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Independent Regions of Adenovirus E1A Are Required for Binding to and Dissociation of E2F-Protein Complexes

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The transcription factor E2F is present in independent complexes with the product of the retinoblastoma susceptibility gene, pRB, and a related gene product, p107, in association with the cyclin A-cdk2 or the cyclin E-cdk2 kinase complex. pRB and p107 can negatively regulate E2F activity, since overexpression of pRB or p107 in cells lacking a functional pRB leads to the repression of E2F activity. The products of the adenovirus E1A gene can disrupt E2F complexes and result in free and presumably active E2F transcription factor. The regions of E1A required for this function are also essential for binding to a number of cellular proteins, including pRB and p107. Through the use of a number of glutathione *S*-transferase fusion proteins representing different regions of E1A, as well as in vivo expression of E1A proteins containing deletions of either conserved region 1 (CR1) or CR2, we find that CR2 of E1A can form stable complexes with E2F. E1A proteins containing both CR1 and CR2 also associate with E2F, although the presence of these proteins results in the release of free E2F from its complexes. In vitro reconstitution experiments indicate that E1A-E2F interactions are not direct and that pRB can serve to facilitate these interactions. Complexes containing E1A, p107, cyclin A, and E2F were identified in vivo, which indicates that E1A may associate with E2F through either p107 or pRB. Peptide competition experiments demonstrate that the pRB-binding domain of the human E2F-1 protein can compete with the CR1 but not CR2 domain of E1A for binding to pRB. These results indicate that E1A CR1 and E2F-1 may bind to the same or overlapping sites on pRB and that E1A CR2 binds to an independent region. On the basis of our results, we propose a two-step model for the release of E2F from pRB and p107 cellular proteins.

The products of the adenovirus E1A gene are the first polypeptides synthesized after viral infection (43, 50, 67). Two major transcripts of the E1A gene are the 12S and the 13S mRNA species, which encode 243- and 289-amino-acid proteins, respectively, which differ by the presence of 46 amino acids in the middle of the 13S product (45, 55). The E1A proteins are essential for the activation of adenovirus early gene expression (50). In cooperation with the adenovirus E1B gene product as well as other viral proteins, such as the polyomavirus middle T antigen, or a cellular oncogene, such as *ras*, E1A is capable of transformation of cultured cells (24, 35, 58, 70, 79). The regions of E1A required for cellular transformation are contained within the 12S products and encompass two discrete regions of the protein (25, 46, 47, 59, 65, 71, 73, 79). These same regions are also essential for the interaction of E1A with a number of cellular proteins that were initially identified by their respective molecular weights (in thousands) as p300, p130, p107, p105 (the retinoblastoma protein [pRB]), p60 (cyclin A), and p33 (cdk2) (21, 23, 68, 72, 74). The correlation between binding to these host cell proteins and the requirement for transforming activity has led to the suggestion that E1A induces transformation by targeting a number of these cellular proteins.

The 12S product of E1A can also activate transcription from promoters containing DNA-binding sites for the transcription factor E2F (for reviews see references 51 and 52). E2F was originally identified as a DNA-binding protein that bound specific sequences within the adenovirus E2 gene promoter (41, 76). The activity of E2F in the cell is tightly regulated, partly through complex formation with a number of key regulators of cellular proliferation and cell cycle control. The retinoblastoma tumor suppressor gene product, pRB, and a closely related protein, p107, negatively regulate E2F activity (26, 33, 60, 78, 80). For pRB, this inhibitory effect is mediated through direct binding of E2F (30). The cyclin A-cdk2 and cyclin E-cdk2 kinase complexes also interact with E2F in a temporal fashion across the cell cycle (3, 5, 10, 42, 48, 53, 62). The stoichiometric interaction of these two kinase complexes with p107 suggests that their association with E2F is in part mediated through p107 (17, 20, 22). The regions of E1A required for enhancement of E2F activity are the same regions required for binding to pRB, p107, and p130. It is therefore believed that by binding to proteins which regulate E2F, E1A causes the release of free and presumably active E2F, which in turn can enhance transcription of genes whose promoters contain E2F DNA-binding sites.

E2F binding sites have been identified in the promoters of a number of cellular genes involved in DNA synthesis and cellular proliferation, such as the DNA polymerase α (54), thymidine kinase (40), dihydrofolate reductase (4, 44, 63), c-*myc* (34, 66), N-*myc* (32), and *cdc2* (8) genes. This finding suggests that E2F may play an important role in promoting cell growth and proliferation. In addition, regulation of E2F activity may be equally as important for maintaining cell growth control. This notion is consistent with the targeting of E2F regulators by oncoproteins such as E1A. Small DNA tumor viruses such as adenoviruses, the polyomaviruses, and the human papillomaviruses rely on their host cells for a number of functions required for their replication and propagation (15, 67). Similar to adenovirus E1A, the large tumor antigens of simian virus 40 (SV40) and other polyomaviruses and the E7 protein product of the high-risk-group human papillomaviruses also bind to pRB and p107 and lead to activation of transcription of genes containing E2F DNA-binding sites (9, 11, 16, 18, 49, 56). Therefore, modulation of

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E2F activity within the cell may be an integral part of the life cycle of these viruses.

An important property of these viral oncoproteins is their involvement in the transformation of affected cells. The regions of these proteins that are essential for transformation are also required for binding to pRB and p107 and the activation of E2F activity. In an effort to understand the mechanism by which E1A can disrupt E2F-protein complexes, we have analyzed E1A and determined the contribution of each of its conserved regions to this dissociation process. We demonstrate that E1A can be detected in stable complexes with E2F and that discrete regions of E1A are required for this property. On the basis of our results, we suggest a mechanism by which E1A can disrupt E2F-containing protein complexes.

MATERIALS AND METHODS

Cells and transfections. ML-1 (a human myeloid leukemia cell line), T98G (human glioblastoma), and C-33A (an RB^- human cervical carcinoma) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. HL-60 (human promyelocytic leukemia) cells were grown in RPMI 1640 containing 15% heat-inactivated fetal bovine serum. 293 cells (human embryonic kidney epithelial cells transformed by the adenovirus E1A and E1B genes) were grown in DMEM containing 10% calf serum. T98G and C-33A cells were transfected as previously described by the calcium phosphate precipitation method and harvested 36 h after addition of the precipitate (30).

Preparation of cell extracts. Extracts from ML-1 and HL-60 cells were prepared as previously described (31). Whole cell extracts from transfected T98G and C-33A cells were prepared by a modification of this protocol. Dishes (100-mm diameter) of transfected cells were washed twice with phosphate-buffered saline (PBS), scraped, and pelleted in microcentrifuge tubes. Cell pellets were then resuspended in 200 μl of 5× extraction buffer (100 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.4], 500 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 35% glycerol, 5 mM NaF, 1 mM dithiothreitol, 100 μg of phenylmethylsulfonyl fluoride per ml, and 5 μg each of aprotinin and leupeptin per ml) and subjected to three freeze-thaw cycles in liquid nitrogen. After incubation on ice for 30 min, cellular debris was removed by centrifugation at 100,000 × *g* for 45 min. Supernatants were then used as the source of whole cell extracts.

Gel retardation assays. Gel retardation assays using ML-1 cell extracts were performed as previously described (77). Where applicable, 0.5 μg of the indicated glutathione *S*-transferase (GST) fusion protein was incubated with the whole cell extract for 10 min prior to the addition of the ^{32}P-labeled oligonucleotide probe. Gel retardation assays using whole cell extracts prepared from transfected T98G and C-33A cells were performed by incubating 2 μl of extract with 0.2 μg of sheared salmon sperm DNA in a 9-μl total volume for 10 min at room temperature; 1 μl of ^{32}P-labeled oligonucleotide probe (0.2 ng, approximately 20,000 cpm) was then added, and the mixture was incubated at room temperature for a further 20 min. The DNA-protein complexes separated on 4% polyacrylamide gels containing 0.25× Tris-borate-EDTA buffer. Where indicated, 1 μl of the specified antibody (as tissue culture supernatant) was added during the first 10-min incubation. The sequences of the oligonucleotides used for gel retardation assays were 5′-ATT TAAGTTTCGCGCCCTTTCTCAA-3′ (wild type) and 5′-AT TTAAGTTTCGATCCCTTTCTCAA-3′ (mutant). Double-stranded probe (wild-type oligonucleotide) was end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase and purified on 12% polyacrylamide gels.

Release of proteins from immune complexes. Two hundred microliters (approximately 1 mg) of whole cell extracts from one 100-mm-diameter dish of T98G or C-33A cells transfected with the indicated expression plasmid construct was immunoprecipitated with the appropriate antibody in a 1-ml total volume. Immune complexes collected on protein G-Sepharose (Pharmacia) beads were treated with 0.8% sodium deoxycholate (DOC) as previously described (1, 6), and the released proteins were analyzed by gel retardation assays after the addition of Nonidet P-40 to a final concentration 1%. In experiments using GST fusion proteins as probes, 2 mg of HL-60 whole cell extracts was incubated with 5 μg of the indicated GST fusion protein for 2 h at 4°C. Protein complexes were then recovered by using glutathione-agarose (Sigma) affinity beads, treated with 0.8% DOC, and analyzed by gel retardation.

Construction of E1A plasmids. Plasmids containing the 12S or 13S cDNA product of adenovirus type 5 E1A were used as templates to amplify various regions of E1A by the polymerase chain reaction. The primers were p1 (5′-GCTAGGATCCATGAGACATATTATCTGCCAC-3′), p50 (5′-GCTAGAATTCTAAATCATACAGTTCGTGAAT-3′), p75 (5′-GCTAGAATTCCTGCACCGCCAACATTACAGA-3′), p120 (5′-GCTAGAATTCCACCTCCGGTACAAGGTT TGG-3′), p139 (5′-GCTAGAATTCCTCTTCATCCTCGTC GTCACT-3′), p30 (5′-GCTAGGATCCGATAATCTTCCAC CTCCTAGC-3′), p76 (5′-GCTAGGATCCGAAGGGATTG ACTTACTCACT-3′), pR3 (5′-ATGCGGATCCAGGTGAG GAGTTTGTGTTA-3′), and p289 (5′-ATGCGAATTCTTAT GGCCTGGGGCGTTTACA-3′). These primers were used for amplification of the indicated regions of E1A as follows: amino acids 1 to 50 (p1 plus p50), amino acids 1 to 75 (p1 plus p75), amino acids 1 to 120 (p1 plus p120), amino acids 1 to 139 (p1 plus p139), amino acids 30 to 289 (p30 plus p289) (13S), amino acids 76 to 289 (p76 plus p289) (13S), amino acids 139 to 289 (pR3 plus p289) (13S), and amino acids 76 to 243 (p76 plus p289).

The amplified DNA fragments were then digested with *Bam*HI and *Eco*RI and subcloned into pGEX-2T vector (Pharmacia) except for the fragment corresponding to E1A amino acids 139 to 289, which was subcloned into pGEX-3X (Pharmacia). GST-large T and GST-large T (K1 mutant) plasmids were a kind gift of V. Kraus (Duke University) and GST-E7 plasmid was a kind gift of K. Munger (Laboratory of Tumor Virology, National Cancer Institute). GST fusion proteins were purified from *Escherichia coli* XA90 cells as previously described (31). Primers p1 and p289 were also used in polymerase chain reactions with the E1A *dl*646 plasmid (for the E1A-Δconserved region 1 [CR1] construct) (74), the E1A 922/947 plasmid (for the E1A-ΔCR2 construct) (73), and the 10S E1A cDNA plasmid (64) as templates. Amplified DNA fragments from these polymerase chain reactions along with the DNA fragment corresponding to wild-type 12S E1A sequences were then digested with *Bam*HI and *Eco*RI, blunt ended, and subcloned into the pCMV/neo-BAM expression vector (2).

Immunoprecipitation. Transfected T98G cells were labeled with [^{35}S]methionine for 4 h in methionine-free DMEM. Cells were then washed with PBS and lysed in ELB buffer (50 mM HEPES [pH 7.0], 250 mM NaCl, 0.1% Nonidet P-40) containing 100 μg of phenylmethylsulfonyl fluoride per ml, 5 μg each of aprotinin and leupeptin per ml, 1 mM dithiothre-

itol, 1 mM EDTA, and 10 mM NaF for 30 min on ice. After removal of cell debris by centrifugation, the supernatants were used for immunoprecipitation as previously described (29), using the indicated antibodies. The antibodies used were PAb419 (monoclonal anti-SV40 large-T-antigen antibody [27]), M73 (monoclonal anti-E1A antibody [28]), XZ77 and XZ104 (monoclonal anti-pRB antibodies [36]), SD4, SD6, SD9, and SD15 (monoclonal anti-p107 antibodies [12]), and C160 (monoclonal anti-cyclin A antibody [72]). Polyclonal anti-E2F-1 antibodies were used to immunoprecipitate in vitro-translated E2F-1 protein (30).

In vitro binding. Plasmid p-βglobin/E2F-1 was constructed by subcloning a DNA fragment corresponding to the complete coding sequence of E2F-1 into pBSK-βglobin (37), using *Nco*I and *Bam*HI linkers. Plasmid pBSK, containing wild-type E1A coding sequences, or plasmid pGEMHE, containing E1A with deletions of sequences corresponding to amino acids 30 to 85 (E1A-ΔCR1) or amino acids 120 to 140 (E1A-ΔCR2) (both kind gifts of K. Lam, Massachusetts General Hospital Cancer Center, Boston), were used as templates. T7 RNA polymerase (Promega) was used to in vitro transcribe cRNAs from linearized plasmids according to the manufacturer's suggested protocols. The resulting cRNAs were used to program rabbit reticulocyte lysates (Promega) in the presence of [^{35}S]methionine. In a typical binding reaction, 10 μl of the indicated in vitro translation product was mixed for 2 h with 5 to 10 μg of GST fusion protein in ELB buffer as indicated in the figure legends. Protein complexes were collected on glutathione-agarose (Sigma) affinity beads for 30 min and washed three times with ELB buffer. After addition of SDS sample buffer, labeled proteins were analyzed on 8 or 10% polyacrylamide gels and visualized by fluorography. Affinity-purified pRB60 was a kind gift of A. Oliff (Merck Sharp & Dohme Research Laboratories). Plasmid GST-pRB60 was a kind gift of W. Kaelin (Dana Farber Cancer Institute, Boston, Mass.). For peptide competition experiments, the indicated quantities of the peptides were included in the binding reactions. The peptides used in these experiments have been described previously (13, 31) and correspond to E1A and E2F-1 sequences as follows: CR1 peptide, E1A amino acids 30 to 62; CR2 peptide, E1A amino acids 115 to 132; CR1+CR2 peptide, E1A amino acids 37 to 49-Q-117 to 132 (Q = glutamine); control peptide, mutated version of the CR1+CR2 peptide; and E2F-1 peptide, E2F-1 amino acids 409 to 427.

RESULTS

In vitro association between E2F and E1A. Previous experiments have demonstrated that sequences within both CR1 (amino acids 40 to 80) and CR2 (amino acids 120 to 140) of E1A are essential to disrupt E2F-containing protein complexes (57). We have produced various regions of E1A as GST fusion proteins expressed in bacteria and examined the effects of each fusion protein on E2F complexes in gel mobility shift assays. In these assays, the p107-E2F protein complex, which also includes the cyclin A-cdk2 and independently the cyclin E-cdk2 kinase complexes, is typically the lowest-mobility band observed (Fig. 1B, lane 1). As can be seen in Fig. 1B, addition of GST fusion proteins containing both CR1 and CR2 domains of E1A (lanes 5 and 6) resulted in an increase in the level of free E2F with a concomitant loss of the p107/E2F and a minor reduction of the pRB/E2F protein complexes. In contrast to previous experiments (6, 57) which used in vitro-translated E1A proteins to dissociate E2F complexes in gel mobility shift assays, we have not observed a complete disruption of pRB-E2F complexes with use of these E1A preparations. This may reflect differences between the reagents used for these assays. A new DNA-protein complex with a mobility slower than that of the p107-E2F band was also observed when these GST fusion proteins were included in the reaction. Addition of GST-E1A 1–75 neither altered the pattern of E2F protein complexes observed nor gave rise to an increase in the level of free E2F (lane 2). These results are consistent with previous reports that E1A CR1 alone is insufficient to disrupt E2F complexes in gel mobility shift assays. Addition of GST-E1A 76–243 fusion protein resulted in the loss of the p107-E2F complex without an increase in the level of free E2F observed. However, a new, more slowly migrating band was observed upon the addition of this GST-E1A protein to cell extracts. The appearance of the slower-mobility protein complex indicated either that GST-E1A 76–243 as well as GST-E1A 1–139 and GST-12S E1A fusion proteins induced the formation of new, slower-mobility E2F protein complexes or that these E1A proteins were present in E2F protein complexes and therefore resulted in their slower mobility.

To test whether E1A was present in association with E2F, we used distinct regions of E1A as GST fusion proteins and examined the ability of each fusion protein to interact with E2F when mixed with cell extracts. In this assay, GST-E1A proteins were mixed with human HL-60 cell extracts and adsorbed onto glutathione-agarose beads. Proteins associated with E1A were then released with DOC, and the supernatants were examined for the presence or absence of E2F DNA-binding activity in gel retardation assays. Figure 1C demonstrates that GST-E1A proteins representing successively longer fragments of the E1A amino terminus from amino acids 1 to 120 did not precipitate any E2F activity (lanes 3, 5, and 7). Addition of a further 20 amino acids of E1A in the GST fusion (from amino acids 120 to 139; E1A 1–139), however, resulted in the precipitation of E2F activity from these cell extracts (lane 9). Through further mapping, we have determined that the CR2 domain of E1A, amino acids 120 to 139, was the only region required for the association of E1A with E2F in this assay (Fig. 1A and C).

The SV40 large T antigen and the human papillomavirus E7 protein share high sequence homology with E1A CR2 and to a lesser degree with E1A CR1. Large T antigen and E7 bind to a number of the E1A-associated cellular proteins, and, like E1A, they can disrupt E2F protein complexes in gel retardation assays (7, 9, 11, 16, 18, 49, 56). Since E1A CR2 contains the sequences necessary for association with E2F, we examined the ability of SV40 large T antigen and human papillomavirus type 16 E7 protein to interact with E2F. Affinity precipitation and DOC release experiments similar to those described above were performed with GST-large T, GST-large T K1 mutant (mutated in the large T CR2 homology region), and GST-E7 fusion proteins. As can be seen in Fig. 1D, both GST-large T (lane 5) and GST-E7 (lane 9) fusion proteins precipitated specific E2F DNA-binding activity from HL-60 cell extracts in a manner similar to that of GST-12S E1A (lane 3). The K1 mutant of large T antigen as a GST fusion protein was unable to associate with cellular E2F in this assay (lane 7). These results indicate that the ability of CR2 sequences to associate with E2F is a conserved property among these viral oncoproteins.

E1A can associate with E2F in vivo. To address the possibility that improper folding of bacterially expressed E1A may have caused an artifactual association between

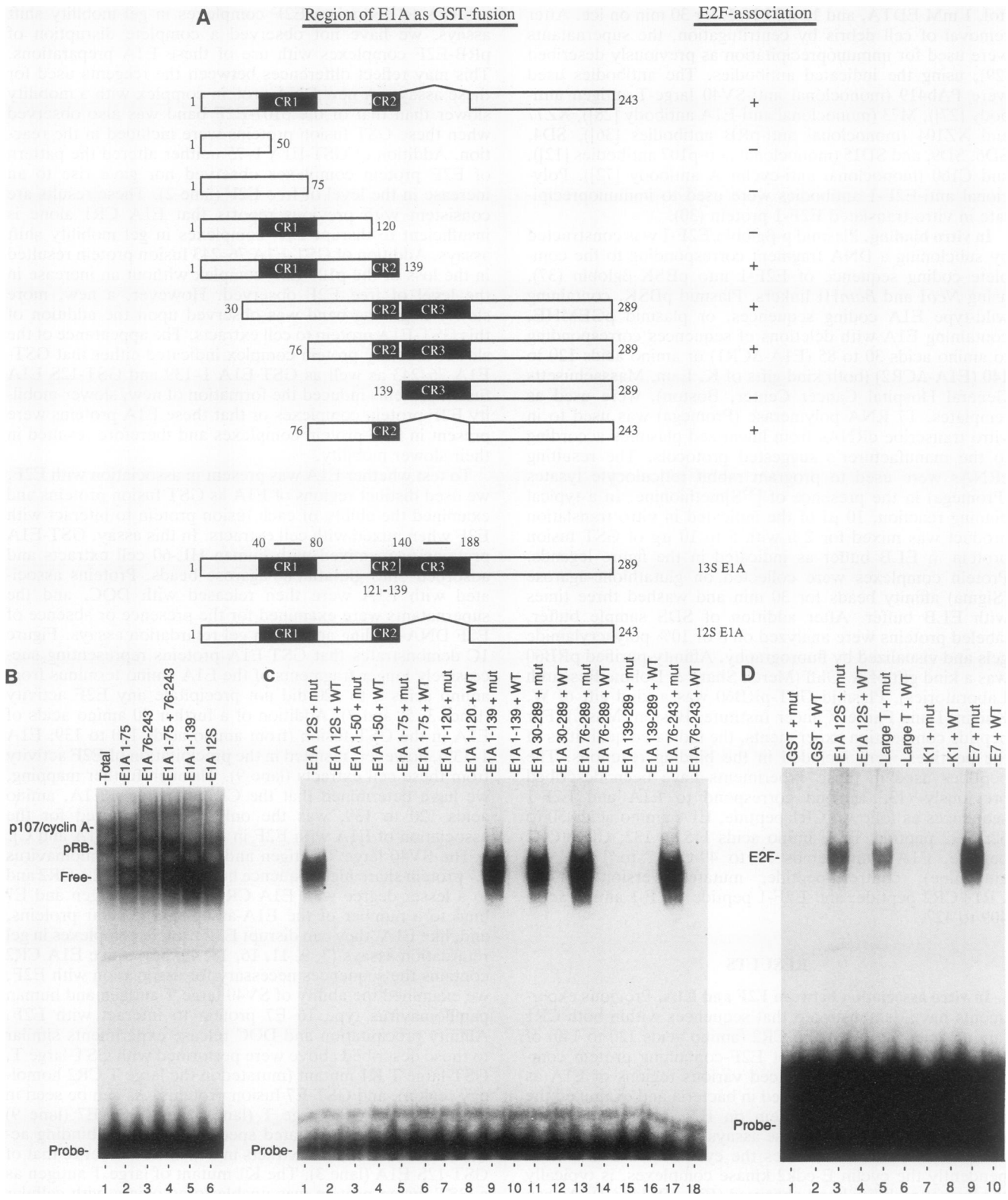

FIG. 1. E1A associates with E2F in vitro. (A) Line diagram representing the various GST-E1A fusion proteins used to map the regions of E1A required for association with E2F. Line diagrams of the 12S and 13S E1A are shown at the bottom as a reference for the boundaries of the different conserved regions of E1A. (B) ML-1 whole-cell extracts (15 μg) were used in band shift assays with 0.2 ng of ^{32}P-labeled E2F oligonucleotide probe; 0.5 μg of GST fusion proteins corresponding to wild-type 12S E1A (lane 6) or E1A amino acids 1 to 75 (lane 2), 76 to 243 (lane 3), or 1 to 139 (lane 5) were added to each reaction. In lane 1, no exogenous GST fusion protein was added; in lane 4, 0.5 μg of each GST-E1A fusion protein was coincubated with the cell extract. (C) HL-60 whole-cell extracts (2 mg) were incubated with 5 μg of the indicated

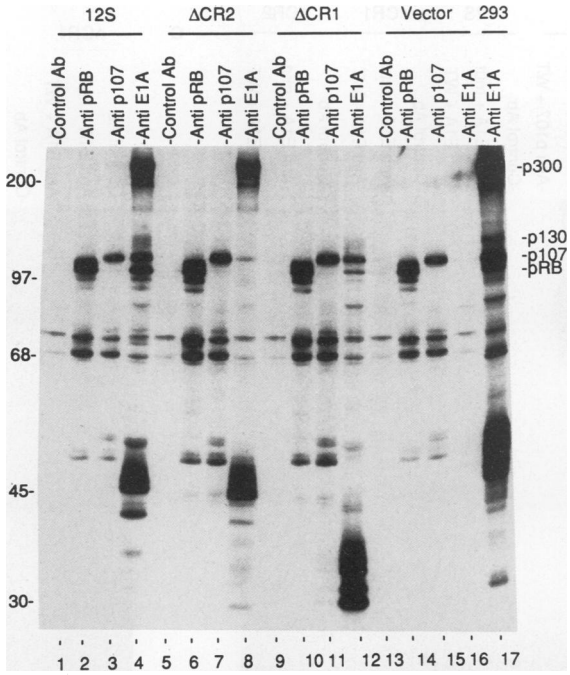

FIG. 2. Immunoprecipitation of transfected T98G cells. T98G cells were transfected with the indicated plasmid and labeled with [^{35}S]methionine 36 h after transfection. Cell lysates were immunoprecipitated with either anti-large T PAb419 as a control antibody (Ab) (lanes 1, 5, 9, and 13), anti-pRB XZ104 (lanes 2, 6, 10, and 14), a mixture of anti-p107 SD4, SD6, SD9, and SD15 (lanes 3, 7, 11, and 15), or anti-E1A M73 (lanes 4, 8, 12, and 16) antibodies. Immune complexes were collected on protein G-Sepharose beads, washed, dissociated by using SDS sample buffer, and resolved on an 8% polyacrylamide gel. Cell lysates prepared from [^{35}S]methionine-labeled 293 cells were immunoprecipitated with an anti-E1A M73 antibody (lane 17). Positions of size markers (in kilodaltons) and E1A-associated proteins are indicated.

E1A and E2F and to eliminate the possible effects of the GST moiety as a fusion to the amino terminus of E1A, we examined the ability of in vivo-expressed E1A proteins to associate with E2F. For this, we constructed plasmid constructs which contain wild-type 12S or E1A with deletions of the CR1 (E1A-ΔCR1, deletion of amino acids 30 to 85) or CR2 (E1A-ΔCR2, amino acids 122 to 129 deleted) domain for in vivo expression in transient transfection assays. Transfection of these plasmids into T98G glioblastoma cells resulted in the expression of similar levels of the expected-size E1A proteins (Fig. 2, lanes 4, 8, and 12). The previously characterized E1A-binding proteins (p300, p130, p107, and pRB) were readily detected in anti-E1A immunoprecipitations of labeled extracts of cells transfected with the 12S E1A expression plasmid (Fig. 2, lane 4). Consistent with previous reports which delineated the regions of E1A required for binding these cellular proteins, anti-E1A immunoprecipitations of cell extracts transfected with E1A-ΔCR2 lacked the p130- and pRB-associated proteins and demonstrated a drastically reduced level of p107 associated with E1A (lane 8). Deletion of E1A CR1 resulted in the loss of p300 and marginally reduced the levels of pRB and p107 observed in anti-E1A immunoprecipitates (lane 12). These results indicate that similar levels of E1A are expressed under these transfection conditions and that these proteins function as expected in terms of interaction with the previously characterized cellular proteins.

The ability of these E1A proteins to associate with E2F in transfected cells was then examined by an immunoprecipitation-DOC release assay (see Materials and Methods). As expected, E2F DNA-binding activity was detected when extracts of T98G cells transfected with the expression vector alone were immunoprecipitated with an anti-pRB or anti-p107 antibody (Fig. 3A, lanes 2 and 4). As a control, immunoprecipitation of these same cell extracts with an anti-E1A antibody did not release any detectable E2F activity (lane 1). However, E2F DNA-binding activity was recovered from anti-E1A immunoprecipitates of T98G cell extracts transfected with the E1A-ΔCR1 expression plasmids (lane 10). Anti-E1A immunoprecipitations of 12S E1A-containing cell extracts also released E2F activity (lane 7). Cell extracts containing E1A-ΔCR2 protein did not demonstrate any E2F DNA-binding activity released from anti-E1A immunoprecipitations (lane 13). These data are consistent with the results presented above in that E1A and E1A CR2 were capable of association with E2F.

To examine the ability of E1A to associate with E2F in the absence of pRB, we repeated the immunoprecipitation-DOC release experiments with cell extracts prepared from the RB$^-$ cervical carcinoma cell line C-33A which were transfected with the same E1A expression plasmids. From control vector-transfected cell extracts, the anti-p107 but not anti-pRB antibody precipitated specific E2F DNA-binding activity (Fig. 3B, lanes 2 and 4). Similar to what was found for T98G cells, E2F DNA-binding activity was detected from anti-E1A immunoprecipitations of 12S E1A- and E1A-ΔCR1-transfected C-33A cell extracts (lanes 7 and 10). Associated E2F DNA-binding activity was not detected in anti-E1A immunoprecipitations of E1A-ΔCR2-transfected C-33A cells (lane 13). These results demonstrate that the same regions of E1A are required for the E1A-E2F association in the absence of a functional pRB protein.

The 10S transcription product of E1A encodes a 30-kDa protein that accumulates late in adenovirus infection (64, 69). This transcript is produced as a result of an alternate splicing event which results in the deletion of amino acids 27 to 101 of E1A (in the 12S background). This naturally occurring E1A gene product resembles the E1A-ΔCR1 protein in that both contain E1A CR2 in the absence of CR1. For this reason, we sought to examine the ability of 10S E1A to associate with E2F. As shown in Fig. 3C, anti-E1A immunoprecipitations of 10S E1A-transfected T98G cell extracts contained E2F DNA-binding activity (lane 5). These results demonstrate that the protein product of the 10S E1A transcript can associate with E2F in a fashion similar to that of the E1A-ΔCR1 protein (Fig. 3C, lane 3). The role of the 10S protein in the adenovirus life cycle is currently unclear. The

GST-E1A fusion proteins. mut, mutant; WT, wild type. (D) HL-60 whole-cell extracts (2 mg) were mixed with 5 μg of GST (lanes 1 and 2), GST-12S E1A (lanes 3 and 4), GST-SV40 large T (lanes 5 and 6), GST-K1 mutant of large T (lanes 7 and 8), or GST-E7 (lanes 9 and 10). For panels C and D, protein complexes collected on glutathione beads were released by 0.8% DOC, and the supernatant was assayed by gel retardation for E2F DNA-binding activity. Excess cold wild-type or mutant oligonucleotide was incubated in the reactions as indicated to demonstrate the specificity of the DNA-protein complexes. Retarded complexes and free probe are indicated on the left.

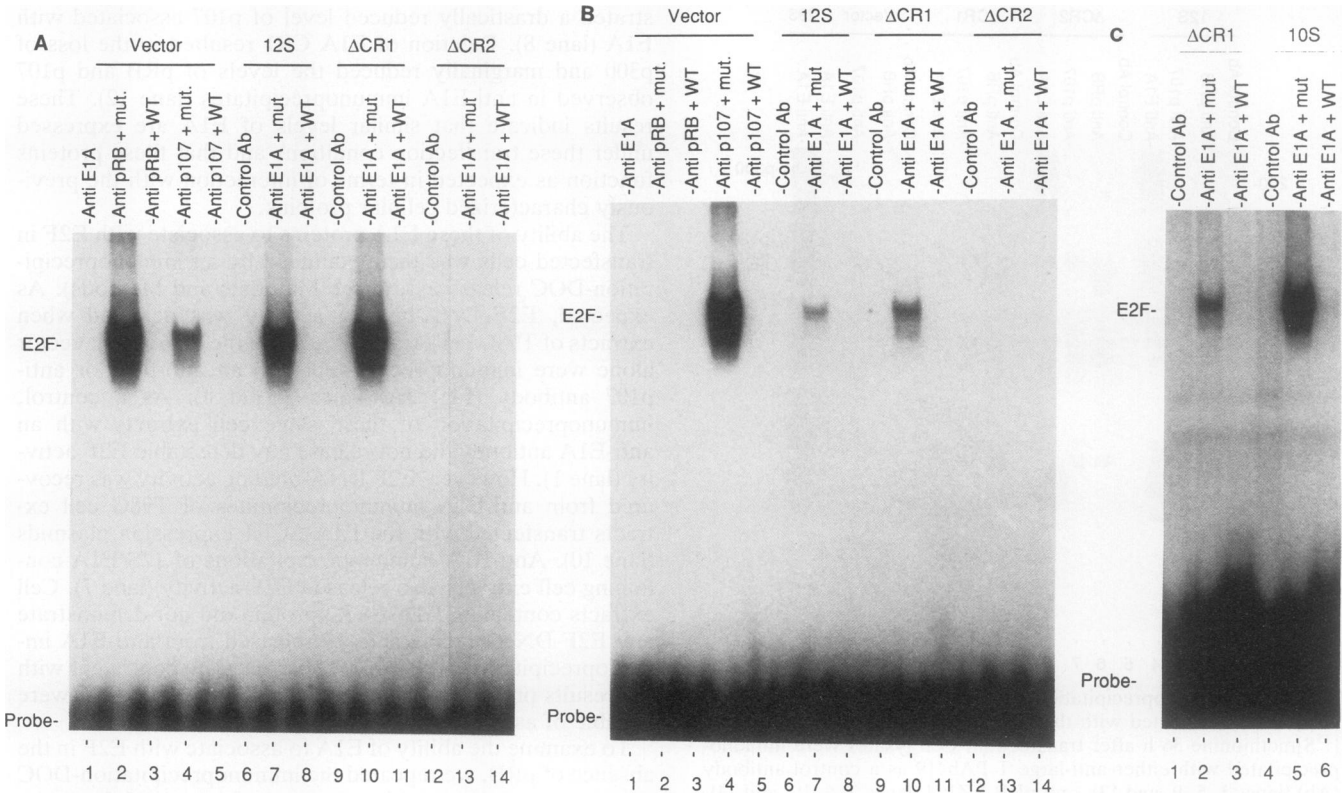

FIG. 3. E1A associates with E2F in vivo. Whole-cell extracts prepared from T98G (A) and C-33A (B) cells transfected with the pCMV/neo-Bam vector were immunoprecipitated with either anti-E1A M73 (lane 1), anti-pRB XZ77 (lanes 2 and 3), or anti-p107 SD4 (lanes 4 and 5) antibody (Ab). Extracts of these cells transfected with the pCMV/12S E1A, pCMV/E1A-ΔCR1, and pCMV/E1A-ΔCR2 expression plasmids were immunoprecipitated with PAb419 (lanes 6, 9, and 12) or anti-E1A M73 antibody (lanes 7, 8, 10, 11, 13, and 14). (C) Cell extracts prepared from T98G cells transfected with plasmid pCMV/E1A-ΔCR1 (lanes 1 to 3) or pCMV/10S E1A (lanes 4 to 6) were immunoprecipitated with PAb419 (lanes 1 and 4) or anti-E1A M73 antibody (lanes 2, 3, 5, and 6). Immune complexes were treated as described in the legends to Fig. 1B. Excess unlabeled wild-type (WT) and mutant (mut) E2F oligonucleotides were used as competitors to demonstrate the specificity of the DNA-binding activity.

10S E1A protein, however, is devoid of any transformation activity, and mutant adenoviruses, encoding only the 10S protein, are highly impaired in their growth potential (64).

p107 and cyclin A are present in the same complex with E1A and E2F. The above results demonstrate that stable E1A-E2F complexes can be detected in vitro and in vivo and that this association requires the E1A CR2 domain. However, we have not been able to demonstrate the direct binding of E1A with E2F (see below). It is therefore likely that this interaction is indirect and possibly mediated through contact with other cellular proteins. To determine whether such protein complexes exist, we performed the following gel retardation assays with extracts prepared from cells transfected with the E1A expression plasmids. As seen in Fig. 4A, when extracts of T98G cells transfected with the E1A-ΔCR1 expression plasmid were used in gel shift assays, a supershifted band was observed with the addition of an anti-E1A antibody (lanes 4, 7, and 10). Addition of a control antibody did not alter the mobility of any of the observed E2F complexes (lane 2). Whereas the addition of an anti-pRB antibody resulted in a supershifted pRB-E2F complex (to a position close to the p107-cyclin A-E2F complex; lane 3), incubation of both anti-E1A and anti-RB antibodies together did not result in the appearance of any new, retarded complexes (lane 5). However, the addition of anti-E1A and anti-p107 antibodies together resulted in a new, more slowly migrating complex (lane 8). This protein complex represents p107, E1A, and E2F in one DNA-protein complex. Similar results were observed when anti-E1A and anti-cyclin A antibodies were incubated together with E1A-ΔCR1-containing cell extracts (lane 11). Previous reports have demonstrated that cyclin A is present in the same complex with p107 and E2F (5, 10, 62). We therefore conclude that the most slowly migrating supershifted band observed in Fig. 4A, lanes 8 and 11, represents protein complexes that contain p107, cyclin A, E1A, and E2F.

When similar experiments were performed with extracts prepared from E1A-ΔCR2 plasmid-transfected T98G cells, additional more slowly migrating protein complexes were not detected with the anti-E1A antibody alone (Fig. 4B, lanes 4, 7, and 10) or in combination with anti-p107 and anti-cyclin A antibodies (lanes 8 and 11). This finding is consistent with the previous results in which E2F DNA-binding activity could not be detected in anti-E1A immunoprecipitation-DOC release experiments using extracts containing E1A-ΔCR2 protein (Fig. 3B, lane 13).

pRB can mediate the association between E1A and E2F. The regions of pRB that are required for binding to E1A, termed the pRB pocket, have been mapped to the carboxy-terminal two-thirds of the protein. The E1A-associated protein p107 (19), as well as the p130 protein (71a), share extensive sequence homology to the pRB pocket. This region in p107

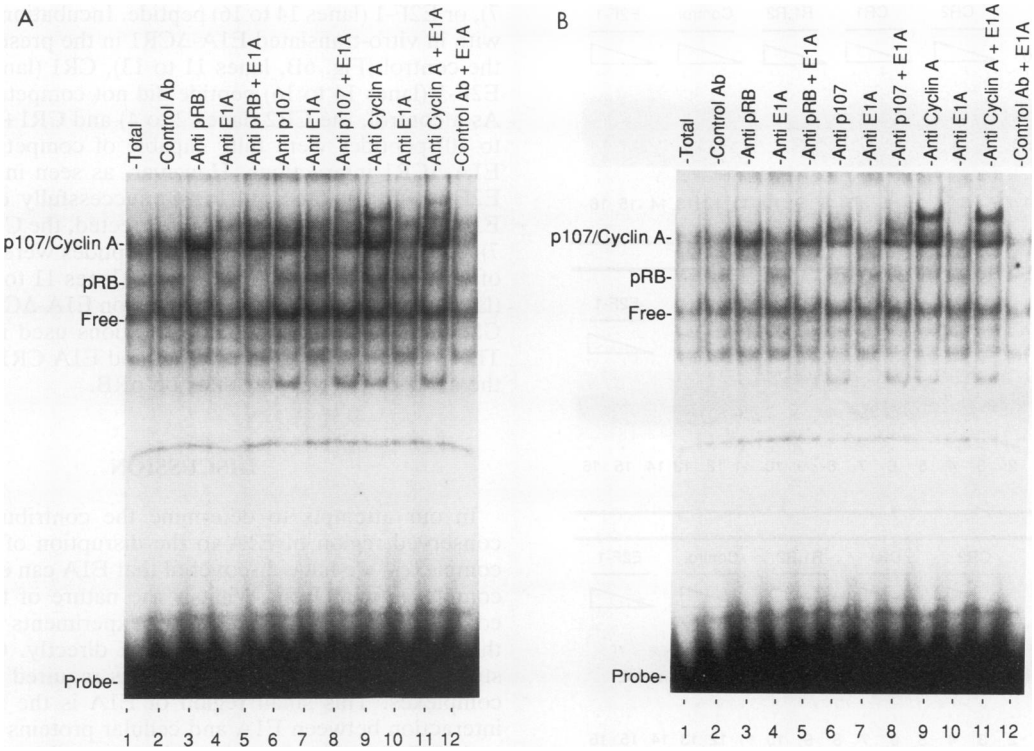

FIG. 4. E1A associates with E2F in complexes containing p107 and cyclin A. Extracts prepared from T98G cells transfected with either the pCMV/E1A-ΔCR1 (A) or pCMV/E1A-ΔCR2 (B) expression plasmid were used in gel retardation assays as described in Materials and Methods. One microliter of each antibody (Ab) (pAb419, XZ77, SD4, M73, or C160) indicated above the lanes was incubated with the extracts to supershift the appropriate E2F-protein complexes. DNA-protein complexes were resolved on 4% polyacrylamide gels.

is also required for interaction with E1A. pRB, p107, and p130 may therefore belong to the pocket protein family, members of which interact with E1A in similar manners. We have been interested in determining whether E1A can interact directly with E2F or whether this interaction is mediated only through the E1A-associated pocket proteins. We and others have recently isolated a gene, termed *E2F-1*, whose protein product displays many of the properties of the transcription factor E2F (31, 39, 61). We used E2F-1 to examine the possibility of its direct interaction with E1A in vitro. As shown in Fig. 5, GST (lane 4) and GST-E1A 1–75 (lanes 7) did not bind to in vitro-translated E2F-1. GST-E1A 76–243 (lane 9) and GST-12S E1A (lane 5) exhibited a minor capacity to bind to in vitro-translated E2F-1. This binding of E1A observed in lanes 5 and 9 may be due to the contribution of endogenous pRB present in rabbit reticulocyte lysates used for the in vitro translation of E2F-1. However, the interaction between GST-E1A 76–243 and E2F-1 was greatly enhanced when purified pRB60 (containing the pRB pocket) was coincubated in the reaction mixture (compare lanes 9 and 10). Similarly, the binding of GST-12S E1A to E2F was also augmented with the addition of purified pRB60 (lane 6). Inclusion of pRB60 in the reaction mixture did not result in the ability of GST-E1A 1–75 to interact with E2F (lane 8). These results indicate that the pRB pocket (pRB60) can facilitate E1A-E2F interactions, possibly by serving as a bridge in the complex.

E2F-1 and E1A CR1 bind to pRB in similar manners. Dyson and colleagues have previously demonstrated that peptides corresponding to sequences within the CR1 and CR2 domains of E1A make independent contacts with pRB and p107 (13, 14). This finding suggests that pRB and p107 contain two nonoverlapping surfaces for interaction with E1A. The major site of human E2F-1 protein needed for pRB binding has been mapped to an 18-amino-acid region near its carboxy terminus (31). We have shown that E2F and E1A

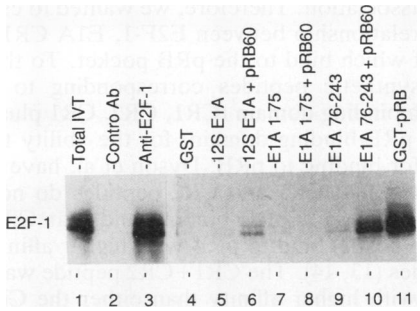

FIG. 5. pRB facilitates the association between E1A and E2F-1. Ten microliters of in vitro-translated (IVT) [^{35}S]methionine-labeled E2F-1 (lane 1) was mixed with 5 μg of GST (lane 4), GST-12S E1A (lanes 5 and 6), GST-E1A 1–75 (lanes 7 and 8), GST-E1A 76–243 (lanes 9 and 10), or GST-pRB as a positive control (lane 11). In lanes 6, 8, and 10, 0.5 μg of affinity-purified, bacterially expressed pRB60 was also included in the binding reaction. Ten microliters of the in vitro-translated E2F-1 protein was immunoprecipitated with the control monoclonal antibody (Ab) (PAb419; lane 2) or anti-E2F-1 antibody (lane 3). Protein complexes collected on glutathione-agarose affinity beads (lanes 4 to 11) or protein A-Sepharose beads (lanes 2 and 3) were dissociated in SDS sample buffer, analyzed on 10% polyacrylamide gels, and visualized by fluorography.

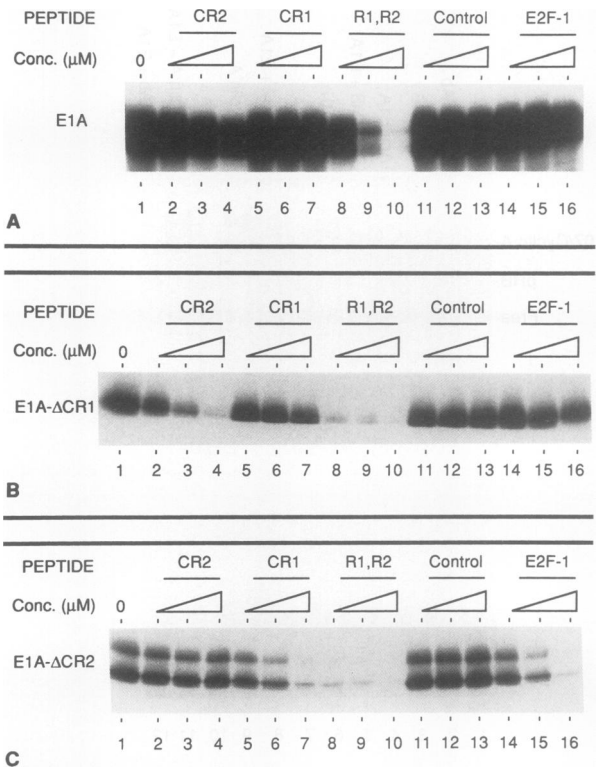

FIG. 6. The pRB-binding domain of E2F-1 can compete with E1A CR1 but not CR2 for binding to GST-pRB. Ten micrograms of GST-pRB60 was mixed with in vitro-translated [^{35}S]methionine-labeled wild-type E1A (A), E1A-ΔCR1 (B), or E1A-ΔCR2 (C) in the presence of increasing concentrations (0.5, 5, and 50 μM) of the peptides indicated at the top. Protein complexes were then adsorbed onto glutathione-agarose affinity beads and processed as described in the legend to Fig. 5.

CR2 can simultaneously exist in one protein complex with p107. Our results also indicate that E1A does not bind directly to E2F and that the pRB pocket can mediate the E1A-E2F association. Therefore, we wanted to establish the structural relationship between E2F-1, E1A CR1, and E1A CR2, all of which bind to the pRB pocket. To this end, we analyzed synthetic peptides corresponding to sequences within each binding domain (CR1, CR2, CR1 plus CR2, and the E2F-1 pRB-binding domain) for the ability to compete with E1A for binding to pRB. Dyson et al. have previously demonstrated that CR1 and CR2 peptides do not compete with one another for pRB binding and that CR2 peptides (coupled to beads) bind to pRB with higher affinity than do CR1 peptides (13, 14). The CR1+CR2 peptide was shown to bind pRB with higher affinity than either the CR1 or CR2 peptide, presumably because of the presence of two pRB-binding sites present on the same peptide. For our experiments, GST-pRB60 fusion proteins were mixed with in vitro-translated wild-type E1A or E1A containing a deletion of the CR1 or CR2 domain in the presence of increasing concentrations of each peptide.

As can be seen in Fig. 6A, the CR1+CR2 peptide (lanes 8 to 10), and less effectively the CR2 peptide (lanes 2 to 4), competed with wild-type E1A for binding to GST-pRB. No significant competition was noted when GST-pRB was incubated with wild-type E1A in the presence of increasing concentrations of the control (lanes 11 to 13), CR1 (lanes 5 to 7), or E2F-1 (lanes 14 to 16) peptide. Incubation of GST-pRB with in vitro-translated E1A-ΔCR1 in the presence of either the control (Fig. 6B, lanes 11 to 13), CR1 (lanes 5 to 7), or E2F-1 (lanes 14 to 16) peptide did not compete for binding. As a control, the CR2 (lanes 2 to 4) and CR1+CR2 (lanes 8 to 10) peptides were fully capable of competing for pRB–E1A-ΔCR1 interactions. However, as seen in Fig. 6C, the E2F-1 peptide (lanes 14 to 16) successfully competed for E1A-ΔCR2 binding to pRB. As expected, the CR1 (lanes 5 to 7) and CR1+CR2 (lanes 8 to 10) peptides were also capable of competing, whereas the control (lanes 11 to 13) and CR2 (lanes 2 to 4) peptides had no effect on E1A-ΔCR2 binding to GST-pRB at any of the concentrations used in this assay. These results suggest that E2F-1 and E1A CR1 may bind to the same or overlapping sites on pRB.

DISCUSSION

In our attempts to determine the contribution of each conserved region of E1A to the disruption of E2F protein complexes, we have discovered that E1A can exist in stable complexes with E2F. What is the nature of the E1A-E2F complexes? Our reconstitution experiments demonstrate that E1A and E2F do not interact directly. Our mapping studies revealed that CR2 of E1A is required to form such complexes. This small region of E1A is the major site of interaction between E1A and cellular proteins such as pRB and p107. It is therefore plausible to speculate that E1A-E2F interactions are mediated through pocket proteins. To this end, using extracts prepared from cells transfected with either wild-type 12S E1A or E1A with a deletion of the CR1 domain (E1A-ΔCR1), we have demonstrated the presence of protein complexes containing p107, cyclin A, E1A, and E2F. In addition, association between E1A and E2F was obtained when purified pRB was included in the reaction mixture. Furthermore, E2F DNA-binding activity was detected in E1A immunoprecipitations from transfected cell extracts that were precleared by using anti-p107 antibodies (data not shown), indicating that E1A-E2F complexes can exist in the absence of p107. These results demonstrate that cellular proteins such as pRB and p107 (which bind to E1A and E2F) can bridge the interaction between E1A and E2F.

Previous experiments have demonstrated that both the CR1 and CR2 domains of E1A are required to disrupt E2F protein complexes in gel retardation assays (57). Dyson et al. have also shown that CR1 and CR2 bind to pRB in an independent, nonoverlapping fashion (13). Each region can also bind to both p107 and the p130 proteins (14). The E1A-associated p130 protein has recently been identified in E2F complexes (7a). A possible mechanism by which E1A can dissociate E2F complexes containing pRB, p107, or p130 is through direct competition with E2F for binding to each of these cellular proteins. We have previously mapped the region of E2F-1 that is sufficient for direct pRB binding to an 18-amino-acid motif near its carboxy terminus. In this study, we have used pRB as a representative pocket protein and demonstrated that E2F-1 peptides can compete for binding of pRB pocket to the E1A CR1 but not CR2 domain. This finding indicates that the E1A CR1 domain and the E2F-1 pRB-binding domain bind to the same or an overlapping region of pRB. We also demonstrated that the CR2 domain of E1A binds to a region of pRB that is distinct from the E2F-1-binding surface on pRB. These conclusions are consistent with recent reports by Huang et al. (38) and Wu et al. (75) which suggest that E2F and the CR2 domain of the

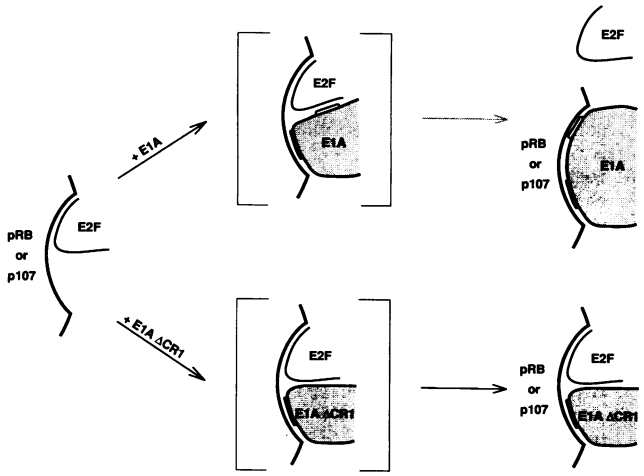

FIG. 7. Two-step model for the disruption of E2F complexes. The initial binding of E1A through its CR2 domain (high-affinity binding) would juxtapose CR1 to the same region of pRB-p107 that binds to E2F. CR1 binding would then lead to the competitive release of E2F from pRB-p107 and occupy the binding site. E1A proteins lacking CR1 would form stable complexes with pRB-p107 and E2F but would not release E2F.

human papillomavirus E7 protein bind to different regions of pRB.

Assuming that E1A CR1 and E2F bind to the same region of the pRB pocket, it is plausible to hypothesize that dissociation of E2F from pocket proteins may occur through direct competition between these two binding domains. What functional role may CR2 then play in disruption of E2F complexes? To answer this question, it may be important to consider the difference in affinities with which CR1 and CR2 domains of E1A bind to pRB. From direct peptide competition experiments, Dyson et al. have previously estimated that CR2 binds to pRB with an affinity 10-fold higher than that of CR1 (13). Our peptide competition experiments indicate that CR1 and E2F-1 bind to pRB with approximately the same affinity. In addition, E1A CR2 and E2F bind to distinct regions of pRB. Taking these two facts into consideration, we propose a model (Fig. 7) whereby the initial binding of CR2 to pRB may serve as an anchor to juxtapose and increase the local concentration of CR1 in the region of pRB that is bound by E2F. This would in turn increase the avidity of a CR1- and CR2-containing molecule (E1A) to a level which is higher than the affinity value of E2F for the same site on pRB and hence result in the competitive release of E2F with the subsequent occupation of the site by CR1. The 12S E1A-E2F complexes may represent intermediates in such a dissociation process (Fig. 7, top). E1A-ΔCR1 would therefore behave as a highly stable intermediate that is fully capable of binding pRB yet unable to dissociate pRB-E2F complexes (bottom). This is consistent with our results, since we reproducibly detect quantitatively more E2F capable of association with E1A-ΔCR1 than with 12S E1A. An alternate possibility is that a small subset of pRB-E2F or p107-E2F complexes can stably interact with E1A while the remaining complexes are disrupted. Whether a stable association between E1A-pRB or p107 and E2F has a functional role is unknown at present.

Interestingly, the 10S gene product of E1A, which lacks the entire CR1 domain, is capable of stable association with E2F. The function of this E1A protein is not known; however, 10S protein product accumulates late during adenovirus infection (64). The 10S E1A cannot cooperate with other oncogenes in transformation assays (64). Whether the association between 10S E1A and E2F is a functionally important step in the adenovirus life cycle remains to be resolved.

The large T antigens of SV40 and the polyomaviruses and the E7 protein products of the high-risk human papillomaviruses share regions of sequence homology with E1A CR2. Large T antigen and E7 bind to a number of the E1A-associated proteins, including pRB and p107. These oncoproteins have also been demonstrated to disrupt E2F complexes in gel mobility shift assays. We were able to demonstrate that SV40 large T antigen and human papillomavirus E7 were also capable of association with cellular E2F. These results indicate that the ability to associate with E2F is a conserved feature of small DNA tumor virus oncoproteins. On the basis of our two-step (binding-and-release) model, we suggest a common mechanism for the disruption of E2F protein complexes by these viral oncoproteins. For E1A, this may be visualized by the initial binding of CR2 to proteins such as p107 and pRB and the subsequent release of E2F through competition between CR1 and E2F. Large T antigen and E7 are also capable of interaction with these proteins through their respective CR2 homology regions. However, the mechanism whereby these two oncoproteins are capable of dissociating E2F-pRB and E2F-p107 complexes is less clear.

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