Identification of Specific Adenovirus E1A N-Terminal Residues Critical to the Binding of Cellular Proteins and to the Control of Cell Growth

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Adenovirus early region 1A (E1A) oncogene-encoded sequences essential for transformation- and cell growth-regulating activities are localized at the N terminus and in regions of highly conserved amino acid sequence designated conserved regions 1 and 2. These regions interact to form the binding sites for two classes of cellular proteins: those, such as the retinoblastoma gene product, whose association with the E1A products is specifically dependent on region 2, and another class which so far is known to include only a large cellular DNA-binding protein, p300, whose association with the E1A products is specifically dependent on the N-terminal region. Association between the E1A products and either class of cellular proteins can be disrupted by mutations in conserved region 1. While region 2 has been studied intensively, very little is known so far concerning the nature of the essential residues in the N-terminal region, or about the manner in which conserved region 1 participates in the binding of two distinct sets of cellular proteins. A combination of site-directed point mutagenesis and monoclonal antibody competition experiments reported here suggests that p300 binding is dependent on specific, conserved residues in the N terminus, including positively charged residues at positions 2 and 3 of the E1A proteins, and that p300 and pRB bind to distinct, nonoverlapping subregions within conserved region 1. The availability of precise point mutations disrupting p300 binding supports previous data linking p300 with cell cycle control and enhancer function.

The adenovirus (Ad) early region 1A (E1A) gene is required for normal transcriptional activation of the remainder of the viral genes (4, 42, 46, 55) and is sufficient to induce extended proliferation in quiescent rodent cells such as primary baby rat kidney (BRK) epithelial cells (36). The E1A gene encodes three regions of highly conserved amino acid sequence in exon 1 (72), which also appears to contain three independent active sites (for reviews, see references 6 and 51); the sequence of the E1A protein is illustrated schematically in Fig. 1. Conserved region 3, specifically required for efficient viral gene transactivation (8, 26, 70), can be removed selectively by differential splicing (11, 56). Exon 1 sequences in the absence of region 3 are sufficient for the cell growth control activities of the E1A products (48, 50, 83). However, the two active sites in this region are not limited to sequences within conserved regions 1 and 2; growth control activity requires the relatively nonconserved region at the extreme N terminus as well (41, 66, 69, 79). The active site linked with conserved region 2 is supported by sequences in conserved region 1 (17, 79) (Fig. 1). These noncontiguous sequences form an apparently direct binding site for the product of the retinoblastoma (RB) tumor susceptibility gene (15, 17, 79). A pRB-related gene product, designated p107 (20), likewise binds via region 2 (17, 79) and is itself complexed with cyclin A and p33^{cdk2} (19, 21, 37). Binding of

a less well-characterized cellular product, p130, is also dependent on sequences in region 2 (25).

In addition to supporting the binding of region 2-associated products, conserved region 1 participates in the formation of a second active site, one that requires noncontiguous sequences at the extreme N terminus of the E1A proteins. The active site linked with the N terminus is involved, directly or indirectly, in binding a 300-kDa cellular product, p300 (17, 66, 79).

Binding of these various cellular products is closely linked with the cell growth control functions of the E1A products. E1A region 2 is a discrete genetic element that is shared by the products of the transforming proteins of several classes of small DNA tumor viruses (22, 49, 57, 65) and by cellular pRB-binding proteins as well (13). These all contain the conserved amino acid motif (D)-L-X-C-X-E, and the integrity of this motif is essential for the transforming and pRB-binding functions of the E1A, simian virus 40 (SV40) large T antigen, and human papillomavirus (HPV) E7 transforming gene products (3, 12, 49). Most of the region 2-associated proteins have also been identified as components of the cellular transcription factor E2F (2, 7, 9, 14, 63).

The N terminus of the E1A products is likewise strictly required for E1A transforming functions (41, 66, 69, 79). In addition, the N-terminal function has been linked specifically with E1A-mediated repression of viral enhancers (41) and tissue-specific gene expression (66). Moreover, p300 has been identified as a nucleus-localized phosphoprotein (81) with sequence-specific DNA-binding activity and affinity for enhancer element-related DNA motifs (59). Nevertheless, little is known about the exact amino acid sequences involved in E1A N-terminal function. Since the extreme N-terminal sequence is not strongly conserved among the E1A products of the various Ad serotypes (Fig. 2), there are

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FIG. 1. Schematic diagram of E1A active sites and associated proteins. The E1A regions required for cell growth-regulating functions (shown as unshaded regions in this diagram) are the binding sites for several cellular proteins. These regions constitute two independent active sites that can be inactivated selectively by mutations confined to either the extreme N terminus or region 2. The activity of either site is sufficient to induce the G₁ and S phases in resting cells, although both sites are required for the E1A immortalization function (38, 77, 84). While both sites can induce cell cycle functions, the ability of E1A to repress enhancers controlling tissue-specific gene expression is linked with the N-terminal active site and the binding of p300 (41, 66). A.A., amino acids.

few clues to the nature of the potentially essential residues. However, since these sequences are implicated in the E1A functions targeting control of cell growth and differentiation, it is of significant interest to determine whether specific E1A residues are essential for N-terminal function, especially as these sequences could conceivably contain motifs homologous to important cellular proteins.

MATERIALS AND METHODS

Cells and viruses. Monolayer cultures of HeLa, 293 (29), and COS (27) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 100 µg of penicillin per ml, and 100 µg of streptomycin per ml. Primary baby rat kidney (BRK) cells were prepared from 6-day-old Fisher rats as described previously (50) and infected 2 to 3 days after plating.

Ad2 E1A	MRHII-CHGGVITEEMAASLLDQLIE
Ad5	MRHII-CHGGVITEEMAASLLDQLIE
Ad4	MRHLRDLPEEIIIASGSEILELVVN
Ad7	MRHLRFLPQEIISSETGIEILEFVVN
Ad12	MRTEMTP--VLSYQEADDILEHLVD
Ad40	MRMLPDDFTGNWDDMFQ-GLLETEYV
Ad41	MRMLPDDFTGNWDDMFQ-GLLEAEHP
SA7	MRHL-ALEM--ISELLD-LGLDTIDG
SV40 TAg	MDKVLNREE--SLQLMDLLGLERSAW

FIG. 2. Comparison of N-terminal E1A protein sequences from various primate Ads. The first 25 amino acid residues of Ad5 E1A are compared with the N-terminal sequences of E1A from other serotypes. The sequences and alignments of Ad serotypes Ad5, Ad7, Ad12, and SA7 are presented as reported by Kimelman et al. (43); dashes indicate gaps introduced to maximize homology. The Ad2, Ad4, and Ad40 and Ad41 sequences are taken from Tokunaga et al. (71), Ishino et al. (40), and Allard and Wadell (1), respectively. Conserved features as noted by Kimelman et al. (43) are boxed. The first 24 residues of SV40 large T antigen (TAg), taken from Seif et al. (62), are also shown for comparison.

All viruses were propagated on 293 monolayers. The 12S.WT (50, 83) and 12S.928 (53) viruses have been described previously. The deletion mutant viruses used for the monoclonal binding site mapping experiments are 12S variants of constructs described previously, as indicated: 12S.2-36, 12S.15-35, 12S.51-116, 12S.81-120 (66); 12S.86-120, formerly called 12S.NCdl (53); 12S.38-67 (58). The mutant deleted for residues 1 to 14 is the virus *dl309-68* (32a), which encodes an altered initiation codon such that translation begins at the residue 15 methionine. The single-residue substitution mutants p12S.RG2, p12S.HN3, p12S.LS20, p12S.DA21, p12S.YG47, p12S.YH47, p12S.LP49, and p12S.EV55 were constructed by standard oligonucleotide-directed mutagenesis techniques. Subsequently, restriction fragments containing the point mutations were subcloned into a 12S.WT parent pUC plasmid, and the presence of the mutations directing the single-residue substitutions was verified by dideoxy sequencing through the entire restriction fragment.

The 12S double mutants 12S.RG2/928 and 12S.YH47/928 were constructed by first isolating a 773-bp *EcoRI* (bp 1 of Ad type 2 [Ad2])-to-*StyI* (bp 875 of Ad2) fragment from genomic constructs pE1A.RG2 and pE1A.YH47, containing the substitution, and ligating each to the larger *EcoRI*-to-*StyI* fragment of p12S.928 containing the vector sequences. The structures of the recombinant plasmids were confirmed by restriction digest analysis and selective hybridization with the oligonucleotides used to generate the mutations.

Plasmid p12S.T:1-24/E:25-243 is a 12S variant of pT.1-24/E.25-289 described previously (80). It was constructed by ligating equimolar amounts of the following four gel-purified restriction fragments and a synthetic double-stranded DNA oligomer: a 146-bp *SfiI-EcoRI* (SV40 nucleotide positions 5240 to 5094) restriction fragment from pT.1-100/E.121-289 (80); a 13-bp synthetic oligonucleotide with *EcoRI-RsaI*-complementary ends,

CCTGGGAAGAGGT
CTTCTCCA,

a 239-bp *RsaI-StyI* (E1A nucleotide positions 638 to 877) restriction fragment from p12S.WT (53); a 208-bp *StyI-XbaI* (E1A nucleotide positions 877 to 1336, minus the 12S intron) restriction fragment from p12S.WT; and a 4,420-bp *XbaI-SfiI* (E1A nucleotide position 1336 to SV40 nucleotide position 5240) restriction fragment from pT.1-100/E.121-289.

Plasmid p12S.T:1-14/E:20-243 was constructed by ligating equimolar amounts of the following three gel-purified restriction fragments: the 923-bp *EcoRI-AvaI* restriction fragment from pT.1-100/E.121-289 and the 257-bp *AvaII-StyI* and 3,319-bp *StyI-EcoRI* restriction fragments from p12S.WT.

The structures of the recombinant plasmids were confirmed by restriction digest analysis and sequencing through mutated regions. Mutant and chimeric E1A regions were rebuilt into virus constructs by the method of Stow (68) as described in detail previously (50).

Immunoprecipitations. Immunoprecipitations were performed as described previously (77). HeLa or BRK cells were infected at multiplicities of 10 to 50 PFU per cell. Electrophoretically separated immunocomplex components were visualized by autoradiography. E1A-specific mouse monoclonal antibodies M73, M1, M29, and M37 (31) and pRB-specific mouse monoclonal antibody XZ133 (39) were generously provided by Ed Harlow and Qianjin Hu. The results presented are representative of experiments repeated at least three times.

Virus immortalization assay. In all experiments, primary BRK cells were infected at a multiplicity of 10 PFU per cell. After adsorption at 37°C for 1 h, nonadsorbed virus and old medium were removed and replaced with fresh antibiotic-supplemented Dulbecco's modified Eagle's medium containing 5% fetal bovine serum. The infected cells were incubated at 37°C for 4 weeks with weekly feedings. At the end of this time, the monolayers were fixed with methanol-acetone (50:50) and stained with 20% Giemsa in phosphate-buffered saline (PBS) solution. Immortalization activity was determined by counting the number of established colonies of Giemsa-staining cells. For each experiment, two to seven plates were averaged per virus isolate.

Analysis of DNA synthesis. Infected BRK cells were labeled with [³H]thymidine (ICN) (25 μ Ci/ml of medium) and assayed for trichloroacetic acid-precipitable counts, as described previously (84).

Enhancer repression assay. For transfection with HeLa cells, 5×10^5 cells were plated on tissue culture plates (100-mm diameter) 24 h before transfection. Cells were transfected by the calcium phosphate coprecipitation method described by Graham and van der Eb (30). The human immunodeficiency virus (HIV) long terminal repeat (LTR)-chloramphenicol acetyltransferase (CAT) reporter plasmid, pBennCAT, has been described previously (24), as was the NF- κ B element deletion plasmid, pNFA-CAT (45). The total plasmid DNA concentration was adjusted with pUC18 to 20 μ g, and the total DNA concentration was adjusted to 40 μ g with salmon sperm DNA as the carrier. The calcium phosphate precipitate remained on the cells for 6 h, at which point the cells were treated with 15% glycerol-PBS for 2 min and then fed with fresh medium and incubated for an additional 30 h.

CAT activity was assayed as described by Gorman et al. (28) and quantified with an AMBIS Beta Scanner. The percent conversion of [¹⁴C]chloramphenicol to acetylated forms was calculated and normalized to the amount of total cell protein in the lysate.

RESULTS

Requirement for specific sequences at the extreme N terminus. As a first approach to identifying the essential N-terminal sequences, we explored the general requirement for specific N-terminal residues in the p300 binding function. Required N-terminal sequences, exclusive of conserved region 1, are known to be limited to the first 25 residues, as a deletion of residues 26 to 35 does not significantly alter the p300 binding ability of E1A, or any of its known biological activities (41). Mutants encoding deletions of residues 4 to 25, 15 to 35, or 2 to 36 make fairly stable proteins but fail to bind p300 or to transform primary cells (17, 41, 66). The only single N-terminal residue so far demonstrated to have an essential role is Arg-2, which, when changed to Gly, results in E1A products defective for transforming activity and p300 binding (79).

Since the N-terminal 25 residues are not well conserved among the E1A products of different Ad serotypes (Fig. 2), we first tested whether there is an overall requirement for specific residues in this region. To do this, we replaced Ad sequences encoding the first 20 N-terminal E1A residues with SV40 sequences encoding the N-terminal 14 residues of large T antigen. This chimeric virus construct (described in detail in Materials and Methods) also exchanges the E1A promoter for the SV40 early promoter. The chimeric virus is designated 12S.T:1-14/E:21-243. A second, similar, con-

FIG. 3. Association of cellular proteins with N-terminal chimeric constructs. Coprecipitation was assayed by immunoprecipitating Tran-³⁵S-labeled infected COS cell lysates with E1A-specific monoclonal antibody M73 (31). The positions of p300, the E1A proteins, and the region 2-associated group of proteins are indicated at the right. The positions of molecular size markers (in kilodaltons) are indicated at the left.

struct, 12S.T:1-24/E:25-243, replaces E1A residues 1 to 24 with T-antigen residues 1 to 24. We compared the T-antigen N-terminal sequence with the E1A N-terminal sequences in Fig. 2. The ability of these chimeric virus proteins to bind p300 was assayed by coimmunoprecipitation in several types of infected cells, including HeLa and primary BRK cells. The chimeric proteins showed no evidence of binding p300 in these cells (data not shown). Association with p300 was also assayed in COS cells, in which expression of the chimeric constructs is optimal as a consequence of their expression from the SV40 early promoter. This promoter is autoregulated by T antigen, which is constitutively expressed in COS cells (27). The chimeric proteins were immunoprecipitated with an E1A-specific monoclonal antibody (series M73; see reference 31) that recognizes an epitope near the C terminus of the E1A products. Even at a high expression level, no p300 binding was detected with the chimeras (Fig. 3, lanes 4 and 5). In the same conditions, the products of 12S wild-type E1A and the pRB binding-defective region 2 mutant, 12S.928 (with a single substitution changing Cys-124 to Gly), retain the ability to bind p300 efficiently (Fig. 3, lanes 2 and 3), even though they are expressed at lower levels relative to the chimeric products. Significantly, the chimeras retain the ability to bind the region 2-associated set of proteins, including pRB, verifying that substitution with the heterologous sequences does not grossly distort the protein structure of the chimeras. These results suggest that there is a specific sequence or structural requirement within the E1A N-terminal sequences for binding p300 that cannot be satisfied simply by substituting N-terminal sequences from a heterol-

FIG. 4. Association of cellular proteins with N-terminal point mutant constructs. Coprecipitation was assayed by immunoprecipitating Tran-³⁵S-labeled infected HeLa cell lysates with E1A-specific monoclonal antibody M73 (31). The positions of p300, the E1A proteins, and the region 2-associated group of proteins are indicated at the right. The positions of molecular size markers (in kilodaltons) are indicated at the left. The experiments shown in Fig. 4 through 8 are representative of results observed multiple times in independent experiments.

ogous protein. Note also that T antigen encodes a biological function that can complement the E1A N-terminal function (80), although it is not known whether any structural homology underlies this activity or where, precisely, in T antigen the complementing function lies. The chimeric construct results, do not, of course, rule out the possibility of a p300-binding site somewhere in T antigen.

Point mutant analysis indicates a requirement in stable p300 binding for specific residues in the N terminus, including positively charged residues at positions 2 and 3. As noted above, a deletion from residues 26 to 35 previously defined a region between the N terminus and conserved region 1 that is not required for p300 binding (41). This deletion demonstrated that p300-binding sequences in the E1A proteins are noncontiguous and indicated that there is some degree of flexibility in the spacing acceptable between the binding sequences at the N terminus and those in conserved region 1. Another deletion, larger by 10 residues, extending the deleted region N terminally to include residue 15, results in an E1A protein with no detectable p300-binding activity (66). This result did not distinguish whether the 15-to-35 deletion loses activity because the region from residues 15 to 25 contains specific essential residues, or more indirectly because the spacing between required N-terminal residues and conserved region 1 was reduced to a level incompatible with p300 binding. We addressed this question by introducing specific point mutations in the region from residues 15 to 25. The only conserved feature that has been noted in this stretch (43) (Fig. 2) is the appearance of a leucine residue immediately followed by a negatively charged residue, either glutamic or aspartic acid. We used oligonucleotide-directed site-specific mutagenesis to change each of these residues selectively. Leucine 20 was changed to serine in a mutant designated 12S.LS20. Aspartic acid 21 was changed to alanine in a mutant designated 12S.DA21. Both of these constructs remained able to bind p300 in HeLa cell lysates

(Fig. 4, lanes 5 and 6); however, while 12S.DA21 showed an essentially wild-type level of p300-binding activity, 12S.LS20 consistently showed a much reduced level, suggesting that stable p300 association does involve specific residues in the small stretch from positions 15 to 25.

Although there is little sequence conservation at the extreme N terminus of the E1A products, it is notable that all serotypically distinct E1A forms so far sequenced have an arginine at position 2, and several encode a histidine at position 3 (1, 40, 43, 71, 72) (Fig. 2). It has already been noted that changing arginine 2 to glycine disrupts p300-binding and -transforming activity (79). The conservation of these positively charged residues is intriguing, especially as the occurrence of positively charged residues in the highly acidic E1A.12S product is rather rare (sequence reviewed in reference 51). To determine whether the histidine residue plays a role in p300 binding in Ad5 E1A, we changed the histidine to the unchanged residue asparagine in a construct designated 12S.HN3. We also made a construct similar to the arginine-to-glycine mutation, which we designate 12S.RG2. The ability to bind p300 is much reduced in the 12S.HN3 mutant and is below the level of detectability in the 12S.RG2 product (Fig. 4, lanes 3 and 4), suggesting that the presence of both positively charged residues is important to stable p300 binding. The HN3 mutation has no discernible effect on pRB, p107, or p130 binding (Fig. 4, lane 4). The RG2 and LS20 mutant products show reduced, but consistently detectable, pRB- and p130-binding activity (see also Fig. 8) and essentially wild-type ability to bind p107.

pRB- and p300-binding sites in conserved region 1 do not appear to overlap. Mapping of the p300- and pRB-binding sites within conserved region 1 has been approached so far with only relatively broad deletion mutants. These constructs indicated that region 1 is important for stable binding of both of these products, but did little to distinguish whether the same specific residues are involved in binding both cellular products. The only distinction so far made between p300 and pRB binding requirements in region 1 is that sequences in the C-terminal half of conserved region 1 are less important for binding pRB than for binding p300; a linker insertion mutation deleting residues 61 to 85 disrupts p300 binding but has little effect on pRB binding (79).

An important consideration when analyzing functional sites that lie very close together is that deletion mutations may affect both sites because of structural constraints even when the deletion lies entirely within a single site. An alternative to using deletion mutations to map binding sites directly is to use antibody competition experiments. This approach has the advantage of permitting the assay of protein interactions on a wild-type molecule after deletion mutants have been used to map the antibody-binding epitopes. For example, it has been shown previously that antipeptide serum raised against an N-terminal peptide blocks p300, but not pRB, binding (82).

To address the question of whether p300 and pRB binding sites in region 1 actually involve overlapping sequences, we tested the ability of a set of monoclonal antibodies binding to different epitopes within this region to block the stable interaction of either product. The feasibility of this approach is supported by previous observations noting that different subsets of E1A-associated proteins could be immunoprecipitated with some of these antibodies (32). The associated protein patterns seen in immunocomplexes with region 1-specific monoclonal isolates were compared with the pattern seen in immunocomplexes with monoclonal isolate M73, which binds near the E1A C terminus and is compat-

FIG. 5. Exclusion of specific cellular protein associations by monoclonal antibody binding to E1A conserved region 1. Coprecipitation was assayed by immunoprecipitating Tran-³⁵S-labeled infected HeLa cell lysates with E1A-specific monoclonal antibody series (31) M73, which binds near the E1A C terminus, or M37, M29, and M1, which bind within conserved region 1. The positions of p300, the E1A proteins, and the region 2-associated group of proteins are indicated at the left.

ible with the binding of all identified E1A-associated proteins. Testing of a variety of monoclonal isolates previously generated by Harlow et al. (31) revealed three whose properties were useful for distinguishing p300 and pRB binding requirements (Fig. 5). Of these, two, M37 and M29, interfered strongly with the binding of pRB and other region 2-associated proteins, but formed complexes containing readily detectable levels of p300 (Fig. 5, lanes 2 and 4). Conversely, monoclonal isolate M1 interferes strongly with p300 binding but has little effect on the binding of the region 2-associated set (Fig. 5, lane 6).

We mapped the epitope-binding sites of monoclonal isolates M1, M29, and M37 using a series of E1A deletion mutants. It has been shown previously that region 2 and exon 2 sequences (i.e., residues C terminal to position 120) are not required for E1A product recognition by these isolates (32; our additional unpublished data). The data obtained from the present screen that are most significant for localizing the epitopes recognized by these antibodies are presented in Fig. 6. Deletion of residues spanning collectively the regions from positions 2 to 67 and 86 to 120 does not delete the epitope recognized by M1, suggesting that the M1 epitope is contained within residues 68 to 85. Consistent with this conclusion, M1 fails to bind an E1A product deleted for residues 81 to 120. By a similar analysis, we conclude that M29 and M37 recognize epitopes contained within residues 37 to 50, or 15 to 50, respectively. In addition, it is likely that the M37 epitope occurs near the middle of the 15-to-50 range because deletions of either 15 to 35 or 38 to 67 disrupt the epitope. Moreover, none of the

FIG. 6. Mapping of monoclonal antibody epitope recognition sites. (A) Ability of a group of monoclonal antibodies to recognize a series of E1A deletion and point mutant products was assayed by immunoprecipitating lysates of Tran-³⁵S-labeled infected HeLa cells. The residues deleted or substituted in the E1A products assayed are indicated above each lane. The presence (+) or absence (-) of the epitope targeted by each monoclonal isolate, indicated by the ability of the monoclonal isolate to precipitate the mutant product, is indicated below each lane. The position of the 12S.2-36 product, which is expressed at rather low levels (77), is highlighted (arrowhead) in the M29 panel to distinguish it from nonspecific background bands. (B) Deduced boundaries of the epitopes recognized by each monoclonal antibody are tabulated, along with the associated protein pattern observed in each specific immunocomplex.

FIG. 7. Association of cellular proteins with region 1 point mutant constructs. Coprecipitation was assayed by immunoprecipitating Tran-³⁵S-labeled infected HeLa cell lysates with E1A-specific monoclonal antibody M73 (31). The positions of p300, the E1A proteins, and the region 2-associated group of proteins are indicated at the right. The positions of molecular size markers (in kilodaltons) are indicated at the left.

point mutations discussed above, and in the section below, interferes with M37 recognition. Since these include alterations of residues 20, 21, 47, and 49, it is very likely that the M37 epitope falls within the more narrow range bordered by residues 22 to 46.

These results suggest strongly that p300 and pRB do not actually bind to overlapping sequences in region 1; more likely, they bind to distinct subregions. Binding of pRB, but not p300, appears to be dependent on sequences in the near vicinity of residues 37 to 50. Conversely, p300 binding, but not pRB binding, appears to be dependent on sequences in the near vicinity of residues 68 to 85. Overall, it is reasonable to propose that pRB utilizes sequences in the N-terminal half of region 1, while p300 binding utilizes sequences limited to the C-terminal half of region 1. A simple model of how this binding pattern may be accommodated is suggested in Fig. 11.

Binding of pRB, but not p300, is disrupted by specific mutation of a tyrosine residue at position 47 within conserved region 1. The limits we propose on the binding sites of p300 and pRB are consistent with the observation cited above (79) suggesting that sequences from residues 61 to 85 are far more important for p300 binding than for pRB binding. They are also consistent with previous suggestions that the region from residues 41 to 49 of E1A region 1 might play a role in pRB binding based on homology to sequences in the HPV type 16 E7 product, which also contains a homolog of E1A region 2 (57) and associates with pRB (16). Within the E7/E1A conserved region represented by E1A residues 41 to 49, there occur three leucine residues with closely conserved spacing (57). There is also a tyrosine residue in both E1A and E7 within this region, which could be significant based on the rarity of occurrence of this residue in general, and in E1A in particular; this is the only tyrosine residue among the 243 residues of the Ad5 12S E1A product.

To assess the involvement of this region in pRB binding, we constructed several additional mutations. Leucine 49 was changed to proline in a construct designated 12S.LP49. Tyrosine 47 was changed to both the somewhat structurally similar residue histidine (12S.YH47) and the less similar residue glycine (12S.YG47). In addition, to probe the involvement of more distant sequences, we changed the strongly conserved glutamic acid at position 55 to valine

(12S.EV55). This last mutation has the effect of introducing a hydrophobic residue into a very hydrophilic region. Association with cellular proteins was assayed by coimmunoprecipitation from infected HeLa cell lysates (Fig. 7). As predicted from the monoclonal antibody competition experiments, none of these mutations had a pronounced effect on p300 binding (Fig. 7, lanes 3 to 7, 10, and 11). In addition, the pRB-binding activity of the 12S.LP49 and 12S.EV55 mutations, although sometimes reduced, was always readily detectable (Fig. 7, lanes 5, 6, and 11). However, pRB binding was not readily detected in these conditions in the mutants with an altered tyrosine residue (Fig. 7, lanes 3, 4, 10, and 17). 12S.YH47 in particular showed a consistent lack of pRB association (Fig. 7, lanes 3, 10, and 17). This mutant is clearly defective in p130 binding as well, but p107 binding remains essentially unaffected. The generation of mutations at position 47 which severely disrupt pRB, but not p300, binding supports the hypothesis that p300 and pRB associate with distinct, nonoverlapping sequences within conserved region 1.

Despite the strong conservation of the leucine residues in this region, the Leu-49 mutation did not severely affect the appearance of the coprecipitating products. This does not, of course, exclude the involvement of the leucines in pRB binding; it is possible, for example, that no single leucine residue is individually essential for this activity, although each may play a role. Overall, these results indicate that pRB binding in region 1 involves contact with specific residues, and that, at the least, Tyr-47 is among these. These results are illustrated schematically in the model proposed in Fig. 11.

The Tyr-47 residue is the second individual residue identified in the E1A products at which substitution severely impairs pRB binding without severely impairing the binding of the pRB-homologous product, p107; mutation of the Cys-124 residue in region 2, in a construct designated pm928, results in a similar phenotype (49, 77) (Fig. 7, lanes 7 and 18). While neither 12S.YH47 nor 12S.928 is severely impaired in the p107 binding property, the combination of these two single point mutations, in a virus designated 12S.YH47/928, abrogates p107 binding (Fig. 7, lane 13). p300 binding remains unimpaired in the 12S.YH47/928 mutant. Curiously, the combination of pm928 and pmRG2 in the virus desig-

TABLE 1. Virus immortalization assay

Virus	No. of colonies, averaged from multiple plates (% of wild type), in expt:							Avg activity (%)
	1	2	3	4	5	6	7	
Mock	ND ^a	0	0	0	0	0	0	
dl312	0	ND	0	0	0	0	0	
12S.WT	73	110	81	36	31	66	66	100
12S.RG2	0	0	0	0	0	0	0	<1
12S.HN3	ND	ND	6 (7.4)	1 (2.8)	2 (6.5)	4 (6)	4.5 (6.8)	5.9
12S.LS20	ND	ND	6 (7.4)	14 (38.9)	6 (19.4)	12 (18)	7 (10.6)	18.9
12S.DA21	ND	18 (16.3)	44 (54.3)	26 (72.2)	29 (93.5)	51 (77)	46 (69.7)	63.8
12S.YH47	89 (122)	37 (33.6)	31 (38.2)	6 (16.7)	19 (61.3)	57 (86)	42 (63.6)	60.2
12S.YG47	29 (39.7)	22 (20.0)	38 (46.9)	20 (55.5)	31 (100)	64 (97)	57 (86.3)	63.6
12S.LP49	41 (56.1)	ND	54 (66.7)	34 (94.4)	60 (196)	75 (106)	76 (115)	100
12S.EV55	87 (119)	ND	58 (71.6)	40 (111)	63 (200)	91 (138)	83 (126)	100
12S.928	0	0	0	0	0	0	0	<1

^a ND, not done.

nated 12S.RG2/928 has a similar effect on p107 binding. While neither of these point mutations alone has a severe effect on p107 association (Fig. 4, lane 3; Fig. 7, lanes 7, 16, and 18), the combination impairs p107 binding severely (Fig. 7, lane 12). The same phenotype was observed previously when the deletion of residues 2 to 36 was combined with the pm928 mutation (77).

Cosegregation of immortalization activity with requirement for specific amino acid residues in p300 binding. In the following experiments, we used the single-residue mutations described above to analyze more precisely the links between the binding of specific cellular products and E1A biological activity. It has already been reported that substitution of residue 2 has severe disruptive effects on both p300 binding and the E1A transformation function assayed in cooperation with the *ras* oncogene (79). Our results extend and confirm this observation. Impairment of the E1A-dependent host-cell immortalization function (Table 1) correlates closely with impairment of p300-binding activity. 12S.HN3 and 12S.LS20, which show clearly reduced p300-binding activity, also show reduced immortalization activity, to levels averaging, respectively, 5.9 and 18.9% of wild-type activity. 12S.RG2, which shows no detectable p300-binding activity, also shows no detectable immortalization activity. The biological phenotype of these mutants correlates more closely with their p300 binding deficiencies than to partial reduction in pRB binding because 12SHN3, at least, has no discernible defect in its region 2 associations (Fig. 4). The 12S.DA21 mutation, although relatively close to important p300-binding residues, shows no disruption of p300 binding and maintains consistently high levels of immortalization activity, on average, more than 60% of 12S.WT activity. 12S.LP49 and 12S.EV55, which also show little disruption of protein associations, show essentially no loss of immortalization function.

Notably, the 12S.YH47 and 12S.YG47 mutations have only a mild effect on the BRK immortalization function, even though they clearly impair the pRB binding pattern in HeLa cells. Their protein association pattern appears as defective for pRB binding as that of 12S.928 (Fig. 7), yet in clear contrast to the loss of immortalization function in 12S.928 (included in Table 1 for comparison), they retain as much as 60% of wild-type activity.

Protein association in BRK cells. We have previously assessed the cellular protein associations of a variety of E1A mutants in both HeLa and BRK cells (49, 66, 77, 80) and found no significant differences dependent on host cell type.

Nevertheless, because the biological activity of the 12S.YH47 and 12S.YG47 mutants is so striking in BRK cells, we assessed the ability of these mutant products to associate with pRB specifically in these cells. While the protein association pattern of most of the single-residue mutant products described here is similar in BRK and HeLa cells (Fig. 8A), the pattern of the 12S.YH47 and 12S.YG47 mutant products is notably different; in BRK cells, these mutants appear readily able to bind pRB (lanes 7 and 8). The 12S.928 product (Fig. 8A, lane 11) remains defective for pRB binding as shown previously (77). Inasmuch as pRB phosphorylation is cell cycle regulated, and only the underphosphorylated forms are generally thought to have growth-suppressing activity (reviewed in reference 78), the ability of the 12S.YH47 and 12S.YG47 products to bind pRB in these conditions may indicate a selective loss of the ability to bind more highly phosphorylated pRB forms, forms that would be expected to predominate in the very rapidly cycling HeLa cells. At the relatively early time after infection (10 h) at which the lysates in Fig. 8A were harvested, the RB products, especially in the mutant-infected cells, are still relatively underphosphorylated, as evidenced by the relative absence of multiple pRB bands in pRB-specific immunoprecipitations (Fig. 8B). At later times after infection, the RB products are largely phosphorylated and show multiple bands in pRB-specific immunoprecipitations (Fig. 8C, lanes 19 to 21). At this time, the 12S.YH47 and 12S.YG47 products still show pRB binding, although with an apparent preference for underphosphorylated forms (Fig. 8C, lanes 14 and 15). However, this result must be considered with caution, as even 12S.WT shows an apparent preference for underphosphorylated forms (Fig. 8C, lane 13).

At early times postinfection in BRK cells, the 12S.928 product shows little ability to bind p107 (Fig. 8A, lane 11). However, at later times this ability is readily apparent (Fig. 8C, lane 16). 12S.YH47 and 12S.YG47 bind p107 readily in BRK cells at both early and late times (Fig. 8A, lanes 7 and 8; Fig. 8C, lanes 14 and 15). Despite the ability of 12S.YH47 and 12S.YG47 to associate with pRB in BRK cells, the phenotype of the 12S.YH47/928 construct is the same in BRK as in HeLa cells: it is severely deficient in both pRB and p107 binding (Fig. 8C, lane 17). Thus, even in BRK cells, the Tyr-47 residue appears to play at least a strong stabilizing role in the region 2 associations. It appears, moreover, that p107 association is not completely independent of region 1 function; it may only be independent of region 1 if region 2 remains unaffected.

FIG. 8. E1A association with cellular products in BRK cells. Coprecipitation was assayed by immunoprecipitating Tran-³⁵S-labeled infected BRK cell lysates with E1A-specific monoclonal antibody M73 (31) (A and C) or pRB-specific antibody XZ133 (39) (B and C). Cells were labeled from either 8 to 10 h (A and B) or 30 to 32 h (C) after infection. The positions of p300, the E1A proteins, and the region 2-associated proteins are indicated at the right of each panel. The positions of molecular size markers (in kilodaltons) are indicated at the left.

Induction of DNA synthesis. One of the most intriguing aspects of E1A biological functions is that E1A-mediated induction of cellular DNA synthesis can be dissociated from the immortalization function (38, 52, 77, 84). Both the p300-linked and the pRB-linked active sites of 12S.WT are required to induce extended proliferation in primary cells, but either site alone is sufficient for the induction of cellular DNA synthesis. The ability to induce expression of the G₁ products necessary to support DNA synthesis is therefore a redundant function in the E1A products. The new point mutations described here confirm and extend this analysis. Each of the single-residue mutations is highly effective in inducing cellular DNA synthesis. This includes 12S.RG2 (Fig. 9A), despite its severe immortalization and p300-binding defects. The kinetics of induction are slightly slower for 12S.RG2 than for 12S.WT (Fig. 9B); a similar delayed induction phenotype was noted previously in region 2 mutants such as 12S.928 (77, 84).

An important question in considering the phenotype of region 2 mutations such as pm928 is whether undetectable residual pRB binding via region 1 could be contributing to the proficiency in induction of DNA synthesis. Strong evidence against this possibility is provided by the 12S.YH47/928 construct. Although 12S.YH47/928 encodes mutations seriously affecting both pRB-binding regions and binding of all the known region 2-associated proteins, it is as proficient in inducing DNA synthesis as is 12S.928 (Fig. 9A), strongly supporting the conclusion that binding of pRB and the other region 2-associated proteins is dispensable for E1A-mediated induction of DNA synthesis. In clear contrast to the abilities of individual N-terminal and region 2 point mutants to induce DNA synthesis, a double point mutant, 12S.RG2/

928, combining pmRG2 with pm928, virtually abrogates this activity (Fig. 9A). This construct demonstrates with great sensitivity the existence in the E1A products of dual mechanisms for intervening in cell cycle control pathways.

FIG. 9. Induction of DNA synthesis in E1A mutant virus-infected primary BRK cells. Primary BRK cells were infected at a multiplicity of 10 PFU per cell. DNA synthesis was measured as the incorporation of [³H]thymidine. (A) Infected cells were labeled with [³H]thymidine for 1 h at 47 to 48 h postinfection (hpi). Incorporation during this period in each infection is indicated as trichloroacetic acid-precipitable counts per minute. (B) Mock-12S.WT-, or 12S.RG2-infected cells were pulsed with [³H]thymidine for 2 h at successive intervals ending at the times postinfection indicated on the x axis. Incorporation during each interval in each infection is indicated on the y axis as trichloroacetic acid-precipitable counts per minute.

FIG. 10. Enhancer repression function. The activity of the HIV LTR enhancer is indicated by the ability of a reporter gene product to convert chloramphenicol (Cm) to its acetylated forms (Ac-Cm). In three independent experiments including the one illustrated here, the average CAT activity measured after cotransfection with 12S E1A mutant plasmids relative to activity in the absence of E1A expression was 0.25, 0.71, 0.72, 0.30, 0.28, and 0.33 for 12S.WT, 12S.RG2, 12S.HN3, 12S.LS20, 12S.DA21, and 12S.YH47/928, respectively. The average standard deviation was 0.07. The approximate locations of the single point mutations are indicated by dots in the upper bar diagram.

Enhancer repression. It has been known for some time that E1A represses the activity of a variety of viral and cellular enhancers (5, 18, 33, 34, 64, 67, 73, 74, 76). While some studies have indicated a link between enhancer repression and region 2 function (47), most studies have found that this activity maps primarily to the N-terminal active site (41, 44, 60, 61, 64, 66, 75). We used the point mutations generated in this study to examine the link between p300 binding and repression of the enhancer activity of the HIV LTR. Expression was assayed with an HIV LTR reporter construct driving expression of the bacterial CAT gene (Fig. 10).

HIV LTR-driven CAT expression in HeLa cells is largely dependent on the presence in the LTR of two sequence motifs closely related to the binding site for NF- κ B (45). In the absence of these sites (pNFA-CAT), the level of expression is very low (Fig. 10, lane 1 versus lane 2). Cotransfection of a 12S.WT expression plasmid, p12S.WT, represses expression of the HIV LTR CAT construct by about 75% (lane 4). Each of the mutant plasmids, p12S.RG2 and p12S.HN3, encoding substitutions of the positively charged N-terminal residues is severely impaired for the enhancer repression function (Fig. 10, lanes 5 and 6). In contrast, p12S.LS20 and p12S.DA21, affected at nearby residues, repress almost as well as wild type (Fig. 10, lanes 7 and 8). The double point mutant 12S.YH47/928 retains a near-wild-type level of repression activity (Fig. 10, lane 9), indicating that binding of the region 2-associated set of cellular proteins is completely dispensable for repression of the HIV enhancer. It is notable that p12S.LS20 retains considerable enhancer repression activity compared with p12S.HN3, when in other ways their phenotypes are more similar: they both bind reduced levels of p300 and they both show seriously reduced immortalization activity.

DISCUSSION

The work described here refines in several ways our understanding of the relationship between E1A protein sequence and the formation of its active sites. While the extreme N-terminal E1A sequences show relatively little conservation in comparison with previously recognized conserved regions designated regions 1, 2, and 3, it is clear from the data described here that the function localizing to the N-terminal region is dependent on specific sequences. Substitution with a heterologous N-terminal sequence of equal length does not result in the maintenance of N-terminal function, and certain single amino acid substitutions greatly impair activity.

The data described here also imply that p300 and the region 2-associated set of cellular proteins do not bind to shared or overlapping sequences within conserved region 1. The monoclonal antibody competition experiments in particular support this hypothesis. Since immunoglobulin molecules bound to the E1A proteins can be expected to cause an appreciable degree of steric hindrance, the ability of the monoclonal antibodies that bind within conserved region 1 to block binding of only one class or the other of E1A-associated proteins argues strongly that the two classes bind to separate residues within conserved region 1. Indeed, conserved region 1 is probably more accurately described as two nearly contiguous subregions with independent functions. Determination of the precise boundary between these subregions will require further analysis, but given the striking specificity of the monoclonal antibody exclusion, it is possible that residues toward the middle of conserved region 1 are not essential for either binding function. These results suggest a simple outline of E1A structure such as that proposed in Fig. 11. The basic feature of this model is the suggestion that conserved region 1 comprises two distinct subregions that participate in the formation of two active sites.

The single-residue substitution mutations described here constitute important new reagents for probing the roles of E1A-targeted cellular proteins. It is already clear that the use of N-terminal-specific and region 2-specific mutations to disrupt E1A active sites has distinct advantages over the use of conserved region 1 versus conserved region 2 mutations. The 12S.RG2/928 double substitution mutant is a particularly elegant example of the precision with which both E1A active sites can be inactivated. In addition, the 12S.YH47/928 mutation is more precisely disruptive than any region 2 mutation studied previously. Together, 12S.RG2, 12S.928, 12S.RG2/928, and 12S.YH47/928 represent the most precise set of inactivating mutations so far available in E1A. Collectively, these mutants represent each of the distinguishable phenotypes so far generated. 12S.RG2 abrogates p300 binding selectively. 12S.928 abrogates pRB binding selectively (p107 binding is relatively unaffected, while p130 binding is intermediate; see also reference 25). 12S.RG2/928 abrogates all known associations dependent on exon 1 of 12S, while 12S.YH47/928 abrogates binding of the entire region 2-associated set without disrupting p300 binding.

The 12S.HN3 and 12S.LS20 mutations show an interesting distinction in E1A function. Both these mutants show reduced p300 binding and marked defects in immortalization activity. Nevertheless, 12S.LS20 retains much of the E1A-mediated enhancer repression function, while 12S.HN3 is as defective in this activity as 12S.RG2. Interestingly, Subramanian et al. (69) reported a linker insertion mutation substituting the sequence Arg-Ile-Arg for Leu-19 that has a

FIG. 11. Schematic model of cellular protein-binding sites in E1A exon 1. The 139 residues of the exon 1 sequences common to the 12S and 13S products, sufficient for E1A-induced proliferation of primary BRK cells, are represented as individual circles in the above diagram. Residues 81 to 120 are shaded gray to indicate that they are not essential for E1A biological activity or protein binding functions (66) and are represented as a loop because the ability to remove them all in a single deletion indicates that they do not contribute essential spacing between conserved regions 1 and 2. The region from residue 26 to 35 can also be removed as a single deletion without impairing E1A function (41), even though residues on either side of it combine to form a binding site for p300. This region, therefore, is also depicted as a loop and shaded gray (residue 35 is partially hidden behind residues 24 and 25 in this diagram). The unshaded regions in this diagram correspond to the unshaded regions in the bar diagrams shown here and in Fig. 1. Conserved region 2 extends from residues 121 to 139. The (D)-L-X-C-X-E motif represented in Ad5 E1A by residues 121 to 126 appears to be a direct target for binding by pRB, p107, and probably p130 (15, 17, 25, 79). The conserved residues in this motif are highlighted by internal dots in the model above. The remainder of conserved region 2 constitutes a casein kinase II substrate recognition site motif separated from the pRB-binding site by two adjacent proline residues. The significance of the casein kinase II motif to E1A function is not known, but this motif apparently contributes to the function of the HPV E7 transforming gene product (3), which contains a direct homolog of E1A region 2. Conserved region 1 is drawn as a reversed loop to represent the genetic evidence implying that the upstream half of conserved region 1 contributes to formation of the pRB-binding site while the downstream half contributes to formation of the p300-binding site. (Residue 79 is partially concealed by residue 37 in this diagram.) Residues 21, 49, and 55 are shaded gray to indicate that nonconservative substitutions at these positions have little or no effect on E1A biological function or protein-binding activity. Residues 2, 3, and 20 are highlighted with internal dots to indicate their apparent involvement in p300 binding. Interestingly, computer analysis supports some features of this schematic outline. The Hopp and Woods (35) method predicts three strong hydrophilic peaks in the E1A 12S product, occurring at residues 133 to 138, 55 to 60, and 100 to 105; the latter two sites are strikingly similar to the peaks of the simple loops postulated from the genetic data. In addition, an interesting pattern emerges when the E1A proline distribution is superimposed on this model. The E1A products are proline-rich; within the first 139 residues, prolines occur at positions 33, 34, 35, 40, 41, 54, 57, 67, 84, 85, 87, 90, 92, 93, 99, 102, 109, 113, 117, 130, and 131. Fully half of these fall within the hypothetical loop postulated to extend from about residue 80 to residue 120 (on average, every fourth residue in this stretch is a proline). Since proline residues are often associated with the presence of β -turns in protein molecules (10), this distribution might indicate that this region forms a tightly twisted structure.

biological phenotype very similar to that of the 12S.LS20 mutation described here. Their mutation was severely defective in a virus immortalization assay, but close to wild type in its ability to repress enhancer activity in CAT assays of the SV40 and immunoglobulin H enhancers; p300 binding was not assessed. It is possible that 12S.LS20 retains the enhancer repression function to a greater extent than 12S.HN3 because of a slightly greater ability to titrate p300, but the wide difference in their enhancer repression activities compared with their more similar p300 binding and virus immortalization activities raises the possibility that the difference in their biological activities may be qualitative, rather than only quantitative. The difference between 12S.LS20 and 12S.HN3 may lie, not in the amount, but in the way by which p300 is bound to the altered E1A products; for example, one mutant may have a more significant effect than another on further p300 protein-protein associations. Resolving the role of p300 in the enhancer repression and immortalization functions will require much further characterization of p300, and substitution mutants such as 12S.HN3 and 12S.LS20 may be particularly useful in distinguishing the individual steps in these pathways.

The involvement of a tyrosine residue in pRB binding to E1A has interesting correlations in HPV E7. The HPV E7 proteins, which contain a direct homolog of E1A region 2, also encode a region strikingly similar to the pRB-binding portion of conserved region 1 (57), including a tyrosine in HPV type 16 (HPV16) E7. Moreover, of the other three tyrosine residues occurring among the 98 residues of the HPV16 E7 product, two occur within the pRB-binding region homologous to E1A region 2. They are presumably not essential for pRB binding since they do not occur in Ad5 E1A region 2, yet it is notable that position 125 in the Ad5 E1A (D)-121-L-122-X-123-C-124-X-125-E-126 motif, while not completely conserved, is a tyrosine in almost every serotypically distinct E1A gene sequenced (sequences compiled in references 1 and 43). Together, these data suggest that tyrosine residues may play a significant stabilizing role in pRB-binding proteins. This may have clinical relevance since it appears that the oncogenicity of HPVs correlates with the stability of pRB binding to the E7 products (3, 23, 54).

The 12S.YH47 and 12S.YH47/928 mutants also reveal novel aspects of p107 interaction with the E1A products. It has previously been thought that conserved region 1 may not be a binding site for p107 since it is not required for p107 association. However, it appears from the data reported here that p107 binding is stabilized by specific interactions in both regions 2 and 1; p107 association may only be independent of region 1 if region 2 remains unaffected. Conversely, p107 binding can also be maintained in the absence of the region 2 cysteine residue, as evidenced by the 12S.928 mutant, even though this residue is critical for pRB association. p107 association with the 12S.928 mutant products shows an interesting kinetic aspect in BRK cells (in which E1A expression induces synchronous progression through the cell cycle). While p107 is bound by the 12S.WT products as soon as they are expressed, the 12S.928 products bind p107 only after the cell cycle has advanced through G₁. As there is a strongly cell-cycle-regulated component of E1A-p107 complexes, i.e., cyclin A (19, 21, 37), these kinetics may reflect an involvement of cyclin A in stabilizing p107 association with the E1A products.

The 12S.YH47 and 12S.YG47 mutants also reveal new details of pRB interaction with the E1A products. For the first time, a marked difference in the protein association

pattern of an E1A mutant has appeared between HeLa and BRK cells. In particular, pRB binding to the 12S.YH47 products is readily detectable in BRK cells, but sharply impaired in HeLa cells. This may reflect a selective loss of the ability to bind phosphorylated pRB forms. However, even when pRB is in a highly phosphorylated state in BRK cells, the mutant residue 47 products still show pRB binding, in contrast to their severe defect in HeLa cells. Thus, the difference may be more related to cell type than to the degree of pRB phosphorylation. We are continuing studies aimed at further defining the role of the Tyr-47 residue in binding pRB.

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