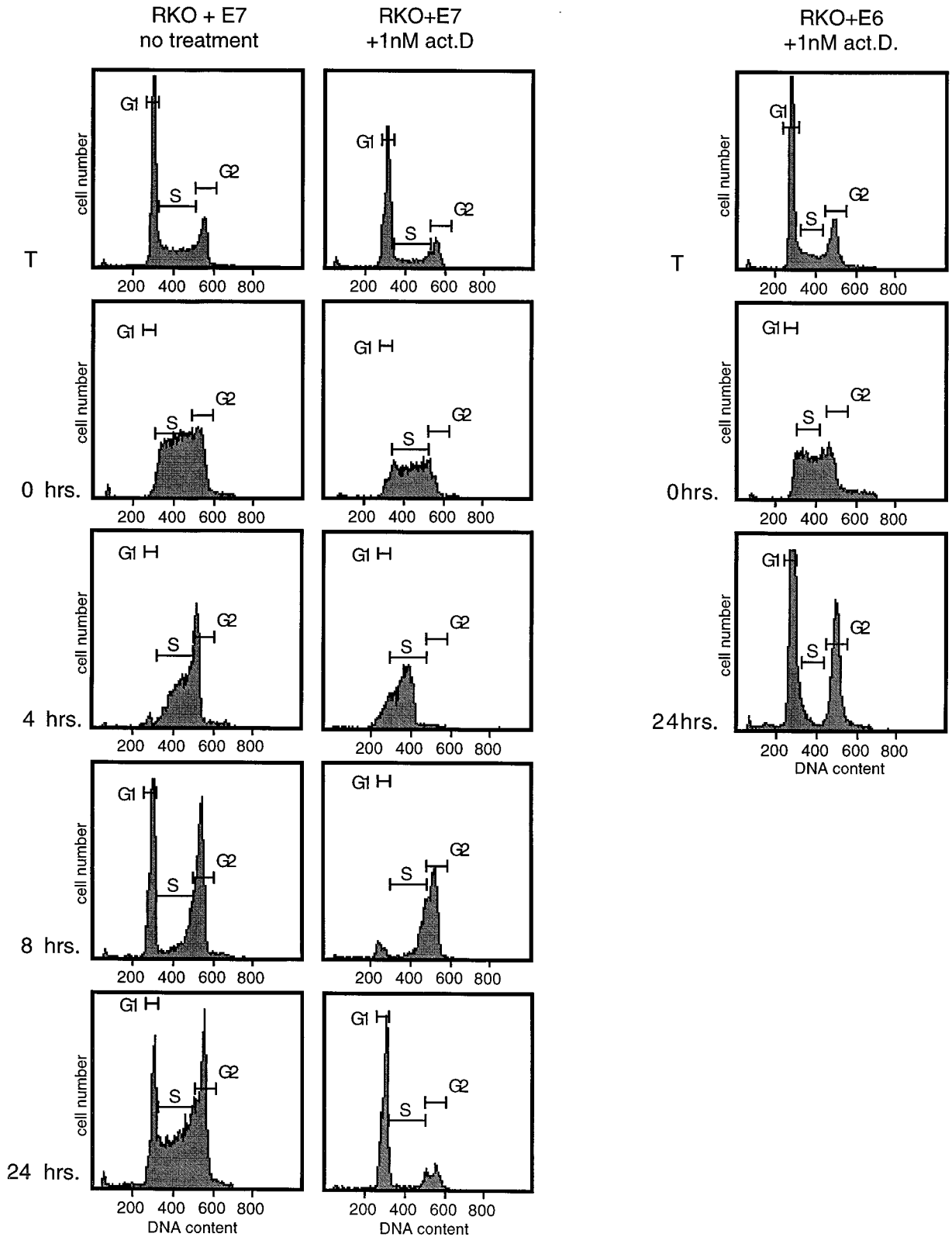

FIG. 1. Cells expressing either E6 or E7 are able to complete mitosis after actinomycin D treatment. E7-expressing cells were grown for 24 h in the presence or absence of 1 nM actinomycin D and then labeled with BrdU. FACS analyses are shown for the total cell populations (T) and for only the BrdU-labeled cells at the indicated time points following the BrdU pulse. Profiles of the E6-expressing lines after treatment are also shown.

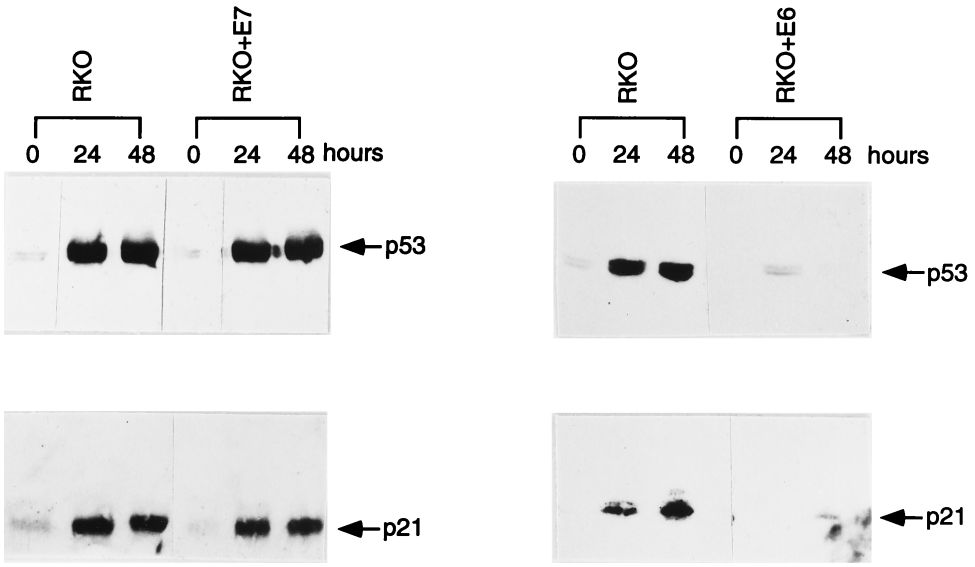

FIG. 2. The immediate p53 response is maintained in E7-expressing lines. Expression of p53 and p21$^{waf1/cip1}$ in control RKO cells and E6- or E7-expressing RKO cells treated with 1 nM actinomycin D for 0, 24, and 48 h is shown.

levels, the kinase activity associated with cyclin E in control MCF-7 and RKO cells was clearly inhibited following DNA damage, consistent with the activation of p21$^{waf1/cip1}$ (Fig. 3C and D). A similar reduction in cyclin E-associated kinase activity was also observed in E7-expressing MCF-7 and RKO cells, indicating that the p21$^{waf1/cip1}$ expressed in these cells remains functional. Nevertheless, low levels of cyclin E-associated kinase activity consistently remained evident in the E7-

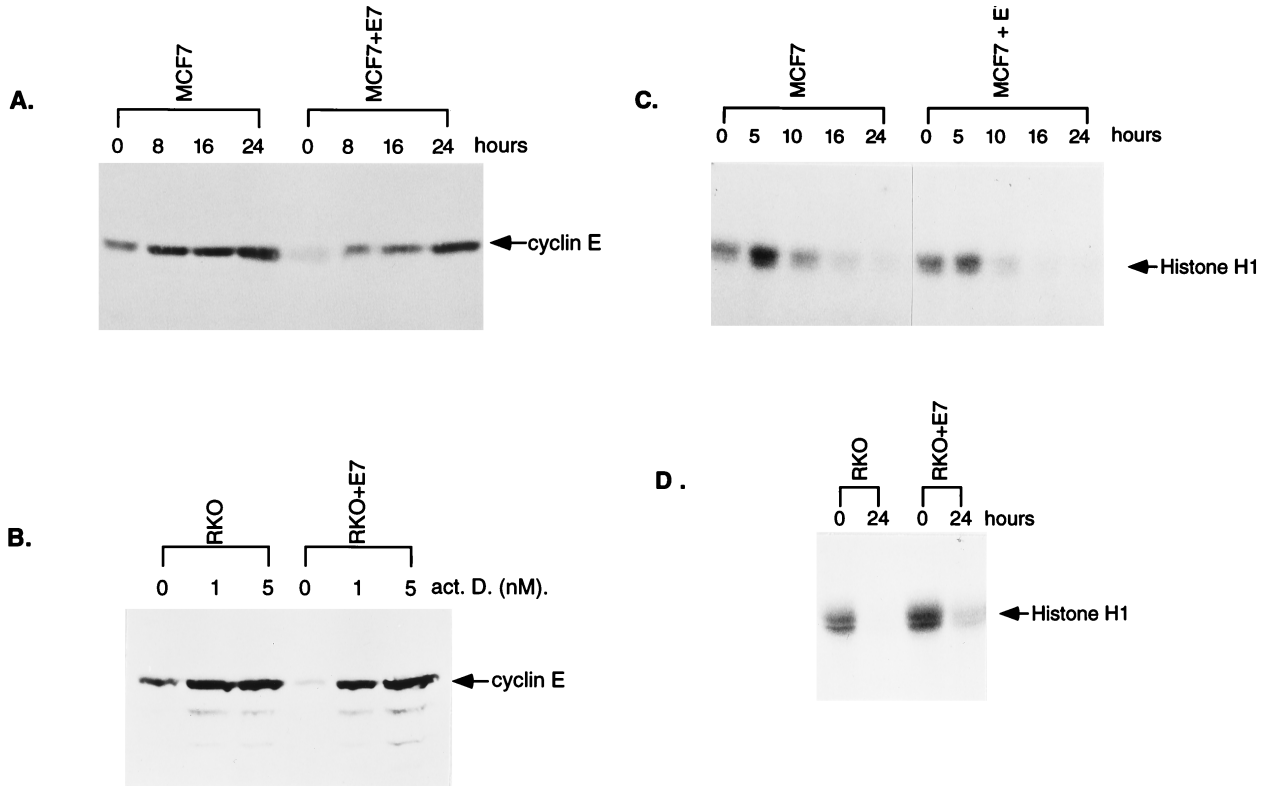

FIG. 3. Effects of actinomycin D treatment on cyclin E expression levels and activity. (A and B) Cyclin E protein levels are increased in response to actinomycin D. (A) Western analysis of cyclin E expression in E7- and vector control-transfected MCF7 lines after 0, 8, 16, and 24 h of treatment with 1 nM actinomycin D. (B) Western analysis of RKO cells with and without E7 treated with various concentrations of actinomycin D (act. D.). Cells were grown in the absence of actinomycin D or in either 1 or 5 nM actinomycin D for 24 h. (C and D) Cyclin E-associated kinase activity is reduced following actinomycin D treatment. (C) Cyclin E-associated kinase activity, with histone H1 as a substrate, in MCF7 cells with and without E7 grown in 1 nM actinomycin D for 0, 5, 10, 16, and 24 h. (D) Cyclin E kinase activity in E7-expressing and control RKO lines in the presence or absence of 5 nM actinomycin D. Treatment of these cells with 1 nM actinomycin D over 24 h also reduced cyclin E activity to near background levels (data not shown).

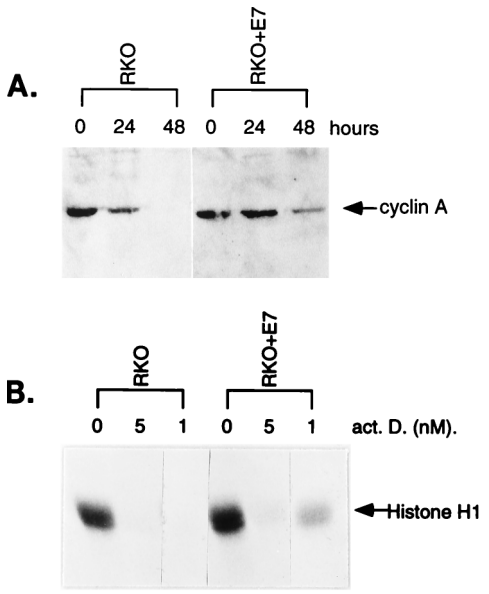

FIG. 4. (A) Cyclin A protein levels are reduced following actinomycin D treatment in both control RKO and E7-expressing lines. Cells were treated with 1 nM actinomycin D for 0, 24, and 48 h. (B) Cyclin A-associated kinase activity is reduced after actinomycin D treatment. E7-expressing and non-E7-expressing RKO cells were grown in 0, 1, or 5 nM actinomycin D (act. D.) for 24 h.

expressing RKO cells, suggesting that E7 may be able to protect some kinase function.

Cyclin A-associated kinase also plays a critical role during cell growth, with functions both at the G_1-S transition and at later stages in cell cycle progression. Unlike the case for cyclin E, cyclin A protein levels decreased following DNA damage in control RKO cells, consistent with the arrest of most of these cells in G_1 (Fig. 4A). Cyclin A levels also declined in E7-expressing RKO cells, although the rate of loss of the protein was somewhat lower and low levels of cyclin A protein were maintained. Immunoprecipitation of cyclin A-associated kinase revealed a reduction of kinase activity in both control and E7-expressing cells following actinomycin D treatment, consistent with the activation of $p21^{waf1/cip1}$ and a reduction in cyclin E-associated kinase activity (Fig. 4B). We did note that the E7-expressing cells reproducibly retained low levels of cyclin A kinase activity following DNA damage, although this was extremely reduced following treatment with 5 nM actinomycin D.

Phosphorylation of pRB is retained after DNA damage in cells expressing E7. The results presented so far indicate that the initial steps of the p53 response are intact in E7-expressing cells, with the activation of functional $p21^{waf1/cip1}$ and consequent inhibition of cyclin-dependent kinase activity. One of the targets for G_1 cyclin-dependent kinase activity is the pRB family of proteins, which are inactivated following phosphorylation. A simple prediction is that E7 allows cell cycle progression in the absence of G_1 cyclin-dependent kinase activities and, by extension, in the presence of hypophosphorylated pRB. However, previous studies (17, 32) demonstrated that at least some degree of hyperphosphorylation of pRB is retained in the cells expressing E7 after DNA damage, and we have confirmed this observation for E7-expressing RKO cells (Fig. 5A).

Analysis of cyclin E- and cyclin A-associated kinase activities showed some evidence that E7 could protect some kinase function following DNA damage. E7 has previously been shown to have associated kinase activity, which has also been identified as a cyclin-dependent kinase, associated with cyclin E or cyclin A (14, 50, 69). Immunoprecipitation of E7-associated kinase complexes from E7-expressing RKO cells showed that, in contrast to cyclin-associated kinase activities, the E7-associated kinase activity was not significantly down-regulated following treatment with 1 nM actinomycin D (Fig. 5B). It is therefore possible that the phosphorylation of pRB is mediated by the E7-associated kinase. Most of the previous studies analyzing this kinase have used histone H1 as a substrate. To ascertain directly whether this kinase could phosphorylate pRB, we used a bacterially synthesized GST-pRB fusion protein as a substrate in this assay and showed that the E7-associated kinase activity isolated from the HPV-positive cervical carcinoma cell line SiHa showed a strong activity in phosphorylating pRB in vitro (Fig. 5C). Although the E7 kinase activity detected in RKO+E7 cells was much weaker than that seen in SiHa cells (compare Fig. 5B and C), GST-pRB was phosphorylated by both the cyclin A-associated kinase and the E7-associated kinase immunoprecipitated from these cells. While cyclin A-associated kinase activity was largely inhibited following treatment of the cells with 5 nM actinomycin D, the ability of the E7-associated kinase to phosphorylate pRB was maintained, although it was slightly decreased (Fig. 5D), consistent with the results shown in Fig. 5B. We noted that the E7-associated kinase activity in cells treated with 5 nM actinomycin D showed some sensitivity to increased stringency of the extraction buffer (data not shown), probably reflecting the indirect nature of the association between E7 and the kinase.

Apoptotic responses in E6- and E7-expressing RKO cells. Although expression of both E6 and E7 can overcome a p53-activated cell cycle arrest, previous studies have indicated that the two viral proteins do not show equivalent functions in their ability to inhibit the apoptotic response to p53 (59, 61, 75). Flow cytometric analysis of control RKO cells treated with actinomycin D shows that following the initial G_1 arrest, cells also show evidence of apoptotic cell death 48 to 72 h after treatment (Fig. 6A and B). Both of these responses are likely to be p53 dependent, since the E6-expressing cells show resistance to apoptosis and cell cycle arrest, consistent with the lack of functional p53 in these cells. By contrast, although RKO cells expressing E7 fail to undergo p53-dependent cell cycle arrest, they demonstrate a striking apoptotic response, in excess of that observed in the control line, which is even stronger in the presence of 5 nM actinomycin D (Fig. 6C). This highlights the clear differences between E6 and E7 with respect to their ability to inhibit p53-dependent suppression of cell growth and indicates that although E7 can facilitate cell cycle progression under conditions of reduced cyclin E- and cyclin A-associated kinase activities, this viral oncoprotein cannot provide signals to ensure cell survival.

DISCUSSION

The two oncoproteins encoded by HPV type 16, E6, and E7, both show transforming and immortalizing activities in cells and together cooperate to immortalize primary human foreskin keratinocytes (72). E6 and E7 are consistently expressed in HPV-positive cervical cancers, and there is evidence that their continued expression is essential for tumor cell growth. The oncogenic activities of these proteins appear to be related, at least in part, to their ability to form complexes with p53 and the pRB protein family. We have shown that both E6 and E7 can prevent cell cycle arrest in response to DNA damage, a p53-dependent response. E6 directly targets the p53 protein, and cells expressing E6 fail to stabilize p53 in response to DNA damage. E7-expressing cells, by contrast, show an apparently

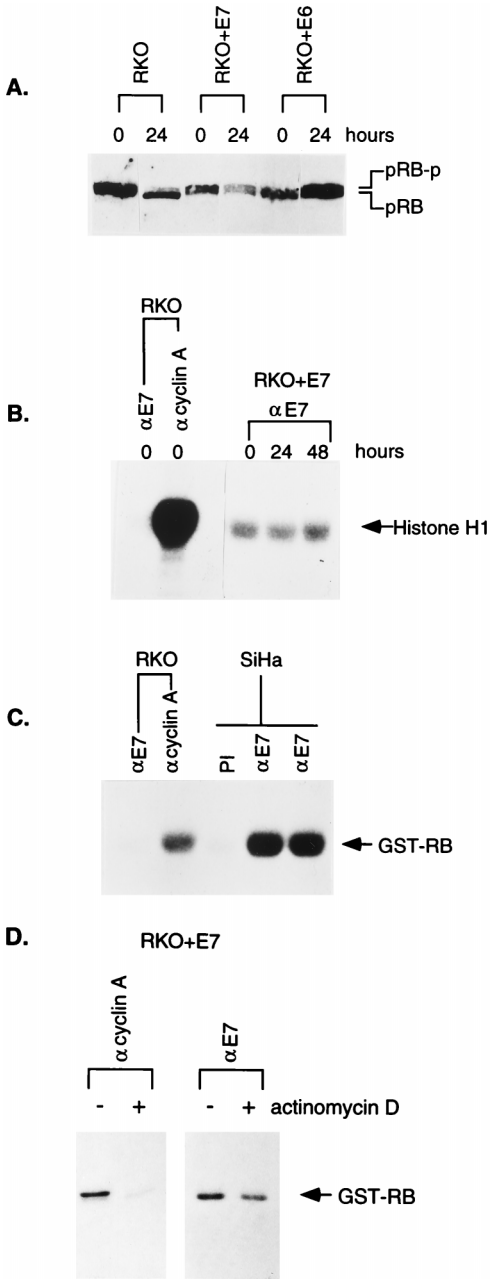

FIG. 5. E7-associated kinase can phosphorylate pRB. (A) Western analysis showing the phosphorylation status of pRB in E6-expressing, E7-expressing, and vector control RKO cells. The slower-migrating band represents the phosphorylated form of pRB and is evident in all untreated lines. The lower, faster-migrating band represents the hypophosphorylated form of pRB and accumulates only in the control cells. (B) In vitro kinase activity of the E7-associated kinase with histone H1 as a substrate. The E7-associated kinase activity was isolated from E7-expressing RKO cells treated with 1 nM actinomycin D for 0, 24, and 48 h. Vector control RKO cells, which do not express E7, were used as a negative control for E7-associated kinase activity, and cyclin A-associated kinase was used as a positive control. (C) The E7-associated kinase activity can phosphorylate GST-RB fusion protein in vitro. E7-associated kinase was isolated from the HPV-positive cervical carcinoma cell line SiHa and used to phosphorylate a bacterially synthesized GST-RB fusion protein in vitro. Negative and positive controls were applied as described in figure 5B. In addition, preimmune serum (PI) failed to precipitate a pRB-directed kinase activity from SiHa cells. (D) Cyclin A- and E7-associated kinases were isolated from RKO+E7 cells with or without treatment with 5 nM actinomycin D for 24 h with GST-RB as a substrate. Different exposures for the cyclin A and E7 kinase experiments are shown to allow a comparison of the relative reduction in activity after treatment. The relative activities of the E7- and cyclin A-associated kinases were similar to those shown in panel B.

normal p53 response, including activation of p53 and p21$^{waf1/cip1}$, and inhibition of cyclin E- and cyclin A-associated kinase activities. Nevertheless, DNA-damaged E7 cells continue cell cycle progression and show evidence for inactivation of pRB through phosphorylation. Uroepithelial cells which have been immortalized by E7 alone show constitutive activation of p53 and elevation of p21$^{waf1/cip1}$, which is not further affected by DNA damage, although these cells still show a DNA damage-dependent apoptotic response (61). Our results with RKO and MCF-7 cells suggest that expression of E7 does not necessarily lead to activation of a p53 response, and it will be of interest to determine the mechanisms underlying the differences in these cell types.

E7 expression clearly allows cells to cycle in the face of elevated expression of the kinase inhibitor p21$^{waf1/cip1}$, and the inhibition of most cyclin-dependent kinase function in E7-expressing cells indicates that the inhibitor is functional. Although the function of cyclin E- and cyclin A-associated kinases is normally essential for entry into DNA synthesis (26, 58), ectopic expression of E2F-1 has been shown to drive cells into S phase without a requirement for these kinases (16). The ability of E7-expressing cells to continue cycling despite a great reduction in cyclin E- and cyclin A-associated kinase activities may reflect an ability of E7 to directly target pRB and release E2F activity (52). However, we have consistently seen that low levels of kinase activity are retained in these cells, an observation reflected in the retention of phosphorylated pRB in E7-expressing cells.

Studies with adenovirus E1A, a protein with some structural similarity to E7 within the pRB binding domain, have shown that E1A binds directly to the p21$^{waf1/cip1}$-related kinase inhibitor p27 (48), and a recent report documents the inhibition of p27 activity by E7 through an apparently indirect interaction (79). It is therefore possible that E7 can inactivate p21$^{waf1/cip1}$ through direct binding, although we have been unable to show E7/p21$^{waf1/cip1}$ interactions either by immunoprecipitation of radiolabeled cell lysates, by immunoblotting from E7-expressing cells following actinomycin D treatment or by a direct binding assay with in vitro-translated proteins. Nevertheless, it is possible that the slight protection of cyclin-dependent kinase function seen in E7-expressing cells reflects an inefficient interaction between E7 and p21$^{waf1/cip1}$. Another possibility is that E7 protects a small proportion of the cyclin E- and cyclin A-associated kinase activities through direct interaction. Unlike the cyclin E- and cyclin A-associated kinases, the E7-associated kinase is not inhibited following actinomycin D treatment, suggesting that it is not affected by p21$^{waf1/cip1}$. The demonstration that this kinase activity can phosphorylate a GST-pRB fusion protein in vitro is consistent with the possibility that E7 directs phosphorylation of pRB in cells. This raises the intriguing possibility that E7 may act catalytically in the inactivation of pRB, as does E6 in p53 degradation. The identity of the E7-associated kinase activity is not entirely clear, and although there is strong evidence that an association with cyclins A and E occurs through p107 (50), a direct interaction between cyclin A and E7 has also been described (69). It has been reported that p107 and p21$^{waf1/cip1}$ bind to cyclin-CDK2 complexes through the same domain (80), and if E7 is associated through p107, the resulting kinase complex may not be subject to p21$^{waf1/cip1}$ inhibition, as seen in our studies. It must be pointed out, however, that the E7-associated kinase activity is low compared to the overall cyclin-dependent kinase activity in the cell, and a physiological role for this function of E7 remains to be established.

A second cyclin A-related cell cycle checkpoint has recently been demonstrated in the requirement to phosphorylate and

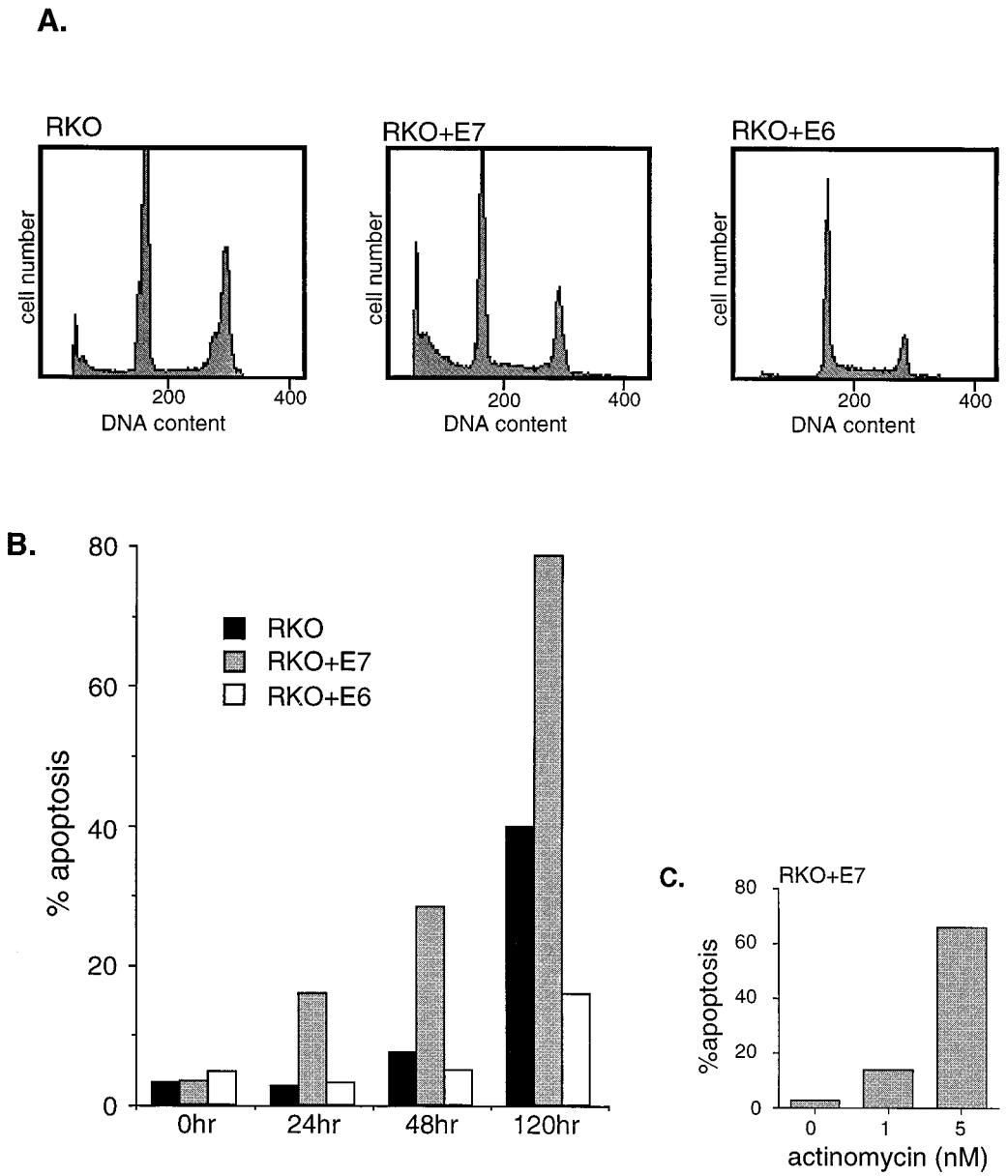

FIG. 6. E7-expressing lines are more susceptible to p53-induced apoptosis. (A) FACS profiles of RKO control and E6- and E7-expressing cells, demonstrating levels of apoptotic cells (sub-G_1 cells) in the respective lines after 48 h of treatment with 1 nM actinomycin D. (B) Levels of apoptosis in each of the cell lines over a 120-h time course in 1 nM actinomycin D. The percent apoptosis is calculated as the percentage of cells in the sub-G_1 population with respect to the total gated population. (C) Increased apoptosis of E7-expressing RKO cells in response to 5 nM actinomycin D. Cells were grown in 0, 1, or 5 nM actinomycin D for 48 h before being harvested for FACS analysis. The percent apoptosis was calculated from the sub-G_1 population.

inactivate E2F function as the cells exit S phase. Loss of cyclin A-mediated regulation of E2F function results in failure to leave S phase and activation of apoptosis (40). It is therefore possible that the enhanced apoptotic rate seen in the E7-expressing cells reflects an inability of E7 to maintain sufficiently high levels of active cyclin A following DNA damage. This suggestion is consistent with the observation that much higher apoptotic rates are seen in E7-expressing cells with extremely low cyclin A-associated kinase activity (such as those treated with 5 nM actinomycin D or those treated with 1 nM actinomycin D for several days) than in cells which retain a larger proportion of cyclin A-associated kinase function (such as those treated with 1 nM actinomycin D for 24 h). The resistance to p53-mediated cell cycle arrest shown by E7-expressing cells is likely to reflect deregulation of E2F and the protection of at least some cyclin-dependent kinase activity. Nevertheless, this is balanced by an increased apoptotic signal, which may be mediated in part by the loss of cyclin A kinase activity and the inability to inactive E7-induced E2F function as the cells complete DNA synthesis.

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