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The E6 protein of human papillomavirus type 16 binds to and inhibits co-activation by CBP and p300

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The co-activators CBP and p300 are important for normal cell differentiation and cell cycle progression and are the targets for viral proteins that dysregulate these cellular processes. We show here that the E6 protein from the oncogenic human papillomavirus type 16 (HPV-16) binds to three regions (C/H1, C/H3 and the C-terminus) of both CBP and p300. The interaction of E6 with CBP/p300 was direct and independent of proteins known to bind the co-activators, such as p53. The E6 protein from low-risk HPV type 6 did not interact with C/H3 or the C-terminus but associated with the C/H1 domain at 50% of the level of HPV-16. HPV-16 E6 inhibited the intrinsic transcriptional activity of CBP/p300 and decreased the ability of p300 to activate p53- and NF-κB-responsive promoter elements. Interestingly, some mutations in HPV-16 E6 abrogated C/H3–E6 interactions, but did not alter the ability of E6 to associate with the C/H1 domain, suggesting that these modified proteins could be used to delineate the functional significance of the C/H1 and C/H3 domains of CBP/p300.

Keywords: CBP/E6 protein/human papillomavirus/p300/transcription co-activators

Introduction

The co-activators CBP and p300 are highly conserved proteins that appear to perform similar activities and were first recognized because of their interaction with the adenovirus E1A protein (for reviews see Shikama *et al*., 1997; Giles *et al*., 1998). These co-activators bind a large number of cellular proteins (see Figure 2A), including transcription factors involved in growth control and differentiation (Eckner *et al*., 1996b; Yuan *et al*., 1996; Puri *et al*., 1997; Giles *et al*., 1998; Kawasaki *et al*., 1998), enzymes that can cause chromatin re-modeling, such as P/CAF (Yang *et al*., 1996), and other co-activators involved in nuclear receptor transcription (Kamei *et al*., 1996; Korzus *et al*., 1998). The regions of CBP/p300 that bind cellular proteins are highly conserved, demonstrating 66 to >90% similarity at the amino acid level distributed throughout the molecule in relatively small defined regions (Giles *et al*., 1998). One of the first described functions given to CBP was its ability to activate the phoshorylated form of c-AMP-regulated enhancer binding protein (CREB) transcription factor through an interaction with the KIX domain in the N-terminal half of the CBP/p300 (Chrivia *et al*., 1993). A number of other proteins bind to this region of CBP/p300 and are co-activated, including c-Jun (Arias *et al*., 1994; Kamei *et al*., 1996; Lee *et al*., 1996), c-Myb (Dai *et al*., 1996; Oelgeschlager *et al*., 1996) and the TAX factor from the human T-cell leukemia virus type I (HTLV-I) (Suzuki *et al*., 1993; Kwok *et al*., 1996). There are other conserved regions of CBP/p300, which bind a number of factors; one in the immediate N-terminus between amino acids 1 and 100 that binds many nuclear receptors (Kamei *et al*., 1996; Korzus *et al*., 1998) and a region in the C-terminus, called the C/H3 domain, that binds cellular transcription factors (Bannister and Kouzarides, 1995; Yuan *et al*., 1996) and two viral proteins, SV40 large T (Eckner *et al*., 1996a) and adenovirus E1A (Eckner *et al*., 1994; Arany *et al*., 1995; Lundblad *et al*., 1995). p53 has been shown to bind to the C-terminal region (Avantaggiati *et al*., 1997; Gu *et al*., 1997) and, in addition, was recently shown to bind the C/H1 domain (Grossman *et al*., 1998). As well as binding transcription factors, CBP/p300 also interacts with proteins important for transcription such as histone acetylases (e.g. P/CAF) (Yang *et al*., 1996), co-activators p/CIP (Torchia *et al*., 1997) and NcoA (Onate *et al*., 1995; Kamei *et al*., 1996), which are necessary for nuclear receptor activation, and members of the basal transcriptional machinery, including the TATA binding protein (TBP) and associated factors such as TFIIB (Kwok *et al*., 1994; Yuan *et al*., 1996). In addition, CBP/p300 have intrinsic histone acetyltransferase (HAT) activity, which is mediated by a region called the HAT domain (Figure 2) and is important in acetylating histones (Bannister and Kouzarides, 1996; Ogryzko *et al*., 1996), and transcription factors such as p53 (Gu *et al*., 1997).

Factors associated with cell proliferation such as c-Fos interact with the C/H3 domain of CBP/p300 (Bannister and Kouzarides, 1995). The E1A oncoprotein of adenovirus also interacts with the C/H3 domain and abrogates the co-activation activity of CBP/p300 (Arany *et al*., 1995; Bannister and Kouzarides, 1995). Since the adenovirus causes cells to proliferate this would seem to be counterproductive; however, these same transcription factors have also been implicated in cellular differentiation (Angel and Karin, 1991) and adenovirus needs to inhibit cellular differentiation in epithelial cells in order to stimulate cells into S-phase. Therefore, E1A may inhibit a subset of genes involved in differentiation, while at the same time stimulate other genes that are important for G_1 progression. The E1A oncoprotein was thought initially to bind only to the conserved C/H3 domain and to inhibit the activation of the Jun family by displacing c-Fos at the C/H3 domain (Bannister and Kouzarides, 1995; Lee *et al*.,

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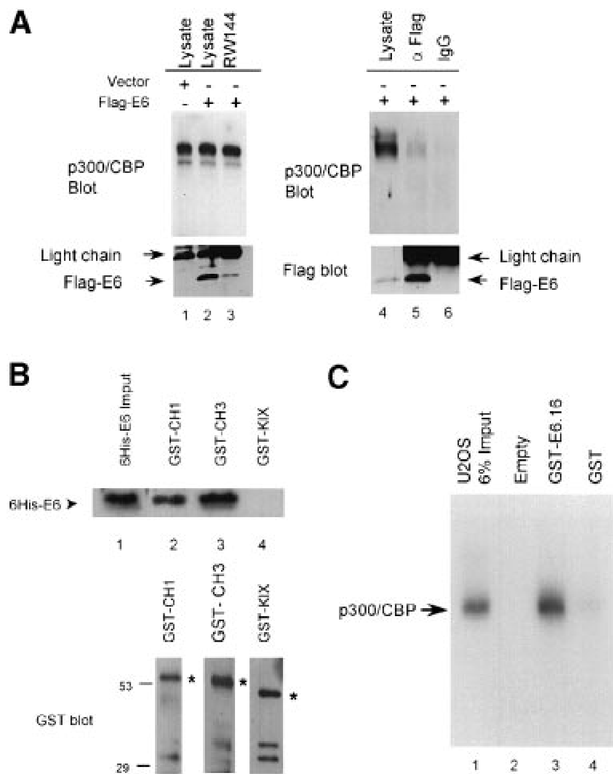


Fig. 1. E6.16 interacts with p300/CBP *in vivo*. (**A**) Cos-1 cells were transfected with an expression vector alone or one that contains E6.16 with a flag tag (Flag-E6). Whole-cell extracts were prepared 48 h after transfection and they were immunoprecipitated with either IgG, RW144 (recognizes both CBP and p300) or anti-flag antibodies. The immunoprecipitated proteins were separated on a 4–20% gradient gel and blotted with either a combination of RW128 and MN11 (upper panels) or anti-flag (lower panels). (**B**) Bacterially derived 6-His-tagged E6 (6His–E6) was incubated with the indicated p300/CBP–GST fusion protein. The bound complexes were removed from solution with glutathione beads, separated on a 15% SDS–polyacrylamide gel and blotted with an anti-histidine antibody. An anti-GST Western blot of the GST–p300 fusion proteins used in the upper panel is shown below. (**C**) Whole-cell extracts from U2OS were incubated with either E6–GST fusion protein or GST alone. The bound complexes were removed with glutathione beads, separated on a 6% SDS–polyacrylamide gel and blotted with a mixture of RW128 and MN11 monoclonal antibodies against p300/CBP.

1996). However, it has since been shown to interfere also with the transcription of CREB (Nakajima *et al*., 1997a,b) and nuclear receptor controlled genes, which require the KIX and the nuclear receptor binding domains, respectively, both in the N-terminal part of CBP/p300. It was shown recently that E1A can in fact bind to the N-terminal region of CBP, between amino acids 1 and 450 and to a C-terminal domain between amino acids 2058 and 2163 (Korzus *et al*., 1998; Kurokawa *et al*., 1998). In addition, binding to the C-terminus was found to have inhibitory effects on the N-terminal co-activation of nuclear receptors through the disruption of CBP complexes containing p/CIP and NcoA, two additional co-activators for nuclear receptor responses. Therefore, in addition to direct inhibition of binding of a transcription factor to CBP/p300 domains, E1A can inhibit indirectly through the disruption of important large transcriptional complexes. Recently E1A has been shown to bind to the HAT domain and inhibit enzymic activity (Chakravarti *et al*., 1999; Hamamori *et al*., 1999). However, others have shown that E1A either has no effect on HAT activity (Bannister and Kouzarides, 1996; Ogryzko *et al*., 1996) or increases it (Ait-Si-Ali *et al*., 1998).

Viral proteins from three distinct viruses associate with CBP/p300, adenovirus E1A, SV40 large T and HTLV-I Tax. Given the important position the CBP/p300 family of co-activators has in the regulation of the cell cycle and differentiation, it would be predicted that other viral proteins may target this family. Here, we show that the human papillomavirus (HPV) E6 protein binds to three domains of CBP/p300 and affects the transcriptional activity of the co-activators. The human papillomaviruses consist of a large group of small DNA viruses, which replicate and cause disease in epithelial surfaces, both mucosal and cutaneous. The viruses causing mucosal disease can be grouped into benign and malignant viruses, with HPV-6 and -11 being the most common benign viruses and HPV-16 and -18 the most common malignant viruses (McCance, 1998). Both groups produce proliferative lesions, which can persist for many months, even years, but only infection with the malignant viruses leads to cancer. The E6 protein is 151 amino acids in length, has a molecular weight of 18 kDa and has two C-X-X-C motifs, which form two zinc fingers that are important for many of the biochemical and biological properties of the protein (Meyers and Androphy, 1995). One of the primary activities of E6 from the oncogenic viruses is the binding to, and degradation of, p53 through a trimeric complex made up of E6, p53 and E6-associated protein (E6AP), a ubiquitin ligase (Scheffner *et al*., 1990, 1993, 1994; Huibregtse *et al*., 1991). Mutations throughout the molecule abrogate binding to p53 and it has been difficult to determine the domain responsible for binding to p53 (Foster *et al*., 1994; Dalal *et al*., 1996). This may be a reflection of the relatively unordered structure of E6 as observed from NMR studies (W.Phelps, personal communication). The E6 from HPV-6, a benign virus, does not bind efficiently to p53 and does not cause its degradation (Crook *et al*., 1991; Foster *et al*., 1994). We show that HPV-16 E6 binds to the C/H1 and C/H3 domains and additionally to a region in the C-terminus. The E6 protein of one of the benign viruses, HPV-6, binds to the C/H1 domain. Binding to the various domains results in an inhibition of the intrinsic transcriptional activity of the domain and its ability to stimulate transcription factor-specific activation. Moreover, E6 binding to CBP/p300 is independent of the ability to bind p53.

Results

In vivo binding of HPV-16 E6 to CBP/p300

Initial studies showed that glutathione *S*-transferase (GST) fusion proteins containing domains of CBP and p300 could bind to HPV-16 E6 (E6.16). To confirm that this was a physiologically relevant interaction, E6.16, tagged at the C-terminus with the flag epitope, was expressed in Cos-1 cells. CBP/p300 and E6.16 were co-immunoprecipitated from Cos-1 cells using anti-flag antibodies and anti-CBP/p300 antibody (RW144) that recognizes both CBP and p300 (Figure 1A). The anti-CBP/p300 antibodies co-immunoprecipitated the E6 protein (Figure 1A, Flag blot, lane 3), and the anti-flag antibodies pulled down a small amount of p300 (Figure 1A, CBP/p300 blot, lane

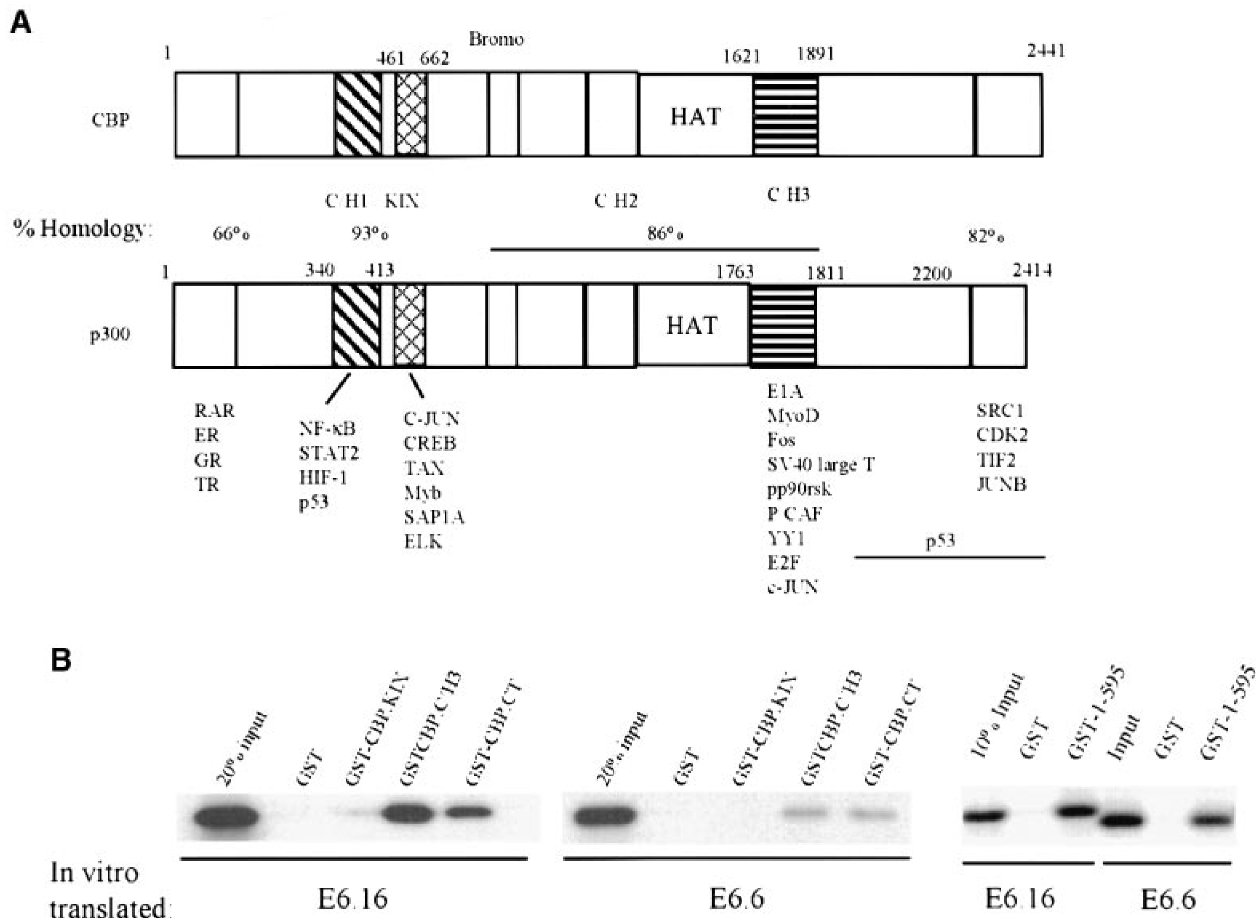


Fig. 2. Interaction of E6.16 and E6.6 with different domains of CBP. (**A**) A diagram of the domains of CBP and p300. The CH1, KIX and CH3 domains are indicated by the hatched boxes. The HAT domain indicates the region that contains the histone acetylase activity. The percentage homology between the two proteins is indicated (Giles *et al*., 1998). The reported interaction regions of the various transcription factors and transcription accessory proteins with p300/CBP are listed below. (**B**) Radiolabeled *in vitro*-translated E6.16 or E6.6 proteins were added to the GST fusion proteins of the regions of CBP indicated: GST–KIX (amino acids 461–662), GST–C/H3 (amino acids 1621–1877) and GST–CT (C-terminal domain, amino acids 1990–2441) (left and center panels). The N-terminal region of p300, amino acids 1–595, was also used (right panel). The bound complexes were removed from solution with glutathione beads, separated on a 15% SDS–polyacrylamide gel and were quantified using a PhosphorImager and Imagequant software (Molecular Dynamics). The radiolabeled imput of each TNT protein is indicated. Note that E6.6 migrates slightly more quickly than E6.16.

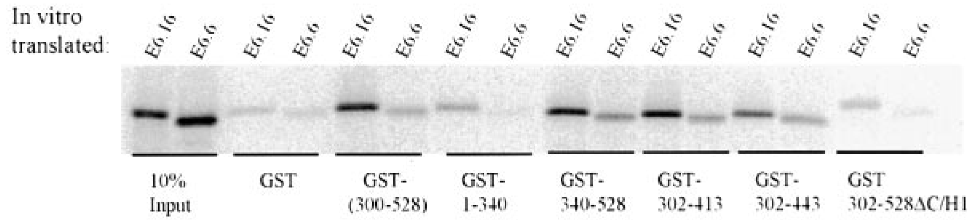
5). The levels of CBP/p300 were similar in cells transfected with either the empty vector (pSG5) or the vector containing E6.16 (Figure 1A, left panel, lanes 1 and 2).

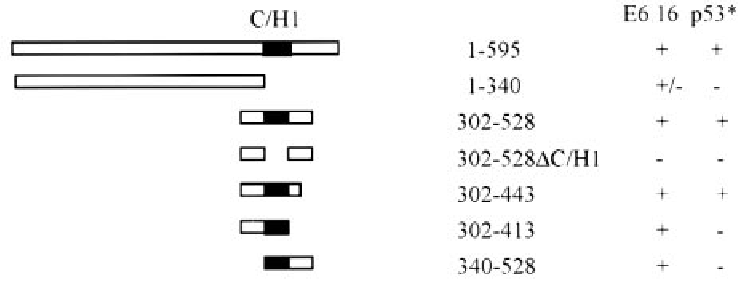
To determine whether binding was direct or through an intermediary protein we used bacterial derived purified proteins. Since full-length p300 is insoluble we produced domains of the p300 as GST fusion proteins. We included the C/H1 and C/H3 domains, which we knew bound E6.16 (see below) and the KIX domain which does not bind E6.16 as a negative control. E6.16 was purified as a histidine-tagged protein. Complexes were precipitated using glutathione beads and E6 was detected by anti-histidine antibodies by Western blotting. Figure 1B shows that E6.16 bound to both the C/H1 and C/H3 domains, but not the KIX domain. The lower panel is a Western blot, using anti-GST antibodies, of the GST fusion proteins used. Finally a GST-E6.16 protein was able to pull down CBP/p300 proteins from extracts of U2OS cells (Figure 1C). Therefore results with the purified proteins and the co-immunoprecipitations from mammalian cells show that E6.16 binds directly to domains of p300. Below, we have mapped the domains more closely and show the binding patterns of a number of E6.16 mutations.

Mapping the binding sites on CBP/p300 for E6.16

We used a series of GST fusion proteins containing domains of both CBP and p300 (Figure 2A) and found that E6.16 bound to three regions of the co-activator proteins. Radiolabeled *in vitro*-transcribed and -translated E6.16 bound to the C/H3 domain (1621–1877 amino acids) and the C-terminal region (1990–2441 amino acids) downstream from the C/H3 domain from CBP (Figure 2B, left panel), but not to the KIX domain of CBP (amino acids 461–662, Figure 2B, left panel). HPV-6 E6 (E6.6) bound poorly to C/H3 and the C-terminal region (Figure 2B, middle panel) and the small amount of binding observed did not appear physiologically relevant (see below, Figure 5). E6.16 also bound to the N-terminal region (1–595 amino acids) of p300 (Figure 2B, right panel). We have further mapped the region in the N-terminus of p300, which binds E6.16, using a number of different p300 N-terminal fragments. The results demonstrated (Figure 3A) that E6.16 bound predominantly to the C/H1 domain between amino acids 340 and 413, although some weak binding to the fragment containing amino acids 1–340 was observed. It has recently been shown that p53 can also bind to the C/H1 domain,

A





B

GST-fusion proteins used in (A)

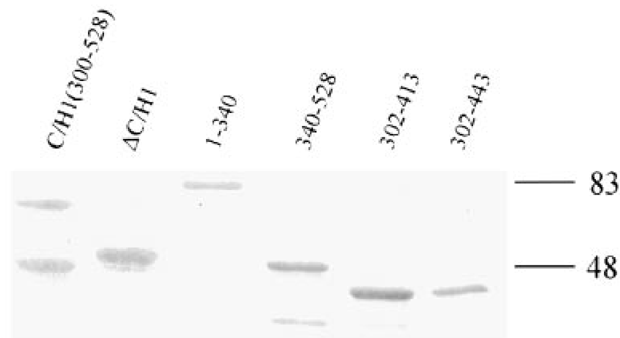


Fig. 3. Mapping the interaction of E6.16 and E6.6 with the N-terminus of p300, which contains the CH1 domain. (**A**) Radiolabeled *in vitro* translated E6.16 and E6.6 were added to the indicated GST–p300 fusion proteins. The bound complexes were separated on a 15% SDS–polyacrylamide gel and were analyzed with phosphoimagery. Summary of the binding of E6.16 and p53 to the p300 C/H1 domain. +/– indicates that binding is above background but greatly reduced over wild-type constructs containing the C/H1 domain. *The results for the binding of p53 to C/H1 domain are taken from Grossman *et al.* (1998). (**B**) A Coomassie Blue stained gel of the GST–p300 fusion proteins used in (A).

although this interaction required more amino acid flanking sequences. E6.16 bound to the 73-amino-acid region making up the C/H1 domain, while E6.6 associated moderately well with C/H1 (Figure 3A) at 50% of the efficiency of E6.16. E6.6 appeared to bind more efficiently to the large N-terminal fragment (1–595 amino acids), than to the smaller C/H1 domain (compare Figure 2B, right panel with Figure 3A), suggesting that E6.6 may require flanking sequences around C/H1 for efficient binding. E6.16 also bound to the C-terminal region of CBP/p300, while E6.6 bound poorly to the same area (Figure 2B). Using two fusion proteins covering the C-terminal of p300 between amino acids 1970 and 2358 [GSTp300.CT3(1970–2220)] and GSTp300.CT4(2170–2358)], we mapped E6.16 binding to the C-terminal region to between amino acids 1970 and 2220, although there was some residual binding to the domain between amino acids 2170 and 2358. There was no detectable binding of E6.6 to either of these p300 domains (Figure 4C).

To further map the interactions between E6.16 and the domains of p300, pull-down assays using GST-p300 and *in vitro*-transcribed and -translated point mutations and small deletions of E6.16 were carried out and the results are shown in Figure 4A–C. These pull-downs were carried out three times with similar binding activities observed. The overall results suggest that the interaction occurs in several regions throughout E6, or because of the unstructured nature of the protein, mutations at one site affect binding at other sites on the molecule. This is a similar situation to the binding of E6.16 to p53, where mutations throughout the molecule reduced binding and degradation of p53 (Crook *et al*., 1991; Foster *et al*., 1994). The levels of the mutated E6.16 proteins precipitated with the GST fusion proteins, relative to input, were measured on a

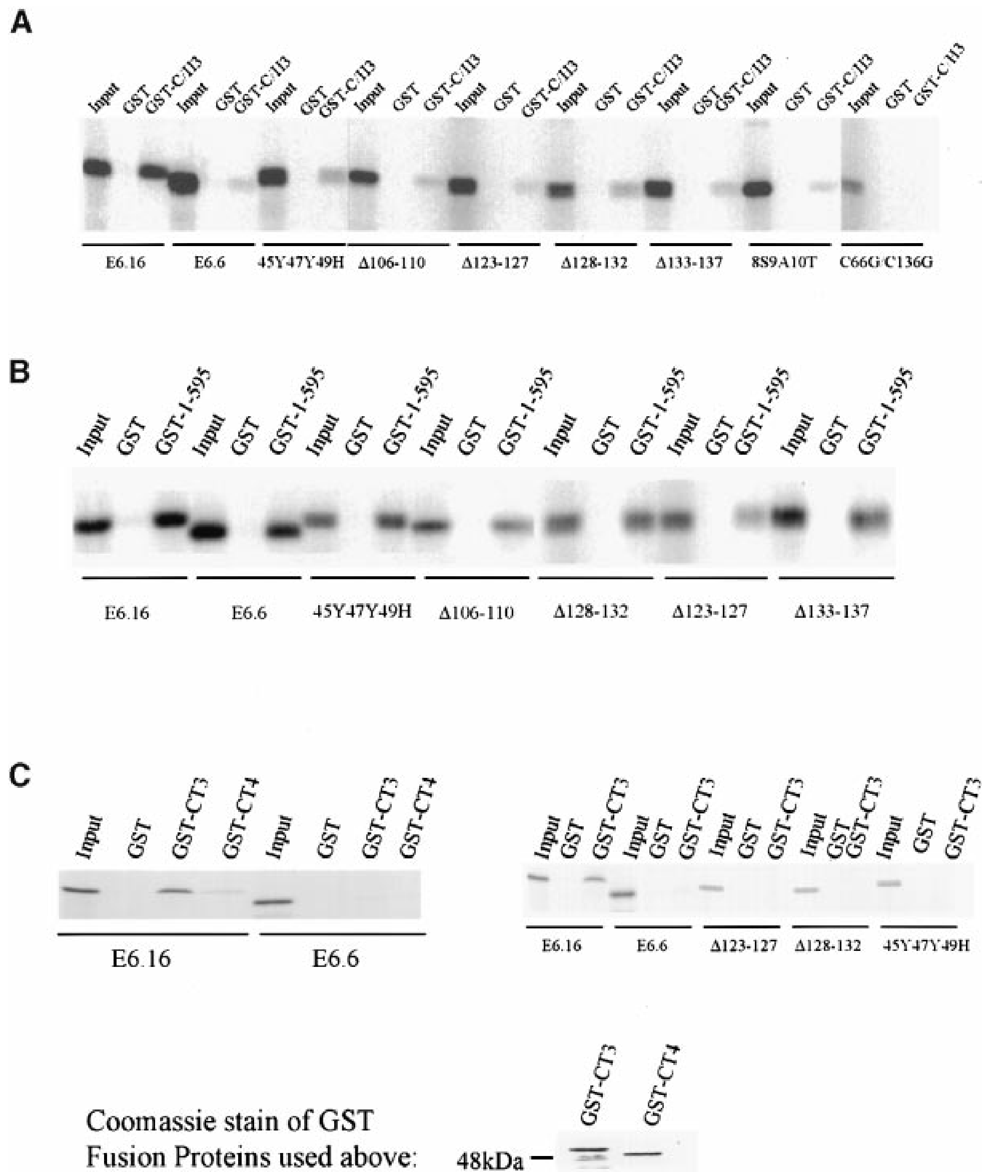


Fig. 4. Interaction of different p300 domains with E6.16 mutants. Radiolabeled *in vitro* translated wild-type E6.16, E6.6 and the indicated E6.16 mutants proteins were incubated with either (**A**) GST–C/H3, (**B**) GST–C/H1 or (**C**) GST–CT3 and –CT4 fusion proteins. The bound complexes were removed with glutathione beads, separated on a 15% SDS–polyacrylamide gel and quantified using a PhosphorImager and Imagequant software (Molecular Dynamics). See Table I. The input is 10% of that added to the test reactions. The Coomassie Blue-stained gel of the GST–CT3 and –CT4 fusion proteins used in (C) is shown below the gel.

PhosphorImager and the values are given in Table I. Of interest was the fact that some mutated proteins bound to the C/H1 domain, but failed to bind to the C/H3 or C-terminal regions and some mutations did not bind to any domain. For example, E6.16Δ106–110, E6.16Δ123–127 and E6.16Δ133–137 bound C/H1 to between 30 and 60% of wild type, but only had residual binding to C/H3, while E6.16Δ128–132 bound to both domains and E6.16C66G/C136G, which disrupts both zinc fingers of E6.16, bound to neither protein domain (Figure 4A, Table I and data not shown). None of the mutations tested were able to bind to the CT3 domain of the C-terminal region (Figure 4C).

E6.16 inhibits CBP/p300 co-activation in vivo

To determine if E6.16 binding had an effect on the ability of the CBP/p300 domains to co-activate transcription we used three different assay systems.

Mammalian two-hybrid assay. In this assay we tested whether E6.16 and E6.6 could disrupt the binding of c-Fos to the C/H3 domain of CBP. c-Fos was used as a bait by fusion to the Gal4 DNA binding domain (Gal4DBD) and the C/H3 domain, serving as prey, was fused to the transactivation domain of VP16 (Figure 5A). The reporter plasmid has five Gal4 binding sites upstream of the luciferase gene. When the reporter plasmid along with Gal4–c-Fos and C/H3–VP16 were transfected into U2OS cells there was 5- to 10-fold activation of the reporter (Figure 5B and C). When E6.16, which binds C/H3, was also transfected into cells, there was a complete inhibition of co-activation (Figure 5B and C). This inhibition was not observed with E6.6, which does not bind the C/H3 region (Figure 5B). To control for a non-specific effect on transcriptional activity we transfected cells with either Gal4–c-Fos or the highly active Gal4–VP16 alone with

Table I. Relative binding of wild-type E6.16, E6.6 and E6.16 mutations to p300 N-terminal, C/H3 and CT3 domains using a PhosphorImager

E6 constructs	p300 constructs					
	Amino acids 1–595		C/H3 domain		C-terminus (CT3)	
	% input bound	Relative binding	% input bound	Relative binding	% input bound	Relative binding
E6.16	11	1	5	1	10	1
E6.6	6	0.5	0.1	0.02	0.1	0.01
E6.168S9A10T	2	0.2	0.3	0.06	NT[a]	NT
E6.1645Y47Y49H	13	1.3	1.1	0.22	0.1	0.01
E6.16C66G/C136G	0.2	0.02	0.3	0.06	NT	NT
E6.16Δ106–110	6	0.5	0.6	0.12	NT	NT
E6.16Δ123–127	3	0.3	0.4	0.08	0.1	0.01
E6.16Δ128–132	8	0.7	2.2	0.44	0.1	0.01
E6.16Δ133–137	6	0.6	0.7	0.14	NT	NT

[a]NT, not tested

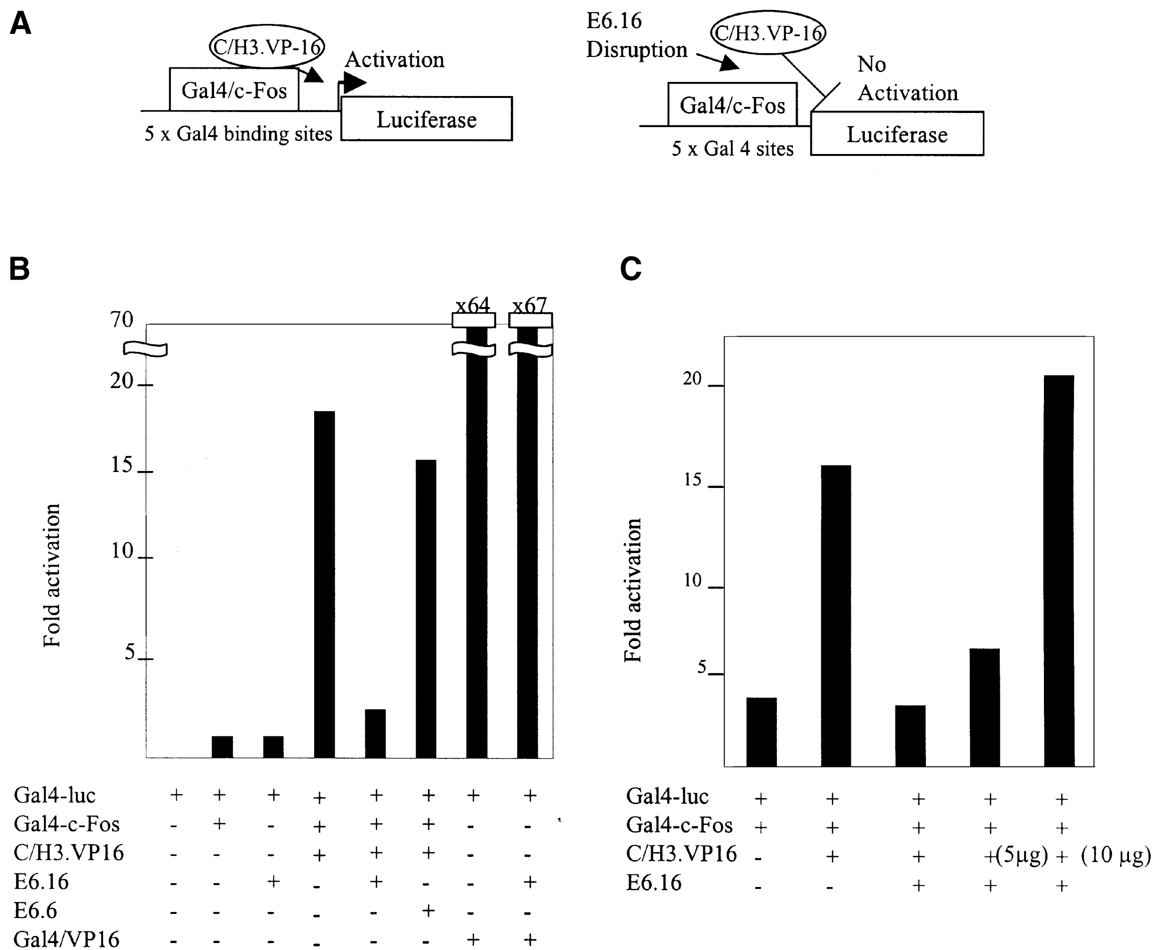


Fig. 5. E6.16 inhibits c-Fos activation in a mammalian two-hybrid system. (**A**) A schematic of the mammalian two-hybrid system used. (**B**) U2OS cells were co-transfected with a reporter (Gal4–Luc) that contains five Gal4 binding sites upstream of the luciferase gene and a construct that has the activation domain of c-Fos fused to the Gal4DBD (Gal4–c-Fos). The co-activation construct contains the C/H3 domain of p300 fused to the VP16 activation domain (C/H3–VP16). The results shown represent one experiment from four carried out. The fold activation is luciferase activity of any test transfection assay over reporter alone. (**C**) The same co-transfections were performed as in (B) but increasing amounts of the C/H3–VP16 construct were added to relieve the inhibitory effect by E6.16.

E6.16 (Figure 5B). No inhibition of Gal4–c-Fos or Gal4–VP16 was observed, suggesting that the abrogation of transcription was not due to a general down-regulation of transcriptional activity. To demonstrate that this inhibition may be due to the displacement of c-Fos by E6.16 from the C/H3 domain, we added increasing amounts of C/H3–VP16 with constant amounts of the other constructs. The results of these experiments showed that inhibition of activation was overcome by the additional C/H3 domains transfected into the cells (Figure 5C). This also suggests that the activity is specific to C/H3 and E6.16.

Inhibition of the intrinsic transactivation activity of p300. p300, when fused to the Gal4DBD, has been shown to

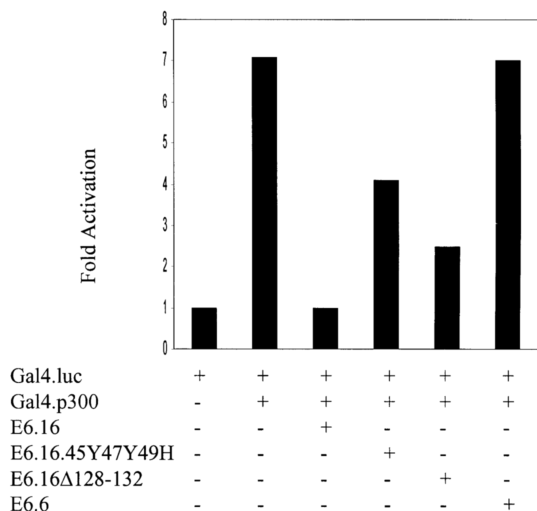


Fig. 6. Full-length p300 has intrinsic transcriptional activation properties which are inhibited by E6.16. U2OS cells were co-transfected with a reporter (Gal4–Luc) that contains five Gal4 binding sites upstream of the luciferase gene and a construct (Gal4–p300) that contains full-length p300 fused in-frame to the Gal4DBD. Wild-type E6.16 and the two E6.16 mutants, E6.1645Y47Y49H and E6.16Δ128–132, are in the pcDNA vector. The results shown represent one experiment from three carried out. The fold activation is luciferase activity of any test transfection assay over reporter alone.

have intrinsic transactivation activity (Yuan *et al.*, 1996). Using full-length Gal4–p300 we found that E6.16, but not E6.6, inhibited intrinsic transcriptional activity (Figure 6). E6.16 mutations that either bound to just the C/H1 domain (E6.16.45Y47Y49H) at wild-type levels, or to both C/H1 and C/H3 domains (E6.16Δ128–132) where binding was 40–65% of that of E6.16 (Table I), were also able to inhibit the activity of the full-length protein but at reduced efficiency compared with E6.16.

To determine if E6.16, E6.6 and E6.16 mutations would inhibit the activity of individual p300 domains we transfected various deletion constructs of p300, which contained either C/H1 or C/H3 domains, into U2OS cells along with the Gal4-responsive reporter, and measured activity in the presence and the absence of E6.16, E6.6 and E6.16 mutations (Figure 7A and B). The first 743 amino acids (Gal4p300.1–743), containing the C/H1 and the KIX domains, activated the reporter 50-fold (Figure 7A). E6.16 completely inhibited the activity, while E6.6 inhibited activity by 40%, which is representative of the binding of E6.6 to the C/H1 domain (Figure 7A and Table I). E6 mutations, E6.16.45Y47Y49H, E6.16Δ123–127 and E6.16Δ128–132, which all bind to the C/H1 domain by varying degrees from 30% to 100% of E6.16, all inhibited activity as well as E6.16. This is in contrast to E6.6, which was only able to inhibit activity by 40%, suggesting that binding, while necessary, is not sufficient for effective repression. The mutation E6.16.C66G/C136G, which did not bind to either C/H1 or C/H3, did not inhibit activity of the N-terminal construct (Figure 7A).

Another p300 construct (p300Δ245–1737) with the portion between amino acids 245 and 1737 spliced out increased the reporter response by >80-fold (Figure 7B). This construct contains the N-terminal region, which binds to nuclear receptors, the C/H3 domain and the downstream C-terminal region, but not the C/H1, KIX or HAT domains. The transcriptional activity of p300Δ245–1737 was inhibited by E6.16 (Figure 7B). The mutations E6.16Δ123–127 and E6.16Δ128–132 were tested, and E6.16Δ128–132 effectively inhibited activity by 65%, while E6.16Δ123–127 did not abrogate activation (Figure 7B). E6.16Δ128–132 binds to the C/H3 domain at 50% of the level of wild-type E6.16 (Figure 4B), while E6.16Δ123–127 binds at 8% of wild type (Table I), and this is reflected in their ability to inhibit p300 transactivation. In addition, the E6.6 protein, which binds at very low levels to the C/H3 or C-terminal domains of CBP/p300, was not able to inhibit transactivation of Galp300Δ245–1737 (Figure 7B). It would appear from the mutations that most of the transcriptional activity of Gal4–p300Δ245–1737 is mediated by the C/H3 domain, since E6.16Δ128–132, which bound only to this domain and not to the C-terminal region, had significant inhibitory activity.

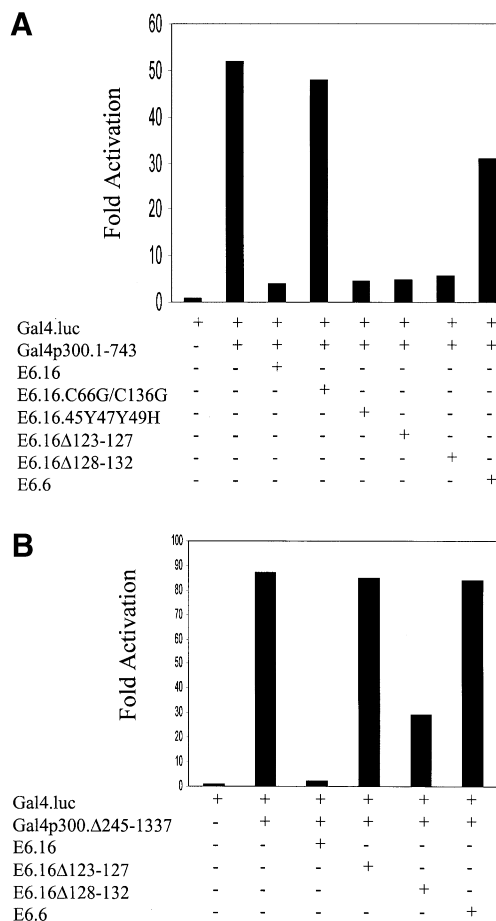


Fig. 7. Co-activation activity of different p300 domains is inhibited by E6.16, E6.6 and E6.16 mutants. (**A**) U2OS cells were co-transfected with a reporter (Gal4–Luc) that contains five gal4 binding sites upstream of the luciferase gene and an activation construct (Gal4–p300.1–743) that contains amino acids 1–743 (contains the C/H1 and KIX domains) of p300 fused in-frame to the Gal4DBD. The wild-type E6.16, E6.6 and the E6.16 mutants are in the pcDNA vector. The results shown represent one experiment from three carried out. The fold activation is luciferase activity of any test transfection assay over reporter alone. (**B**) The same co-transfection assays were performed as in (A) except that the activation construct (Gal4–p300Δ245–1337) used contains the C/H3 and the C-terminal regions of p300 fused in-frame to the Gal4DBD.

The activation of p53 and NF-κB by p300 is inhibited by E6.16

To determine whether E6.16 would inhibit the co-activation of promoters modulated by p300, we used p53- and NF-κB-responsive elements transfected into Saos-2 cells, which do not contain a functional p53. The p53 reporter construct, pG_{13}, which contains 13 p53 binding sites upstream of the luciferase gene, was activated >100-fold by the addition of p53, and this was marginally increased by the addition of p300 (Figure 8A). The control reporter pMG_{15}, with mutated p53 binding sites, was not activated by addition of p53 (Figure 8A). E6.16 expression reduced the activation of p53 by 100% in the absence of any exogenously added p300, although this inhibition was reduced to 75% in the presence of the p300 expression vector (Figure 8A). p53 has been reported to bind to the C-terminal region of CBP and to the C/H1 domain of p300 (Figure 3A; Gu *et al.*, 1997; Grossman *et al.*, 1998), so we chose two E6.16 mutations that bind C/H1 but were shown in one study to bind p53 at reduced levels but are unable to degrade the protein, while another study showed they would not bind to or degrade p53 (Crook *et al.*, 1991; Foster *et al.*, 1994). In our hands the two mutations bound p53 at 20% of the level of wild-type E6.16, but as previously shown were not able to degrade p53 (Figure 8C). These mutations (E6.1645Y47Y49H and E6.16Δ123–127) were still able to inhibit p53 activation by 65–70% (Figure 8A). These results taken together with the fact that E6.16 binds directly to the CBP/p300 domains suggest that the inhibitory activity of E6.16 is independent of p53 and represents another function of the E6 protein. E6.6, which binds the C/H1 domain at 50% of the level of E6.16, did not significantly inhibit activation in three separate experiments (Figure 8A).

NF-κB-mediated transcription is co-activated by CBP/p300. Since NF-κB binds to the C/H1 domain we wished to see whether E6.16 could also inhibit p300 co-activation. The NF-κB reporter construct, which contains three binding sites, was transfected into U2OS cells and was shown to be activated by endogenous NF-κB (Figure 8B). E6.16 abrogated this endogenous activation and this inhibition was overcome by the transfection of increasing amounts of the p300 full-length construct (Figure 8B). Transfection of E6.6, which binds the C/H1 domain but not the C/H3 domain, was not able to inhibit NF-κB transactivation, (Figure 8B). The results with both p53 and NF-κB suggest that either the binding of E6.6 to the C/H1 domain is not sufficient for inhibition of p53 and NF-κB activation, or that binding of NF-κB to the C/H3 and the C-terminal regions of p300 may be more important for its transcriptional activity than for binding to the C/H1 domain. Since E6.16Δ123–127, which only binds C/H1 and inhibits p53 activation, suggests that the former conclusion may be more accurate.

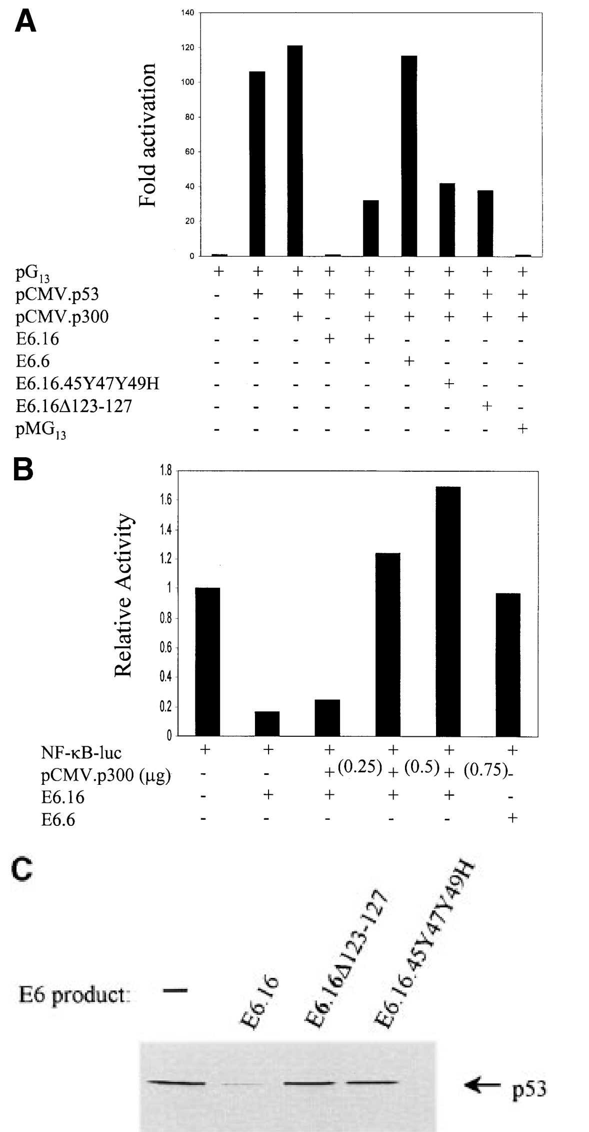


Fig. 8. Both p53- and NF-κB-responsive promoter elements are inhibited by E6.16. (**A**) Saos-2 (does not contain functional p53 proteins) were co-transfected with a reporter construct (pG_{13}) that contains 13 p53 DNA binding sites upstream of the luciferase gene or 13 mutated p53 DNA binding sites (pMG_{13}) and a p53 expression construct (pCMV-p53). The co-activation construct contains full-length p300 (pCMV-p300). The wild-type E6 and the E6 mutants' expression constructs are indicated. The results shown represent one experiment from three carried out. The fold activation is luciferase activity of any test transfection assay over reporter alone. (**B**) U2OS cells were co-transfected with a reporter construct (NF-κB–Luc) that contains three NF-κB sites upstream of the luciferase gene. The co-activation construct contains full-length p300 (pCMV–p300) and different amounts of this expression construct were added as indicated. The results shown represent one experiment. The luciferase activity of the reporter alone is set to one and the results are expressed relative to this value. (**C**) Wild-type E6.16, but not the mutants E6.1645Y47Y49H and E6.16Δ123–127, cause the degradation of p53. Radiolabeled rabbit reticulocyte *in vitro*-translated E6.16, E6.1645Y47Y49H and E6.16Δ123–127 were mixed with radiolabeled rabbit reticulocyte *in vitro*-translated p53. The mixtures were incubated at 25°C for 2 h. The proteins were separated on a 12% SDS–polyacrylamide gel and then quantified using a PhosphorImager and Imagequant software (Molecular Dynamics).

Discussion

CBP and p300 are involved in co-activating, at the transcriptional level, a number of genes that are important for cell growth and differentiation. Both co-activators are targets for viral proteins and the outcome can be inactivation of CBP and p300 in the case of E1A (Arany *et al.*, 1995; Lee *et al.*, 1996; Korzus *et al.*, 1998) or inappropriate stimulation in the case of the HTLV-1 Tax protein (Kwok *et al.*, 1996). The HTLV-I Tax protein interacts with the KIX region, SV40 large T binds the C/H3 domain and

the adenovirus E1A binds to at least three domains. E1A binds to the C/H3 domain and N- and C-terminal regions and inhibits the ability of CBP/p300 to co-activate nuclear receptors, CREB- (Arany *et al*., 1995), STAT-1- (Korzus *et al*., 1998) and c-Jun-responsive (Lee *et al*., 1996) promoters. Recently it was shown that E1A binds to the HAT domain of p300 resulting in the inhibition of HAT activity (Chakravarti *et al*., 1999; Hamamori *et al*., 1999). However, other studies have shown that E1A either has no effect on intrinsic HAT activity (Bannister and Kouzarides, 1996; Ogryzko *et al*., 1996) or actually increases it (Ait-Si-Ali *et al*., 1998). In this study, we show that the E6 protein from the oncogenic virus HPV-16 binds to three regions of CBP/p300 (C/H1, C/H3 and the C-terminal region). Mutations of E6.16 which bind to the C/H1 domain, both the C/H1 and C/H3 domains or neither, were shown to inhibit activation relative to their binding capacity (Figures 6–8). The E6 protein from the benign virus HPV-6 interacts with the C/H1 domain only at 50% of the level of HPV-16 E6 and was consistently shown not to inhibit full-length p300 activity. However, E6.6 could partially inhibit C/H1 domain activity when expressed in isolation from the C/H3 domain (Figure 7A). It is not clear if HPV-16 E6 inhibits binding of transcription factors to the various domains, as suggested by the results of the mammalian two-hybrid assay using c-Fos and the C/H3 domain of CBP, or if it inhibits binding of the chromatin remodeling proteins such as P/CAF or both. We know from our unpublished data that HPV-16 E6 does not bind directly to c-Fos, P/CAF, or the intrinsic HAT domain of p300 (data not shown). Inhibition by HPV-16 E6 could be overcome by the addition of increasing amounts of expression constructs expressing individual CBP/p300 domains or the CBP/p300 full-length protein. p53 has recently been shown to bind the C/H1 domain (Grossman *et al*., 1998) as well as the previously described C-terminal domain (Avantaggiati *et al*., 1997; Gu *et al*., 1997). However, the interaction of E6 with CBP/p300 is independent of the ability of E6 to bind p53, since (i) we have shown the interaction to be direct, and (ii) mutations of E6 which do not bind or degrade p53 still bound to CBP/p300 and inhibited co-activation. It is interesting that two regions of CBP and p300 (amino acids 1–34 and 358–394) have homology with two regions of HPV-16 E6 (amino acids 1–39 and 104–139), with one of the CBP/p300 regions overlapping the C/H1 domain of p300 (Grossman *et al*., 1998). At present the significance of this homology is unclear, and HPV-16 E6 proteins mutated in one of the homology regions (amino acids 106–139) were shown here to bind to the C/H1 domain.

Similar results have recently been published (Zimmermann *et al*., 1999). In this study the authors showed that the E6 protein from HPV-16 and -18, but not from HPV-6 and -11, bound to the CBP2 domain of CBP (equivalent to the C/H3 domain described here), but not to the CBP1 domain (the KIX domain described here). However, other regions of CBP were not tested. The result of binding was the inhibition of transactivation by CBP2. The binding domain of E6 was in the C-terminal domain, between amino acids 100 and 147, while in our study using point mutations and small deletions we were unable to map the domain so precisely. However, although the C-terminus of E6 bound CBP2 as well as full-length E6, it only inhibited transcription at 50% of the efficiency of full-length E6, suggesting that the overall conformation of E6 is important for biological activity.

What are the advantages for the virus to target CBP/p300? The human papillomaviruses infect basal epithelial cells, which are destined to terminally differentiate. Papillomaviruses have a small coding capacity and do not code for any of the replicative machinery and differentiating cells contain very little or none of the cell's replicative components. Therefore the virus needs to stimulate cells into S-phase to have on hand a supply of replicative enzymes. CBP/p300 are known to be important for cell differentiation and transcription factors such as the AP-1 family, which are thought to be important for keratinocyte differentiation, bind to the regions bound by E6. Therefore disruption of AP-1 transactivation may have an effect on keratinocyte differentiation. In addition, mice deficient for IκB kinase-α (IKK-α), which phosphorylates IκB, resulting in the release of active NF-κB, were shown to have a disruption in skin differentiation with hyperproliferation of the stratum spinosum (Hu *et al*., 1999; Takeda *et al*., 1999). In the natural infection HPV-16 causes disruption of differentiation and hyperproliferation of the stratum spinosum and granulosum. In addition, HPV-16 E6 and E7 proteins can independently modulate the ability of primary human keratinocytes to differentiate (Sherman and Schlegel, 1996; Funk *et al*., 1997; Jones *et al*., 1997; Sherman *et al*., 1997). HPV-6 on the other hand does not disrupt differentiation, but does cause a hyperproliferation of the epithelial cells. The HPV-16 E6 protein may therefore have a role in inhibiting differentiation, resulting in hyperproliferation.

The papillomavirus group cause persistent infections with lesions remaining for months, even years. It has been suggested that the viruses can down-regulate the local immune response to allow persistence of the infection. In fact studies have shown a reduced class I MHC expression (Cromme *et al*., 1994a,b; Keating *et al*., 1995; Bontkes *et al*., 1998) and reduced numbers of Langerhans and T cells in the immediate vicinity of the lesion (Tay *et al*., 1987a,b,c,d). The fact that HPV-16 E6 can inhibit the activation of NF-κB by CBP/p300 may help to explain these findings, since this transcription factor is activated by a number of stimuli, including viral infection (for a review see Baldwin, 1996). In addition, binding sites for NF-κB are found in a number of promoters including those for class I MHC, cytokine modulators of the immune response including IL-2, IL-6, IL-8 and GM-CSF. Keratinocytes synthesize a variety of these chemotactic factors, including IL-6, IL-8 and GM-CSF, when stimulated by viral infection (for a review see Tomic-Canic *et al*., 1998). Therefore inhibition of the co-activation of NF-κB may help the virus escape immune recognition in the epithelium.

In summary, we have shown that E6 of HPV-16 binds to three regions of CBP/p300, resulting in the abrogation of the co-activation functions. These co-activators are important for cell differentiation and cell cycle control and are central to the activation of a number of genes that modulate immune responses. Therefore the outcome of the interaction between E6 and CBP/p300 may involve the inhibition of differentiation of the epithelial cells harboring the virus and the down-regulation of the immune

recognition machinery to permit the persistence of the viral infection.

Materials and methods

Plasmids

GST fusions. Constructs containing different regions of p300 fused to GST were provided by Stephen Grossman in D.Livingston's laboratory. These were: pGEXp300(1–595), pGEXp300(1572–2370), pGEXp300-(1708–1913,C/H3), pGEXp300(300–528,C/H1), pGEXp300(Δ300–528), pGEXp300(1–340), pGEXp300(340–528), pGEXp300(302–443) and pGEXp300(302–413). Both pGEXp300(1970–2220) and pGEXp300-(2170–2358), which we call GST.CT3 and GST.CT4, respectively, were a gift of Jasianne Eid in D.Livingston's laboratory. pGEXCBP1(461–662) and pGEXCBP2(1612–1877), which we have called GST–CBP.KIX and GST–CBP.C/H3, respectively, were provided by T.Kouzarides. pGEXCBP3(1990–2441), which we have called GST–CBP.CT, was provided by R.Roeder.

In vitro transcription/translation. E6.16 and E6.6 were cloned in pGEM7z. E6.16Δ106–110 and E6.16Δ133–137 cloned in pGEM7z were provided by E.Androphy. E6.16 45Y/47H/49H (cloned in pTZ19U), E6.16Δ123–127 and E6.16Δ128–132 (cloned in pTZ18U), E6.16 C66G/C136G (from T.Kanda subcloned into pBSKS) were provided by D.Galloway.

Expression plasmids. E6.16 and E6.6 were cloned in pSG5 and pcDNA3, and the E6.16 mutations cloned in pcDNA. Gal4–M2–Luc, CBP2/VP16 and Gal4–c-Fos were provided by T.Kouzarides. Plasmids Gal4–p300, Gal4–p300.1–242, Gal4–p300.1–743 and Gal4–p300Δ242–1737 were provided by A.Giordano. pG_{13}–Luc, pMG_{15}–Luc and pCMV–p300 were provided by D.Livingston. pCMV53 was provided by L.Laimins. NF-κB–Luc was provided by S.Dewhurst.

p53 degradation assay

Constructs containing p53, E6.16 and E6.16 mutants were radioactively labeled, *in vitro* transcribed and translated using the TnT system (Promega, WI). The reaction was carried out in 40-μl volumes containing p53 (20 000 c.p.m.), E6 in buffer 25 mM Tris–HCl pH 7.5, 100 mM NaCl and 3 mM DTT, at 25°C for 2 h. The total amount of rabbit reticulocyte lysate was adjusted by adding unprogrammed rabbit reticulocyte. The reaction was stopped by adding 2× sample buffer, boiled for 5 min, then analyzed by 15% SDS–PAGE. After drying, the gel was exposed to PhosphorImager.

Cells

Soas-2, Cos-1 and U2OS cells were obtained from ATCC and grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum.

Co-immunoprecipitation

Cos-1 cells were transfected with either 6 μg of pSG5 or pSG5 containing flag-tagged E6.16 cloned at the *Bam*HI–*Eco*RI sites (Flag-E6.16) using lipofectamine (Gibco-BRL). The cells were harvested 48 h after transfection, and lysed in buffer (20 mM HEPES pH 7.9, 180 mM NaCl, 0.1% NP-40 supplemented with protease inhibitors). The cell lysates were rotated in the buffer for 1 h at 4°C before the cell debris was removed via centrifugation. Total protein was determined using a Coomassie Blue-based protein assay (Bio-Rad) according to the manufacturer's instructions. The cell lysates (6.8 mg protein per lysate) were precleared with the addition of 2 μg mouse IgG2a and IgG1 and 20 μl anti-mouse IgG magnetic beads (Dynal) for 2 h at 4°C. The appropriate lysate was immunoprecipitated with the addition of either monoclonal antibody against p300/CBP (150 μl of Rw144, a gift from D.Livingston) or 8 μg anti-flag (Sigma) and 30 μl anti-mouse IgG magnetic beads. The immunocomplexes were washed four times in lysis buffer and then separated on a 4–20% gradient gel (Bio-Rad). The lower molecular weight species on the gel were transferred to nitrocellulose using Towbin buffer and the larger molecular weight species were transferred to PVDF membrane in transfer buffer without methanol and the addition of 1% SDS overnight at 50 mA constant current. Both blots were blocked in 5% Blotto PBST (PBS with 0.25% Tween-20) overnight at 4°C. The nitrocellulose blot was then incubated with anti-Flag (1.9 μg/ml PBST with 5% Blotto) and the PDVF membrane was incubated with a mixture of NM-11 and RW128 monoclonal antibodies against CBP/p300. The blots were incubated with a 1:2000 dilution of anti-mouse IgG HRP (Santa Cruz) and the proteins were visualized with the ECL reagent according to the manufacturer's instructions (NEN Dupont).

Binding of bacterially derived proteins

Bacterially derived GST fusion proteins were purified from bacteria and mixed with bacterially (BL21 DE3) grown His-tagged HPV-16 E6, cloned in pET-28a and purified as per the manufacturer's instructions (Novagen, Inc., WI). Proteins were mixed in molar ratios so that 125 ng of E6 and 250 ng of GST fusion proteins were used. The protein preparations were combined and incubated 2–3 h at 4°C in buffer [50 mM Tris pH 8, 180 mM NaCl, 500 μg/ml bovine serum albumin (BSA), 0.5 mM DTT with protease inhibitors]. After incubation the complexes were mixed with glutathione beads to pull down the GST fusion proteins. Western blots were carried out using anti-histidine antibodies (top panel) or anti-GST antibodies (bottom panel).

GST-E6.16 pull-down of p300/CBP from cold U2OS cell extract

A cell lysate from U2OS cells was prepared as described above with the exception that these cells were not previously transfected. The preparation of GST and GST-E6.16 conjugated to glutathione beads is described below. The U2OS cell lysate (1 mg) was precleared with 20 μg GST beads in binding buffer (1× PBS supplemented with 0.1% NP-40 and protease inhibitors) for 2–3 h at 4°C. The sample was divided into two aliquots and another 10 μg GST-bound glutathionine beads were added to one sample and 7.5 μg GST–E6.16 were added to the other. The pull-downs were incubated overnight at 4°C with rotation and then the beads were washed four times with binding buffer and the complexes separated on a 6% SDS–polacrylamide gel and transferred to PVDF as described above. The membrane was blotted with a mixture of NM11 (Pharmigen) and RW128 monoclonal antibodies against p300/CBP followed by anti-mouse–HRP (Santa Cruz). The proteins were visualized using ECL reagent as described by the manufacturer (NEN Dupont).

Transfections

Mammalian two-hybrid transfections. U2OS cells were transfected with 1 μg Gal4–M2–Luc promoter and one or more of the following: 0.5 μg Gal4–Fos, 1 μg CBP2–VP16, 1 μg pSG5–E6.16 and pSG5–E6.6 using lipofectamine (Gibco-BRL) in six-well plates.

Gal4–p300 transactivations. Transfections were carried out in U2OS cells using lipofectamine in 12-well plates. Cells were transfected with Gal4–M2–Luc (0.25 μg) and one or more of the following: Gal4–p300 (0.25 μg), Gal4–p300.1–242 (0.25 μg), Gal4–p300.1–743 (0.25 μg), Gal4–p300Δ242–1737 (0.25 μg), pcDNA–E6.16 (0.5 μg), pcDNA–E6.16Δ123–127 (0.5 μg), pcDNA–E6.16Δ128–132 (0.5 μg).

pG13 Luc and NF-kB–Luc promoter transactivations. Saos2 cells were transfected using the calcium phosphate method in 60-mm dishes with 1 μg pG13–Luc and MG15–Luc promoter and one or more of the following: 100 ng pCMV–p53, 2 μg pCMV–p300, 1 μg pcDNA–E6.16 and 1 μg pcDNA–E6.1645Y/47Y/49H. U2OS cells were transfected using lipofectamine in 12-well plates with 50 ng NF-κB–Luc promoter and 0.125 μg pcDNA–E6.16 with varying amounts of pCMV–p300 as indicated (Figure 7B).

All transfections were carried out in duplicate and repeated at least three times. Empty pSG5 or pcDNA plasmids were used to equalize the amount of DNA in each transfection. Luciferase assays were carried out on lysed extracts 46–48 h after transfection. Equal amounts of extract protein were analyzed in each assay.

GST fusion protein expression and purification

GST fusion proteins were expressed and purified as described in the manufacturer's instructions (Pharmacia Biotech), with the following modifications: GST–p300(1572–2370) was grown at 30°C and induced for 3 h with 0.01 mM isopropylthio-β-D-galactopyranoside (IPTG) before harvest. After glutathione bead purification, fusion proteins were analyzed by SDS–PAGE, stained with Coomassie Blue and compared against BSA standards for quantification.

In vitro transcribed and translation products

All E6 and E6 mutants were *in vitro* transcribed and translated using a Sp6-coupled transcription/translation wheat germ system (Promega) according to the manufacturer's instructions. The proteins were radiolabeled with the addition of [^{35}S]cysteine and percentage incorporation was determined by trichloroacetic acid precipitation according to the manufacturer's protocol.

GST binding assays

Radiolabeled E6 proteins (80 000 c.p.m.) were incubated in buffer A (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40) along

with 1 μg of GST bound to glutathione beads for 1 h at 4°C to preclear. The supernatants were transferred to new tubes and 1 μg of the indicated GST fusions bound to glutathione beads were added and incubated at 4°C overnight. Beads were then washed three times in buffer A, sample buffer added and the proteins analyzed by SDS–PAGE and visualized by autoradiography or the PhosphorImager.

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