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Biochemistry

E2F1 and E1A$_{12S}$ have a homologous activation domain regulated by RB and CBP

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ABSTRACT **The E2F1 transcription factor has a well-characterized activation domain at its C terminus and the E1A protein has a recently defined activation domain at its N terminus. Here we show that these activation domains are highly related in sequence. The sequence homology reflects, at least partly, the conservation of common binding sites for the RB and CBP/p300 proteins, which are preserved in the same relative order along E2F1 and E1A. Furthermore, the interaction of RB and CBP with these two activation domains results in the same functional consequences: RB represses both activation domains, whereas CBP stimulates them. We conclude that the activation domains of E1A$_{12S}$ and E2F1 belong to a novel functional class, characterized by specific protein binding sites. The implication of this conservation with respect to E1A-induced stimulation of E2F activity is discussed.**

The transcription factor E2F1 in combination with DP1 can bind to E2F sites and activate transcription of S-phase specific genes (reviewed in ref. 1). The activity of E2F1/DP1 comes from a small activation domain at the C terminus of E2F1 (2, 3). This domain is required for the ability of E2F1 to induce S phase (4) and is regulated by a number of proteins that bind to it: it is negatively regulated by the retinoblastoma tumour suppressor protein RB (5–7) and positively regulated by the MDM2 oncoprotein and the CBP coactivator (ref. 8; D.T., A. Cook and T.K., unpublished work).

The activation functions of E2F1 are influenced by viral transforming proteins such as the adenovirus E1A protein. E1A has the capacity to stimulate E2F1 activity by displacing the RB repressor from the E2F1 activation domain. E1A can accomplish this in a two-step process: first it binds the RB protein, via conserved region 2 (CR2) sequences and then displaces RB from E2F1 via CR1 sequences (10).

Recently, the E1A protein was shown to possess an activation domain at its N terminus, which spans CR1 (11). This region of the protein is required for activation of a variety of promoters (E2, HSP70, c-*jun*) (12–14) and contains binding sites for the RB protein and the CBP/p300 family of coactivators (refs. 15 and 16; reviewed in ref. 17).

Here we show that the N-terminal activation domain of E1A has sequence similarity to the E2F1 activation domain. Furthermore, these two activation domains bind the RB and CBP/p300 proteins via similar motifs and are regulated by these proteins in a similar way. These results identify a new class of activation domain defined by similarly spaced protein–protein interaction sites.

MATERIALS AND METHODS

Cell Culture, Transfections, and Chloramphenicol Acetyltransferase (CAT) Assays. The U2OS osteosarcoma cell line was maintained at 37°C in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum, 1% glutamine, and 1% penicillin/streptomycin solution. Transfection by calcium phosphate coprecipitation was performed as described (18). The cells were harvested 24 hr after transfection and CAT activity was determined as described (18). Results were quantified with a PhosphorImager.

Plasmids. E2 CAT (gift from N. La Thangue, University of Glasgow) expressed CAT protein under the control of the adenovirus E2 promoter. Cytomegalovirus (CMV)-RB and mutant are gifts from W. Kaelin (Harvard Medical School) (19). Rous sarcoma virus (RSV)-CBP is a gift from R. Goodman (20). Adenovirus type 2 (Ad2) E1A$_{12S}$ proteins are expressed from a RSV-driven expression vector. The E1A$_{12S}$ and E1A-ΔCR1 were kind gifts from H. Land (Imperial Cancer Research Fund, London). E1A p300mut contains a deletion of amino acids 64–68. In E1A RBmut, amino acids 38–44 were converted to alanine.

The CAT reporter vector 1GE1bCAT contained one GAL4 site upstream of E1b TATA box (21). GAL4 E2F-C and GAL4 E1A-N proteins were expressed from a Simian virus 40 promoter-driven pHK plasmid and contain human E2F1 sequences from amino acids 380 to 437 or Ad5 E1A sequences from amino acids 1 to 90 in-frame with the GAL4 DNA binding domain from amino acids 1 to 142. GAL4 E2F-C CBPmut and GAL4 E1A-N p300mut contain a deletion of amino acids 428–431 and 64–68, respectively. E1A-N competitor constructs contain amino acids 1–90 of Ad5 E1A expressed from pHK plasmid. E1A-N p300mut contains a deletion of amino acids 64–68. In E1A-N RBmut, amino acids 38–44 were converted to alanine.

Immunoprecipitation and Western Blotting. Three dishes of U2OS cells were transfected using the E1A expression vectors. Cells were then lysed directly in 1 ml of lysis buffer (22). Extracts were incubated 2 hr in lysis buffer with 50 μl of protein A-agarose beads in the presence of 1 μg of E1A-specific M73 antibody and 2 μg of rabbit anti-mouse IgG. Immunoprecipitates were then analyzed by SDS/PAGE, transferred to a nitrocellulose membrane, and subjected to Western blotting with anti-RB G3-245 antibody (PharMingen). Immunoreactive bands were detected with an ECL kit (Amersham) according to the manufacturer's instructions.

RESULTS

E2F1 and E1A$_{12S}$ Activation Domains Show Homology. The activation domains of E1A$_{12S}$ and E2F1 can be classified as acidic since 26% of their residues (compared to 25% for VP16) have acidic characteristics. However, close inspection of these

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Abbreviations: CR, conserved region; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; RSV, Rous sarcoma virus; Ad5, adenovirus type 5.

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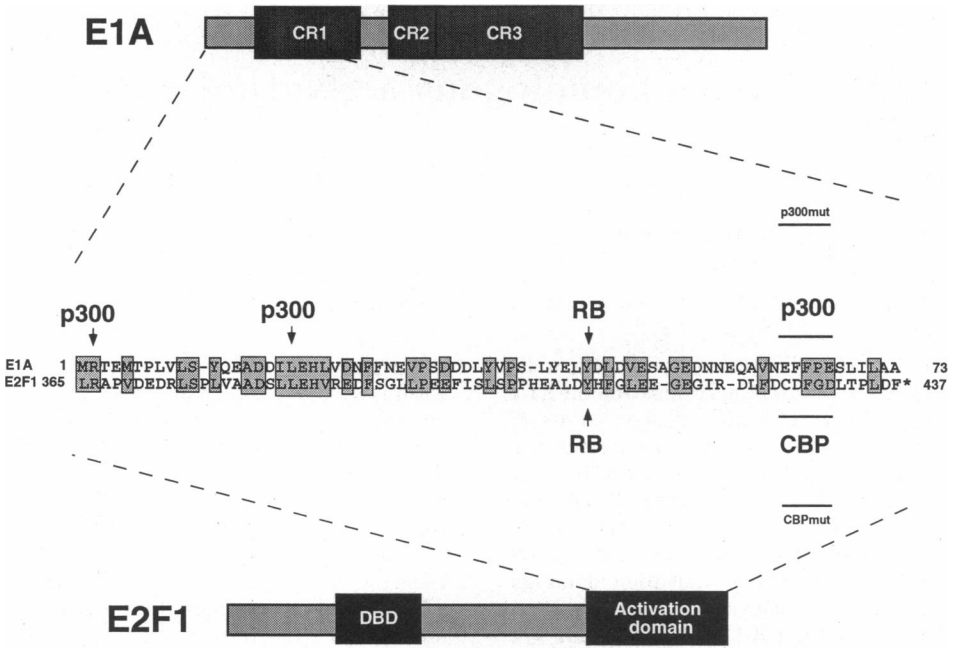

FIG. 1. E1A N terminus shows sequence similarity to the E2F1 activation domain. Sequence alignment between Ad12 E1A N terminus from amino acids 1–73 (upper sequence) and human E2F1 activation domain from amino acids 365–437 (lower sequence). Residues with identity or similarity are boxed. Arrows represent residues essential for p300/CBP and RB binding in E1A (23, 24) and E2F1 (5, 7, 9). p300mut and CBPmut indicate amino acids deleted in E1A and E2F1, respectively.

sequences indicates that they have extensive and colinear sequence identity, which includes mainly nonacidic residues. Furthermore, the similarity overlaps residues of E1A and E2F1 required for RB and p300/CBP binding (Fig. 1). This striking conservation includes a tyrosine residue (Y47 in Ad5 E1A and Y411 in E2F1), which when mutated abolishes the binding of RB to both E1A and E2F1 (5, 7, 23). Short peptides from E1A and E2F1 that span this tyrosine can independently bind the RB protein (7, 10, 25). Residues in E1A involved in binding the p300/CBP family of proteins are also conserved in E2F1. These include the arginine at position 2, the leucine at position 20, and residues 64–68 (Ad5) (23, 24). Deletion of residues 64–68 in E1A (p300mut; Fig. 1) and the analogous deletion in E2F1 (CBPmut; Fig. 1) severely affects the binding of the CBP/p300 family of proteins, consistent with the presence of sequence conservation in this region (9, 24).

CBP Stimulates E2F1 and E1A Activation Domains. The observations that E1A and E2F1 share activation domains linearly similar in sequence indicate that the activation domains of E1A and E2F1 are related in their structure and that they bind similar proteins (RB and CBP/p300) using similar sequence motifs. We next sought to establish whether binding of these proteins resulted in the same functional consequences.

The CBP protein is considered to be a coactivator protein, capable of mediating the activity of the phosphorylated activation domain of CREB (20). We therefore asked whether binding of CBP to E1A and E2F1 was required for their activation functions.

We first tested whether the ability of E1A to activate the E2 promoter was CBP dependent. Fig. 2*A* shows that deletion of five residues within CR1 that abolish CBP/p300 binding *in vivo* (p300mut) (24) decreases the ability of $E1A_{12S}$ to activate the E2 promoter. The loss of activation function of $E1A_{12S}$ p300mut is not due to differences in expression level (Fig. 2*B*) or to loss of RB binding capacity (Fig. 2*C*). However, as shown previously (12), mutating the RB binding site in CR1 (RBmut) also affects activation of the E2 promoter (Fig. 2*A*). These results indicate that loss of p300 binding to E1A reduces E1A's ability to stimulate the E2 promoter.

The $E1A_{12S}$ N-terminal activation domain (E1A-N; residues 1–90) can activate transcription independently when directed to the promoter via the GAL4 DNA binding domain (Fig. 3*A*). The potency of this activation domain is equivalent to that of the E2F1 C-terminal activation domain (E2F-C). Deleting residues required for CBP/p300 binding (p300mut) (Fig. 1) disrupts the activation function of GAL4 E1A-N (Fig. 3*A*). Introducing an analogous mutation in the activation domain of E2F1 (E2F-C CBPmut) (Fig. 1) has the same consequences; it disrupts CBP binding (9) and transcriptional activation (Fig. 3*A*).

Given that the binding of CBP correlates with the activity of the E1A and E2F1 activation domains (Fig. 3*A*), we asked whether the CBP protein would stimulate the activation capacity of these domains, consistent with its role as a coactivator. Fig. 3*B* shows that indeed CBP will stimulate the activation capacity of both the E2F1 and E1A activation domains. Responsiveness to CBP stimulation is not a property exhibited by every activation domain since CBP will not affect the activation domain of Sp1 (9).

To provide further evidence that the E1A and E2F1 activation domains use a similar protein to mediate their function (namely, CBP/p300), we carried out "squelching" studies. Fig. 3*C* shows that E1A-N can repress the activity of GAL4 E2F-C in trans. This sequestration of activity is dependent on an intact CBP binding site in E1A-N since E1A-N p300mut is unable to squelch activity. In contrast, mutating E1A sequences required for RB binding (E1A-N RBmut) does not alleviate the repressive effect of E1A-N.

RB Represses E2F1 and E1A Activation Domains. We next tested whether binding of the RB protein to the E1A and E2F1 activation domains has the same functional consequence. The RB protein has the capacity to repress the E2F1 activation domain when linked to GAL4 (5, 7). Fig. 4 shows that at a similar concentration and in the same cell type the RB protein can repress the activation capacity of both GAL4 E2F-C (Fig. 4*A*) and GAL4 E1A-N (Fig. 4*B*). The level of repression by RB is comparable and is dependent in both cases on the intactness of the RB pocket domain. This repressive effect of RB is

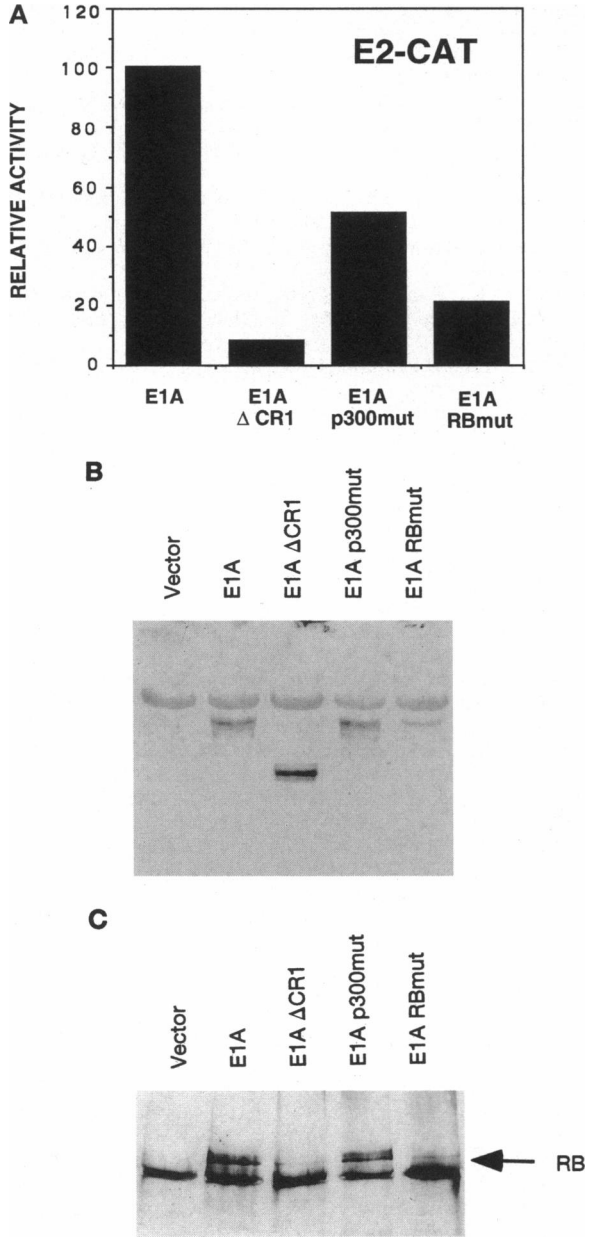

FIG. 2. The CBP binding site in $E1A_{12S}$ is required for activation of the E2 promoter. (*A*) U2OS cells were transiently transfected with 0.5 μg of E2CAT plasmid, 0.05 μg of CMV RB, and 0.5 μg of the indicated $E1A_{12S}$ expression vectors. After a CAT assay, results were quantified with a PhosphorImager (Molecular Dynamics). Results are given as percentage of maximal response (value 0 in the absence of E1A protein and value 100 for wild-type E1A protein). (*B*) Half of the U2OS cells used in *A* were lysed directly with 1× SDS loading buffer and subjected to Western blot analysis using the anti-E1A M73 antibody (Santa Cruz). (*C*) U2OS cells were transfected with the indicated E1A constructs. Whole cell extracts were then immunoprecipitated using the anti-E1A M73 antibody as described (22). Immunoprecipitates were assayed for the presence of RB by Western blot using the anti-RB (G3-245) antibody (PharMingen).

specific since under the same conditions RB will not repress the activation domain of p53 (ref. 26; data not shown).

DISCUSSION

These results indicate that the activation domains of $E1A_{12S}$ and E2F1 are related in the following respects. (*i*) They show considerable colinear sequence similarity. (*ii*) Residues conserved between E1A and E2F1 are required for binding of

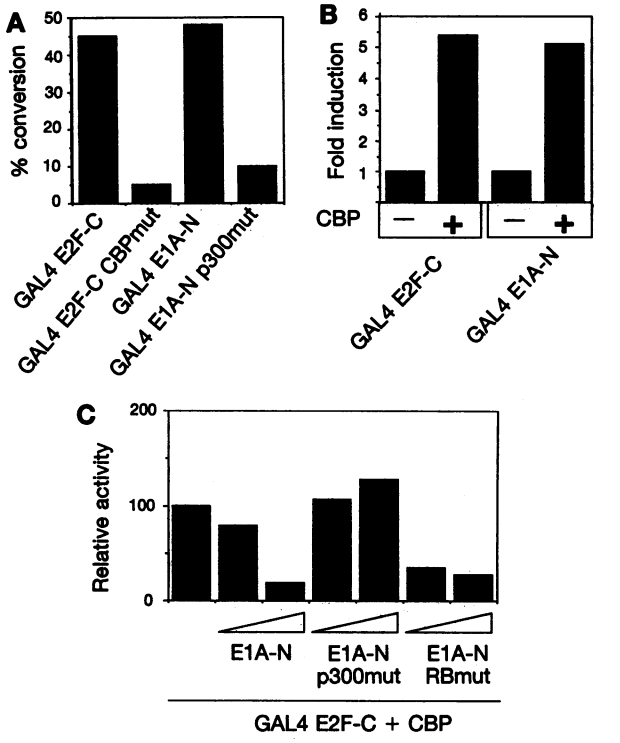

FIG. 3. CBP functions as a coactivator for the E2F1 and $E1A_{12S}$ activation domains. (*A*) U2OS human osteosarcoma cells were transiently transfected with 1 μg of 1GE1bCAT and 1 μg of the indicated GAL4 effector vector. Result of a typical experiment is shown. Similar results were obtained in at least three independent experiments. Note that E2F-C and E1A-N activation domains have similar potency. (*B*) U2OS cells were transfected with 1 μg of 1GE1bCAT, 1 μg of the indicated GAL4 effector vector, and, where indicated, 4 μg of RSV-CBP (+) or 4 μg of empty RSV vector (−). Activity of the effector in the absence of CBP is normalized to a value of 1. (*C*) U2OS cells were transfected using 1 μg of 1GE1bCAT, 1 μg of GAL4 E2F-C, 4 μg of RSV-CBP, and increasing amounts (5 and 10 μg) of the indicated E1A-N competitor. The amount of promoter was kept constant with pHK empty vector. Values were normalized to 100 in the absence of competitor.

similar proteins (RB and CBP/p300). (*iii*) Binding of these proteins leads to a similar functional consequence—either stimulation or repression of activity. Thus, these two activation domains represent a novel functional class characterized by specific protein binding sites. Definition of these novel RB and

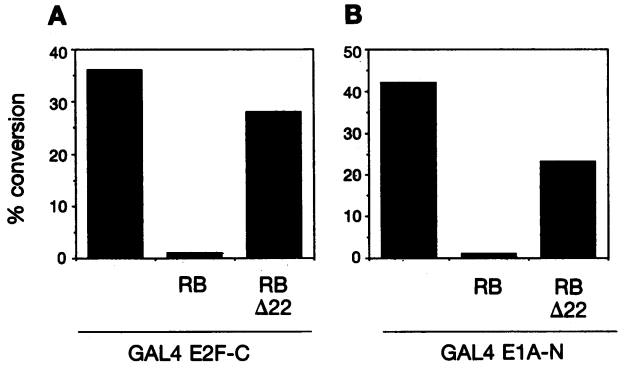

FIG. 4. RB represses the N-terminal activation domain of E1A. U2OS cells were transfected with 1 μg of 1GE1b CAT, 1 μg of GAL4 E2F-C (*A*) or GAL4 E1A-N (*B*), and 0.5 μg of CMV-RB or CMV-RBΔ22 as indicated. The amount of promoter was kept constant by the addition of CMV empty vector. Result of a typical experiment is shown. Similar results were obtained in at least three independent experiments.

CBP binding motifs, highlighted by the conservation between E1A and E2F1, will aid the identification of additional members of this class of activation domain.

Classification of activation domains is commonly restricted to the "richness" of a given residue: acidic, proline-, glutamine-, or serine/threonine-rich. The results presented here and elsewhere (18, 27) suggest that this classification is too simplistic, given the existence of activation domains with more "specific" sequence identity. This identity we show here does not represent a random distribution of a common residue but reflects common protein binding sites found in the same relative order. The binding of these proteins has the same functional consequence for both activation domains. This raises the possibility that activation domains currently placed in a common "residue richness" class may be more related in specific sequence than is recognized. The specific similarity may be difficult to identify since the protein binding sites, which it appears to reflect, are small and degenerate (rather than identical).

The results presented here have implications with respect to the ability of E1A to stimulate E2F activity. In the currently accepted model, E1A removes the RB repressor protein from the E2F1 activation domain, thus making it active. However, evidence presented here suggests that CBP may also be involved in the process of E1A-induced activation of E2F1. First, mutagenesis of the p300 binding site in E1A reduces its ability to activate E2F sites (Fig. 2*A*) even though this mutant can still bind RB. Second, CBP acts as a coactivator for E2F1. Evidence for this comes from experiments in Fig. 3 and in data presented elsewhere (9). These show that CBP can contact the E2F1 activation domain both *in vitro* and *in vivo* and can stimulate the activation capacity of the E2F1/DP1 heterodimer (9). Taken together, these data raise the possibility that E1A activates E2F1 not only by removing the RB repressor but also by providing the CBP adaptor protein. This notion is supported by the fact that E1A-induced cell proliferation requires the interaction of both RB and p300 with E1A (9).

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