Differential Regulation of the Pocket Domains of the Retinoblastoma Family Proteins by the HPV16 E7 Oncoprotein[1]

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Abstract

The human papillomavirus E7 oncoprotein binds to the retinoblastoma (Rb) tumor suppressor protein, and the binding to Rb correlates with the oncogenic potential of E7. Recent studies from several laboratories indicated that the half-life of the Rb protein is reduced in cells that are stably transformed with E7, suggesting that E7 could induce the proteolytic degradation of Rb. To investigate whether the Rb degradation is a primary effect of E7 or a result of altered cell phenotype, we sought to develop assays that can distinguish between the two possibilities. Using recombinant adenovirus expressing the human papillomavirus type 16 E7 protein, we show that the expression of E7 leads to an increased rate of decay of the Rb protein. Moreover, Rb degradation immediately follows the expression of E7, suggesting that it is an early and primary effect. Consistent with a previous study, we observed that the E7-induced degradation of Rb can be blocked by the inhibitors of the 26S proteasome. We have also developed a transient transfection assay for the E7-induced degradation of Rb. Using this assay, we show that the pocket domain of Rb is necessary and sufficient for the E7-induced degradation. However, the proteolysis is relatively specific for Rb because the level of p107 or p130 was not significantly altered by the expression of E7. Thus, although E7 binds to all three members of the Rb family of proteins, the proteolysis is much more efficient in the case of Rb. In the transient transfection assays, adenovirus E1A and SV40 large T antigen failed to induce degradation of Rb, suggesting that the Rb degradation is a unique property of the E7 oncoprotein.

Introduction

HPVs[4] of the high-risk types (16, 18, 31, and 33) have been etiologically linked to malignant cancers of the genital cervix and oral cavities (reviewed in Refs. 1–5). Products of the HPV early genes *E6* and *E7* have been studied extensively in this regard because the biochemical functions of E6 and E7 correlate with the oncogenic potential of the HPVs (6–14). E6 and E7 are found to be expressed in HPV-associated tumors (1–4). Moreover, E6 and E7 could efficiently immortalize primary keratinocytes, which are the natural hosts of HPVs. Biochemical studies revealed that E6 and E7 mediate their immortalizing function by inactivating the cellular tumor suppressors such as p53 and Rb (1–4, 6–9). E6 binds to p53, leading to ubiquitination and proteolytic degradation of the ubiquitinated p53 through 26S proteasome (6, 15, 16). This is consistent with the observation that cells expressing E6 are genetically unstable (17), because p53 is believed to play an important role in maintaining genomic integrity (18). The E7 oncoprotein, on the other hand, exerts its function by regulating the cell cycle inhibitors Rb and the Rb family proteins p107 and p130 (8, 9, 19–23).

E7 is a potent activator of the cell cycle. We observed that the expression of E7 causes quiescent cells (G_0) to enter S phase without requiring the addition of serum or growth factors (23). Cheng *et al.* (24) demonstrated that expression of E7 does not alter the differentiation program in primary keratinocytes; however, the differentiated keratinocytes expressing E7 remain replication competent. The effects of E7 on cellular DNA replication and S phase progression were also observed in cells harboring UV-induced DNA damage. UV-induced DNA damage causes keratinocytes to arrest in G_1. Cells expressing E7, on the other hand, continue to progress through S phase after UV-induced DNA damage (25–28). E7 has been shown to overcome G_1 arrest induced by the cdk2 inhibitor p21 (23, 28). Cells expressing E7 are resistant to p21 inhibition. It has been shown that E7 could stimulate entry into S phase without reversing the inhibitory effect of p21 on the cdk kinases (23, 28). The cell cycle stimulatory function of E7 appears to correlate with its ability to regulate the Rb family of proteins (Rbs) and increase the levels of the activator forms of the E2F family of transcription factors (23, 28).

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[4] The abbreviations used are: HPV, human papillomavirus; cdk, cyclin-dependent kinase; pfu, plaque-forming unit(s); CR, conserved region; CMV, cytomegalovirus.

The E2F family of transcription factors (E2Fs) play a key regulatory role in the expression of a variety of genes required for cellular DNA replication and progression through S phase (29–32). For example, E2F1 has been shown to stimulate expression of dihydrofolate reductase, cyclin A, cyclin E, thymidine kinase, ribonucleotide reductase, cdc2, proliferating cell nuclear antigen, and other replication genes (29–32). The Rb family of proteins binds to the members of E2F and form negative regulatory complexes (29, 33–35). These negative regulatory complexes are believed to repress expression of the above-mentioned replication genes (23–35). Repression of the replication genes by the complexes of E2Fs/Rbs is believed to be critical in the mechanism by which cells are arrested at G_0-G_1 phase by growth factor or mitogen deprivation or by the overexpression of the cell cycle inhibitors such as p21 and p27 (36, 37). Consistent with this notion, expression of E7, which dissociates the interactions between E2Fs and Rbs, overcomes G_1 arrest and induces entry into S phase (23, 28). E7 dissociates the interactions between E2Fs and Rbs by directly binding to the Rb family of proteins (38). The E7-induced dissociation of the complexes of E2Fs/Rbs result in the release of E2Fs in their activator forms, which would stimulate expression of the replication genes. Therefore, it appears that binding to the Rb family of proteins and the consequent conversion of repressor E2Fs to activator E2Fs play a crucial role in the mechanism by which E7 stimulates entry into S phase.

Although binding to the Rb family of proteins is important for the progression into S phase, it is not sufficient for the transforming activity of E7. Binding to Rbs involves the E7 sequences that are homologous to the CR2 of the adenovirus E1A protein (19). However, the transforming function of E7 depends upon the NH_2-terminal sequences and the COOH-terminal zinc-binding motif in addition to the CR2 homology region (39–41). Thus, it would appear that E7 targets other cellular mechanisms through its NH_2- and COOH-terminal sequences to carry out its transforming function. Recent studies indicated that E7 could induce the proteolytic degradation of the Rb protein. Breast epithelial cells and keratinocytes constitutively expressing E7 protein contain greatly reduced levels of the Rb protein (27, 42). Moreover, it was shown that the Rb protein exhibits a much faster decay rate in the cells constitutively expressing E7, suggesting that E7 induces proteolysis of the Rb protein (27, 42).

To investigate the mechanism of Rb degradation in cells expressing E7, we sought to determine whether it is a primary effect of E7 expression or a result of altered cell phenotype. Using recombinant adenovirus expressing the HPV16 E7 protein, we show that the Rb degradation is a primary effect of E7 expression. Rb degradation was observed within a few hours of E7 expression. Moreover, we observed that lactacystin, a specific inhibitor of the 26S proteasome, blocked the E7-induced proteolysis of Rb, suggesting a role for the 26S proteasome in the process. We also describe a simple transient transfection assay for the E7-induced proteolysis of Rb. Using this assay, we show that E7 induces proteolysis of Rb much more efficiently than p107. The E7-induced proteolysis of Rb depends upon the integrity of the pocket domains. Moreover, the pocket domains of Rb are sufficient for the E7-induced degradation. In similar assays, the pocket domains of p107 were not significantly altered by the coexpression of E7, suggesting that E7 differentially regulates the pocket domains of the Rb family of proteins. Analysis of E7 mutants indicates that Rb-binding motif is necessary but not sufficient for the degradation of Rb, suggesting that other functions of E7 are involved in the Rb degradation process. Moreover, we show that the structurally related oncoproteins E1A and T antigen are incapable of inducing proteolysis of Rb.

Results

Rb Degradation Is an Early Effect of E7 Expression. Recent studies with mammary epithelial cells and primary keratinocytes stably expressing the HPV16 *E7* gene indicated that the E7 protein induces a proteolytic degradation of Rb (27, 42). Because these studies were performed using cells that stably expressed E7, we sought to determine whether the induction of Rb degradation is a primary effect of E7 expression. We used a short-term assay using a recombinant adenovirus expressing the HPV16 *E7* gene (hereafter referred to as E7-virus; Ref. 23). This E7-virus allowed us to analyze the effects on the Rb protein immediately following E7 expression. The effects of E7 expression were analyzed by using HaCaT cells because these cells are derived from human keratinocytes that are natural hosts of HPVs (43). Infection of the HaCaT cells with the E7-virus caused an accumulation of E7 that was readily detectable within 16 h of infection (Fig. 1).

To determine the effects of E7 on Rb, HaCaT cells were infected with the E7-virus or a control virus with increasing multiplicities of infection. The cells were harvested 18 h after infection, and lysates of these cells were subjected to Western blot analyses. The blots were probed with a monoclonal antibody against Rb (IF8; Santa Cruz Biotechnology). This antibody always cross-reacted with a M_r 100,000 polypeptide that is not related to Rb because several other Rb-specific antibodies did not recognize this polypeptide (data not shown). We observed that the extracts of cells infected with the E7-virus contained a much reduced level of the Rb protein compared to those infected with a control virus (Fig. 1, *lower panel*). Moreover, the reduction of the level of Rb protein in E7-virus infected cells closely followed E7 accumulation (compare Fig. 1 with the *left panel* of Fig. 2). The HaCaT cells can be synchronized to G_0-G_1 phases by incubating in serum-depleted medium. Stimulation of these cells by serum supplementation leads to the accumulation of the hyperphosphorylated form of Rb that is easily detectable after 16 h of serum addition (Fig. 2). Cells infected with the E7-virus, but not by a control virus, exhibited accumulation of a distinctly different form of the Rb protein at the 16-h time point (Fig. 2); and by 18 h of infection, this form of the Rb protein was reduced to an almost undetectable level (Fig. 2). Reasons for the accumulation of a distinct form of Rb in E7-virus-infected cells at the 16-h time point is unclear. It is possible that E7 binding results in an altered phosphorylation of the Rb protein. This is not unlikely because cyclin Ds, which phosphorylate Rb in serum-stimulated cells, bind Rb

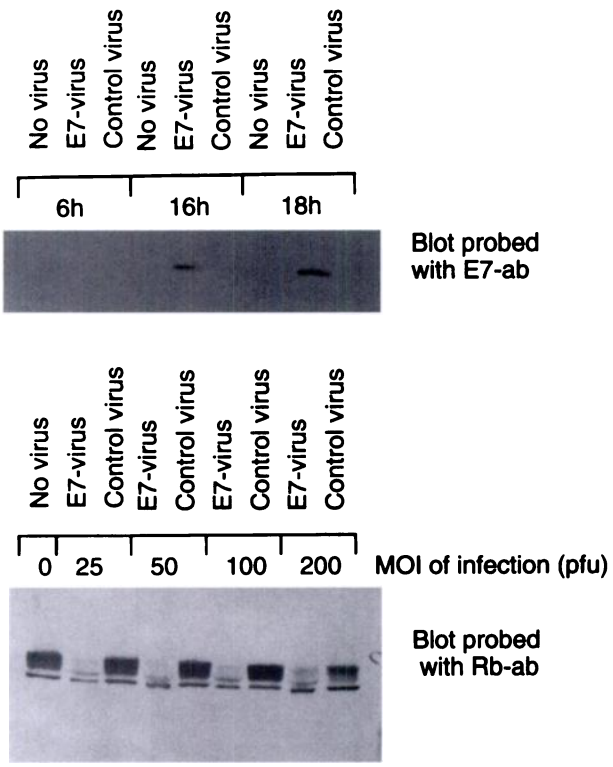

Fig. 1. Recombinant adenovirus-mediated expression of E7 reduces the level of Rb in HaCaT keratinocytes. *Upper panel,* E7 expression in E7-virus-infected HaCaT cells was assayed by Western blot analysis. Fifty μg of proteins from the lysates of cells at the indicated time points postinfection were subjected to SDS-PAGE, followed by Western blot analysis. The blot was probed with a monoclonal antibody against E7 and was developed by ECL. *Lower panel,* HaCaT cells were infected at different multiplicities of infection (25, 50, 100, and 200 pfu/cell) with the E7-virus or the control virus. Cells were harvested 18 h after infection, and 50 μg of protein from cell lysates were subjected to Western blot analysis. The blot was probed with a monoclonal antibody against Rb (IF8) and was developed with ECL. The fastest migrating band in the blot corresponded to a nonspecific band.

through their LXCXE motif (44, 45), a motif also found in E7 and was shown to be involved in the Rb/E7 interaction (19).

Expression of E7 specifically reduced the level of the Rb protein. p130 and p107 are Rb-related proteins, and both of these proteins were shown to interact with E7. However, we did not detect any significant reduction in the levels of the p130 and p107 proteins in the extract that showed a reduction in the Rb protein (Fig. 2). Thus, although Rb, p107, and p130 are related proteins, they are differentially regulated by E7 in keratinocytes. This might be important because Rb regulates cell cycle progression by interacting with four of the five known members of E2F family of transcription factors, whereas p130 and p107 interact with two members of the E2F family (46–48). Therefore, a more drastic reduction of the Rb function might be necessary for E7 to immortalize cells. The specificity was also confirmed by assaying for the cdk2 protein, which did not show any loss in the extracts containing E7 (Fig. 2).

To confirm that the loss of Rb protein was a result of an active proteolysis, we analyzed the half-lives of Rb in the presence and absence of E7. Pulse-chase experiments were performed to determine the decay rate of Rb. HaCaT cells were infected with the control virus or the E7-virus for 12 h at a multiplicity of infection 30 pfu/cell. The cells were then labeled with [^{35}S]methionine for 3 h, followed by chase in medium containing unlabeled methionine. At different time intervals, cells were harvested, and cell lysates were subjected to immunoprecipitation with a monoclonal antibody against Rb. Because we did not detect any E7-induced loss of p130, the supernatants obtained after immunoprecipitation with the Rb antibody were subjected to immunoprecipitation with p130 antibody to use as loading control. The immunoprecipitates were analyzed by SDS-gel electrophoresis and fluorography. During the 3.5-h chase period, the Rb protein in the control virus-infected cells did not exhibit any significant decay, whereas in the E7-virus-infected cells, there was a significant decay of the Rb protein (Fig. 3). These results are consistent with the notion that E7 induces the proteolytic degradation of Rb in HaCaT cells.

Inhibitors of the 26S Proteasome Blocks E7-induced Degradation of Rb. Further evidence for an E7-induced proteolysis of Rb was provided by studies with inhibitors of proteases and the 26S proteasome. 26S proteasome has been shown to be involved in regulating the levels of a variety of cell cycle regulatory proteins (reviewed in Ref. 49). Several inhibitors have been characterized that are highly specific for the 26S proteasome. Included are MG101, MG132, and lactacystin (50, 51). We analyzed the effects of MG101, MG132 (data not shown), lactacystin, and Calpain inhibitor II on the E7-induced degradation of Rb (Fig. 4). Cells were infected with the E7-virus. Fifteen h after infection, the inhibitors were added individually to the culture medium. After an additional 3-h incubation, cells were harvested, and the extracts were analyzed for the Rb protein. As can be seen in Fig. 4, the inhibitors, which are known to block the activity of the 26S proteasome, blocked the E7-induced degradation of Rb. Calpain inhibitor II, which does not inhibit the 26S proteasome, failed to inhibit the E7-induced degradation of Rb. These studies are consistent with the observation of Boyer *et al.* (42), who suggested that E7 reduces the level of the Rb protein by inducing its proteolytic degradation through the 26S proteasome.

Rb Degradation Is a Unique Property of E7: SV40 T Antigen and Adenovirus E1A Are Incapable of Inducing Rb Degradation. We also developed a transient transfection assay for the E7-induced degradation of Rb. Mammalian expression vectors containing the cDNAs of Rb, p107, HPV16 E7, HPV16 E6, and β-galactosidase were used in transfection experiments. Saos2 cells were used for the transfection studies because they lack Rb. After DNA transfection (see "Materials and Methods"), cell lysates of the transfected cells were analyzed for the levels of Rb by Western blot assays. The levels of the β-galactosidase in the transfected cell extracts were also determined. We observed that the E7 oncoprotein, but not E6 oncoprotein, caused a significant reduction in the levels of the Rb protein (Fig. 5*A*). The E6-expressing plasmid used in this experiment was used in functional assays by several laboratories including us (52, 53). The result is also consistent with a previous study

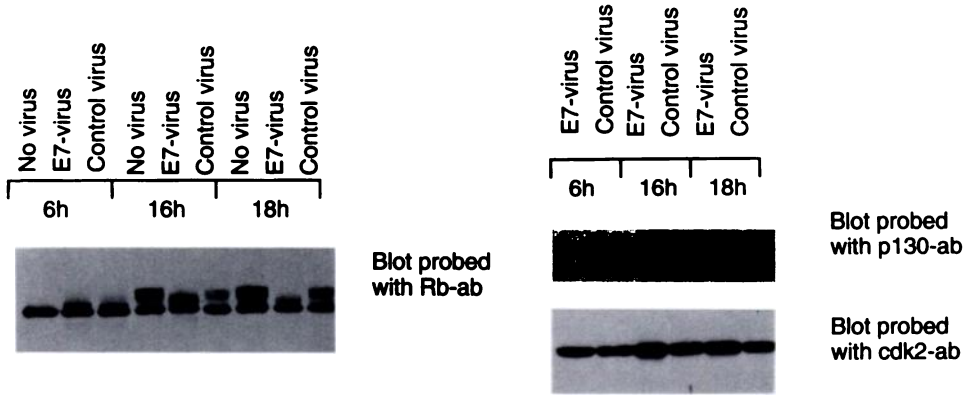

Fig. 2. Rb degradation is an early effect of E7 expression. HaCaT cells were grown to 80% confluence and then maintained in medium containing 0.5% FBS for 48 h. The serum-starved cells were then stimulated to enter the cell cycle by adding serum. The cells were infected with no virus, E7-virus, or control virus during serum stimulation. Cells were harvested at the indicated time points, and the cell lysates were subjected to Western blot analysis. *Left panel,* Western blot was probed with Rb antibody. *Right panel,* Western blots were probed with p130-antibody or cdk2-antibody. All of the blots were developed by ECL.

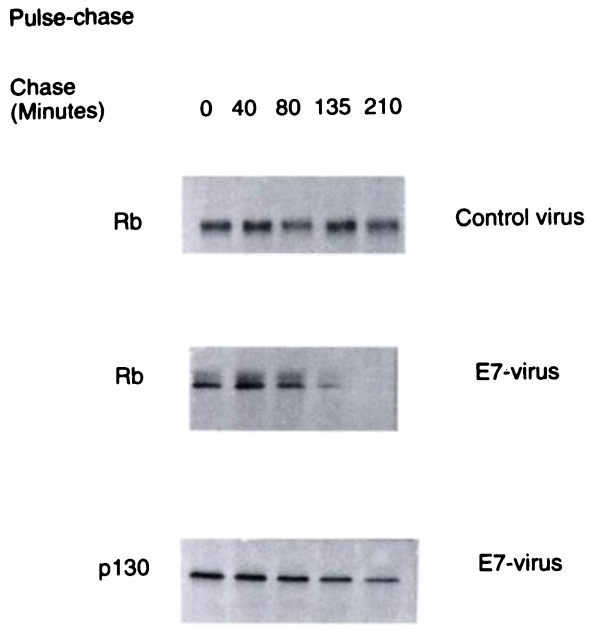

Fig. 3. E7 expression increases the decay rate of the Rb protein. The decay rate of Rb was analyzed by pulse-chase experiment. HaCaT cells were infected with the E7-virus or the control virus. Twelve h after infection, the cells were labeled with [^{35}S]methionine as described in "Materials and Methods." Following 3.5 h of labeling, the cells were incubated in the medium containing 150 mg/ml of unlabeled methionine. Cells were harvested at the indicated time points. Cell lysates containing equal trichloroacetic acid-precipitable counts were subjected to immunoprecipitation using a monoclonal antibody against Rb as described in "Materials and Methods." To control for loading, the supernatants obtained after Rb immunoprecipitation of the indicated samples were subjected to immunoprecipitation with the p130 antibody. The immunoprecipitates were analyzed by 7.5% SDS-gel electrophoresis, followed by fluorography. The Rb polypeptides from the control virus-infected cells migrated with a molecular weight of M_r 110,000–115,000, whereas those from the E7-virus-infected cells were M_r 107,000 and M_r 105,000.

that indicated that E6 is unable to induce degradation of Rb (42).

Previous studies on the HPV16 E7 protein identified the region or sequences of E7 required for its immortalization function. It was shown that the sequences at the NH_2-terminal region of E7, which is homologous to the CR1 of E1A, the LXCXE motif, and the COOH-terminal zinc-binding motif are required for the immortalization function of E7. Studies by several groups (45–46) also indicated that Rb binding alone is not sufficient for the immortalization function of E7. The LXCXE motif of E7 is required for Rb binding; however, the functions of the other two regions have remained elusive. To investigate E7 sequences involved in Rb degradation, mutants of E7 were analyzed in transient transfection assays. Expression of E7 mutants in Saos 2 cells was confirmed by Western blot experiments (data not shown). We were unable to detect expression of the COOH-terminal mutants in these cells; therefore, the COOH-terminal mutants were not analyzed.

To analyze the NH_2-terminal mutants for their ability to degrade Rb, the mutants were individually transfected into Saos 2 cells along with an Rb expression plasmid. Lysates of the transfected cells were analyzed for the levels of Rb protein by Western blot assay as described in "Materials and Methods." The mutants 16E7(C24-S) and 16E7(E26-Q) that are defective in Rb binding were also impaired in their ability to induce degradation of Rb (Fig. 5*B*), suggesting that Rb binding is required for the degradation process. The CR2 domain mutant 16E7 (delEDE) with mutation outside the Rb-binding domain can also induce Rb degradation efficiently. Moreover, a mutant harboring changes in the NH_2-terminal sequences, 16E7 (del PTLHE), which binds Rb, was also defective in inducing the degradation of Rb (Fig. 5*B*). The E7 mutants used in this study were characterized previously (39). To rule out the possibility that the results are artifacts of unequal expression of the mutants, the experiments were performed several times using different levels of mutant E7-expressing plasmids (data not shown). These results clearly indicated that Rb binding is not sufficient for its degradation (Fig. 5*B*). It is also noteworthy that the NH_2-terminal mutants that are defective in Rb degradation are also defective in the immortalization function.

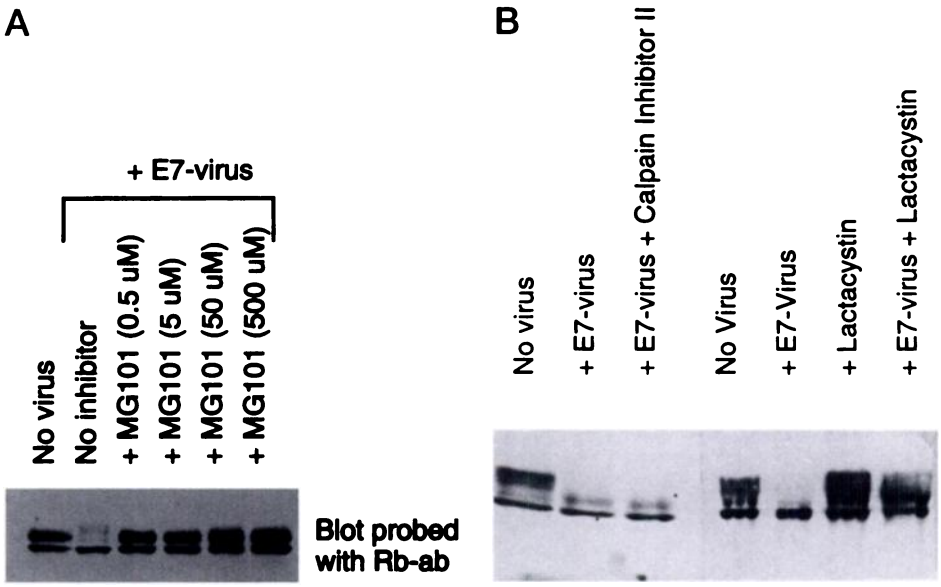

Fig. 4. Inhibitors of the 26S proteasome block E7-induced reduction of Rb. HaCaT cells were infected with 25 pfu/cell of the E7-virus or the control virus. In *A*, after 14 h of infection, the E7-virus-infected cells were treated with increasing concentrations of the protease inhibitor MG101 (0.5–500 μM). Following 4 h incubation, the cell lysates were subjected to Western blot analysis with the Rb antibody. *B*, after 14 h of infection, the E7-virus-infected cells were treated with lactacystin (10 μM) or Calpain inhibitor II (50 μM), and following 4 h of incubation, cells were harvested. Cell lysates (50 μg) were subjected to Western blot analysis with a monoclonal antibody against Rb and developed with ECL.

The Rb protein is regulated by at least three DNA tumor virus oncoproteins: the adenovirus E1A, SV40 large T antigen, and human papillomavirus E7 (reviewed in Ref. 54). Genetic analyses clearly indicated that the ability to bind Rb is critical for the transforming activities of E1A, T antigen, and E7 (54). These oncoproteins contain a common motif (LXCXE), which is involved in binding to the Rb protein (54). It is believed that this common motif in the oncoproteins encoded by the DNA tumor viruses is important to inhibit the growth-regulatory function of Rb and stimulate expression of the E2F-regulated replication genes of the host cells, which is also important for the viral DNA replication. To determine whether E1A and T antigen, like E7, can induce degradation of Rb, Saos 2 cells were transfected with mammalian expression plasmids containing cDNA of E1A and T antigen along with an Rb expression plasmid. Expression of E1A and T antigen was confirmed by Western blots that were probed with specific antibodies (Fig. 6*A*). These plasmids were shown to produce functionally active E1A and T antigen (38, 55–58). The lysates of the transfected cells were also assayed for the Rb protein. As can be seen in Fig. 6*A*, expression of E1A or T antigen had no detectable effect on the steady-state level of the Rb protein. Moreover, infection of HaCaT cells with E1A-expressing adenovirus dl313 did not result in a loss of the Rb protein (Fig. 6*B*). Thus, although T antigen and E1A possess the CR1 and CR2 homology regions, these oncoproteins were unable to induce degradation of Rb.

The Pocket Domains of Rb, but not of p107, Are Necessary and Sufficient for the E7-induced Proteolysis. Rb and p107 exhibit sequence homology within their pocket domains, and the sequences outside the pocket domains are comparatively less homologous (54). E7 binds to Rb and p107 through their pocket domains (19). Because the level of Rb was specifically reduced by E7, we sought to determine what region within the Rb protein is important for the E7-induced proteolysis. The pocket domains of Rb lie between residues 379 and 792. We observed that an NH_2-terminally truncated Rb protein containing the pocket domains and the COOH-terminal sequences (containing sequences between 379 and 928) was efficiently degraded by the coexpression of the E7 protein (Fig. 7*A*). To analyze the role of the pocket domains, we used three mutants of Rb that harbor changes within the pocket region. Two deletion mutants lacking sequences encoded by the exons 21 and 22, respectively, as well as a point mutant (706 C-F), which was defective in pocket domain function, were analyzed. These mutants are impaired in binding to the DNA virus oncoproteins as well as to the E2F family of transcription factors (55–59). Coexpression of E7 did not alter the steady-state levels of these mutant Rb proteins, whereas an Rb protein containing the intact pocket domains was degraded by the E7 expression (compare lanes in Fig. 7*A*). To investigate whether the pocket domains of Rb are sufficient for the E7-induced proteolysis, a mutant Rb protein that contains the sequences between amino acid residues 379 and 792 was used. The pocket domains of Rb were expressed at a high steady-state level. Coexpression E7 clearly reduced the level of the Rb pocket domains (Fig. 7*B*). These results are consistent with the notion that the pocket domains of Rb are necessary and sufficient for the E7-induced proteolysis. These results also suggest that the pocket domains of Rb and p107 are differentially regulated by the E7 oncoprotein of HPV. We also analyzed the wild-type and pocket domain mutants of the p107 protein in side-by-side assays. As can be seen in Fig. 7*C*, expression of E7 had very little effect on the steady-state levels of the p107 proteins. These results suggest that the pocket domains of Rb are critical for the E7-induced degradation.

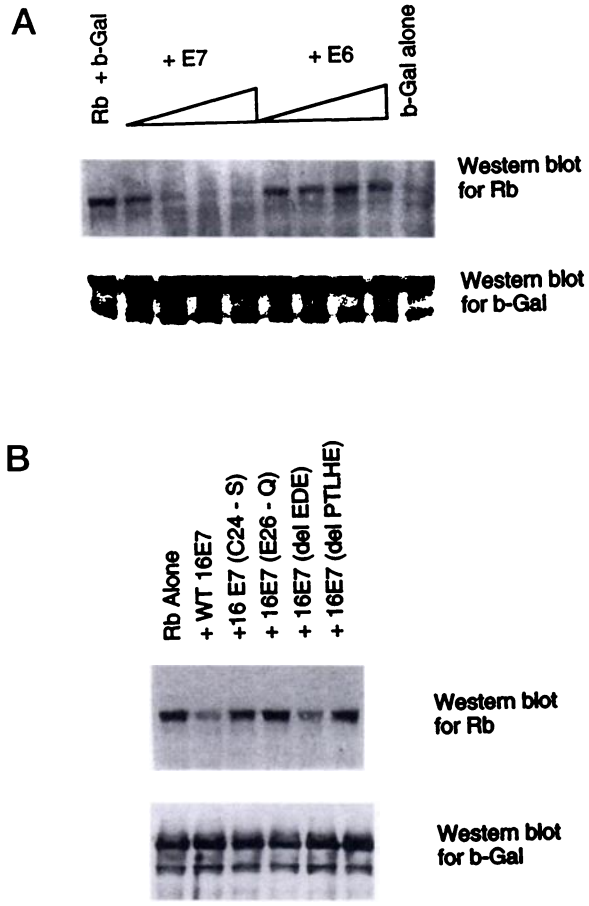

Fig. 5. A transient transfection assay for the E7-induced degradation of Rb. Sequences in the CR1 and CR2 regions of E7 are essential for Rb degradation. *A,* Saos 2 cells were transfected with a plasmid expressing Rb (15 μg) along with 1, 2, 4, or 10 μg of an E7 or an E6 expression plasmid. All transfection mixtures contained 1 μg of a plasmid expressing β-galactosidase. DNA transfection was carried out using the calcium-phosphate precipitation method as described in "Materials and Methods." Extracts of the transfected cells containing equal β-galactosidase were analyzed for the Rb protein (*upper panel*) by a Western blot assay. *Lower panel,* a Western blot assay for the β-galactosidase. The blots were developed with ECL. *B,* Saos 2 cells were transfected with a plasmid expressing Rb (15 μg) along with plasmids expressing the indicated mutants of E7 (7 μg each). Mutants 16E7 (C24-S), 16E7 (E26-Q) and 16E7 (delEDE) harbor changes in the CR2 region of E7, whereas the mutant 16E7 (delPTLHE) corresponded to a deletion in the CR1 region of E7. Extracts of transfected cells containing equal β-galactosidase were analyzed for the Rb protein by a Western blot assay (*upper panel*). A Western blot for β-galactosidase is shown in the lower panel.

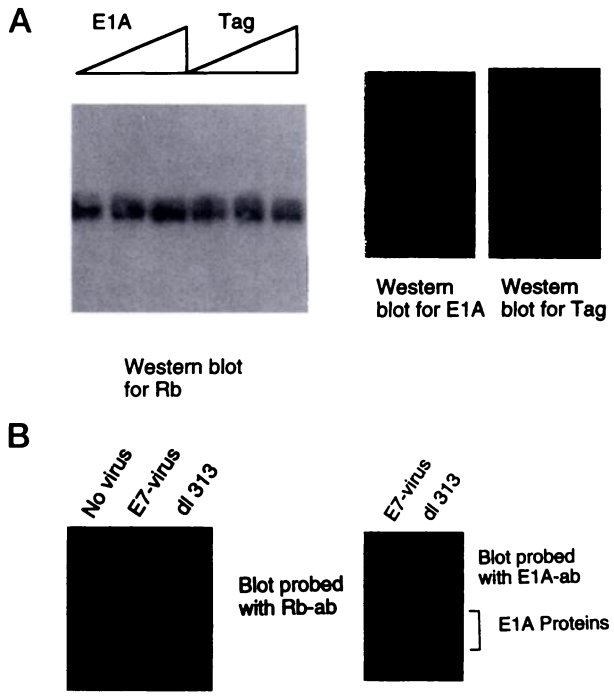

Fig. 6. Adenovirus E1A and SV40 T antigen are incapable of inducing degradation of Rb. *A,* Saos 2 cells were transfected with plasmid expressing Rb (15 μg) along with 1, 2.5, or 5 μg of E1A of T-antigen expression plasmids. Extracts of the transfected cells containing equal β-galactosidase were assayed for the Rb protein by a Western blot assay (*left panel*). Extracts from the 2.5 and 5 μg E1A or T-antigen-transfected cells were also assayed for the expression of E1A (*middle panel*) and T antigen (*right panel*) by Western blot assays using specific antibodies. *B,* HaCaT cells were infected with 25 pfu/cell of the E7-virus or the E1A-encoding adenovirus dl313. Eighteen h after infection, cells were harvested, and 50 μg of cell lysates were analyzed by Western blot analysis. *Left panel,* the blot was probed with Rb antibody and developed with ECL. The fastest migrating band is unrelated to Rb. *Right panel,* the blot was probed with E1A antibody and developed with ECL.

Discussion

The work described here is significant in several ways: (*a*) it demonstrates that the degradation of the Rb tumor suppressor protein is an early effect of E7 expression; (*b*) this study clearly demonstrates that E7 differentially regulates the pocket domains of Rb, p130, and p107; and (c) this study also demonstrates that structurally related oncoproteins E1A and T antigen that bind Rb are incapable of inducing proteolysis of Rb.

To understand why Rb exhibits a faster decay rate in E7-expressing cells, it was important to investigate whether the effect of E7 on the stability of Rb is a primary effect of E7 expression or an effect of an E7-induced altered phenotype, such as a faster growth rate of the E7-transformed cells. We used recombinant adenovirus to express E7 and assay the reduction of the endogenous Rb in HaCaT keratinocytes. The advantage of this system is that one can assay Rb at various time points after E7 expression. We showed that the Rb degradation follows very closely the expression kinetics of E7. Therefore, these results will be consistent with the notion that Rb degradation is an early effect of E7.

We observed that the steady-state levels of p107 and p130 were not altered by the expression of E7. It is important to note that, although Rb, p107, and p130 are considered related proteins, the biochemical function of Rb is significantly different from those of p107 and p130. Mice harboring null homozygous mutation in the *RB* gene (*RB−/−*) are embryonically lethal, suggesting that p130 or p107 cannot compensate for the loss of Rb function (60, 61). Mice lacking both copies of the *p107* and *p130* genes are developmentally impaired and undergo postnatal death (62). The defects in *RB−/−* embryos and *p107−/−*,

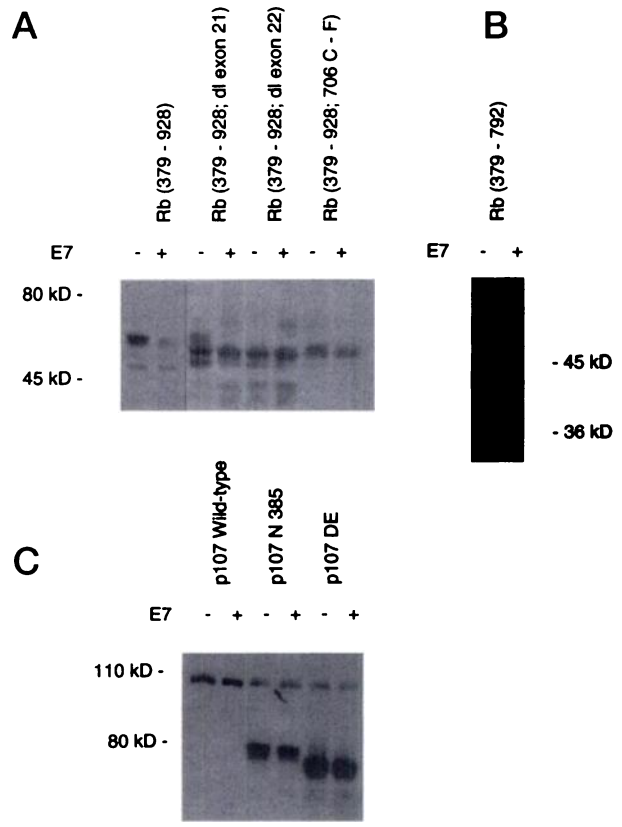

Fig. 7. Pocket domains of Rb are necessary and sufficient for the E7-induced degradation of Rb. In *A* and *B*, Saos 2 cells were transfected with plasmid (10 μg) expressing the indicated mutants of Rb along with 6 μg of the wild-type HPV 16 E7-expressing plasmid. Transfection mixture also contained 1 μg of β-galactosidase expression plasmid. Extracts of the transfected cells containing equal β-galactosidase were assayed for the Rb protein by a Western blot assay. The mutant Rb (379–792) contained the only the pocket domains of the Rb protein. In *C*, Saos 2 cells were transfected with plasmid (10 μg) expressing the indicated mutants of p107 along with 6 μg of the wild-type HPV16 E7-expressing plasmid. Transfection mixture also contained 1 μg of β-galactosidase expression plasmid. Extracts of the transfected cells containing equal β-galactosidase were assayed for the p107 protein by a Western blot assay. p107N385 corresponded to an NH_2-terminal deletion mutant lacking the first 385 amino acid residues, and it contains intact pocket domains of p107. p107DE corresponded to the deletion mutant that lacks amino acid residues 407 to 827 of the pocket domains of p107.

p130−/− embryos are different (60–62), which is consistent with the notion that the functions carried out by Rb are different from those performed by p130 and p107. Studies on the interactions of the Rb, p107, and p130 with the members of the E2F family also indicated differences. For example, Rb interacts with E2F1, E2F2, E2F3, and E2F4, whereas p107 and p130 interact with E2F4 and E2F5 (46–48). Thus, Rb could regulate transcription through four of the five E2Fs, which is believed to be important for its tumor suppression function. It is also noteworthy that mutations in the *RB* gene are found in human tumors more frequently than mutations in the *p107* and *p130* genes. Therefore, it is not surprising that HPVs, which associate with human tumors, have evolved several mechanisms to eliminate the functions of the Rb protein. Recent studies also showed that E7 could stimulate expression of cyclin E and cyclin A, which could theoretically cause phosphorylation and inactivation of Rb (63). The E7-induced proteolysis of Rb would be potentially important under conditions of low cyclins, such as in resting or quiescent cells.

In this study, we also show that the pocket domain of Rb is necessary and sufficient for the E7-induced degradation of the Rb protein. E7 binds to p130 and p107 through their pocket domains. Therefore, it is surprising that p130 and p107 are not efficiently degraded by the expression of E7. It cannot be due to lack of binding or low-affinity binding because stable complexes containing E7 and p107 or p130 can be easily detected in extracts of cells expressing E7 (19–23). It is possible that binding of cyclins (E or A) to the spacer region of p107 and p130, which lies between the pocket domains, prevents the E7-induced proteolysis of p107 and p130. p107 and p130, unlike Rb, forms stable complexes with cyclin-cdk2 in dividing cells, and the cyclin-cdk2 are not displaced by E7 binding (20). Thus, it is possible that cyclin-cdk2-mediated phosphorylation or binding of cyclin-cdk2 prevents E7-induced degradation of p107 or p130. On the other hand, it is possible that the pocket domain of Rb contains a specific sequence element, which is not present in p107 and p130, that is critical for the E7-induced proteolysis of the Rb protein. Clearly, further work is necessary to determine the basis of the specificity of Rb degradation by E7.

We observed that the Rb degradation depends on the Rb-binding motif (CR2 motif) of E7, along with sequences NH_2-terminal (CR1 motif) to the Rb-binding motif. These sequences are also found in other viral oncoproteins, such as the SV40 T antigen and the adenovirus E1A. T antigen and E1A were shown to efficiently bind Rb. However, we were unable to detect proteolytic degradation of Rb by overexpressing either E1A or T antigen, suggesting that these two oncoproteins are incapable of reducing the half-life of Rb. Obviously, these observations indicate differences between E7 and E1A or T antigen. The COOH-terminal sequences of E7 do not possess any significant homology with E1A or T antigen. It is possible that this region is essential for degradation of Rb through the 26S proteasome pathway. It is noteworthy that the COOH-terminal sequences of E7 possess homology with the E6 protein that is known to interact with the ubiquitin-proteasome pathway. The major problem of the COOH-terminal sequences of E7 is that mutations in this region reduce the stability of the E7 protein inside cell and, therefore, are difficult to analyze. We have recently identified an interaction between E7 and the S4 subunit of the 26S proteasome (64). *In vitro* this interaction between E7 and the 26S proteasome depends on the COOH-terminal Cys-X-X-Cys motif of E7 (64). It is possible that the interaction between E7 and the 26S proteasome is involved in the mechanism by which E7 induces degradation of Rb. Development of an *in vitro* system for an E7-dependent proteolysis of Rb by the 26S proteasome will be crucial in determining the mechanism of Rb degradation by E7.

Materials and Methods

Cell Cultures and Viruses. HaCaT cells were maintained in DMEM containing 10% fetal bovine serum. Construction of the recombinant adenovirus expressing HPV16 E7 has been described elsewhere (23). The control virus used in this study expresses a mutant E7. However, the mutant E7 was produced at a much reduced level (23) and is, therefore, used as an infection control. The recombinant viruses as well as the wild-type adenovirus (type 5) and the mutant adenovirus dl313 were grown in 293 cells. The virus titers were determined following a procedure described previously.

Virus Infection. HaCaT cells were infected with the adenoviruses in 2 ml of medium containing no serum for 1 h at 37°C. After infection, cells were maintained in DMEM containing 10% fetal bovine serum. During a 12–22-h infection period with the recombinant viruses and the dl313 virus, we did not detect any cytopathic effect. The infected cells were harvested and were washed three times with PBS before protein extraction.

Western Blot Analysis. For Western blots, cell lysates were prepared by using RIPA buffer that contained SDS, sodium deoxycholate, phosphatase inhibitors, and protease inhibitors. The Western blots were performed using standard procedure, and the blots were developed with ECL reagents (Amersham). The monoclonal antibody against E7 was obtained from Triton Diagnostics (Alameda, CA). The Western blots of Rb were carried out using a monoclonal antibody (IF8; Santa Cruz Biotechnology, Santa, Cruz, CA). This antibody always cross-reacts with an unrelated M_r 100,000 polypeptide in HaCaT cell extracts. The antibodies against p130 and cdk2 were purchased from Santa Cruz Biotechnology.

Pulse-Chase Experiment. HaCaT cells were infected with recombinant adenoviruses at a multiplicity of infection 20 pfu/cell. Twelve h after infection, the medium was replaced by 2 ml of medium (per 10-cm dish) lacking methionine. The methionine-free medium also contained 10% dialyzed fetal bovine serum. After a 30-min incubation with the methionine-free medium, 0.5 mCi of [^{35}S]methionine was added to the medium. The labeling was continued for 3.5 h. The medium was then replaced by the chase medium, which contained 150 mg/l of unlabeled methionine. At different time intervals, cells were harvested for further analysis. The labeled cells were lysed in buffer containing 1% NP40, 0.25 M NaCl, 50 mM Tris-HCl (pH 8.0), 50 mM NaF, 1 mM orthovanadate, and 2 mM phenylmethylsulfonyl fluoride. The lysates were precleared and subjected to immunoprecipitation using 2 μg of a monoclonal antibody (G3-245) against Rb (PharMingen) or a polyclonal antibody against p130 (C-18; Santa Cruz Biotechnology).

Plasmids and Expression Constructs. The wild-type and mutant CMV driven Rb and p107 expression plasmids have been described previously (57, 65). The CMV-driven expression plasmids for E6, E7, SV40 T antigen, and E1A have been described previously.

DNA Transfection. Saos-2 cells were transfected at 40–50% confluence, using the calcium phosphate coprecipitation method as described previously (66). DNA precipitates were removed 12 h after transfection, and the cells were replenished with fresh medium. The cells were harvested 30 h later. In each transfection experiment, 1 μg of β-galactosidase (lacZ) expression plasmid driven by CMV promoter was included as internal control. The cells were lysed in buffer containing 20 mM HEPES (pH 7.9), 0.1% NP40, 0.4 M NaCl, 1 mM EDTA, 2.5 mM DTT, 20% glycerol, and supplemented with 1 μg each of aprotinin, leupeptin, and pepstatin per ml and 10 μM phenylmethylsulfonyl fluoride. Five μl of the cell extract was used to assay for the β-galactosidase. The level of the β-galactosidase activity was not affected by E6 or E7 or Rb expression in Saos-2 cells and, therefore, the cell lysates normalized against the β-galactosidase were subjected to Western blot analysis.

Antibodies. The Rb monoclonal antibodies G3-245 and XZ55 were from PharMingen. The Rb monoclonal antibody IF8 was from Santa Cruz Biotechnology. The p107 polyclonal antibody (C-18) and the p130 polyclonal antibody (C-20) were from Santa Cruz Biotechnology as well. The E1A monoclonal antibody (M73) and the SV40 T antigen monoclonal antibody (PAb 419) were from Oncogene Science. The β-galactosidase monoclonal antibody (clone D19-2F3-2) was from Boehringer Mannheim.

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