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E7 Protein of Human Papilloma Virus-16 Induces Degradation of Retinoblastoma Protein through the Ubiquitin-Proteasome Pathway[1]

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Abstract

Rb protein is a critical regulator of entry into the cell cycle, and loss of Rb function by deletions, mutations, or interaction with DNA viral oncoproteins leads to oncogenic transformation. We have shown that the human papilloma virus (HPV)-16 *E7* gene is sufficient to induce the immortalization of mammary epithelial cells (MECs). Surprisingly, the steady-state level of Rb protein in these immortal cells was drastically decreased. Here, we used pulse-chase analysis to show that the *in vivo* loss of Rb protein in E7-immortalized MECs is a consequence of enhanced degradation. Expression of HPV16 E7 in a cell line with a temperature-sensitive mutation in the E1 enzyme of the ubiquitin pathway demonstrated that degradation of Rb was ubiquitin dependent. Treatment of E7-immortalized MECs with aldehyde inhibitors of proteasome-associated proteases led to a marked stabilization of Rb protein, particularly the hypophosphorylated form. Taken together, our results provide evidence for HPV-16 E7-induced enhanced degradation of Rb protein via a ubiquitin-proteasome pathway and suggest a second mechanism of oncogenic transformation by E7, in addition to its previously identified ability to sequester Rb from E2F. Our analyses also show that normal Rb levels are regulated by the ubiquitin-proteasome degradation pathway.

Introduction

The Rb[3] protein, first identified as a tumor suppressor protein, plays a critical role to control the length of the G_1 phase of cell cycle (1, 2). Rb protein is found in cells in a hyperphosphorylated (p112-114) and hypophosphorylated (p110) form (3). The p110 Rb protein complexes with and sequesters the E2F family of transcription factors, thereby preventing transcription of critical genes, the products of which are essential for cell cycle progression (4). These E2F targets include prominent cell cycle regulatory genes, such as *cdc2, thymidine kinase, myb, dihydrofolate reductase,* and the *E2F1* gene itself (reviewed in Ref. 1). Rb protein phosphorylation by G_2-M and S-phase cyclin-dependent kinases releases E2F and allows it to transcribe its target genes, resulting in cell cycle progression (1, 4).

The Rb protein has also emerged as a target of cellular transformation mediated by a number of DNA viruses. For example, the adenovirus E1A and SV40 large T have been demonstrated to form complexes with pRb and inactivates its function (5, 6). In recent years, both *in vitro* and *in vivo* analyses have demonstrated that the E7 proteins of high-risk HPVs bind to Rb protein (7). The E7 protein specifically binds to hypophosphorylated Rb protein, thereby allowing E2F to be constitutively active; this is thought to result in uncontrolled cell cycle progression (reviewed in Refs. 1 and 7).

Recently, we demonstrated that introduction of the HPV-16 *E7* gene into normal human MECs led to their immortalization in the absence of any cooperating oncogenes (8). These cells allowed an assessment of the nature of E7-induced aberrations in the Rb protein. Surprisingly, these cells expressed dramatically lower levels of Rb protein with normal levels of Rb mRNA (8). Decreased levels of Rb protein have also been reported in E7-expressing keratinocytes and uroepithelial cells (9, 10). Thus, a decrease in the levels of Rb protein represents an additional potential mechanism of cellular transformation by HPV E7 oncoprotein. However, the mechanism of the E7-induced loss of Rb protein is unknown.

Here, we used pulse-chase analyses to demonstrate that *in vivo* loss of Rb in E7-immortalized MECs is a result of its enhanced rate of degradation. Using a cell line with a temperature-sensitive defect in the ubiquitin pathway and treatment of cells with peptide aldehyde inhibitors, we show that E7-induced Rb protein degradation is mediated by ubiquitin-proteasome pathway. These analyses reveal a novel mechanism by which a DNA tumor virus oncoprotein, such as HPV-16 E7, can induce cellular transformation.

Materials and Methods

Cells and Cell Culture. Reduction mammoplasty-derived normal MECs (76N, 81N, 70N, 7VN, and 8VN; Refs. 8 and 11), HPV-16 E7-immortalized MECs (76E7, 81E7, 70E7, 7VE7, and 8VE7), and E6-immortalized MECs (76E6, 81E6, 70E6, 7VE6, and 8VE6; Ref. 8) have been described earlier. All cell lines were grown in DFCI-1 medium (11). The ts20b cell line, which carries a temperature-sensitive mutation in ubiquitin activating enzyme (E1), was kindly provided by Dr. Harvey Ozer (UMDNJ-New Jersey Medical School, Newark, NJ) (12). The ts20b cells were retrovirally infected with an HPV-16 E7 expression vector (9) to derive E7-expressing cell line tsE7. The ts20b cells were grown in α-MEM (Life Technologies, Inc.) supplemented with 10% FCS. tsE7 cells were grown in the same medium supplemented with G418 (1 mg/ml).

Western Blot Analysis. Fifty μg of each cell lysate prepared in SDS-PAGE sample buffer (quantitated by bicinchoninic acid protein assay; Pierce) was resolved on 7.5% PAGE and transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore). Membranes were incubated with mAb against p53 (PAb 1801; Neomarkers) or Rb protein (mAb 245; PharMingen), followed by goat antimouse antibody conjugated to horseradish peroxidase (Pierce). Enhanced chemiluminescence (ECL) detection was performed according to the manufacturer's instructions (Amersham Corp.).

Determination of Rb Half-Life. Fifty to 70% confluent monolayers of cells were prestarved for 30 min in cysteine- and methionine-free α-MEM medium (Life Technologies, Inc.) and pulse-labeled for 15 min with 250 μCi of [^{35}S]methionine and [^{35}S]cysteine (Expre^{35}S^{35}S; DuPont NEN) followed by chase for the indicated time periods. At each time point, cells were washed with cold PBS and lysed in NETN lysis buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8, 0.5% NP40, and 1 mM phenylmethylsulfonyl fluoride). Lysates were precleared, and equal cpm of lysates were immunoprecipitated with an anti-p53 mAb, PAb 122 (hybridoma obtained from American Type Culture Collection), or anti-Rb antibody (Rb1; Neomarkers) and resolved by SDS-7.5% PAGE as described earlier (8).

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[3] The abbreviations used are: Rb, retinoblastoma; HPV, human papillomavirus; MEC, mammary epithelial cell; mAb, monoclonal antibody.

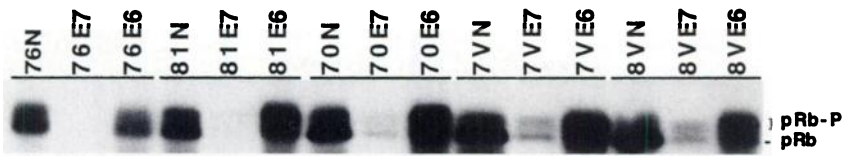

Fig. 1. Western blot analysis of Rb protein in normal (*76N, 81N, 70N, 7VN,* and *8VN*), E6 (*76E6, 81E6, 70E6, 7VE6,* and *8VE6*), and E7 (*76E7, 81E7, 70E7, 7VE7,* and *8VE7*) immortalized cells. Equal amounts of cell lysate (50 μg) were resolved by SDS-PAGE and subjected to Western blotting with an anti-Rb mAb. Blots were visualized using goat antimouse horseradish peroxidase conjugate followed by ECL. Note a dramatic decrease in the levels of Rb protein in E7-expressing cells. *pRb-P,* hyperphosphorylated Rb protein; *pRb,* hypophosphorylated Rb protein.

Results

Decrease in the Levels of Rb Protein Is a General Feature of E7-immortalized MECs. Earlier, we noted a dramatic decrease in the levels of Rb protein in HPV16 E7-immortalized MECs. To further extend these initial findings, we used anti-Rb immunoblotting of whole-cell lysates to assess the relative levels of Rb protein in a panel of normal MECs and their E6- or E7-immortalized derivatives. As shown in Fig. 1, in each case the E7-expressing cells showed a drastic decrease in the levels of Rb protein as compared to their paired normal or HPV16 E6-immortalized cells. As expected from our earlier studies (8, 13), p53 protein was selectively lost in E6-immortalized cells (data not shown).

Decrease in the Levels of Rb Protein Is Not a Consequence of Selection for Immortalization. Because emergence of E7-immortalized MECs is preceded by a period of crisis, during which a large proportion of cells die, we wanted to rule out the possibility that E7 immortalization selected a subpopulation of cells with intrinsically lower levels of Rb. Therefore, we introduced the HPV16 E7 into MEC strain 76N using retroviral infection and examined their Rb protein levels after 48 or 72 h after infection. A substantial time-dependent decrease in the levels of the Rb protein was seen following transient E7 expression (Fig. 2), directly supporting the role of E7 in inducing a decrease in the Rb protein levels.

Enhanced Degradation of Rb Protein in E7-immortalized MECs. A marked decrease in the steady-state levels of Rb protein in E7-immortalized MECs, together with unchanged levels of Rb mRNA (8), suggested the possibility of decreased Rb protein synthesis or its rapid degradation. To distinguish between these possibilities, normal or E7-immortalized MECs were metabolically pulse-labeled with [^{35}S]methionine and cysteine for 15 min, followed by chase for various time periods. Equal amounts of radiolabeled lysates (based on cpm) were immunoprecipitated with anti-Rb antibody and analyzed by SDS-PAGE, followed by fluorography. Relatively similar levels of hypophosphorylated Rb protein were observed at time "0" in both normal and E7-immortalized cells, indicating similar Rb protein synthesis (Fig. 3). A 12-h chase analysis showed that Rb signals remained essentially unchanged in normal MECs. In contrast, Rb protein was nearly undetectable in E7-immortalized cells after an 8-h chase period. Notably, the hypophosphorylated form of Rb was nearly undetectable in these cells at 3 h of chase. Taken together, these results demonstrate that Rb protein is much more rapidly degraded in E7-immortalized cells compared to that in normal MECs. As expected (8, 13), p53 protein showed similar half-life in normal and E7-immortalized cells.

The Ubiquitin Pathway Is Required for E7-induced *in Vivo* Loss of Rb. The levels of a number of cell cycle control proteins, such as p53 and p27, are regulated by targeting them for proteolytic degradation by the ubiquitin pathway (14, 15). This mechanism is used by the HPV-16 E6 oncoprotein to target p53 for enhanced degradation (16). These results, together with the evidence of enhanced Rb degradation in E7-immortalized MECs (above results), prompted us to assess the role of the ubiquitin pathway in E7-induced loss of Rb. For this purpose, we used a murine fibroblast cell line (ts20b) that carries a temperature-sensitive mutation in ubiquitin-activating enzyme E1 (12). This cell line has been used to demonstrate the role of the ubiquitin pathway in *in vivo* p53 degradation (12). We obtained a cell line tsE7 by stable transfection of ts20b cells with HPV16 E7. E7 expression was confirmed by immunoprecipitation with anti-E7 antibody (data not shown). Control ts20b and tsE7 cells were incubated for 15 h at 33°C (permissive temperature for E1 function) or 39°C (restrictive temperature), and the levels of Rb protein were examined by anti-Rb immunoblotting of whole-cell lysate. Rb protein was easily detectable in ts20b cells grown at 33°C. In contrast, the level of Rb protein in tsE7 cells grown at 33°C was substantially lower, as expected from E7-induced enhancement of Rb degradation (Fig. 4). Strikingly, when cells were shifted to nonpermissive temperature (39°C), rendering E1 enzyme inactive, an increase in the level of Rb protein was observed in both ts20b and tsE7 cells; however, the increase in tsE7 cells was more dramatic, further substantiating the conclusion that lower levels of Rb protein in tsE7 cells grown at 33°C were due to ubiquitin-dependent degradation (Fig. 4). In addition to demonstrating the role of the ubiquitin pathway in E7-induced degradation of Rb, these results also suggest a role for this pathway in determining the turnover of Rb protein in the absence of E7. Over the duration of the assay, no significant change in the cell cycle distribution was observed at either temperature, indicating that changes in Rb levels were not a consequence of changes in cell cycle (data not shown). The analysis of p53 protein in ts20b and tsE7 cells showed that E7 did not enhance p53 degradation, as demonstrated by comparable low levels of p53 protein at 33°C; the expected accumulation of p53 protein (14) was seen in both cells at 39°C.

Increase in the Levels of Rb Protein by Peptide Aldehyde Inhibitors of Proteases in the Proteasome Pathway. Ubiquitination is known to target cellular proteins for degradation by proteasome pathway (17). Recent studies have identified a series of peptide aldehydes that inhibit the chymotryptic and peptidylglutamyl peptidase activities of the 26S proteasome (18). We used these agents to investigate the role of the proteasome pathway in E7-induced Rb degradation. For this purpose, we analyzed levels of Rb protein in normal and E7-immortalized cells before and after their treatment

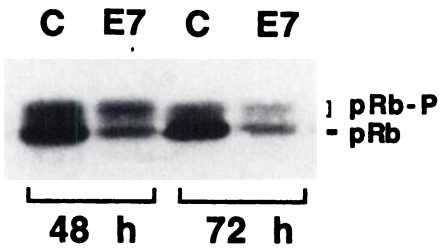

Fig. 2. Western blot analysis of cells after transient infection with retroviral HPV-16 E7. 76N normal MECs were infected with retroviral supernatants of the HPV-16 E7 producer cell line and analyzed for Rb protein after 48 and 72 h. Note a time-related decrease in the levels of Rb protein with a drastic loss at 72 h following infection. *C,* control (vector-infected) cells; *E7,* HPV-16 E7-infected cells.

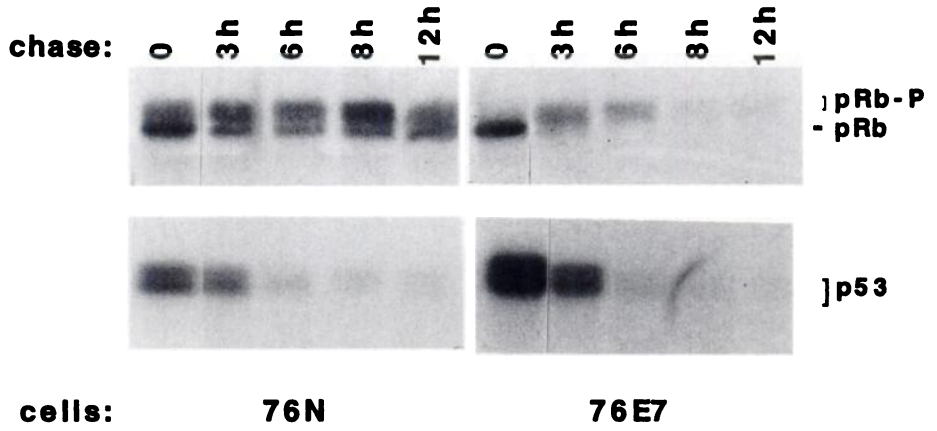

Fig. 3. Pulse-chase analysis of Rb protein in normal and E7-immortalized cells. 76N and 76E7 cells were metabolically pulse-labeled with [^{35}S]methionine plus cysteine for 15 min and chased for various time periods (shown in hours) as described in "Materials and Methods." Equal amounts of cell lysates (cpm) were immunoprecipitated using anti-Rb antibody and separated on SDS-7.5% polyacrylamide gels. Note that Rb signals decrease with a markedly faster kinetics in 76E7 cells as compared to a relatively stable Rb protein in 76N cells. Analysis of p53 immunoprecipitates from paired lysates shows a comparable time course of the loss of radioactivity in both cells.

with increasing concentrations of two peptide aldehydes, MG132 and LLnL, used previously to assess the role of the ubiquitin-proteasome pathway in *in vivo* degradation of other proteins (14, 18). The treatment of E7-immortalized MECs with both inhibitors led to a dramatic concentration-dependent increase in the steady-state levels of Rb protein (Fig. 5, *A* and *B*). This was accompanied by a substantial stabilization of the newly synthesized Rb protein, as determined by pulse-chase analysis (data not shown). As expected (14), the levels of p53 protein were increased with this treatment (Fig. 5, *A* and *B*). Comparison of Rb levels in inhibitor-treated normal and E7-immortalized MECs showed a dramatic accumulation of hypophosphorylated Rb in both cells, consistent with the role of the ubiquitin-proteasome pathway in the normal turnover of Rb (Fig. 5, *C* and *D*).

Taken together, these studies demonstrate that Rb protein is degraded by the ubiquitin-proteasome pathway and that E7 enhances Rb degradation by using this pathway.

Discussion

The Rb tumor suppressor gene product plays a seminal role in controlling cell cycle progression, and recent studies have demonstrated that the ability of the oncoproteins of DNA tumor viruses such as adenovirus E1A, SV40 large T, and human papilloma virus E7 to complex with Rb contributes to their transforming ability (5–7). This conclusion was further substantiated by the observation that Rb mutations in human cancers are concentrated within the Rb pocket, the area where proteins from DNA tumor viruses bind (reviewed in Ref. 1). Based on the inability of tumor virus oncoprotein to induce degradation of Rb in *in vitro* rabbit reticulocyte lysates, it has been suggested that complex formation is sufficient to inactivate Rb function (19). A plausible mechanism of the functional inactivation of Rb is provided by the selective complexing of DNA tumor viral oncoproteins with hypophosphorylated Rb, which in normal cells complexes with and sequesters the E2F family of transcription factors (1, 4, 6). Binding of E7 to Rb is thought to release the E2Fs, thus inducing the transcription of genes involved in promoting cell cycle progression, the promoters of which have binding sites for E2Fs (1, 4). Although the proposed sequestration of hypophosphorylated Rb by complex formation with DNA tumor viruses is well supported, additional mechanisms have not been fully explored. Interestingly, the HPV E7 mutants defective in Rb binding were still able to transform rodent cells in cooperation with ras and human keratinocytes in conjunction with the E6 protein (20, 21). These studies suggested that either E7 binding to Rb was not important for its transforming ability or that additional mechanisms of E7-induced functional inactivation may occur in cells.

One such mechanism was suggested by our recent studies of E7-immortalized human MECs, which unexpectedly showed a dramatic decrease in the levels of Rb protein in the absence of any change in Rb mRNA expression (8). Interestingly, the levels of Rb protein were also decreased in keratinocytes and uroepithelial cells in which E7 was expressed by retroviral infection (9, 10). Together, these studies suggested that *in vivo* loss of Rb protein may represent an additional mechanism by which E7 may eliminate Rb function. Here, we used analyses of stably as well as transiently E7-transfected MECs to provide clear evidence that HPV-16 E7 induces a marked *in vivo* loss of Rb protein by enhancing its degradation. Because expression of E7 alone is sufficient to immortalize these cells, our results indicate that the degradation of Rb contributes to E7-induced cellular transformation.

Recent studies have shown a role for the ubiquitin pathway in the degradation of a number of cell cycle-regulated proteins (14, 15). In this pathway, a series of cellular enzymes participate in ubiquitinating the cellular protein, thus targeting it for degradation by the multicatalytic proteolytic machinery associated with the 26S proteasome particle (17, 18). Using a cell line with a temperature-sensitive mutation in ubiquitin-activating enzyme E1 (12) and by using peptide aldehyde inhibitors of proteasome proteases (14, 18), we show that E7-induced degradation of Rb protein is mediated by the ubiquitin-proteasome

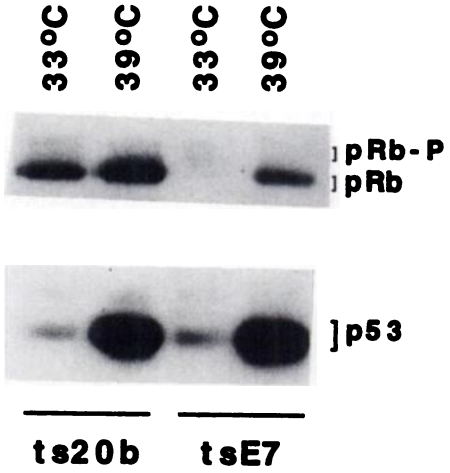

Fig. 4. Western blot analysis of Rb and p53 proteins in ts20b and tsE7 cells grown at permissive and nonpermissive temperatures. Parental ts20b cells (with a temperature-sensitive mutation in ubiquitin-activating enzyme E1) and its E7-expressing transfectant tsE7 were grown at 33°C (permissive temperature for E1 activity) or at 39°C (nonpermissive temperature) and analyzed for Rb and p53 protein by Western blot analysis. Note a marked decrease in Rb levels in tsE7 cells grown at 33°C compared to ts20b cells at 33°C, indicating enhanced degradation of Rb. Also note the accumulation of Rb and p53 proteins at 39°C.

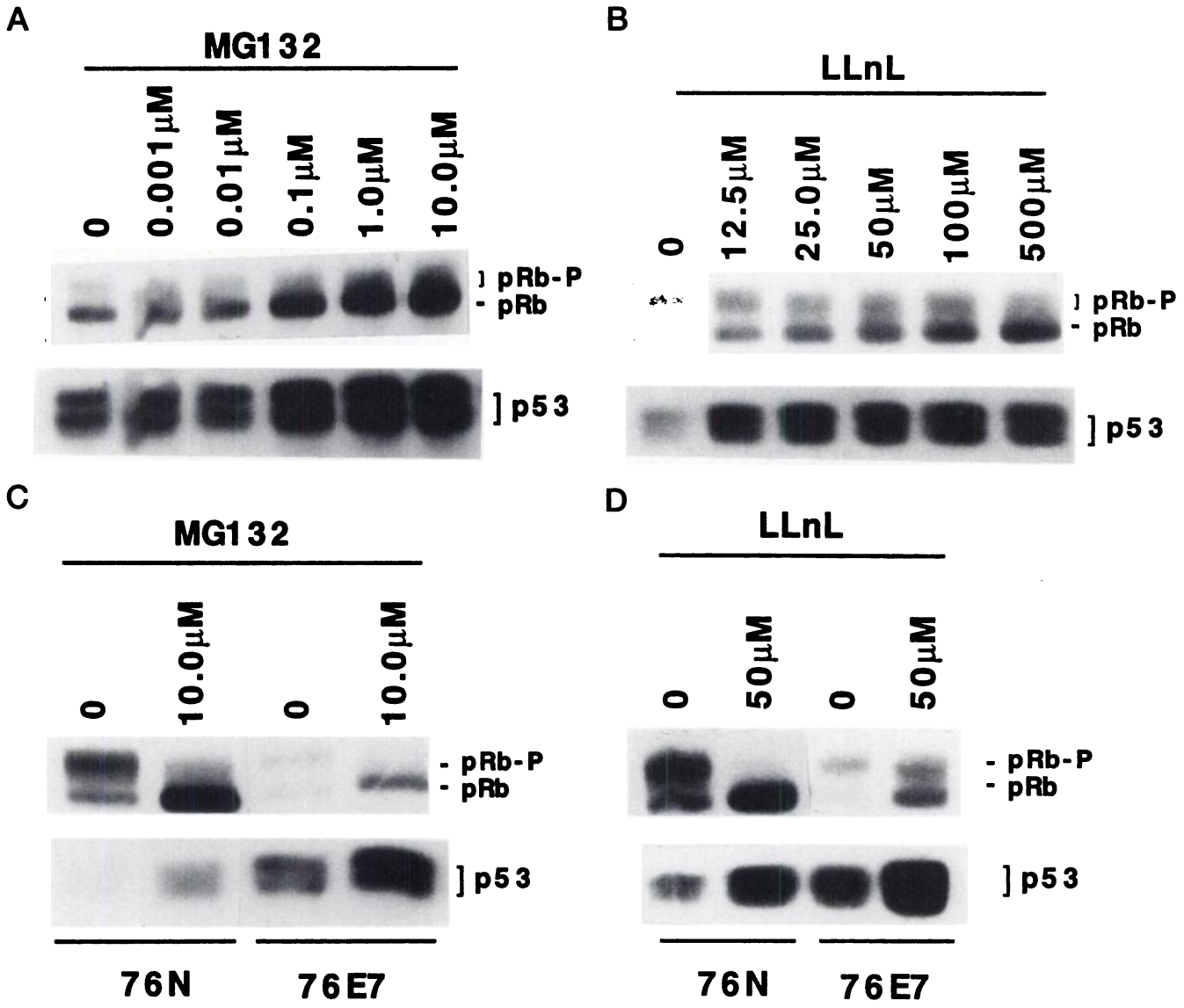

Fig. 5. Effect of peptide aldehyde inhibitors treatment on the levels of Rb protein. *A* and *B*, lysates from untreated 76E7 cells or cells treated with different concentration of MG132 (*A*) or LLnL (*B*) for 6 h were analyzed for Rb protein using anti-Rb antibody (*upper panel*) or anti-p53 antibody (*lower panel*) by Western blot analysis. Note a dose-dependent increase in the levels of Rb as well as p53 protein in cells treated with MG132 and LLnL. *C* and *D*, 76N and 76E7 cells were treated with 10 μM MG132 (*C*) or 50 μM LLnL (*D*) for 6 h and analyzed for the levels of Rb protein and p53 protein by Western blot analysis. Note an accumulation of hypophosphorylated Rb and p53 in both 76N and 76E7 cells treated with either MG132 or LLnL.

pathway. Similar approaches have been used to demonstrate that *in vivo* degradation of p53 protein in normal cells is mediated by the ubiquitin-proteasome pathway (12, 14). *In vitro* analyses have shown that HPV-16 E6 induces p53 protein for enhanced degradation by engaging this same pathway (16). However, similar *in vitro* assays have not detected E7-induced degradation of Rb protein (19). It is possible that this discrepancy reflects the deficiency of some critical cofactors in rabbit reticulocyte lysates commonly used for *in vitro* degradation assays. Alternatively, the sensitivity of *in vitro* degradation assays may be limiting. For example, we have shown earlier that HPV-6 E6, bovine papillomavirus (BPV)-1 E6, and certain HPV-16 E6 mutants, which failed to induce p53 degradation in *in vitro* rabbit reticulocyte lysates, were fully able to induce enhanced degradation of p53 protein *in vivo* when MECs immortalized with these oncoproteins were examined (13, 22).

In addition to demonstrating that the HPV-16 E7-induced enhanced degradation of Rb protein is mediated by the ubiquitin-proteasome pathway, our results also indicate the role of this pathway in regulating the turnover of Rb protein in normal cells. It is of interest that a recent report suggests the possible role of the interleukin converting enzyme protease in the cleavage of Rb during apoptosis (23). Under our experimental conditions, we did not observe apoptosis of cells (data not shown). However, it remains possible that the ubiquitin-proteasome degradation pathway may be involved in apoptosis-induced change of Rb protein as well. Whether interleukin converting enzyme plays a role in mediating degradation of Rb in normal or E7-transformed cells is not known at present. However, these results are consistent with the possibility that multiple proteolytic pathways may regulate the levels of Rb protein in normal and oncogenically transformed cells.

In conclusion, we have demonstrated that the HPV-16 E7-induced immortalization of MECs is accompanied by enhanced degradation of Rb protein, and that this degradation involves ubiquitin-proteasome-mediated proteolysis. We suggest that enhanced degradation provides a novel mechanism of E7-induced inactivation of Rb function in these

cells. Future experiments using E7 mutants should clarify the role of Rb degradation in MEC immortalization.

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References

1. Weinberg, R. A. The retinoblastoma protein and cell cycle control. Cell, *81:* 323–330, 1995.
2. Hollingsworth, R. E., Chen, P-L., and Lee, W-H. Integration of cell cycle control with transcriptional regulation by the retinoblastoma protein. Curr. Opin. Cell Biol., *5:* 194–200, 1993.
3. DeCaprio, J. A., Ludlow, J. W., Lynch, D., Furukawa, Y., Griffin, J., Piwnica-Worms, H., Huang, C. M., and Livingston, D. M. The product of the retinoblastoma gene has properties of a cell cycle regulatory element. Cell, *58:* 1085–1095, 1989.
4. Chellappan, S. P., Hiebert, S., Mudryl, M., Horowitz, J. M., and Nevins, J. R. The E2F transcription factor is a cellular target for the RB protein. Cell, *65:* 1053–1061, 1991.
5. Whyte, P., Buchkovich, K. J., Horowitz, J. M., Friend, S. H., Raybuck, M., Weinberg, R. A., and Harlow, E. Association between an oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature (Lond.), *334:* 124–129, 1988.
6. Ludlow, J. W., DeCaprio, J. A., Huang, C. M., Lee, W. H., Paucha, E., and Livingston, D. M. SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. Cell, *56:* 57–65, 1989.
7. Dyson, N., Howley, P. M., Munger, K., and Harlow, E. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science (Washington DC), *243:* 934–940, 1989.
8. Wazer, D. E., Liu, X. L., Chu, Q., Gao, Q. and Band, V. Immortalization of distinct human mammary epithelial cell types by human papilloma virus 16 E6 or E7. Proc. Natl. Acad. Sci. USA, *92:* 3687–3691, 1995.
9. Demers, G. W., Foster, S. A., Halbert, C. L. and Galloway, D. A. Growth arrest by induction of p53 in DNA damaged keratinocytes is bypassed by human papillomavirus 16 E7. Proc. Natl. Acad. Sci. USA, *91:* 4382–4386, 1994.
10. Yeager, T., Stadler, W., Belair, C., Puthenveettil, J., Olopade, O., and Reznikoff, C. Increased p16 level correlate with pRb alterations in human uroepithelial cells. Cancer Res., *55:* 493–497, 1995.
11. Band, V., and Sager, R. Distinctive traits of normal and tumor-derived human mammary epithelial cells expressed in a medium that supports long-term growth of both cell types. Proc. Natl. Acad. Sci. USA, *86:* 1249–1253, 1989.
12. Chowdary, D. R., Dermody, J. J., Jha, K. K., and Ozer, H. L. Accumulation of p53 in a mutant cell line defective in the ubiquitin pathway. Mol. Cell. Biol., *14:* 1997–2003, 1994.
13. Band, V., Dalal, S., Delmolino, L., and Androphy, E. J. Enhanced degradation of p53 protein in HPV-6 and BPV-1 E6-immortalized human mammary epithelial cells. EMBO J., *12:* 1847–1852, 1993.
14. Maki, C. G., Huibregtse, J. M., and Howley, P. M. *In vitro* ubiquitination and proteasome-mediated degradation of p53. Cancer Res., *56:* 2649–2654, 1996.
15. Pagano, M., Tam, S. W., Theodoras, A. M., Beer-Romano, P., Del Sal, G., Chau, V., Yew, P. R., Draetta, G. F., and Rolfe, M. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science (Washington DC), *269:* 682–685, 1995.
16. Scheffner, M., Huibregtse, J. M., Vierstra, R. D., and Howley, P. M. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell, *75:* 495–505, 1993.
17. Ciechanover, A. J., DiGiuseppe, J. A., Bercovich, B., Orian, A., Richter, J. D., Schwartz, A. L., and Brodeur, G. M. Degradation of nuclear oncoproteins by the ubiquitin system *in vitro*. Proc. Natl. Acad. Sci. USA, *88:* 139–143, 1991.
18. Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell, *78:* 761–771, 1994.
19. Scheffner, M., Munger, K., Huibregtse, J. M., and Howley, P. M. Targeted degradation of the retinoblastoma protein by human papilloma virus E7–E6 fusion proteins. EMBO J., *11:* 2425–2431, 1992.
20. Banks, L., Edmonds, C., and Vousden, K. H. Ability of the HPV16 E7 protein to bind RB and induce DNA synthesis is not sufficient for efficient transforming activity. Oncogene, *5:* 1383–1389, 1990.
21. Jewers, R. J., Hildebrandt, P., Ludlow, J. W., Kell, B., and McCance, D. J. Regions of HPV16 E7 oncoprotein required for immortalization of human keratinocytes. J. Virol., *66:* 1329–1335, 1992.
22. Dalal, S., Gao, Q., Androphy, E. J., and Band, V. Mutational analysis of HPV16 E6 demonstrates that p53 degradation is necessary for mammary epithelial cell immortalization. J. Virol., *70:* 683–688, 1996.
23. An, B., and Dou, Q. P. Cleavage of retinoblastoma protein during apoptosis: an interleukin 1β-converting enzyme-like protease as candidate. Cancer Res., *56:* 438–442, 1996.