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Rb binds c-Jun and activates transcription

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The retinoblastoma protein (Rb) acts as a critical cell-cycle regulator and loss of Rb function is associated with a variety of human cancer types. Here we report that Rb binds to members of the AP-1 family of transcription factors, including c-Jun, and stimulates c-Jun transcriptional activity from an AP-1 consensus sequence. The interaction involves the leucine zipper region of c-Jun and the B pocket of Rb as well as a C-terminal domain. We also present evidence that the complexes are found in terminally differentiating keratinocytes and cells entering the G_1 phase of the cell cycle after release from serum starvation. The human papillomavirus type 16 E7 protein, which binds to both c-Jun and Rb, inhibits the ability of Rb to activate c-Jun. The results provide evidence of a role for Rb as a transcriptional activator in early G_1 and as a potential modulator of c-Jun expression during keratinocyte differentiation.

Keywords: AP-1 factors/retinoblastoma protein/transactivation

Introduction

The retinoblastoma protein (Rb) is known to regulate progression from G_1 to S phase of the cell cycle (for review see Weinberg, 1995) and has been implicated in the differentiation of muscle cells (Gu *et al.*, 1993) and adipocytes (Chen *et al.*, 1996a). Regulation of the G_1 phase of the cell cycle entails the binding of Rb to a number of cellular proteins and modulation of their activity. Many of these proteins are transcription factors such as the E2F family (Helin *et al.*, 1992), ATF-2 (Kim *et al.*, 1992), c-Myc (Rustgi *et al.*, 1991b) and N-Myc (Rustgi *et al.*, 1991a), and their activities are important for the progression of the cell through G_1 and into S phase. The most studied activity is the ability of Rb to bind to E2F. Rb binds the transactivation domain of E2F through the A/B pocket and inhibits the transcriptional activity of this family (Weintraub *et al.*, 1992, 1995). Phosphorylation of Rb by cyclin-associated kinases (Lukas *et al.*, 1994; Bremner *et al.*, 1995) during the latter part of G_1 results in the release of E2F and the up-regulation of E2F-responsive genes, many of which are required for DNA synthesis (Rustgi *et al.*, 1991b; Dou *et al.*, 1992; Nevins, 1992). Phosphorylation of Rb is necessary for E2F activation; however, activation of E2F can also be achieved without phosphorylation through the interaction of Rb with viral oncoproteins, such as adenovirus E1A, SV40 large T (LT) and the human papillomavirus E7 protein (Chellappan *et al.*, 1992). The oncoproteins are thought to compete for the binding of E2F to Rb resulting in the release of free active E2F. Rb has also been shown to bind co-activators of transcription and up-regulate their activity. The human homolog of the yeast SNF2/SW12, hBrm–BRG1 binds to and is activated by Rb, and in one study was shown to activate glucocorticoid receptors (Singh *et al.*, 1995).

The differentiation of muscle cells and adipocytes also appears to require Rb activity. In muscle cells Rb binds MyoD and mediates differentiation and the co-operation can be inhibited by SV40 LT or genetic alterations in Rb (Gu *et al.*, 1993). In adipocytes Rb binds to C/EBPs (CCAAT/enhancer-binding-proteins) only in differentiating cells and enhances C/EBP binding to and transactivation of a C/EBP responsive reporter (Chen *et al.*, 1996a). Genetic alterations of Rb resulted in inhibition of binding and differentiation of adipocytes.

The AP-1 family of transcription factors is ubiquitously expressed and is involved in the early stage of mitogenic signalling, although these proteins play a critical role in the differentiation of myeloid and keratinocyte cells (for review see Angel and Karin, 1991; Briata *et al.*, 1993). The AP-1 family is also stimulated under stress situations (Angel and Karin, 1991) and some members have been shown to be involved in cell-cycle arrest (Angel and Karin, 1991; Pfarr *et al.*, 1994), both properties being compatible with a role in differentiation. There are a number of genes regulated by AP-1 factors (Angel and Karin, 1991) and their control is complex since members of the Jun family (c-Jun, JunB and JunD) can homodimerize or heterodimerize with Fos and ATF family members. Different Jun–Jun or Jun–Fos complexes bind to the recognition site (TGAC/GTCA) with different affinities, which is reflected in the transactivation ability of each.

During investigations on the interaction of human papillomavirus type 16 (HPV-16) E7 protein with cellular proteins, we observed that Rb could bind c-Jun *in vitro* and hence initiated a study to determine the significance of these interactions. Here we show that Rb binds c-Jun *in vitro* and *in vivo* during early G_1, stimulates its binding to the AP-1 consensus site and up-regulates c-Jun transcriptional activity. The HPV-16 E7 protein, which binds both Rb and c-Jun, can inhibit this Rb up-regulation of Jun. In addition, using human keratinocytes we show that the

Rb–c-Jun complexes are only observed during differentiation of cells and that the hypophosphorylated form of Rb is involved.

Results

In vivo detection of Rb–c-Jun complexes

Immunoprecipitations from crude nuclear extracts of G_1-synchronized HaCaT cells, which are a spontaneously immortalized keratinocyte cell line containing wild-type Rb, using anti-c-Jun antibodies, were performed. The associated proteins were examined for the presence of Rb by Western blots. A Rb band was clearly seen in the lanes where anti-RB and anti-c-Jun were used for the immunoprecipitation (Figure 1A). The anti-c-Jun antibody used for immunoprecipitations recognizes c-Jun phosphorylated on serine 63, which is the transcriptionally active form of c-Jun. Immunoprecipitations using anti-Rb antibodies, and subsequent blotting for c-Jun, were also performed and c-Jun was co-immunoprecipitated (Figure 1B). The co-precipitated Rb band appeared to consist of the hypophosphorylated form of Rb (pRb), and this is the predominant form at this stage in G_1 (Ludlow *et al.*, 1990; data not shown). Rb–c-Jun complexes were not detected in asynchronized cultures of HaCaT cells (data not shown) or in primary human keratinocytes (Figure 8).

To determine if the interaction observed is direct or mediated by a third partner we purified Rb and purchased purified c-Jun from a commercial source. A silver-stained gel of each of the protein preparations is shown in Figure 1C, upper panels. Using anti-Rb antibodies it was possible to co-immunoprecipitate c-Jun (Figure 1C, lower panels, c-Jun Blot, lane 2) from a mixture of both proteins indicating that there is a direct interaction between Rb and c-Jun. The interaction was specific since Rb antibodies did not co-immunoprecipitate the control protein ovalbumin (Figure 1C, lower panels, ova Blot, lane 6). Also, since the bacterially derived c-Jun will not be phosphorylated to the transcriptionally active form, this means that Rb can bind to different phosphorylated states of c-Jun, although in early G_1 the transcriptionally active form does predominate.

Mapping the binding domains of Rb and c-Jun

In vitro binding reactions using glutathione *S*-transferase (GST) fusion proteins encoding various domains of Rb (Kaelin *et al.*, 1991) were performed to further characterize the interaction between Jun proteins and Rb. Binding was localized to within the small pocket of Rb (Figure 2A), and in particular to the B pocket region between amino acids 612 and 657 (Figure 2B). The A pocket did not bind to c-Jun (Figure 2B, lane 6). A mutation in amino acid 706 (Kaye *et al.*, 1990), in the context of the small pocket (Figure 2A, lane 3), which abrogates complex formation of several Rb binding proteins, and a larger deletion in the same region from amino acids 703–737 (Figure 2B, lane 5), exhibited strong c-Jun binding. Faint binding was observed with GST–Rb712–767 (Figure 2B, lane 8), suggesting that a C-terminal region of the B pocket may play a role in binding. Binding of c-Fos to Rb was localized to the same region (Figure 2C). The other Jun members JunD and JunB were also able to bind to Rb although the sites of binding were not mapped (data not shown). Independent of binding to the B pocket, c-Jun was observed to bind to the C-terminal domain (Figure 3A). A summary of the Rb domains important for binding to c-Jun is shown in Figure 2D.

The c-Jun regions involved in the interactions with Rb were examined using a previously described series of c-Jun deletion mutations (Alani *et al.*, 1991) (Figure 3) and GST fusions containing either C-terminal domain of Rb (Figure 3A), or the small pocket region (Figure 3B). Deletion of the N-terminus of c-Jun from amino acids 1–124 or 1–223 had no effect on binding to Rb (Figure 3A and B, lanes 7 and 8), indicating that the transcriptional activation domain of c-Jun is not involved in the interaction with pRb. Deletion of most of the leucine zipper and the C-terminus (Figure 3A and B, lane 9), eliminated binding to the Rb domains, as did deletion of the entire basic and leucine zipper region (Figure 3A and B, lane 10). However, the Rb small pocket region (A and B pocket, Figure 3C, lane 4), but not a Rb fusion containing the A pocket alone of Rb (Figure 3C, lane 3), bound to a region of c-Jun containing only the leucine zipper. Identical results were observed with the C-terminal domain (data not shown), indicating that both the small pocket and C-terminal domains of Rb interact with the c-Jun leucine zipper independently. A summary of the domains of c-Jun which are necessary for Rb binding is shown in Figure 3D.

The experiments described above were carried out using either cell extracts or TNT reactions, which contain variable amounts of DNA. Since c-Jun can bind specifically to AP-1 sites present in DNA and Rb has a tendency to bind non-specifically to DNA, it was important to determine if the *in vitro* binding of Rb–c-Jun was mediated by binding to DNA. Therefore, we carried out GST-pull down experiments in the presence of ethidium bromide, which is known to inhibit protein–DNA interactions. Figure 4 shows the result of these experiments in the presence of 100 μg/ml of ethidium bromide, an amount at the high end of the range normally used (Lai and Herr, 1992). There was no reduction in either the binding of Rb and c-Jun, or a known protein–protein interaction such as c-Jun–c-Jun in the presence of ethidium bromide (Figure 4). However, only 10 μg/ml of ethidium bromide was able to disrupt a protein–DNA interaction, as shown by the inhibition of c-Jun binding to an AP-1 consensus site (Figure 4, right-hand panel). The results indicate that DNA is not mediating the *in vitro* interactions observed.

Rb up-regulates the transcriptional activity of c-Jun

Transient transfection assays were performed to determine what effect expression of Rb has on Jun-mediated transcription driven by a single AP-1 consensus binding site from the collagenase gene promoter (Figure 5A). Addition of exogenously expressed c-Jun caused, on average, a 10-fold increase in luciferase activity over endogenous levels with the vector alone in both primary human keratinocytes and CV-1P cells (Figure 5A and B). When c-Jun and Rb were co-transfected, transactivation increased another 5- to 6-fold compared with using c-Jun alone. Wild-type Rb as well as Rb small protein (RbSP) with and without a mutation at amino acid 706 are able to activate transcription, while the N-terminal domain (pRbNT amino acids 1–329), which did not bind c-Jun,

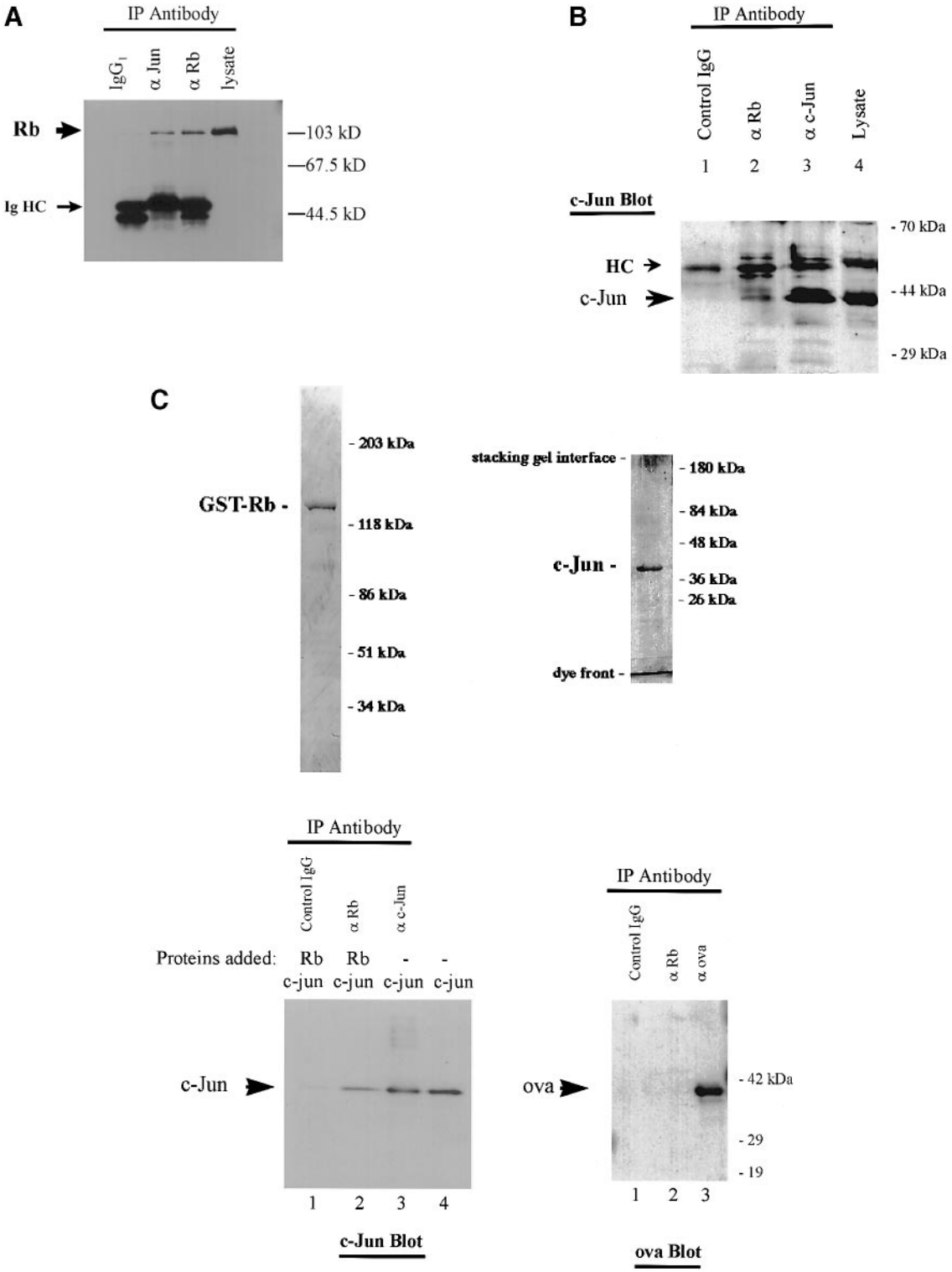

Fig. 1. Formation of pRb–Jun complexes *in vivo*. (**A**) Nuclear extracts (40 μg) of HaCaT cells in the G_1 phase of the cell cycle were subjected to immunoprecipitation with either, mouse IgG1 (lane 1), monoclonal anti-c-Jun (KM-1, Santa Cruz) (lane 2), or monoclonal anti-Rb (Oncogene Science, C36) (lane 3). The immune complexes and 20 μg of nuclear extract (lane 4) were resolved on a 7.5% SDS–polyacrylamide gel, transferred to nitrocellulose and blotted with monoclonal anti-Rb (PharMingen, 245). The blot was then incubated with anti-mouse-HRP and the immune complexes were visualized with ECL reagent. The Rb band is indicated with the arrow and is present in the anti-c-Jun, anti-Rb and lysate lanes (lanes 2–4) but not in the IgG1 negative control lane (lane 1). The mouse IgG heavy chains present in the original immunoprecipitations that are recognized by the anti-mouse-HRP are indicted (Ig HC). (**B**) Rb from the nuclear extracts were immunoprecipitated using anti-Rb antibodies and the resulting complexes run on a 10% SDS–polyacrylamide gel, transferred to nitrocellulose and blotted with anti-c-Jun antibodies. (**C**) Co-immunoprecipitation of purified c-Jun with anti-Rb antibodies. The upper panel is a silver-stained gel of 1 μg of purified GST–Rb and c-Jun (Santa Cruz, CA) showing the lack of significant contaminating proteins. The lower left panel (c-Jun blot) shows a series of co-immunoprecipitations after addition of 300 ng of each of c-Jun and GST–Rb using anti-IgG1 (lane 1), anti-Rb (lane 2) and anti-c-Jun (lane 3), or with c-Jun alone (lane 4). The filter was blotted with anti-c-Jun antibodies. Rb was also mixed with purified ovalbumin (right lower panel, ova blot) and complexes co-immunoprecipitated with anti-IgG1 (lane 1) or anti-Rb antibodies (lane 2) and the filter blotted with rabbit anti-ovalbumin antibodies. No interaction was observed with the latter co-immunoprecipitation. Lane 3 is ovalbumin alone.

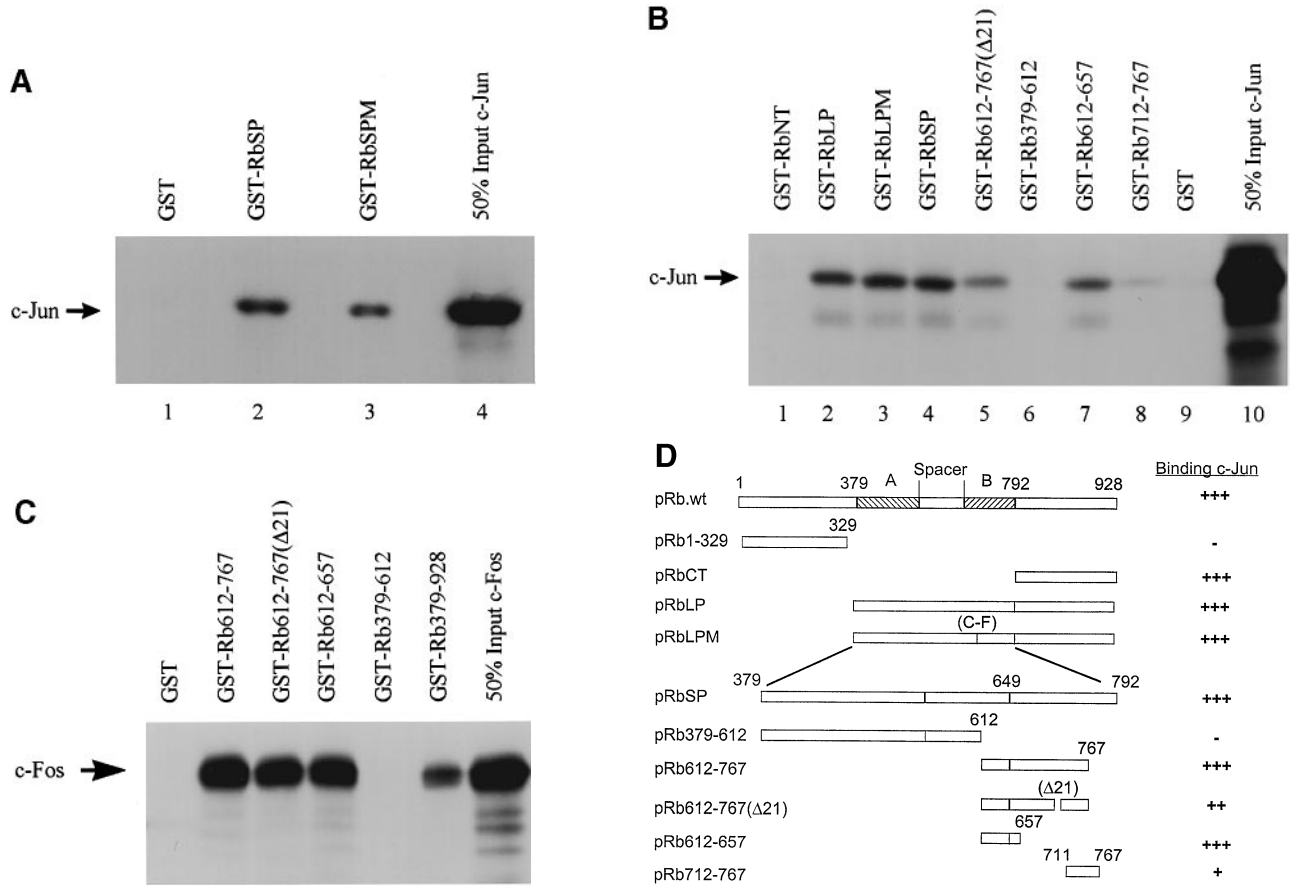

Fig. 2. Retinoblastoma protein domains that bind *in vitro* to c-Jun. (**A**) A Rb small pocket–GST fusion (RbSP–GST) protein bound to glutathione–agarose beads binds to *in vitro* translated ^{35}S-labeled c-Jun protein (lane 2) but not to GST alone (lane 1). The radiolabeled c-Jun protein can also bind to a RbSP–GST fusion protein mutated at residue 706 (lane 3). The input radiolabeled c-Jun protein is shown in lane 4. (**B**) Mapping of the B pocket region of the small pocket of Rb that binds to c-Jun. *In vitro* transcribed and translated ^{35}S-labeled c-Jun was incubated with the indicated RbSPand small pocket deletion GST fusion proteins (lanes 4–8). Binding reactions were also performed with the N-terminus of Rb (amino acids 1–379) (lane 1), large pocket of Rb (lane 2), mutation in large pocket (lane 3) and GST (lane 9). One-half of input c-Jun is shown for comparison (lane 10). (**C**) c-Fos also binds *in vitro* to the B pocket of the small pocket of Rb. *In vitro* transcribed and translated ^{35}S-labeled c-Fos was incubated with the indicated small pocket deletion GST fusion proteins bound to glutathione–agarose beads (lanes 2–6) and to GST alone (lane 1). The input c-Fos is shown for comparison (lane 7). (**D**) Diagram of the GST–Rb constructs used to map the binding of c-Jun to Rb indicate that a region of the B pocket and the C pocket bind to c-Jun.

was unable to activate c-Jun (Figure 5C). c-Jun is auto-regulated through an AP-1 site (Angel *et al.*, 1988), and similar activation by Rb was observed using a region of the c-Jun promoter containing the AP-1 site (data not shown).

Rb facilitates binding of c-Jun to an AP-1 consensus site

To investigate if a pRb–c-Jun complex could bind DNA containing an AP-1 site, we carried out gel shift assays using a 21mer oligonucleotide containing the consensus AP-1 binding sequence TGACTCA. While we were able to observe a super-shift complex bound to the AP-1 oligonucleotide using anti-c-Jun antibodies, we did not observe consistent super-shifted bands using three different Rb antibodies (data not shown). To investigate whether Rb might in some way facilitate or stabilize binding of c-Jun to an AP-1 site, we added sub-optimum binding levels of either GST–c-Jun or purified c-Jun to gel shift reactions and added increasing amounts of Rb. As the amount of Rb increased, there was specific binding of c-Jun to the oligonucleotide (Figure 6A, lanes 3–5). The shifted oligonucleotide contained c-Jun because the complex could be super-shifted with anti-Jun antibodies (Figure 6A, lane 7). The binding was mediated through the AP-1 site because binding was inhibited by excess wild-type, but not mutated competitor (Figure 6A, lanes 9 and 10). Rb alone did not bind to the AP-1 site containing oligonucleotide (Figure 6A, lane 6). While anti-Rb antibodies did not super-shift the complex, the amount of Rb–c-Jun complex detected was reduced in the presence of the antibody presumably because of the removal of Rb (Figure 6A, lane 8). Similar results using less input protein were observed using commercially available pure c-Jun protein and GST–RbLP fusion proteins, but not with c-Jun and GST–RbNT or GST protein (Figure 6B, compare lanes 3–6 with 7–10 and 11–14), demonstrating the specificity of the interaction.

HPV-16 E7 protein inhibits Rb activation of c-Jun transcription

Since the HPV-16 E7 protein can inhibit the binding of the transcription factor E2F1 to Rb, we investigated the possibility that E7 may have an effect on the transcriptional activity of Rb–c-Jun complex. The c-Jun promoter was used in this set of experiments with the distal ATF-

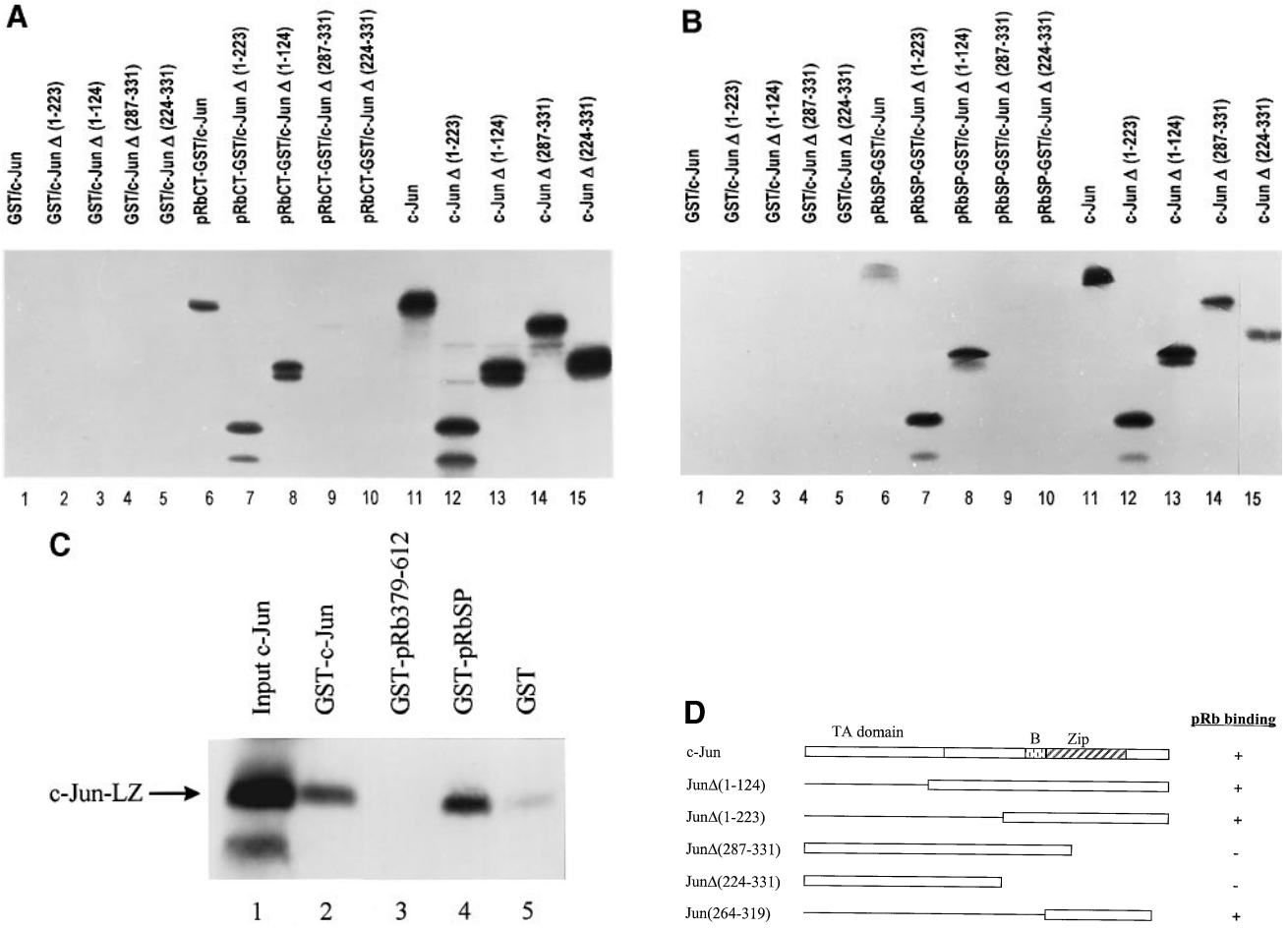

Fig. 3. Mapping the c-Jun region involved in the interaction with retinoblastoma protein. (**A**) ^{35}S-labeled c-Jun deletion mutations were produced in *in vitro* transcription and translation reactions and were tested for binding to GST alone (lanes 1–5) and retinoblastoma C-terminus–GST fusion protein bound to glutathione beads (GST–RbCT) (lanes 6–10). The c-Jun input is shown in lanes 11–15. Deletion of the N-terminus of c-Jun [c-JunΔ(1–223) and c-JunΔ(1–124)] did not affect binding to GST–RbSP. Partial deletion of the leucine zipper [c-JunΔ(287–331)] diminished binding while deletion of the entire bZIP domain [c-JunΔ(224–331)] abolished binding to GST–RbCT. (**B**) The c-Jun deletion mutants described in (A) were tested for binding to GST alone (lanes 1–5) and retinoblastoma small pocket-GST (GST–RbSP) (lanes 6–10). The c-Jun input is shown in lanes 11–15. The results of these binding reactions are the same as in (A). (**C**) A region of c-Jun containing the leucine zipper (amino acids 264–319) was ^{35}S-labeled *in vitro* and tested for binding to GST alone (lane 5), GST–c-Jun (lane 2), GST–RbSP (lane 4) and GST–Rb379–612 (A pocket, lane 3). The c-Jun leucine zipper was able to bind to the small pocket region but not the A pocket (compare lane 4 with lane 3). Binding to GST–c-Jun is included as a positive control and demonstrates that the leucine zipper TNT product can dimerize with the full-length GST–c-Jun fusion (lane 2). (**D**) Diagram of the c-Jun domains that interact with pRb. The schematic of the c-Jun constructs and their pRb binding properties shows that deletions that affect the leucine zipper motif disrupt binding, while an isolated leucine zipper is able to bind. Lines represent deletions, boxes represent coding regions. TA, transactivation; B, basic region; Zip, leucine zipper domain.

2/c-Jun responsive site deleted, leaving only one AP-1 proximal site, which has been shown to be activated by c-Jun/c-Fos (van Damm *et al.*, 1993). In Figure 7A we show that wild-type E7 inhibits the ability of Rb to activate c-Jun by 75%, while a mutated E7 (E7.24), which has a mutation in the LXCXE motif and is unable to bind Rb, has no inhibitory properties. Both E7 genes were driven by a SV40 promoter and equal amounts of the E7 wild-type and E7.24 were produced, as indicated by immunoprecipitations of lysates from transfected cells (Figure 7B).

Rb–c-Jun complexes formed during human keratinocyte differentiation

To investigate if this E7 inhibition is physiologically relevant, we examined c-Jun expression in keratinocytes during differentiation, since HPV oncogenic types cause inhibition of keratinocyte differentiation. c-Jun is up-regulated when normal keratinocytes are induced to differentiate and both Rb and c-Jun have been individually localized to the upper layers of the epithelium (Szekely *et al.*, 1992; Briata *et al.*, 1993). In addition, several differentiation specific proteins, such as keratin 1 and involucrin, contain AP-1 sites in their promoters (Angel *et al.*, 1987; Rothnagel, *et al.*, 1993; Welter, *et al.*, 1995). Western blots of lysates from keratinocytes induced to differentiate show that Rb becomes progressively hypophosphorylated (Figure 8, lysate lanes). In addition, Rb was detected in the anti-c-Jun immunoprecipitates 24 and 48 h into differentiation (t = 24 and 48 h; Figure 8, IP α-c-Jun lanes), but not from non-synchronized cycling keratinocytes (t = 0 h; Figure 8). The induction of differentiation was confirmed by immunohistochemically staining keratinocytes for filaggrin, a differentiation

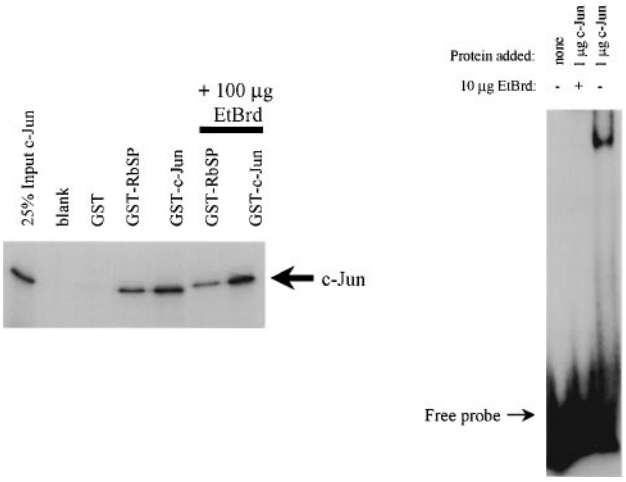

Fig. 4. c-Jun and pRb interact in a DNA-independent manner. GST pull-down reactions were performed in the presence or absence of 100 μg/ml ethidium bromide. The binding of the small pocket of pRb to c-Jun was not affected by the presence of the ethidium bromide (compare lane 4 with lane 6), suggesting that a DNA bridge does not mediate the pRb–c-Jun interaction. Since c-Jun homodimerization occurs in the absence of DNA, it was included as a positive control. An additional control shows that a gel shift complex using 1 μg of purified c-Jun and a ^{32}P-labeled oligonucleotide containing the c-Jun consensus binding site is disrupted by only 10 μg/ml of EtBrd.

specific marker (data not shown). The data show that Rb–c-Jun complexes are also detected in differentiating keratinocytes.

Discussion

Rb has been shown to bind and modulate the activity of a number of different transcription factors and co-activators. Modulation can take the form of repression of transcription as with E2F (Weintraub *et al.*, 1992), or activation as with NF-IL6 (Chen *et al.*, 1996b) and the hBrm–BRG1 complex (Singh *et al.*, 1995). Here we show that Rb can bind and activate the AP-1 family of transcription factors, in particular the Jun family and c-Fos. The AP-1 family has been shown to be activated early in G_1 and they are thought to have a mitogenic stimulus on the cell. In addition, the AP-1 family can be activated upon stress stimuli such as UV irradiation, heat shock and during differentiation of keratinocytes and myeloid cells (Angel and Karin, 1991; Briata *et al.*, 1993; Szabo *et al.*, 1994). The AP-1 factors have been implicated in the regulation of keratin genes (Rothnagel *et al.*, 1993) and involucrin (Welter *et al.*, 1995), which are involved in keratinocyte terminal differentiation. Like AP-1 factors, Rb has been implicated in differentiation, in particular of muscle cells (Gu *et al.*, 1993) and adipocytes (Chen *et al.*, 1996a), so the possibility exists that Rb–c-Jun interactions may be important for differentiation of human keratinocytes.

We have shown the binding of Rb and AP-1 transcription factors using GST fusion proteins and co-immunoprecipitations from HaCaT cells and primary human keratinocytes. However, formation of the complex was only observed in the G_1 phase of the cell cycle or during differentiation of primary human keratinocytes. The complex contains the transcriptionally active form of c-Jun, since the antibody used for the immunoprecipitations recognizes a peptide phosphorylated on serine 63, and is

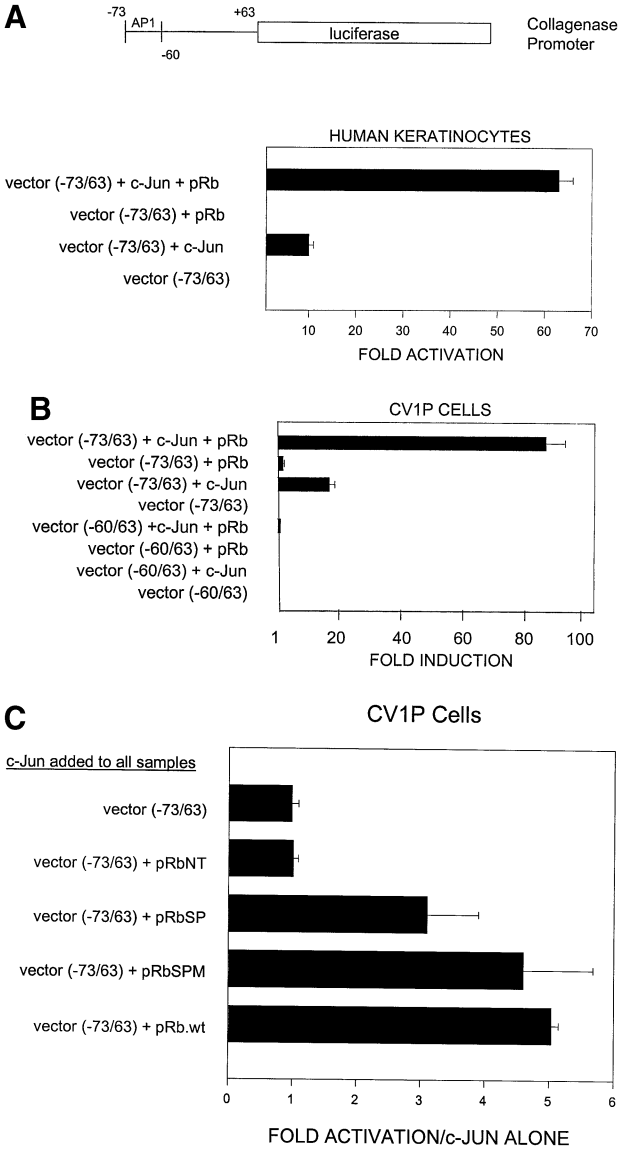

Fig. 5. Retinoblastoma protein enhances c-Jun transactivation from an AP-1 site. (**A**) pRb (expressed from a SV40 promoter) and c-Jun (expressed from an RSV promoter) were transfected into primary keratinocytes alone or together using poly-L-ornithine (Nead and McCance, 1995). The results are an example in each case of one experiment; however, several experiments were carried out and the statistics for each are shown below. A diagram of the collagenase promoter/luciferase reporter construct is shown at the top of the figure. pRb transactivates c-Jun mediated transcription when both expression constructs (1 μg of each) are transfected into primary keratinocytes. Results are expressed as the fold increase over the activity of –73/+63 Coll–Luc promoter ± SD alone. Three experiments were carried out and Rb transactivated c-Jun significantly in all cases ($P < 0.01$). (**B**) The same experiment described above was carried out in CV-1P cells using 1 μg of each expression plasmid, pRb and c-Jun. Eight experiments were carried out, and all showed significant pRb transactivation ($P < 0.0065$). (**C**) RbSP and RbSPM, but not the N-terminus of Rb, are involved in the transactivation of c-Jun. Experiments were carried out four times, and all plasmids except the N-terminus of Rb (amino acids 1–329) transactivated c-Jun ($P < 0.05$). Note that in this experiment, results are expressed as fold increase over that seen with c-Jun alone.

one of two amino acids in the transactivation domain which need to be phosphorylated for transactivation. However, bacterially derived c-Jun binds Rb indicating that other phosphorylated forms of c-Jun do bind as well,

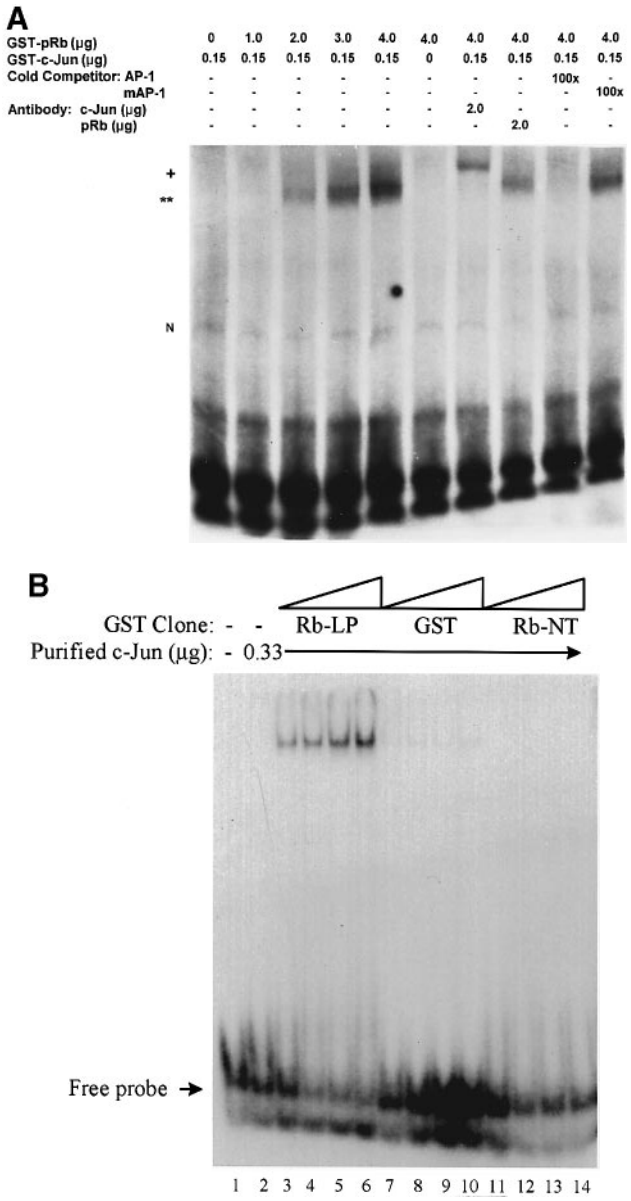

Fig. 6. pRb enhances the binding of c-Jun to an AP-1 site. (**A**) Gel shift assays were performed with a constant amount of GST–c-Jun added to a 21 bp ^{32}P-labeled AP-1 oligonucleotide such that no shift of the oligonucleotide was observed (lane 1). Increasing amounts of GST–Rb was added to the gel shift reaction shown in lane 1 (lanes 2–5). The addition of the GST–Rb lead to an increased level of labeled oligonucleotide shifted, while the highest GST–Rb concentration in the gel shift reaction without GST–c-Jun did not shift a complex (lane 6). The shifted oligonucleotide was specific to the presence of the AP-1 site because 100× cold AP-1 competitor decreased the complex (lane 9), while the 100× cold mutant AP-1 oligonucleotide had no effect on the shifted complex (lane 10). GST–c-Jun is present in the shifted complex because the addition of anti-c-Jun (lane 7) super-shifted the AP-1 oligonucleotide. The addition of anti-pRb did not result in a super-shift (lane 8). (**B**) Increasing amounts of eluted GST–RbLP (lane 3–6) facilitates binding of purified c-Jun (0.33 μg) to a radiolabeled AP-1 oligonucleotide compared with c-Jun alone (lane 2). The same amounts of GST–RbNT (amino acids 1–329) (lanes 11–14) and GST alone (lanes 7–10), neither of which bind c-Jun, did not have an effect on c-Jun binding.

although in early G_1 the transcriptionally active form of c-Jun predominates. The complex also contains the hypophosporylated form of Rb observed during early G_1 and during keratinocyte differentiation (Figure 8). Therefore, binding is potentially between the transcriptionally active forms of both proteins. c-Jun binds independently to two regions of Rb, one in the B pocket region and the other in the C-terminal domain, or C pocket. Therefore, one molecule of Rb could conceivably bind two of the AP-1 factors. Rb binds to c-Jun through the leucine zipper region, and it is possible that Rb could facilitate dimerization and DNA binding of homo- and heterodimers of the AP-1 factors. This is supported by experiments that show that in the presence of increasing amounts of Rb, suboptimal levels of pure c-Jun bind efficiently to an oligonucleotide containing an AP-1 consensus site (Figure 6). A similar result has been obtained with NF-IL6, a member of the family of C/EBP transcription factors, in the presence of Rb (Chen *et al.*, 1996b). Again, in this study, Rb was not detected complexed to NF-IL6 on DNA as determined by gel shift assays and super-shifts. A previous investigation of Rb-binding AP-1 factors was confined to a report that Rb could bind ATF-2 and activate the transcription of the TGF-β2 gene (Kim *et al.*, 1992). Binding was thought to be through the transactivation domain of ATF-2, although this was not directly tested. It is clear from our results that for c-Jun and c-Fos the binding of Rb is through the leucine zipper domain. The HTLV I Tax protein has been shown to bind to ATF through the leucine zipper domain, and increased the DNA-binding activity of ATF-containing complexes (Wagner and Green, 1993). It was suggested that Tax promoted dimerization and increased the concentration of dimerized factors, which resulted in more efficient binding. A similar conclusion could be presented here for the Rb–c-Jun interaction. However, at present it is not clear if there is any specificity of Rb for certain homo- or heterodimers of the AP-1 family.

The consequence of the Rb–c-Jun complex formation is that Rb can activate c-Jun 5- to 6-fold above c-Jun alone. This has been shown in human keratinocytes, CV-1P and NIH 3T3 cells using two AP-1 responsive promoters, the collagenase and c-Jun promoter, with very similar results. Since binding was observed in early G_1 and during the differentiation of keratinocytes, Rb may have a role in activating genes at these two stages in the life cycle of certain cells. While c-Jun has historically been thought of as associated with positive cell growth signaling, it is also involved in apoptosis (Bossy-Wetzel *et al.*, 1997) and differentiation (Gu *et al.*, 1993; Chen *et al.*, 1996a), where cell growth is arrested. Also, JunD has been shown to negatively regulate fibroblast growth (Pfarr *et al.*, 1994) and JunB has been shown to be inhibitory to c-Jun transactivation (Chiu *et al.*, 1989). Therefore, depending on the complexes formed between different AP-1 factors, there are a variety of effects on cell growth.

HPV-16 E7, which can bind to both Rb and c-Jun, is able to inhibit the ability of Rb to activate c-Jun, while a mutation in E7, which abrogates Rb binding, but has retained c-Jun binding has no effect. It should be remembered that E7 binds to Rb at two sites, one in the small pocket (B pocket), and the other in the C pocket (in a region between amino acids 803 and 841) in a manner similar to c-Jun, so it is possible that E7 will compete at both sites for binding to Rb. Certain AP-1 members including c-Jun, JunB, JunD and Fra1, but

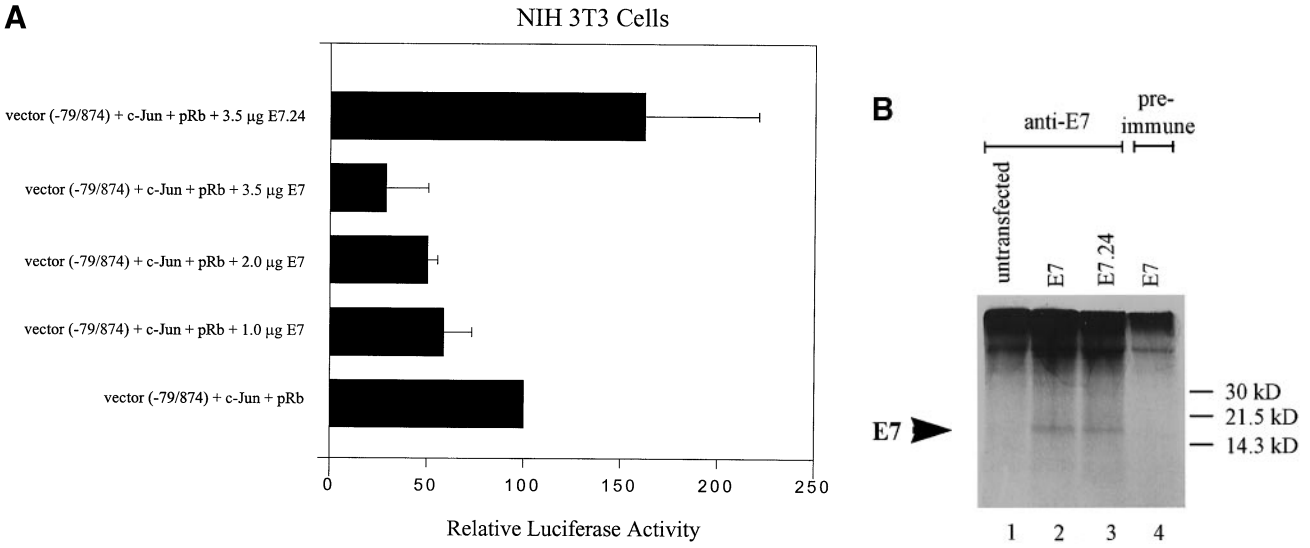

Fig. 7. HPV-16 E7 inhibits the ability of Rb to complex with c-Jun. (**A**) The addition of wild-type E7 inhibited the ability of Rb to activate c-Jun in transfection experiments. NIH 3T3 cells were transfected with increasing amounts of wild-type E7 and a mutated form of E7 (E7.24), which no longer binds pRb. One μg of the Rb and c-Jun expression plasmids was added, along with the indicated amounts of an E7 wild-type or E7.24 expression plasmid and 1 μg reporter plasmid, c-Jun promoter (–79/874) luciferase. (**B**) Immunoprecipitations using rabbit polyclonal anti-HPV-16 E7 (lanes 1 to 3) or preimmune antibodies (lane 4), of wild-type E7 and E7.24 proteins from transfected cells. Lane 1, control untransfected cells; lane2, cells transfected with wild-type E7; lane 3, cells transfected with E7.24 and lane 4, cells transfected with wild-type E7 and immunoprecipitated with IgG control antibodies.

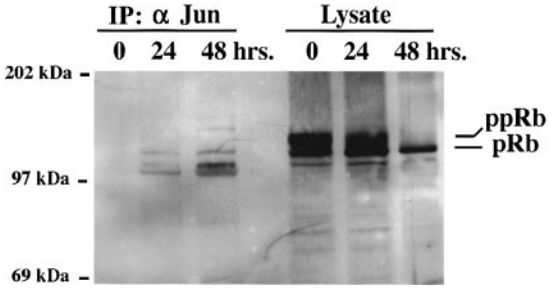

Fig. 8. pRb and c-Jun form a complex when primary keratinocytes are induced to differentiate. Human keratinocytes were induced to differentiate for 0, 24 or 48 h and samples were subjected to either Western blotting (Lysate) or co-immunoprecipitation (IP). The Western blot was incubated with anti-pRb (245, PharMingen and XZ-61). The same sample was immunoprecipitated with anti-c-Jun (Ab-1, Oncogene Science) and protein A beads. The immune complexes were separated on a 7.5% SDS–polyacrylamide gel and transferred to nitrocellulose. The blot was incubated with anti-pRb (245, PharMingen and XZ-61). pRb co-immunoprecipitates with anti-c-Jun at 24 and 48 h after the start of differentiation (lane 2 and 3), but not at $t = 0$. The lower bands have been observed previously during embryonic carcinoma and muscle cell differentiation and represent truncated forms of Rb (Slack *et al.*, 1993; Savatier *et al.*, 1994; Corbeil *et al.*, 1995).

not c-Fos, are active during keratinocyte differentiation (Welter *et al.*, 1995), and we have recent evidence that the DNA-binding activities of these four AP-1 members in E7-expressing keratinocytes, as measured by gel shifts, are low compared with normal keratinocytes (L.A.Baglia and D.J.McCance, manuscript in preparation). This would be consistent with the results presented here, that E7 can inhibit the activation of c-Jun by Rb and perhaps is a mechanism by which HPV can inhibit keratinocyte differentiation. However, to confirm this, it will be necessary to show that AP-1 activity is essential for keratinocyte differentiation by using specific inhibitors of AP-1 activity (Krylov *et al.*, 1995) and to determine which members of the family members are required. Recent studies have shown that c-Jun null mutations in mice are lethal, with *in utero* death occurring at 12.5 days gestation (Johnson *et al.*, 1993). Unfortunately, this time of death is too early to indicate if epithelial cell differentiation was normal.

In summary, we have shown that Rb binds and activates c-Jun responsive promoters possibly by facilitating binding of AP-1 factors to their site on certain cellular promoters. HPV-16 E7 inhibits the ability of Rb to activate c-Jun and this depends on the binding of E7 to Rb, since a mutation in the Rb binding domain of E7 has no effect. A Rb–c-Jun complex is only observed during G_1 phase of the cell cycle and during keratinocyte differentiation. In the latter situation, the presence of wild-type E7 down-regulates c-Jun levels, while a mutation, which does not bind to Rb, has little effect on c-Jun levels. These results show that Rb can modulate c-Jun activity and this modulation can be disrupted by a viral oncoprotein.

Materials and methods

Cells and cell culture

Primary keratinocytes were isolated from fresh foreskin pieces and maintained in keratinocyte growth medium (KGM) medium (Clonetics, San Diego, CA). CVIP African Green monkey kidney cells and HaCaT, an immortalized keratinocyte cell line, were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS). NIH 3T3 mouse fibroblasts were obtained from ATCC and were maintained in DMEM supplemented with 10% bovine calf serum (BCS).

Constructs

The plasmids encoding GST fusion proteins of pRbLP, pRbLPM, pRbSP and pRbSPM were obtained from Dr William Kaelin and have been described previously (Kaelin *et al.*, 1991). pGSTRbNT encodes amino acids 1–379 of Rb. GST fusion constructs for pRb379–612, pRb612–767, pRb612–767(Δ21), pRb612–657 and pRb712–767 were provided by Dr Tony Kouzarides. pGSTRbCT was created by PCR amplification from a Rb cDNA template and encodes amino acids 772–928 with *Bam*HI and *Eco*RI sites at the 5′ and 3′ ends, respectively. The resulting

product was digested with *Eco*RI and *Bam*HI and cloned in-frame into pGEX 2T (Pharmacia, Piscataway, NJ).

pSG5-Rb, which produced full-length, wild-type pRb from a T7 promoter, was kindly provided by Dr William Kaelin. The 1 kb open reading frame of mouse c-Jun was amplified by PCR from the full-length cDNA clone, pRSV–c-Jun, (gift of Dr Michael Birrer) and was cloned 5′ at the *Eco*RI site and 3′ at the *Bam*HI site of pGEM7Zf (Promega, Madison, WI), such that SP6 yields sense mRNA. The pRSV–c-Fos expression vector containing murine c-Fos was obtained from Dr Tom Curran.

Wild-type E7 cloned into pSG5 (Promega, Madison, WI) was a gift from Dr J.Icenogle. The point mutation HPV16 E7.24 was a gift from Dr Karen Vousden and was cloned in pKV, a vector with the mouse Moloney LTR as promoter. The E7 point mutation was removed from pKV by *Bam*HI–*Eco*RI digestion and was cloned into an altered pSG5 vector which has a 5′ *Bam*HI and a 3′ *Eco*RI site (Promega, Madison WI). Plasmids –73/+63 Coll–Luc and –60/+63 Coll–Luc have been described previously (Antinore *et al.*, 1996). The –79/874 c-Jun promoter cloned into a CAT vector was obtained from Dr Robert Chiu (Chen *et al.*, 1994). The c-Jun promoter was removed from the parent construct with *Sca*I at the 5′ end and *Hin*dIII at the 3′ end and was religated into the same restriction sites in pXP1 (Nordeen, 1988) resulting in the c-Jun promoter being placed 5′ to the luciferase gene. RSV–c-Jun was obtained from Dr Michael Birrer. pSG5–c-JunLZ was made by PCR amplifying mouse c-Jun cDNA from base pairs 2050–2218 of the cDNA which encodes the leucine zipper (amino acids 264–319). The PCR primers have a 5′ *Bam*HI site and a 3′ *Eco*RI site which allowed directional cloning into the modified pSG5 vector described above.

Protein extract preparation and Western blots

Nuclear extracts were prepared from G_1-synchronized HaCaT cells and differentiating keratinocytes for co-immunoprecipation experiments. To synchronize HaCaT cells, 70% confluent 100 mm plates of cells were washed in PBS and then methionine- and cysteine-free DMEM supplemented with 2% FBS was added for 48 h. To obtain cells in G_1, the methionine- and cysteine-free DMEM was removed and DMEM with 10% FCS was added for 4 h before cell harvest.

To obtain *in vitro* differentiated human keratinocytes, we used the method described by Poumay and Pittelkow (1995). Briefly, primary human foreskin keratinocytes were grown on 100 mm plates to confluence in KGM (Clonetics, San Diego, CA) which contains growth factors. The KGM was removed and keratinocyte basal medium (KBM), which does not contain growth factors, was added to the cultures. At various times between a 48 h period after KBM addition, cells were harvested.

HaCaT or keratinocytes cultures were harvested via trypsinization. The cell pellets were washed twice in PBS. Nuclear extracts were prepared as described previously (Osborn *et al.*, 1989) but with larger volumes. Each cell pellet was washed twice in 500 µl Buffer A (20 mM HEPES pH 7.9, 1.5 mM Mg Cl_2, 10 mM Kcl, 0.5 mM DTT), counted and resuspended in 80 µl Buffer A with 0.1% NP-40/10^7 cells. After 10 min on ice, the samples were vortexed briefly and centrifuged in an eppendorf microcentrifuge at 13 000 r.p.m. at 4°C for 10 min. The supernatant was discarded and the pellet was resuspended in 28 µl Buffer C (20 mM HEPES pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5mM PMSF, 0.5 mM DTT) for 15 min on ice. The samples were vortexed and centrifuged in an eppendorf microcentrifuge at 13 000 r.p.m. and 140 µl of Buffer D (20 mM HEPES pH 7.9, 20% glycerol, 0.1 mM KCl, 0.2 MM EDTA, 0.5 mM PMSF, 0.5 mM DTT) were added to the supernatant. The protein concentration of each sample was determined using the Bio-Rad protein assay reagent, according to the manufacturer's instructions. The extracts were aliquoted and stored at –85°C.

Whole cell extracts were prepared for Western blots. Briefly, 48 h after transfection, the cells were removed from 100 mm plates via trypsinization. The resulting pellet was washed once with PBS and the pellet was resuspended in one pellet volume of EBC buffer [50 mM Tris–HCl pH 8.0, 120 mM NaCl, 0.5% NP-40, 10 µg/ml phenylmethylsulfonyl fluoride (PMSF), and 20 µl/ml aprotinin (A-6279, Sigma, St Louis, MO)]. The resuspended pellet was incubated on ice for 30 min. Cellular debris was pelleted by centrifugation and the protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacturer's instructions.

Equal amounts of protein were resolved on a 7.5% SDS–polyacrylamide gel, transferred to 0.2 µm nitrocellulose and blocked in 5% powdered milk in PBS–Tween 20 (PBST). The membrane was incubated with the indicated primary antibody in 2% powdered milk in PBST. The membrane was washed extensively with PBST and then incubated with a 1:1000 anti-rabbit-HRP (Southern Biotechnology Associates, Birmingham, AL). Proteins were visualized with ECL reagents according to the manufacturer's instructions.

Co-immunoprecipitations

HaCaT nuclear extracts (40 g) were incubated with either mouse IgG1, monoclonal anti-Rb (Oncogene Science, C36) or monoclonal anti-c-Jun (Santa Cruz, KM-1) and precoated sheep anti-mouse IgG magnetic beads (Dynal, Oslo, Norway) in gel shift buffer [20 mM HEPES pH 7.9, 60 mM KCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 20 µl/ml aprotinin (A-6279, Sigma, St Louis, MO), 10 µg/ml PMSF] overnight at 4°C on a rotator. The immune complexes were washed three or four times with 1× gel shift buffer supplemented with 120 mM NaCl before loading onto a SDS–polyacrylamide gel. After electrophoresis, the gel was transferred to 0.2 µm nitrocellulose, blocked in 5% powdered milk in PBST (pH 7.3). The blots were incubated with either 1:1000 dilution of monoclonal anti-Rb (PharMingen, 245) or 1:1000 dilution of polyclonal anti-c-Jun (N) (Santa Cruz Biotechnology, Santa Cruz, CA). The nitrocellulose was then probed with the appropriate horse radish peroxidase-conjugated secondary antibody (Southern Biotechnology, Birmingham, AL) and developed for chemiluminescent detection (Amersham Life Sciences, Arlington Heights, IL) according to manufacturer's instructions.

Co-immunoprecipitations from keratinocyte extracts were performed as described above, except 100 µg of total cell lysate was used for cell lysate lanes and protein A beads were used to precipitate the immune complexes.

For the *in vitro* co-immunoprecipitation, Rb was purified as a full-length GST fusion protein from *Escherichia coli* strain BL21pLys using glutathione–Sepharose beads as described recently (Zarkowska *et al.*, 1997a,b). The GST–Rb was then eluted from the beads using excess glutathione. Three hundred and thirty nanograms each of purified GST–Rb and c-Jun (Santa Cruz, CA) were added to 500 µl of IP buffer (50 mM HEPES pH 7.9, 150 mM NaCl, 0.1% NP-40, 1 mM PMSF and 1 mM aprotinin) and incubated at 4°C for 1 h with rotation before addition of 20 µl of either anti-Rb (C36), anti-c-Jun (KM-1) or anti-IgG1 antibody-coated magnetic beads (1 µg of each antibody was used). As a control, GST–Rb was mixed with 300 ng of purified ovalbumin (Sigma, St Louis, MO) and the co-immunoprecipitation carried out with anti-Rb (C36) or anti-IgG1 antibodies. The reactions were incubated overnight at 4°C. The beads were washed three times with IP buffer before loading onto a 10% SDS–polyacrylamide gel. The gel was transferred onto 0.2 µM nitrocellulose, and the filter blocked in 5% powdered milk in PBST for 1 h at room temperature. The blots were then probed with either mouse anti-c-Jun, or rabbit anti-ovalbumin antibodies (gift of Dr Edith Lord), and developed as above.

Immunoprecipitations of wild-type E7 and E7.24 from transfected cells were performed as above for the keratinocytes except that the cells were labeled with [^{35}S]cysteine for 3 h prior to being harvested 24 h after transfection. The cells were lysed in RIPA buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% Na deoxycholic acid, 0.1% SDS and 0.5mM PMSF) and E7 precipitated with a rabbit polyclonal anti-E7 antibody. Samples were then separated on a 15% SDS–polyacrylamide gel and processed for autoradiography.

GST pull-down experiments

All *in vitro* reactions were performed using [^{35}S]methionine in TNT Coupled Transcription/Translation Systems with wheat germ lysate (Promega, Madison, WI) according to the manufacturer's instructions. GST fusion proteins were expressed and purified as described previously (Smith and Johnson, 1988). For each pull-down experiment, 100 000 c.p.m. of ^{35}S-labeled protein were diluted in 200 µl of ice cold NET-N [20mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM EDTA pH 8.0, 0.5% NP-40, 0.5mM PMSF, 1 µg/ml leupeptin and 2 µg/ml aprotinin (Sigma, St Louis, MO)], cleared with GST–glutathione beads and then incubated at 4°C with rotation, with ~2 g of fusion protein attached to glutathione–agarose beads for 1 h. Unbound proteins were removed from the beads by washing five times with NET-N, and the bound proteins were analyzed by SDS–PAGE and autoradiography.

Transactivation assays

Transfections were performed in CVIP and NIH 3T3 cells using Lipofectamine (Gibco-BRL, Gaithersburg, MD) as per the manufacturer's instructions. CV-1P cells were harvested 48 h (or 24 h for NIH 3T3) after the start of transfection and assayed for luciferase activity as recommended by the manufacturer (Promega, Madison, WI). Assays were performed in triplicate with either a fourth plate stained *in situ* for β-galactosidase activity or samples normalized for protein content to

control for transfection efficiency. Primary human keratinocytes were transfected as described previously (Nead and McCance, 1995).

Gel shift assays

HaCaT nuclear extracts were prepared as described while fusion proteins were expressed in bacteria and eluted from glutathione–agarose beads as per the manufacturer's recommendations (Pharmacia Biotechnology, Piscataway, NJ). Purified c-Jun was obtained from Promega. Binding reaction mixtures contained modified buffer D (Osborn *et al.*, 1989), 2.5 μg dI–dC, antibody if specified, and 5000–7000 c.p.m. ^{32}P-end-labeled 21 bp AP-1 olionucleotide (Santa Cruz Biotechnology, Santa Cruz, CA). Antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA (c-Jun; sc-822) and Oncogene Science, Cambridge, MA (pRb; OP28). Complex formation occurred on ice, with analysis of complexes bound to the oligonucleotide being performed by electrophoresis on a 4.5% native polyacrylamide gel followed by autoradiography.

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