E1A Promotes Association between p300 and pRB in Multimeric Complexes Required for Normal Biological Activity

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The oncogenes of the small DNA tumor viruses encode transforming proteins with multiple domains that influence the cell cycle and aspects of the transformed phenotype. Like other gene products of this type, the adenovirus E1A proteins influence the cell by binding to specific cell growth control proteins. These include members of the retinoblastoma gene product (pRB) family, which are bound by the E1A region 2-specific site, and p300, which is bound at the E1A amino terminus. Binding at these two sites is largely independent, and discrete transcription-regulating functions remain intact in E1A products when only one or the other binding site is functional. In this report, immunoprecipitation with p300 antibodies reveals the presence of the pRB family proteins in p300 complexes when E1A is expressed in host cells, indicating that E1A can mediate physical contact between p300 and the pRB-related proteins. The ability of E1A to induce proliferation efficiently in quiescent primary cells correlates closely with the ability to bind p300 and individual members of the pRB family simultaneously in multimeric complexes, even though the E1A active sites can bind their target proteins efficiently when separated on different molecules. Conservation of a spacer region between the two binding sites that is required for simultaneous binding and efficient induction of proliferation supports the concept that the E1A protein structure has evolved to facilitate simultaneous binding. These results indicate that the E1A proteins are designed not merely to sequester these cellular products, but also to bring them into proximal association with each other in biologically significant complexes.

The oncogenes of the small DNA tumor viruses encode transforming proteins with multiple domains that influence the cell cycle and aspects of the transformed phenotype. Like other gene products of this type, the adenovirus E1A proteins influence the cell by binding to specific cell growth control proteins (reviewed in references 3, 4, 9, 24, 25, 30, and 31). Principal among these is the retinoblastoma gene product, pRB (39), which appears to function as a regulator of cellular transcription factors, in particular E2F (reviewed in references 17, 19, 29, 30, and 41). The E1A proteins have at least two independent protein binding domains concerned with cell cycle regulation (Fig. 1). One of these (inactivated specifically by mutations in E1A region 2) binds pRB and the related cellular products p107 and p130. The other (inactivated specifically by mutations at the E1A amino terminus) binds a large cellular product designated p300 (10, 11, 13, 37, 40). The p300 binding function, like the pRB binding function, is closely linked with E1A-mediated cell cycle induction (18, 36, 37). In addition, through its E1A association, p300 is implicated in mechanisms regulating tissue-specific gene expression and cell differentiation (5, 18a, 28, 35). Some functions of the p300 and pRB binding sites overlap, but their functions are not completely redundant. While each of these binding sites in E1A retains an independent ability to induce G₁ gene expression and cellular DNA synthesis in primary rodent cells, both regions are re-

quired for E1A-mediated induction of proliferation in these cells or for the ability of E1A to cooperate with other oncogenes such as activated *ras* to transform these cells more fully (18, 23, 36, 37, 44).

An important consequence of pRB binding by E1A is the activation of the cellular transcription factor E2F. pRB and the pRB-related proteins p107 and p130 exist in cellular complexes with E2F and appear to act as negative regulators of E2F activity until they are dissociated by a mechanism such as E1A binding (reviewed in references 19, 25, 29, and 30). p300 has not been linked with E2F activation. However, the cDNA sequence of p300 (10) predicts a protein highly related to a coactivator of CREB, the cyclic AMP (cAMP)-responsive element binding protein. The strong relationship (discussed in reference 1a) between p300 and CREB binding protein (CBP) (7) suggests that p300 itself may act as a regulator of cAMP-responsive pathways. E1A, by binding p300, may act to sequester p300 from cellular transcription complexes, much as E1A acts to sequester pRB from E2F complexes.

The results presented here suggest, however, that the specific role of E1A in binding p300 and the pRB family involves significantly more than passive sequestering of these cellular proteins. We show here that the E1A proteins bind p300 and individual members of the pRB family simultaneously in multimeric complexes. Moreover, the biological activity of the E1A products is greatly enhanced when both binding sites are present on the same molecule, bringing these two classes of cellular proteins into close proximity, even though the E1A binding sites can bind their target proteins efficiently when separated on different molecules. These results demonstrate that the E1A proteins are designed not just to sequester these cellular products but also to bring them into proximal association with each other in biologically significant complexes.

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FIG. 1. Two protein binding sites are required for E1A-stimulated cell proliferation. The E1A proteins induce quiescent cells to enter the cell cycle through the functions of two active sites that serve as binding sites for specific cellular proteins. The E1A active sites are formed by sequences in the regions left unshaded on the bar representing the 289-amino-acid-residue protein that is the product of the 13S (largest) Ad5 E1A splice product. Regions 1, 2, and 3 (indicated in the figure) are regions of high-amino-acid conservation among the E1A products of the various adenovirus serotypes. In Ad5 E1A, conserved region 3 is 13S-specific; the 12S splice product represents an in-frame deletion of these sequences. Regions 1 and 2 plus the relatively unconserved amino terminus (N) are required for host cell growth-regulating functions. Region 1 sequences contribute to both binding sites. On a linear representation of the E1A molecule, the sequences required for binding the p300 or pRB family appear to overlap. However, small deletions or single-amino-acid substitutions within region 1 readily distinguish subregions contributing different sequences to each binding function (reviewed in references 3 and 25). Moreover, antibody competition experiments indicate that the native E1A structure permits the formation of two binding sites that do not compete (37). The loop in the figure above is drawn arbitrarily as a schematic representation of a native structure that orients region 1 sequences towards the binding sites with which they interact. The products associated with the E1A region 2-specific binding site include the retinoblastoma tumor suppressor gene product (pRB) and the pRB-related proteins p107 and p130 (22), as well as their associated cyclins and kinases (represented by the smaller, unlabeled shapes in the diagram above) (3, 25). The amino-terminal binding site binds the large cellular protein p300, which has recently been found to be homologous to CBP, a coactivator of CREB, the cAMP response element binding protein. (The relationship between p300 and CBP is discussed in reference 1a.) The relative positions of the single-amino-acid substitutions used in this report are indicated by the three dots in the figure above. From the amino terminus, they are, respectively, RG2, an Arg→Gly substitution at amino acid position 2; YH47, a Tyr→His substitution at position 47; and 928, a Cys→Gly substitution at position 124.

MATERIALS AND METHODS

Purification of p300 for monoclonal antibody production. p300 was obtained from 293 cells (cells that constitutively express the E1A products [see reference 14]) by selective elution with a high-detergent buffer from E1A complexes immobilized with E1A-specific monoclonal antibody M73 (15) as described previously (42) and further purified as described here. After elution of p300 with radioimmunoprecipitation assay buffer, the p300-enriched fraction was passed through a 1-ml protein A-Sepharose column to remove contaminating antibodies and then was dialyzed overnight against dialysis buffer containing 10 mM Tris (pH 7.5), 50 mM sodium chloride, 1 mM dithiothreitol, and 10% glycerol at 4°C. The dialyzed p300 was loaded on a 1-ml primed Q-Sepharose Fast Flow (Pharmacia) column with a flow rate of 6 ml/h. The column was washed with 3 ml of dialysis buffer, and p300 was eluted with dialysis buffer containing 500 mM sodium chloride. Fractions thus enriched for p300 were used directly to immunize female BALB/c mice. Mice with a high-titer antibody response to p300 were sacrificed to obtain splenocytes for hybridoma construction by standard procedures (16). Individual hybridoma supernatants positive for mouse immunoglobulin production were screened by immunoprecipitation for specificity to p300. Two independent fusions yielded hybridomas producing p300-specific antibodies. NM1 and NM2 were obtained from the first fusion; NM3 through NM11 were obtained from the second fusion.

Immunoprecipitations and proteolytic peptide mapping procedures. Cells were metabolically labeled and immunoprecipitated essentially as described previously (36, 42). Typically, a lysate from a single 10-cm-diameter monolayer culture was immunoprecipitated with 100 μ l of monoclonal antibody supernatant. Immunoprecipitated proteins were separated on 10% polyacrylamide gels and analyzed by autoradiography or fluorography.

Protein digests were performed as described by Cleveland et al. (8) with *Staphylococcus aureus* V8 protease (Boehringer Mannheim) on proteins excised from wet gels. Photographic exposures were taken to facilitate the most direct comparison between M73- and NM11-derived material.

Cells and viruses. Monolayer cultures of HeLa, 293, and WI-38 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 100 μ g of penicillin per ml, and 100 μ g of streptomycin per ml. Primary baby rat kidney (BRK) cells were prepared from 6-day-old Fisher rats as described previously (27) and infected 2 to 3 days after being plated. All viruses were propagated on 293 cell monolayers. 12S.WT (26, 43), 12S.NCdl (44), and 12S.RG2 and 12SYH47/928 (37) have been described previously. BRK cell

infections and proliferation assays were performed as described previously (37) and in the text.

CAT reporter assays. Primary BRK cells were transfected with 5 μ g of an E2-CAT plasmid construct (21) and 1 μ g of pON260 β -galactosidase plasmid (34), and 16 h later, the cells were infected at a multiplicity of infection 10 PFU per cell with E1A variant adenoviruses as indicated in the text. Cells were harvested at 24 h postinfection, and transfection efficiency was normalized according to β -galactosidase activity. Chloramphenicol acetyltransferase (CAT) assays were performed with normalized lysates as described in reference 33.

RESULTS

p300-specific antibody coprecipitates members of the pRB family when E1A is present. We have developed a series of monoclonal antibodies raised against p300 immunoaffinity purified from E1A protein complexes as described in Materials and Methods. All NM series antibodies are able to immunoprecipitate p300 defined by prior elution from E1A complexes (data not shown). Among the best-producing lines is NM11, which we have used for most subsequent studies.

In the absence of E1A, NM11 antibodies precipitate the p300 species with no associated cellular proteins (Fig. 2, lane 3). When E1A is present, NM11 will precipitate a number of protein species in addition to p300 (Fig. 2, lane 4). Bands comigrating with the E1A proteins appear, and we have verified that these are E1A products by specifically reprecipitating them from denatured complexes with the E1A-specific antibody M73 (data not shown). Unexpectedly, but also readily apparent in p300-specific immunoprecipitations in the presence of E1A, are species comigrating with the E1A region 2-associated proteins pRB, p107, and p130 (Fig. 2, lane 4 compared with lane 2). These protein bands are not seen in p300-specific immunoprecipitations in the absence of E1A

FIG. 2. Immunoprecipitation with p300-specific monoclonal antibodies (α) in the presence of E1A. 32 P-labeled cell lysates were immunoprecipitated as described in Materials and Methods with the p300-specific monoclonal antibody NM11 or the E1A-specific monoclonal antibody M73, as indicated in the figure. Immune complex formation in the presence of E1A was analyzed in 12S.WT-infected (+) HeLa cell lysates (lanes 2 and 4). Complex formation in the absence of E1A expression was assayed in mock-infected (-) cell lysates (lanes 1 and 3). The migration positions of the E1A products, as well as those of p300, p130, p107, and pRB, are indicated in the figure.

FIG. 3. Peptide digest comparison of pRB, p107, and p130 from E1A-specific versus p300-specific complexes. Partial proteolytic digests of ^{35}S -labeled E1A region 2-associated proteins pRB, p107, and p130 (coprecipitated with the E1A-specific monoclonal antibody M73 from 293 cell lysates) were compared with digests of the respective comigrating products recovered from complexes brought down with the p300-specific monoclonal antibody NM11. Protein species were treated with 5, 50, or 500 ng of V8 protease, as indicated.

(Fig. 2, lane 3). These experiments were done with virus-infected cells, but the appearance of other E1A-associated proteins is not dependent on virus infection; the same results are seen when 293 cells (cells that express E1A constitutively) are compared with non-E1A-expressing cells (not shown).

To confirm that these comigrating proteins are indeed the E1A region 2-associated products, we compared the peptides resulting from partial V8 protease digestion of E1A-associated pRB, p107, and p130 with the digestion products of the comigrating proteins found in p300-specific immunocomplexes (Fig. 3). Each pair of comigrating bands generated essentially identical patterns. The high level of similarity of the comigrating species contrasts with the readily distinguishable differences among the pRB-related proteins and confirms that the 105- to 130-kDa species seen in p300 complexes in the presence of E1A are indeed pRB, p107, and p130.

The presence of the pRB family of proteins in p300 complexes is surprising, because the E1A binding sites are known to have genetically separable functions. The joint presence of these molecules in E1A complexes means that there is an unexpected potential for them to interact with each other as well as with the E1A proteins. In further experiments described below, we have examined the biological significance of the ability of E1A to promote multimeric complex formation.

Normal biological activity depends on the ability of E1A to form multimeric complexes, not merely the sequestering of E1A-targeted proteins. The relative independence of the two E1A binding sites is represented by several lines of data. The function of each protein binding site remains largely operative in the absence of the other, as shown by numerous mutant studies (2, 5, 37, 40), including the immunoprecipitation shown in Fig. 4 (see, for example, lanes 2 and 3). Likewise, certain gene-regulating effects of either active site remain intact when the other site is functionally inactivated. For example, either site alone is able to induce G_1 gene expression and DNA synthesis in primary rodent cells (18, 23, 36, 37). Nevertheless, unless both E1A active sites are present, these cell cycle effects

are incomplete and the stimulated cells do not go on to divide successfully. In plasmid transformation assays, the E1A active sites can cooperate from separate molecules, affirming the separable functions of these active sites, but E1A transforming activity is about 10-fold more efficient when the two active sites are present on the same molecule (23, 43).

The recognition of the ability to form multimeric complexes now reveals a molecular mechanism consistent with the much greater biological activity of a *cis* arrangement of the E1A binding sites. We have explored this model in the experiments shown in Fig. 4 and 5. Infection of primary BRK cells with an adenovirus expressing the wild-type E1A.12S product (12S.WT) results in the outgrowth of numerous colonies of proliferating cells whose presence is readily detectable after application of a vital stain (Fig. 5, plate 1). As discussed above, 12S.WT proteins will coprecipitate pRB, p107, p130, and p300 when brought down with E1A-specific antibodies (Fig. 4, lane 1). A single-residue substitution at the amino terminus results in an E1A mutant product (12S.RG2) that fails to coprecipitate p300 but readily coprecipitates pRB, p107, and p130 (Fig. 4, lane 2). This mutant fails to induce colony formation (Fig. 5, plate 2). A converse pattern is seen with a double-residue substitution mutant altering two key residues in the pRB family member binding site. This mutant, 12S.YH47/928, fails to co-

FIG. 4. Analysis of p300-specific complexes formed with E1A products carrying binding-site mutations. HeLa cells were infected at a multiplicity of infection of 10 with 12S.WT virus or with viruses expressing one of the mutant E1A products, 12S.RG2 or 12S.YH47/928. An additional set of cells was coinfecting at a multiplicity of infection of 10 plus 10 with both mutant viruses. The virus infection or coinfection from which each ^{32}P -labeled cell lysate was derived is shown above the lanes. Each lysate was divided into two aliquots and immunoprecipitated with either the E1A-specific monoclonal antibody M73 or the p300-specific monoclonal antibody NM11, as indicated in the figure (αE1A and αp300 , respectively).

FIG. 5. Virus immortalization assay. Primary BRK cells were infected (or coinfectd) at a multiplicity of infection of 10 (or 10 plus 10) with the wild-type and/or mutant viruses described in the legend to Fig. 4. At 6 weeks postinfection, cells were stained with 20% Giemsa stain to highlight colonies of actively proliferating cells.

precipitate pRB, p107, and p130 but readily coprecipitates p300 (Fig. 4, lane 3). This mutant also fails to induce colony formation (Fig. 5, plate 3). A coinfection with mutants 12S.RG2 and 12S.YH47/928 results in combined protein expression that shows a coprecipitating protein profile essentially the same as that seen in 12S.WT-infected cells (Fig. 4, compare lane 4 with lane 1). In spite of the ability of such a combination of mutant E1A proteins to bind and sequester the full profile of E1A-targeted cellular proteins, the coinfection fails almost completely to induce colony formation (Fig. 5, plate 4, and Table 1). (Increasing the multiplicity of infection fivefold, to 50 PFU per cell, does not increase the efficiency of colony outgrowth [additional data not shown].)

While the combination of mutant proteins appears to sequester the same profile of cellular proteins as 12S.WT, examination of the protein complexes formed specifically with p300 reveals a striking difference. Immunoprecipitation with p300-specific antibodies reveals that in 12S.WT-infected cells, the pRB-related proteins are in the same complexes as p300 (Fig. 4, lane 5), while no pRB-related proteins appear in p300-specific complexes derived from the mutant coinfectd cells (Fig. 4, lane 6). The ability of E1A to induce primary cell proliferation correlates strikingly with the ability to bind p300 and individual members of the pRB family in the same complex. Simple sequestering of the E1A-targeted proteins on separate E1A molecules is not sufficient to induce cell proliferation.

A requirement for multiple integration events does not explain the inefficient biological activity of separated E1A binding sites. The ability to measure the outgrowth of colonies of proliferating cells in the assay described above requires stable integration of the E1A genes. Therefore, it is possible that the failure of colony formation seen in cells infected with the mutant virus pair versus cells infected with 12S.WT virus results not from initial failure of the mutant E1A products to cooperate, but rather from the lower statistical chance of successfully stably integrating two E1A genes versus one. To explore this possibility, we examined the extent of proliferation at 3 days after infection, in a transient assay not dependent on stable integration. These results are shown in the micrographs in Fig. 6. At 3 days after infection, untreated (mock-infected) cells are found as small patches of epithelial cells among occasional fibroblasts (Fig. 6A). 12S.WT-infected cells are actively proliferating, as indicated by the intensity of the absorbance of the vital stain and the large number of epithelial cells crowded together in each patch (Fig. 6B). Cells coinfectd with

the mutant virus pair (Fig. 6C) show little difference from mock-infected cells; the cells remain uncrowded, in small patches, and do not take up more stain than mock-infected cells. This inability of the mutant coinfection to induce even transient proliferation indicates that the loss of biological activity is intrinsic to the mutants themselves and is not due to the decreased likelihood of integrating two constructs versus one.

Loss of biological activity in the separated binding sites is not correlated with defective region 2 function or lack of E2F induction. It is apparent from immunoprecipitations such as that shown in Fig. 4 that abrogation of the amino-terminal binding site by a mutation such as RG2 weakens the affinity of the region 2-specific binding site for pRB and its related proteins. This effect has been quantified in some experiments (for example, see reference 2). These observations, combined with the realization that these binding sites are commonly occupied simultaneously, make it likely that binding at the amino-terminal site actually stabilizes binding at the region 2-specific site. Such an interaction is consistent with an unexpected effect we have noted previously that the individual mutants, 12S.928 and 12S.RG2, alone have little deleterious effect on p107 binding, but the combination abrogates p107 binding to E1A, even though the residue affected by the RG2 substitution is not near the p107 binding sites (37).

We have considered the possibility that the failure of mutant coinfection to restore the biological activity lost in the single-site mutants is attributable to the weaker binding of the 12S.RG2 product to members of the pRB family rather than to a requirement for simultaneous binding. To test this, we assayed for activity attributed specifically to binding of the pRB

TABLE 1. Virus immortalization assay

Virus	No. of colonies in expt ^a :					
	1	2	3	4	5	6
12S.WT	82	61	70	30	334	253
12S.NCdl (residues 86–120 deleted)	ND ^b	ND	ND	1	3	0
12S.81–120	ND	ND	2	1	3	4
12S.RG2 + 12S.YH47/928	1	2	ND	ND	ND	ND

^a Values represent total from multiple plates (three to five plates per virus per experiment) stained 4 to 6 weeks postinfection.
^b ND, not done.

FIG. 6. Transient assay of E1A-induced proliferation. Primary BRK cells, infected as described in the legend to Fig. 5, were stained at 3 days postinfection with 20% Giemsa stain and photographed under $\times 200$ magnification.

family: activation of E2F promoter elements. Previous results have shown that deletion of 35 residues at the E1A amino terminus, including the residue altered in the RG2 mutant, does not impair the ability of E1A to alter E2F complexes in gel shift assays. A similar amino-terminal deletion mutant that also abrogates p300 binding functions likewise impairs neither the mobility shift activity nor the E1A-mediated ability to activate expression from an E2-CAT reporter plasmid in mouse L cells (32 [reviewed in references 25 and 30]).

To test this biological endpoint in our system, BRK cells were transfected with an E2-CAT plasmid and then infected as described above with 12S.WT or mutant viruses to probe E2F activity (Fig. 7). The 12S virus activates E2-CAT expression (lane 2 versus lane 1). Abrogation of the pRB, p107, and p130 binding function in the 12S.YH47/928 mutant abrogates the ability to activate E2-CAT (lane 3). However, although the 12S.RG2 mutant proteins have a weakened affinity for the pRB family, their binding function in vivo is sufficient to activate the E2-CAT reporter construct similarly to 12S.WT (lane 4). Thus, it is unlikely that the failure of the mutant coinfection to induce colony outgrowth in these cells is due to a failure of the amino-terminal mutant to titrate the members of the pRB family sufficiently.

A number of other lines of evidence indicate that the pRB family binding activity is functionally intact in 12S.RG2 or more extensive N-terminal deletion mutants (for review, see references 3 and 25). The most important finding in the present context is the observation that such mutants induce DNA synthesis at wild-type levels with only a brief delay in primary BRK cells (18, 37). This phenotype requires that numerous cell cycle-related genes be activated in the proper order and involves a number of distinct transcription complexes. This activity indicates strongly that all of the pre-S-phase functions attributable to pRB family binding are working at physiological levels in the 12S.RG2 mutant. This view is also supported by the observation that increasing multiplicities of infection do not increase biological activity in the experiment described in Fig. 5. The results are far more consistent with the interpretation that certain initial cell cycle effects can be induced by either site independently but that the full biological effects of E1A depend on the ability to bind these cellular proteins in multimeric complexes formed on the same E1A molecules. The biological effects recorded here and previously all suggest that 12S E1A mutants with a single first exon binding activity intact can induce S phase successfully in primary cells but that they fail to continue the cell cycle successfully

after S phase. Expression of the two binding sites on two different peptides does not overcome the S phase block efficiently, but the cell cycle proceeds normally when both sites are active in *cis*.

Conservation of a spacer region within E1A correlates with the requirement for simultaneous occupation of both binding sites. Between conserved regions 1 and 2 in the Ad5 E1A

FIG. 7. E1A mutant activation of E2-CAT. Primary BRK cells were transfected with the E2-CAT plasmid construct and infected 16 h later at a multiplicity of infection of 10 PFU per cell with the E1A variant viruses indicated in the figure. Cells were harvested 24 h postinfection. CAT activity (conversion of chloramphenicol to acetylated forms) was determined as described in Materials and Methods. In repeated experiments, conversion induced by 12S.RG2 did not differ distinguishably from that induced by 12S.WT, while conversion induced by 12S.YH47/928 did not differ distinguishably from the background level seen with dl312.

FIG. 8. Analysis of p300-specific complexes formed with E1A products expressing an internal deletion mutation. HeLa cells were infected at a multiplicity of infection of 10 with 12S.WT virus or with 12S.NCdl, a mutant expressing a deletion of the sequences encoding amino acid residues 86 to 120 (the shaded area between conserved regions 1 and 2 in the schematic diagram shown in Fig. 1). The virus infection from which each 32 P-labeled cell lysate was derived is shown above the lanes. Each lysate was divided into two aliquots and immunoprecipitated with either the E1A-specific monoclonal antibody M73 or the p300-specific monoclonal antibody NM11, as indicated in the figure (α E1A or α p300, respectively).

molecules is a nonconserved stretch of about 35 residues that is not required for efficient binding at either of the two protein binding sites in the E1A products (35, 40) (Fig. 1). Although the sequence of this region is not conserved, the existence of a spacer region larger than either the amino terminal or the region 2 active sites is a feature of all sequenced E1A genes. The correlation of biological activity with the ability to occupy both E1A binding sites simultaneously suggests that the need for simultaneous binding is the selective pressure promoting conservation of the spacer region. A region of some minimal length but no definite sequence may be required to provide enough flexibility in the E1A proteins to allow simultaneous binding at both sites. That the spacer region is indeed required for stable simultaneous binding of pRB-related proteins and p300 is shown in Fig. 8. The E1A mutant 12S.NCdl lacks residues 86 through 120. An E1A-specific immunoprecipitation (Fig. 8, lane 2) suggests that 12S.NCdl binds the E1A-targeted proteins much like 12S.WT (lane 2 compared with lane 1). However, examination of the complexes specifically containing p300 reveals that the actual binding profile of 12S.NCdl is very different from that of 12S.WT (Fig. 8, lane 4 compared with lane 3). 12S.NCdl binds both sets of proteins, but not readily in the same complexes, such that the pRB-related proteins are not brought together stably with p300.

We have considered the region deleted from the mutant lacking residues 86 to 120 largely dispensable for E1A transforming activity on the basis of *ras* cotransformation assays in

which this mutant shows about 53% of wild-type activity (27). However, as noted above, *ras* cotransformation is less dependent on the *cis* arrangement of the E1A binding sites than is virus immortalization. This may be a consequence of *ras* function itself. In virus infection assays, 12S.NCdl has a phenotype intermediate between those of 12S.WT and the coinfection of 12S.RG2 plus 12S.YH47/928. The 12S.NCdl virus induces initial proliferation much like the wild type (not shown), but it is very defective in establishing colonies of continuously proliferating cells (Table 1). A mutant with deletion slightly larger than that of 12S.NCdl (residues 81 to 120) and with a similar protein binding profile (35) has a similar biological activity (Table 1). It is possible that the spacer region deletion mutants retain transient proliferation activity, because with individually functional binding sites present on the same molecule, they remain able to promote at least a transient interaction between the cellular products targeted by the two binding sites, one that is too unstable to be detected in immune complexes. Nevertheless, the severe defect in immortalization activity relative to the essentially wild-type ability of 12S.NCdl to bind the p300 and pRB-related products individually (Fig. 8) argues strongly that the immortalization activity of E1A depends not on passive sequestering of these cellular targets but on actively promoting an association between them. In addition, the immortalization defect in the spacer region deletion mutants argues against a possibility that the lack of biological activity in the mutant coinfection results from inefficient coinfection rather than an inability of the E1A proteins to cooperate readily in *trans*.

In a previous publication, we noted the presence of pRB family members in p300-specific complexes with the Ad12 serotype E1A proteins (38). Although we did not examine the biological consequences of this interaction, the conservation of this binding profile across serotypes supports the suggestion that promoting proximity between p300 proteins and members of the pRB family is an important part of E1A function.

DISCUSSION

The demonstration that p300 and the region 2-associated proteins are present in the same E1A complexes reveals an unexpected potential for interactions between these growth regulatory proteins. Several lines of evidence support the implication that an interaction between these two families of proteins has important consequences in cell cycle regulation.

The conservation in E1A of a spacer region whose only known role is to allow simultaneous occupation of both binding sites implies strongly that the ability of E1A to promote physical proximity between the p300 proteins and members of the pRB family is central to the cell growth-stimulating functions of the E1A products. In addition, the ability of E1A to induce even transient cell proliferation is sharply reduced when the two E1A binding sites are present only in *trans*. This does not appear to result from indirect effects at either site when the distal site is altered, because most biological activities attributed to these sites are only minimally affected when the other site is inactivated. Either site can independently induce G_1 gene expression and cellular DNA synthesis to wild-type levels; the major discernable effect of single-site activity is the delayed kinetics seen with the mutants relative to that of the wild type (18, 23, 36, 37). Also, the ability of the 12S E1A protein to repress expression from the human immunodeficiency virus long terminal repeat is not impaired when the pRB binding site is inactivated (37). In addition, as shown here, inactivation of the p300 binding site has little detrimental effect on the ability of E1A to induce E2-CAT expression in infected cells.

These small effects contrast sharply with the large biological effects seen downstream of DNA synthesis in the cell cycle. When cells are induced with E1A constructs expressing only one functional first exon active site, the cell cycle fails after DNA synthesis and few cells go on to complete mitosis successfully (18, 23, 36, 37). As seen in Fig. 6, coexpression of the two active sites in *trans* has very little effect beyond what is seen with one active site alone; infected cells still fail to show significant proliferation. The low level of biological activity contrasts sharply with the extensive proliferation seen when the first exon active sites are present on the same molecule and are able to promote formation of multimeric complexes.

The simplest interpretation of these results is that the E1A molecule specifically promotes association between members of the two groups of proteins represented by pRB and p300. This association has a major influence on the course of events following DNA synthesis in the cell cycle. Possibly, since the pRB and p300 families are both strongly implicated in transcription pathways, there are specific late transcriptional effects that are facilitated by physical proximity between these cellular proteins. Representatives of both sets of proteins might participate together in transcription complexes. This kind of model is more consistent with recent data supporting the concept that pRB and p300 play active roles in transcription than with models envisioning species such as pRB as simple negative regulators that are targeted only for sequestering. The possibility that E1A promotes cooperative transcription complex formation via simultaneous binding at the amino terminus and region 2 also has precedents to some extent in the realization that E1A region 3 promotes interactions between the TATA binding protein (TBP) and upstream transcription factors by binding them simultaneously at distinct sites within region 3 (6, 12). In addition, genetic and biochemical evidence now indicates that E1A promotes a physical and functional interaction between p300, bound at the E1A amino terminus, and the transcription factor YY1, bound at a site within E1A region 3 (20).

Among the questions that arise from the recognition that p300 and pRB are brought into association by E1A is where the association actually occurs and whether it has any effect on E2F complexes. Our evidence does not in itself suggest that p300 is a regulator of E2F. When p300 associates with pRB on the E1A molecule, pRB is presumably already dissociated from E2F complexes. Moreover, formation of the multimeric complexes does not appear to affect E2F activity substantially, at least prior to S phase. The effects of the multimeric complexes are seen only after cells have completed DNA replication. It remains possible that the multimeric complexes modulate the function of E2F complexes that are active specifically after S phase.

It is also an interesting possibility that the p300-E1A-pRB complexes form on specific DNA sites. We have shown previously that most of the cellular p300 binds DNA and that this activity is not disrupted by E1A expression (32a). In addition, p300 can be detected in stable complexes with the basal transcription factor TBP, an interaction that is also not disrupted by E1A expression (1). These properties suggest that p300 is an integral part of active transcription complexes. This suggestion is consistent with the amino acid structure derived from the p300 cDNA (10). It is possible that E1A enters these complexes on promoter sites and is able to bring pRB into them. As indicated above, this type of interaction has precedents in the region 3 associations.

The correlation between cell cycle activity and E1A promotion of multimeric complexes bringing pRB and p300 family members into physical proximity suggests that proteins in

p300- and pRB-regulated pathways normally have some interaction in the absence of E1A, although such interactions have not yet been detected in uninfected cells. It is possible, of course, that such interactions could involve proteins that do not bind E1A directly or that the interactions are normally so transitory that they would not be detected readily. Although such interactions have not been observed *in vivo*, it is likely that E1A is providing valuable clues to the potential for such interactions. The strong effects on E1A biological activity that correlate with the ability to form these multimeric complexes suggest that they have highly important consequences for cell cycle progression.

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