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The E7 oncoprotein associates with Mi2 and histone deacetylase activity to promote cell growth

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E7 is the main transforming protein of human papilloma virus type 16 (HPV16) which is implicated in the formation of cervical cancer. The transforming activity of E7 has been attributed to its interaction with the retinoblastoma (Rb) tumour suppressor. However, Rb binding is not sufficient for transformation by E7. Mutations within a zinc finger domain, which is dispensable for Rb binding, also abolish E7 transformation functions. Here we show that HPV16 E7 associates with histone deacetylase *in vitro* and *in vivo*, via its zinc finger domain. Using a genetic screen, we identify Mi2β, a component of the recently identified NURD histone deacetylase complex, as a protein that binds directly to the E7 zinc finger. A zinc finger point mutant which is unable to bind Mi2β and histone deacetylase but is still able to bind Rb fails to overcome cell cycle arrest in osteosarcoma cells. Our results suggest that the binding to a histone deacetylase complex is an important parameter for the growth-promoting activity of the human papilloma virus E7 protein. This provides the first indication that viral oncoproteins control cell proliferation by targeting deacetylation pathways.

Keywords: histone deacetylase/human papilloma virus E7 oncoprotein/Mi2/transformation

Introduction

Many human papilloma viruses (HPVs) infect the mucosa of the anogenital tract (reviewed in zur Hausen, 1996). 'High-risk' HPVs (e.g. types 16 and 18) are found frequently in cervical carcinoma and are considered to be essential for full malignant transformation. In contrast, 'low-risk' HPVs (types 6 and 11) are associated with benign genital warts and are very rarely detected in tumours. The HPV16 E6 and E7 genes play a crucial role in transformation and bind cell cycle regulators such as p53 and Rb, respectively (Tommasino and Crawford, 1995; Jansen-Durr, 1996). E7 is the major transforming protein of HPV and shares sequence and structural homology with adenovirus E1A (Figure 1A). Based on this homology, the E7 protein can be divided into three domains: conserved region 1 (CR1, residues 2–15), conserved region 2 (CR2, residues 16–38), which contains the LXCXE motif required for high-affinity binding to Rb and other 'pocket proteins', and conserved region 3 (CR3, residues 39–98). CR3 shows little sequence homology to E1A but, like CR3 of E1A, forms a zinc finger structure. E7 shares transformation and transactivation functions with E1A (Chellappan *et al.*, 1992).

Several *in vitro* transformation assays reflect the transforming potential of the HPV16 E7 protein: for example, HPV16 E7 can cooperate with activated Ras to transform rodent primary fibroblasts (Chesters *et al.*, 1990) and can also cooperate with HPV16 E6 to immortalize human keratinocytes, the natural host cells of HPV (Hawley-Nelson *et al.*, 1989; Jewers *et al.*, 1992). The ability to bind pocket proteins is important for transformation, and mutation of the LXCXE motif of HPV16 E7 abrogates high-affinity binding to Rb and transformation activity (Edmonds and Vousden, 1989; Chesters *et al.*, 1990; Jewers *et al.*, 1992; Phelps *et al.*, 1992). Furthermore, HPV6 E7 and HPV11 E7, which bind Rb with lower affinity than does HPV16 E7, display much lower efficiencies in transformation assays (Storey *et al.*, 1988; Munger *et al.*, 1989). However, it is clear that high-affinity binding to Rb is not sufficient for transformation. Several HPV16 E7 mutants which retain the ability to bind Rb are transformation deficient (Edmonds and Vousden, 1989; Banks *et al.*, 1990; Chesters *et al.*, 1990; Jewers *et al.*, 1992; Phelps *et al.*, 1992). Therefore, regions outside the Rb-binding domain must be important for the transforming potential of HPV16 E7. How these regions contribute to transformation is poorly understood.

In recent years, it has become increasingly apparent that cellular transcription is regulated at the chromatin level. Actively transcribed genes are rich in acetylated histones, while repressed genes show little chromatin acetylation (reviewed in Struhl, 1998). Acetylation of specific lysine residues in the N-termini of histones is believed to weaken their interaction with DNA. This results in a more open chromatin structure which is conducive to transcription, for example by facilitating transcription factor access to their binding sites. Conversely, deacetylation of histones is thought to favour the formation of compact, repressive chromatin. In agreement with this view, co-activators of transcription (CBP/p300, P/CAF and GCN5) and components of the basal transcription machinery ($TAF_{II}250$) have been shown to possess intrinsic histone acetyl transferase activity. Co-repressors, such as Sin3, NCoR and SMRT, on the other hand, bind enzymes which catalyse the deacetylation of histones (Pazin and Kadonaga, 1997; Wolffe, 1997), such as HDAC1 (Taunton *et al.*, 1996) and HDAC2 (Yang *et al.*, 1996).

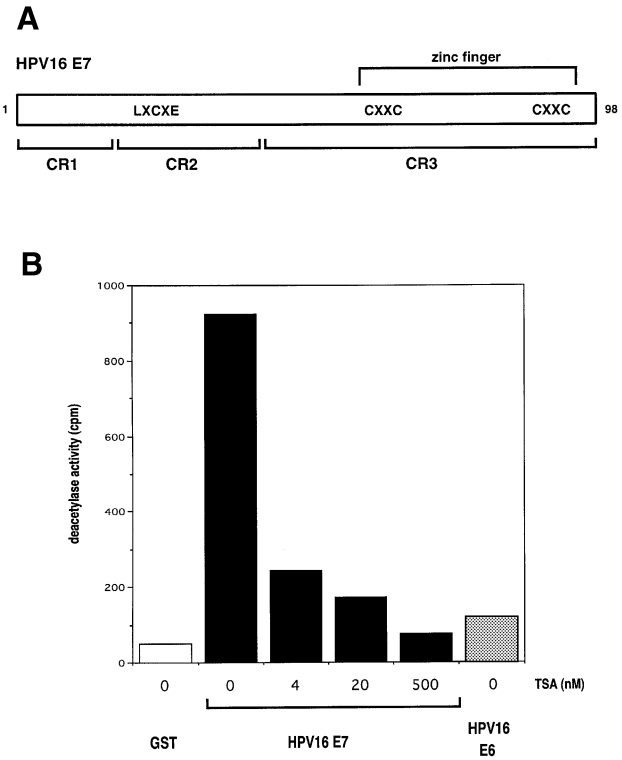


Fig. 1. (**A**) Structure of the HPV16 E7 protein. Regions of conservation between E7 and adenovirus E1A are shown (CR1, CR2 and CR3). LXCXE, pocket protein-binding motif; CXXC, cysteine pairs involved in zinc finger formation. (**B**) HPV16 E7 associates with histone deacetylase activity. Equivalent amounts of GST, GST–E7 (HPV16 E7) and GST–E6 (HPV16 E6) bound to glutathione–Sepharose beads were used to purify histone deacetylase activity from HeLa nuclear extract as indicated (see Materials and methods). Histone deacetylase assays were performed in the absence or presence of the indicated concentration of trichostatin A (TSA). Deacetylase activity is expressed as radioactivity (c.p.m.) released from a ^{3}H-labelled acetylated histone H4 peptide (Taunton *et al.*, 1996; Brehm *et al.*, 1998).

Recently, several independent findings suggest that histone deacetylases play an important role in cell cycle regulation. We and others have shown that the tumour suppressor Rb recruits histone deacetylase (HDAC) to repress E2F-regulated S-phase-specific genes, and that viral oncoproteins disrupt the Rb–HDAC complex (Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). Furthermore, translocations found in promyelocytic leukaemias result in the production of hybrid proteins (PML-RARα and PLZF-RARα) with altered histone deacetylase complex-binding properties (Grignani *et al.*, 1998; Lin *et al.*, 1998). The involvement of histone deacetylases in cell proliferation suggests that these enzymes represent likely targets of viral oncoproteins that deregulate the cell cycle.

Using *in vitro* and *in vivo* interaction assays, we show here that HPV16 E7 binds histone deacetylase in an Rb-independent manner. Histone deacetylase binding maps to the zinc finger region of E7. This zinc finger region specifically binds Mi2β, a component of a recently identified histone deacetylase complex. A point mutation in the zinc finger region shown previously to abrogate transformation functions results in the loss of Mi2β and histone deacetylase binding. Furthermore, we show that this Mi2β/deacetylase binding mutant fails to overcome cell cycle arrest efficiently in Saos2 cells. These data link the binding of a histone deacetylase complex to the oncogenic potential of the HPV16 E7 protein.

Results

HPV16 E7 associates with histone deacetylase

To establish whether HPV16 E7 can associate with histone deacetylase activity, a glutathione *S*-transferase (GST)–E7 fusion protein bound to glutathione–Sepharose beads was incubated with HeLa nuclear extract, extensively washed and subjected to a histone deacetylase assay using an acetylated peptide corresponding to the N-terminus of histone H4 (Taunton *et al.*, 1996; Brehm *et al.*, 1998). Figure 1B shows that GST–E7 purified a deacetylase that was sensitive to nanomolar concentrations of the potent histone deacetylase inhibitor trichostatin A (TSA). In contrast, GST alone or GST fused to the HPV16 E6 protein failed to bind significant levels of activity, demonstrating the specificity of the E7–HDAC interaction.

Next we tested whether E7 interacted with any of the known histone deacetylases present in the extract. To this end, cellular proteins associated with GST–E7 were subjected to Western analysis using HDAC1- and HDAC2-specific antibodies. GST–E7 bound cellular HDAC1 (Figure 2A, lane 3), while GST failed to retain detectable levels of HDAC1 protein (lane 2). In agreement with our previous results, GST–Rb also bound HDAC1 (lane 4). Similar results were obtained when binding to cellular HDAC2 was assessed (Figure 2B). Both GST–E7 (lane 7) and GST–Rb (lane 8) affinity purified HDAC2 from nuclear extract, whereas no interaction between GST and HDAC2 was detected (lane 6). These findings suggest that binding of HDAC1 and HDAC2 to E7 accounts, at least in part, for the histone deacetylase activity detected.

To assess whether HPV16 E7 and HDAC1 form a complex *in vivo*, we performed a co-immunoprecipitation experiment. Epitope-tagged E7 and HDAC1 were expressed in human osteosarcoma (U2OS) cells, extracts were precipitated with an antibody recognizing epitope-tagged E7, and the presence of HDAC1 in the immunoprecipitate was analysed by Western blotting with an antibody recognizing epitope-tagged HDAC1. Figure 2C shows that HDAC1 was detected in the immunoprecipitate only when E7 and HDAC1 were co-expressed (lane 4). These results argue that E7 can bind histone deacetylase both *in vitro* and *in vivo*.

Histone deacetylase binding to E7 is Rb independent

Both E7 and HDAC1 have been demonstrated to interact physically with Rb (Munger *et al.*, 1989; Chellappan *et al.*, 1992; Phelps *et al.*, 1992; Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). It is therefore conceivable that deacetylase binding to E7 in our pull-down assays was mediated by Rb present in the nuclear extract. We employed two Rb-binding mutants of E7 to address this question (Figure 3A and B): we reasoned that if E7 binds to deacetylase through Rb, then E7 mutants which fail to bind Rb would also fail to bind to deacetylase.

The E7 C24 mutant carries a single point mutation in the LXCXE motif that disrupts the interaction with Rb

(Phelps *et al.*, 1992). The E7 39–98 deletion mutant lacks CR1 and CR2, including the LXCXE motif (residues 22–26; Figure 3A). We analysed the ability of these E7 mutants to purify histone deacetylase activity from nuclear extract. As shown in Figure 3C, neither point mutation of the LXCXE motif (C24) nor deletion of CR1 and CR2 (39–98) had a significant effect on deacetylase binding.

We also employed peptide competitors to assess the role of the LXCXE motif in E7–HDAC complex formation. We have shown previously that an LXCXE motif-containing peptide can abrogate the binding of Rb to histone deacetylase (Brehm *et al.*, 1998). If E7 bound histone deacetylase through Rb, then the LXCXE peptide would be expected to interfere with the E7–HDAC interaction. However, when wild-type GST–E7 (E7 WT) was incubated with nuclear extract in the absence or presence of a control peptide (HA) or the LXCXE motif-containing peptide (LXCXE), the same amount of histone deacetylase activity was recovered and no adverse effect on HDAC binding was detected (Figure 3C).

Taken together, the analysis of Rb-binding mutants and peptide competition experiments strongly suggest that E7 binding to histone deacetylase does not require the LXCXE

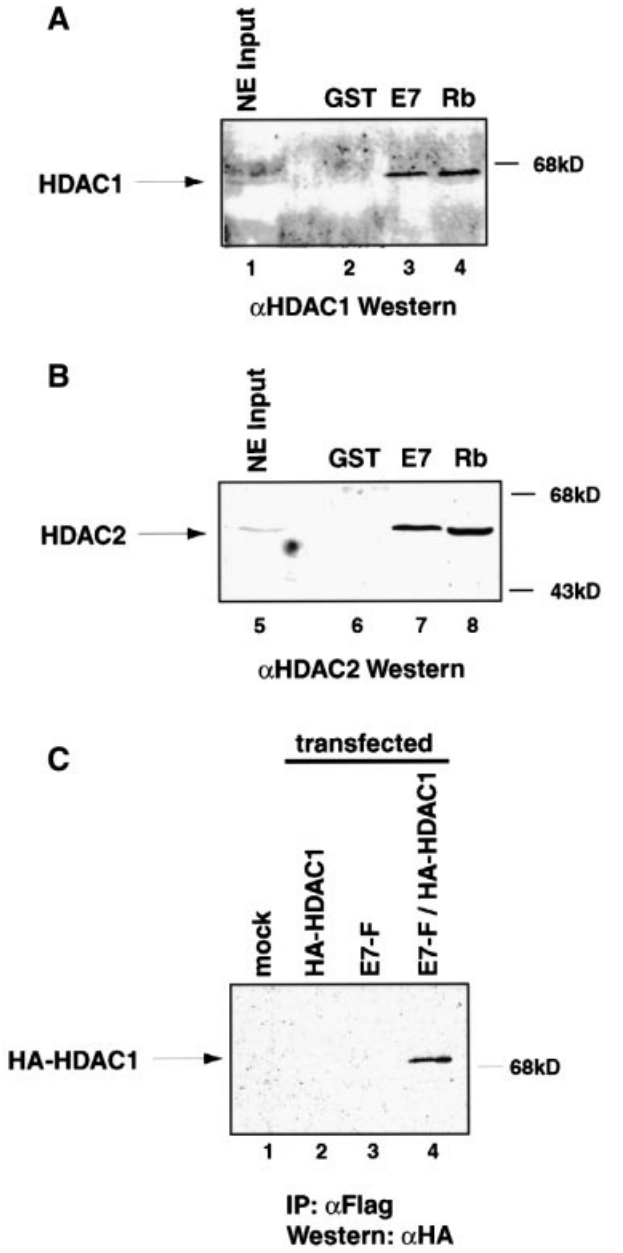


Fig. 2. E7 binds the histone deacetylases HDAC1 and HDAC2. GST–E7 binds cellular HDAC1 (**A**) and HDAC2 (**B**). GST, GST–E7 (E7) and GST–Rb 379–928 (Rb) bound to glutathione–Sepharose beads were used to purify histone deacetylases from HeLa nuclear extract as indicated. Bound proteins were subjected to SDS–PAGE and Western analysis using αHDAC1 antiserum ('1123') and αHDAC2 antibody (C19, Santa Cruz) as shown. The positions of HDAC1 and HDAC2 are indicated by arrows; molecular weights are shown on the right. Lanes 1 and 5: 5% of nuclear extract (NE) input. (**C**) E7 and HDAC1 co-immunoprecipitate. U2OS cells were transfected with 10 μg each of pCMV-HA-HDAC1 (HA-HDAC1), pcDNA3-E7-F (E7-F) and empty expression vectors (mock) as indicated. The total amount of DNA transfected was kept at 20 μg using empty expression vectors. Whole-cell extracts were prepared 36 h after transfection. Flag-tagged E7 was precipitated using αFlag antibody (M2). Immunoprecipitates were subjected to SDS–PAGE and Western analysis using αHA antibody (12C5A, Boehringer Mannheim). The position of HA-tagged HDAC1 is shown by an arrow; molecular weight is indicated on the right.

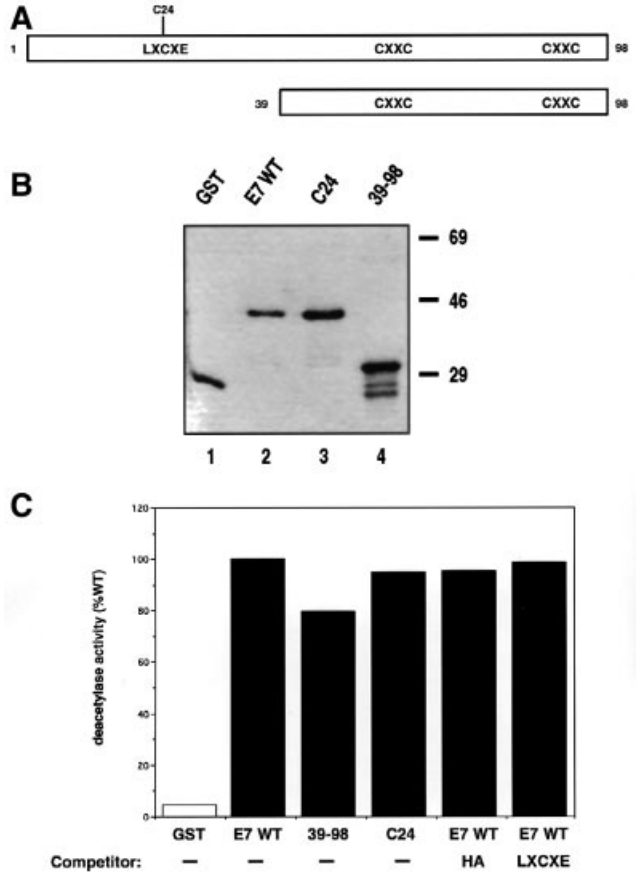


Fig. 3. The Rb-binding motif of E7 is dispensable for histone deacetylase binding. (**A**) Schematic representation of E7 mutants used. The position of the C24 mutation which disrupts Rb interaction with the LXCXE motif is shown. LXCXE denotes the Rb-binding motif; CXXC shows the positions of cysteine pairs that form the zinc finger. (**B**) Coomassie-stained SDS–polyacrylamide gel showing the GST fusion proteins used. Lane 1, GST; lane 2, GST–E7 wild-type (E7 WT); lane 3, GST–E7 C24G (C24); and lane 4, GST–E7 (39–98). Molecular weight markers are indicated on the right. (**C**) GST, GST–E7 wild-type (E7 WT), GST–E7 39–98 (39–98) and GST–E7 C24G (C24) were used to purify histone deacetylase activity from nuclear extract in the absence or presence of peptide inhibitors (20 μg/ml) as indicated. Deacetylase activity is expressed relative to activity bound by wild-type E7 WT, which is set to 100%.

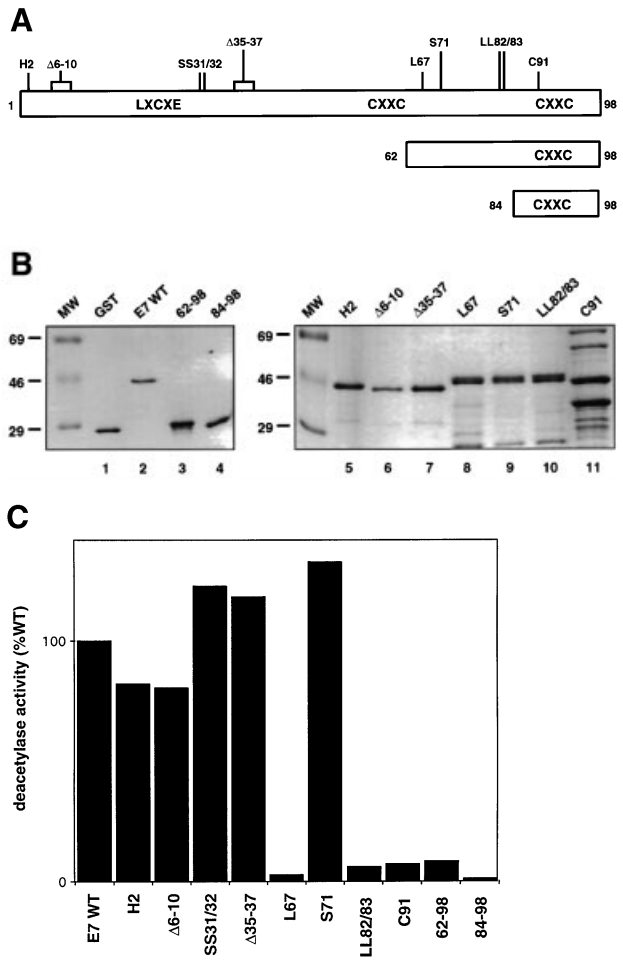


Fig. 4. The zinc finger domain of E7 is important for histone deacetylase binding. (**A**) Schematic representation of the HPV16 E7 mutants used. The positions of point mutations are indicated. LXCXE denotes the Rb-binding motif; CXXC shows the positions of cysteine pairs that form the zinc finger. (**B**) Coomassie-stained SDS–polyacrylamide gel showing the GST fusion proteins used. Lane 1, GST; lane 2, GST–E7 wild-type (E7 WT); lane 3, GST–E7 62–98 (62–98); lane 4, GST–E7 84–98 (84–98); lane 5, GST–E7 H2G (H2); lane 6, GST–E7 Δ6-10 (Δ6-10); lane 7, GST–E7 Δ35-37 (Δ35-37); lane 8, GST–E7 L67R (L67); lane 9, GST–E7 S71C (S71); lane 10, GST–E7 LL82/83RR (LL82/83); and lane 11, GST–E7 C91G (C91). Molecular weight markers are indicated on the left. (**C**) GST–E7 fusion proteins were used to purify histone deacetylase activity from HeLa nuclear extract as indicated. Abbreviations for mutants are given in (B). Deacetylase activity is expressed relative to the activity bound by wild-type GST–E7 (E7 WT) which is set to 100%.

motif. Therefore, Rb is unlikely to be involved in the E7–HDAC interaction. Rb binding and histone deacetylase binding appear to be two separable functions of E7.

E7 binds histone deacetylase via the zinc finger domain

We analysed further E7 deletion and point mutants in order to identify residues important for histone deacetylase binding (Figure 4A and B). Mutations outside the zinc finger region of E7 (H2, Δ6-10, SS31/32 and Δ35-37) had little effect on the ability of GST fusion proteins to bind histone deacetylase activity from nuclear extract (Figure 4C). This agrees well with our finding that the N-terminal 38 amino acids of E7 (CR1 and CR2) are dispensable for the interaction with histone deacetylase (Figure 3). In contrast, three point mutations located within the CR3 zinc finger region tested (L67, LL82/83 and C91) resulted in the loss of histone deacetylase interaction. The S71 zinc finger mutant bound as much histone deacetylase activity as did wild-type E7. N-terminal deletions which extend into the zinc finger region (62–98 and 84–98) also abolished histone deacetylase binding. Thus, all mutations that decreased histone deacetylase binding impinge on or map to the C-terminal zinc finger domain of E7. The mutants that show a defect in binding of deacetylase activity are also unable to bind HDAC1 from nuclear extract (Figure 7B and data not shown).

The zinc finger mutants chosen for the analysis of deacetylase binding have been characterized previously for their effect on the transformation functions of E7 (Jewers *et al.*, 1992; Phelps *et al.*, 1992; Demers *et al.*, 1996). Comparing the transformation capacity of E7 (Jewers *et al.*, 1992; Phelps *et al.*, 1992; Demers *et al.*, 1996) with the deacetylase-binding capacity (Figure 4) of the mutants, it becomes apparent that mutants which are unable to bind deacetylase activity (L67, LL82/83 and C91) are also defective in transformation. In contrast, mutant S71, which binds wild-type levels of deacetylase activity, is also fully transformation competent (Phelps *et al.*, 1992; Demers *et al.*, 1996).

We conclude from this mutant analysis that the histone deacetylase-binding activity maps to the zinc finger domain of E7 and that residues important for deacetylase binding are also important for cell transformation.

E7 does not contact HDAC1 directly

Given that histone deacetylase binding to E7 is not mediated by Rb, we sought to establish if the association of HDAC1 with E7 was the consequence of a direct interaction between the two proteins. To this end, we tested whether purified recombinant proteins expressed in *Escherichia coli* could interact in the absence of any cellular proteins. Glutathione–Sepharose beads loaded with GST, GST–E7 or GST–Rb were incubated with histidine-tagged HDAC1 (hisHDAC1) and subjected to extensive washing. Binding of HDAC1 was monitored by SDS–PAGE and Western analysis using an HDAC1-specific antibody. We did not detect binding of HDAC1 to GST and GST–E7 in this assay (Figure 5A, lanes 1 and 3, respectively). GST–Rb has been suspected to contact HDAC1 directly since the recombinant proteins can interact in reticulocyte lysate. Indeed, GST–Rb bound HDAC1 in our direct interaction assay, formally demonstrating for the first time that Rb can contact HDAC1 directly in the absence of other factors (lane 2). We considered the possibility that we failed to detect a direct interaction between HDAC1 and GST–E7 because of limiting amounts of GST–E7 protein. To test this possibility, we assessed direct binding of HDAC1 to increasing amounts of GST fusion proteins. As shown in Figure 5B, HDAC1 binding to 1 μg of GST–Rb was clearly detectable (lane 6). In contrast, up to 100 μg of GST–E7 failed to retain significant levels of HDAC1 (Figure 5A, lanes 2–5). In agreement with these results, we find that reversing the tags on the bacterially expressed proteins does not affect the result: GST–HDAC1 can bind histidine-tagged Rb but fails to interact with histidine-tagged E7 (data not shown). We conclude that the association of E7 and

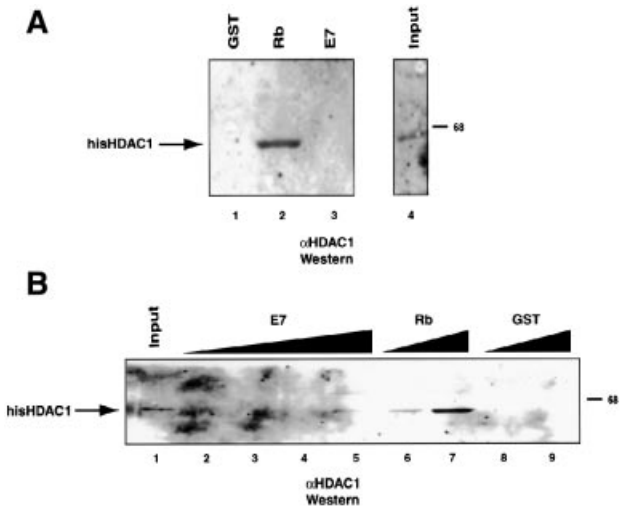


Fig. 5. E7 does not bind HDAC1 directly. (**A**) Recombinant purified GST, GST–Rb 379–928 (Rb) and GST–E7 (E7) bound to glutathione–Sepharose beads were incubated with recombinant purified histidine-tagged HDAC1 (hisHDAC1). Bound material was subjected to SDS–PAGE and Western analysis using HDAC1-specific antiserum ('1123'). Lane 4: 5% input. The position of HDAC1 is shown by an arrow; molecular weight is indicated on the right. (**B**) Increasing amounts of GST fusion proteins were incubated with recombinant purified histidine-tagged HDAC1. HDAC1 binding was followed by SDS–PAGE and Western analysis as in (A). Lane 1, 5% input; lanes 2–5, 1, 5, 20 and 100 μg of GST–E7, respectively; lanes 6 and 7, 1 and 5 μg of GST–Rb 379–928, respectively; lanes 8 and 9, 1 and 5 μg of GST, respectively.

HDAC1 observed in extracts is indirect and that it is mediated by a cellular factor.

The E7 zinc finger binds Mi2β directly

Since E7 does not bind to HDAC1 directly, we sought to identify cellular proteins that could mediate E7 binding to HDAC1. We employed a yeast two-hybrid screen using the zinc finger of E7 (residues 39–98) as a bait, as this region is sufficient for binding of histone deacetylase in nuclear extracts (Figure 3). The screen identified a sequence derived from a Mi2β cDNA (amino acid residues 1015–1118) as a possible E7 interaction partner. We performed several control experiments to verify that the interaction between the E7 zinc finger and Mi2β is specific (Figure 6A): the LexA DNA-binding domain on its own and fusions to lamin, P/CAF or MDM2 all failed to bind Mi2β-VP16. This strongly argues that Mi2β interacts specifically with the E7 zinc finger. Mi2β is a member of a multigene family and contains an HMG-like box, two PHD fingers, two chromo domains and a SWI2/SNF2-related helicase/ATPase domain (Figure 6B). Four groups recently have shown that Mi2β is a component of a novel multisubunit complex with nucleosome remodelling and histone deacetylase activity (NURD complex; Tong *et al.*, 1998; Wade *et al.*, 1998; Xue *et al.*, 1998; Zhang *et al.*, 1998). The HDAC1 and HDAC2 deacetylases and the Mi2β protein are stoichiometric components of the NURD complex. Thus, Mi2β is likely to be the cellular factor that mediates the association of E7 with histone deacetylase. Mi2β has been shown to contact HDAC1 directly through the PHD fingers located in the N-terminus of Mi2β (Zhang *et al.*, 1998). If Mi2β was mediating HDAC binding to E7, it would be expected also to bind to E7 directly. Although the yeast two-hybrid data suggest that the interaction between LexA-E7 (39–98) and Mi2β (1015–1118)-VP16 is direct, we cannot rule out the possibility that binding was mediated by a factor present in yeast. Therefore, we assessed the binding of recombinant histidine-tagged E7 to a GST–Mi2β (1015–1118) fusion protein in the direct interaction assay. As shown in Figure 7A, lane 4, bacterially expressed GST–Mi2β and histidine-tagged E7 interact in the absence of any other proteins. This demonstrates formally that E7 and Mi2β can interact directly.

Thus, Mi2β displays binding characteristics (direct binding to both HDAC1 and E7) expected of a cellular factor that mediates an interaction of E7 with HDAC1 and HDAC2. These data indicate that E7 associates with the NURD complex via a direct interaction with Mi2β. Consistent with E7 binding the NURD complex is the finding that E7 binds other components of this complex (such as pRbAp48) from nuclear extract (data not shown).

Rb and Mi2β recognize distinct residues of E7

Having established that Mi2β binds to the E7 zinc finger, we went on to verify that (i) in the full-length E7 protein, Mi2β recognizes residues also required for histone deacetylase binding and (ii) that loss of Mi2β binding does not disrupt Rb binding to E7 (and vice versa). To this end, we characterized a zinc finger mutant which disrupts the binding to histone deacetylase activity (L67; Figure 4) and a point mutant which disrupts binding to Rb (C24; Jewers *et al.*, 1992; Phelps *et al.*, 1992; Demers *et al.*, 1996). Figure 7A shows that the L67 mutation abolishes the direct interaction between Mi2β and E7 (compare lanes 4 and 6). In contrast, the C24 mutant still interacted with Mi2β (lane 5).

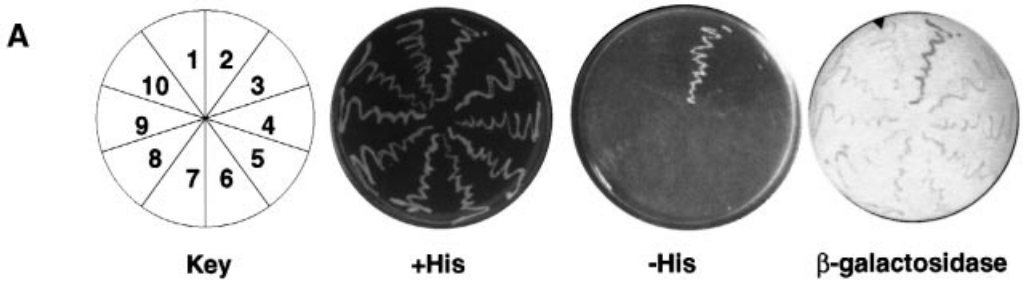
The C24 and L67 mutants were also compared for their ability to interact with Rb and HDAC1 in nuclear exctract (Figure 7B). As expected, E7 C24 was deficient for Rb binding whereas E7 L67 bound the same levels of Rb as wild-type E7 (compare lanes 2, 3 and 4). Conversely, E7 C24 bound as much HDAC1 as wild-type E7 whereas E7 L67 failed to bind to HDAC1 in nuclear extract (compare lanes 6, 7 and 8). These results are consistent with the differential binding of histone deacetylase activity displayed by the two mutants (Figures 3 and 4).

This comparative binding analysis shows that the L67 and the C24 mutants have opposite binding profiles: E7 L67 has lost the ability to interact with both HDAC1 and Mi2β but has retained full Rb-binding activity. E7 C24, on the other hand, interacts with HDAC1 and Mi2β but is defective for Rb binding.

These results are consistent with the idea that Mi2β bridges E7 to histone deacetylase activity and that this association is distinct and separable from Rb binding.

Mi2β/HDAC-binding and growth-promoting functions of E7

The E7 mutants C24 and L67 shown above to abolish either Rb or Mi2β/HDAC binding, respectively, have both been shown to fail to transform rodent fibroblasts efficiently (Phelps *et al.*, 1992). This raises the possibility that both Rb binding and Mi2β/HDAC binding are necessary for E7 to promote growth. To test this hypothesis in a distinct biological assay which monitors proliferation



Key	LexA-fusion	YP16-fusion	Growth on -His	β-galactosidase activity
1	E7 39-98	none	–	–
2	E7 39-98	Mi2β 1015-1118	+	+
3	MDM2 1-130	none	–	±
4	MDM2 1-130	Mi2β 1015-1118	–	±
5	Lamin	none	–	–
6	Lamin	Mi2β 1015-1118	–	–
7	p/CAF 489-581	none	–	–
8	p/CAF 489-581	Mi2β 1015-1118	–	–
9	none	none	–	–
10	none	Mi2β 1015-1118	–	–

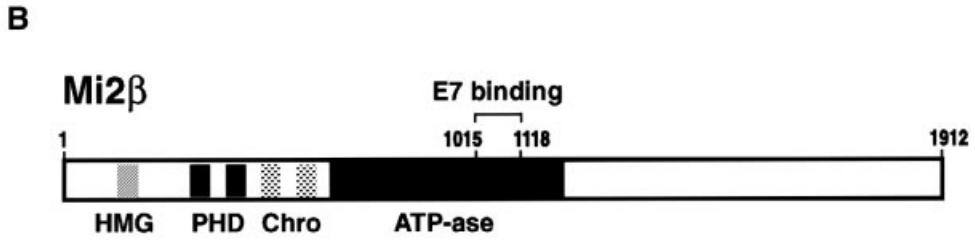


Fig. 6. Mi2β is an E7 zinc finger-binding protein. (**A**) L40 yeast cells were co-transformed with the indicated plasmids and plated onto –THULL plates for assaying β-galactosidase activity or onto –THULL plates containing 5 mM 3-AT in order to examine the interaction between bait and prey proteins as shown. (**B**) Schematic representation of the Mi2β protein. The positions of the HMG-like box (HMG; hatched), PHD finger motifs (PHD; black), chromo domains (chro; stippled) and the ATPase domain (ATPase; black) are shown. Numbers indicate amino acid residues. The sequence found to interact with the E7 zinc finger in the yeast two-hybrid screen (residues 1015–1118) is indicated.

potential, we assessed the ability of the C24 and the L67 mutants to overcome growth arrest in the flat cell assay.

In the flat cell assay, Rb overexpression in Rb-negative Saos2 cells leads to an arrest in the G_1 phase of the cell cycle and a pronounced change in cell morphology (flat cell phenotype) (Hinds *et al.*, 1992; Figure 8A). These effects can be relieved by the co-expression of viral oncoproteins such as E1A (Hinds *et al.*, 1992). In Figure 8B, we show that HPV16 E7 likewise can rescue the flat cell phenotype in a dose-dependent manner when co-expressed with Rb. We then compared the activity of wild-type E7 with that of the C24, L67 and S71 mutants in this assay. All these mutants are expressed at equivalent levels in mammalian cells (Phelps *et al.*, 1992; data not shown). Figure 8C shows that the L67 mutant which fails to bind Mi2β and histone deacetylase displays a 5-fold reduction in flat cell rescue compared with wild-type E7. Mutation of the Rb-binding site of E7 (C24) led to a comparable decrease of flat cell rescue activity. In contrast, the S71 zinc finger mutant which retains both deacetylase- and Rb-binding activity (Phelps *et al.*, 1992; Figure 4C) was able to release cell cycle arrest almost as efficiently as wild-type E7.

These results demonstrate that E7 mutants which are unable to bind either Mi2β/HDAC (L67) or Rb (C24) are unable to relieve cell cycle arrest and rescue the flat cell phenotype. Thus, an interaction of E7 with the NURD complex (via Mi2β) as well as an interaction with Rb are important for the growth-promoting potential of E7.

Discussion

Several groups have shown that the zinc finger domain is important for the transactivation and transformation functions of HPV16 E7 (Edmonds and Vousden, 1989; Chesters *et al.*, 1990; Jewers *et al.*, 1992). It is not clear,

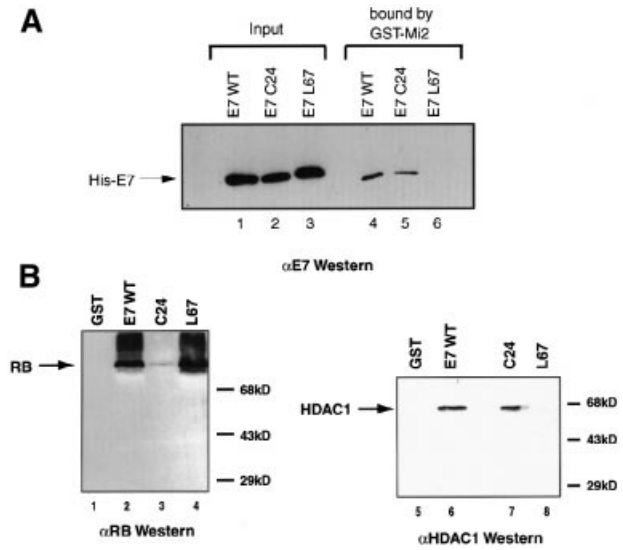


Fig. 7. Comparative analysis of binding of E7 mutants to Mi2β, Rb and HDAC1. (**A**) E7 binds directly to Mi2β. Recombinant purified GST–Mi2β (1015–1118) was incubated with recombinant purified histidine-tagged E7 wild-type (wt), E7 C24G (C24) or E7 L67R (L67) as indicated. Bound material was subjected to SDS–PAGE and Western analysis using an HPV16 E7-specific antibody (TVG710Y, Santa Cruz). The position of histidine-tagged E7 is indicated on the left. Lanes 1, 2 and 3: E7 wild-type, E7 C24G and E7 L67R input, respectively. (**B**) Equivalent amounts of GST fusion proteins bound to glutathione–Sepharose beads were incubated with nuclear extract [lanes 1 and 5, GST; lanes 2 and 6, GST–E7 wild-type (E7 WT); lanes 3 and 7, GST–E7 C24G (C24); lanes 4 and 8, GST–E7 L67R (L67)] and subjected to SDS–PAGE and Western analysis. The α-Rb C36 antibody (PharMingen) was used to detect Rb (lanes 1–4); α-HDAC1 antiserum ('1123') was used to detect HDAC1 (lanes 5–8). The positions of Rb and HDAC1 are shown by arrows; molecular weights are indicated on the right.

however, whether the zinc finger, like the LXCXE motif, targets cellular regulators of the cell cycle. Recently, histone deacetylases have been shown to play an important role in cell cycle regulation (Brehm *et al.*, 1998; Grignani *et al.*, 1998; Lin *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). This property makes histone deacetylases potential targets for viral oncoproteins. We have found that the zinc finger region of E7 can associate with histone deacetylase and have identified three point mutations in this region that abolish histone deacetylase binding (L67, LL82/83 and C91). Using a yeast two-hybrid screen, we have identified Mi2β, a component of a recently identified complex with histone deacetylase and chromatin remodelling activity, as a protein that binds the E7 zinc finger directly. The L67 point mutant, which fails to bind to Mi2β and histone deacetylase, also fails to overcome cell cycle arrest in Saos2 cells. This result correlates Mi2β and histone deacetylase, binding with the growth-promoting potential of E7.

Previous analysis of the transformation capacity of the E7 zinc finger mutants used here further supports the conclusion that the binding of a histone deacetylase complex by E7 is involved in its cell proliferation function. Mutants L67 and LL82/83, which cannot bind deacetylase activity, are defective in transformation of rodent fibroblasts, whereas the S71 mutant, which still binds deacetylase, transforms as efficiently as wild-type E7 (Phelps *et al.*, 1992). In a separate assay, the C91 mutant, which is unable to bind deacetylase activity, fails to immortalize primary human keratinocytes (Jewers *et al.*, 1992). Double point mutation of residues C91 and C58 results in the loss of the ability to mediate escape from transforming growth factor-β (TGF-β)-induced growth inhibition (Demers *et al.*, 1996). In the same assay, mutation of residue S71, which does not affect deacetylase binding, has no effect on release from the TGF-β block (Demers *et al.*, 1996).

How does the binding of Mi2β and histone deacetylase by E7 contribute to its cell proliferation functions? Our binding data suggest that E7 binds an Mi2β-containing histone deacetylase complex such as the recently described NURD complex (Tong *et al.*, 1998; Wade *et al.*, 1998; Xue *et al.*, 1998; Zhang *et al.*, 1998). The NURD complex combines ATP-dependent chromatin remodelling activity (supplied by the Mi2β subunit) and histone deacetylase activity (supplied by HDAC1 and HDAC2). The chromatin remodelling activity of this complex is thought to facilitate access of the histone deacetylases to their substrates in nucleosomal templates. The NURD complex is believed to play a role in transcriptional repression of specific target genes. Aberrant regulation of the NURD complex has been implicated in malignant transformation (Xue *et al.*, 1998; Zhang *et al.*, 1998). One NURD complex subunit, the metastasis-associated factor (MTA), is highly expressed in rapidly dividing cells (Zhang *et al.*, 1998). Moreover, expression of MTA1 correlates with the metastatic potential of several human cancer cell lines and tissues (Toh *et al.*, 1994, 1997). In addition, Mi2β itself is the nuclear autoantigen for the autoimmune disease dermatomyositis (Ge *et al.*, 1995; Seelig *et al.*, 1995). Dermatomyositis patients face an increased risk of malignancy, with 15–30% of patients developing cancer (Airio *et al.*, 1995). These findings suggest that the NURD complex is involved in the regulation of genes that are important for cell cycle control, although specific target genes for the NURD complex have not been identified so far.

It is possible that the binding of the E7 zinc finger to the Mi2β subunit of the NURD complex results in the deregulation of genes that govern the cell cycle, thus promoting inappropriate cell growth.

There are several ways in which E7 might interfere with an Mi2β-containing histone deacetylase complex. For example, E7 might sequester the complex away from its target genes or E7 might dissasociate it in a manner analogous to the disruption of Rb-containing complexes by E7. Alternatively, E7 might alter the enzymatic properties of the complex. We have noted that the E7 zinc finger-binding site identified in the two-hybrid screen maps to the SWI2/SNF2-related helicase/ATPase domain of Mi2β. This domain is thought to be essential for the chromatin remodelling activity of the NURD complex. It is tempting to speculate that E7 binding inhibits the ATPase and chromatin remodelling activity, thus preventing the histone deacetylases from gaining access to their substrates. The failure to deacetylate chromatin would then result in an inappropriate derepression of genes encoding cell cycle regulators.

Our results suggest that E7 uses multiple mechanisms to interfere with cell cycle control. One mechanism is the well-documented inactivation of the Rb tumour suppressor protein through an LXCXE motif-mediated interaction.

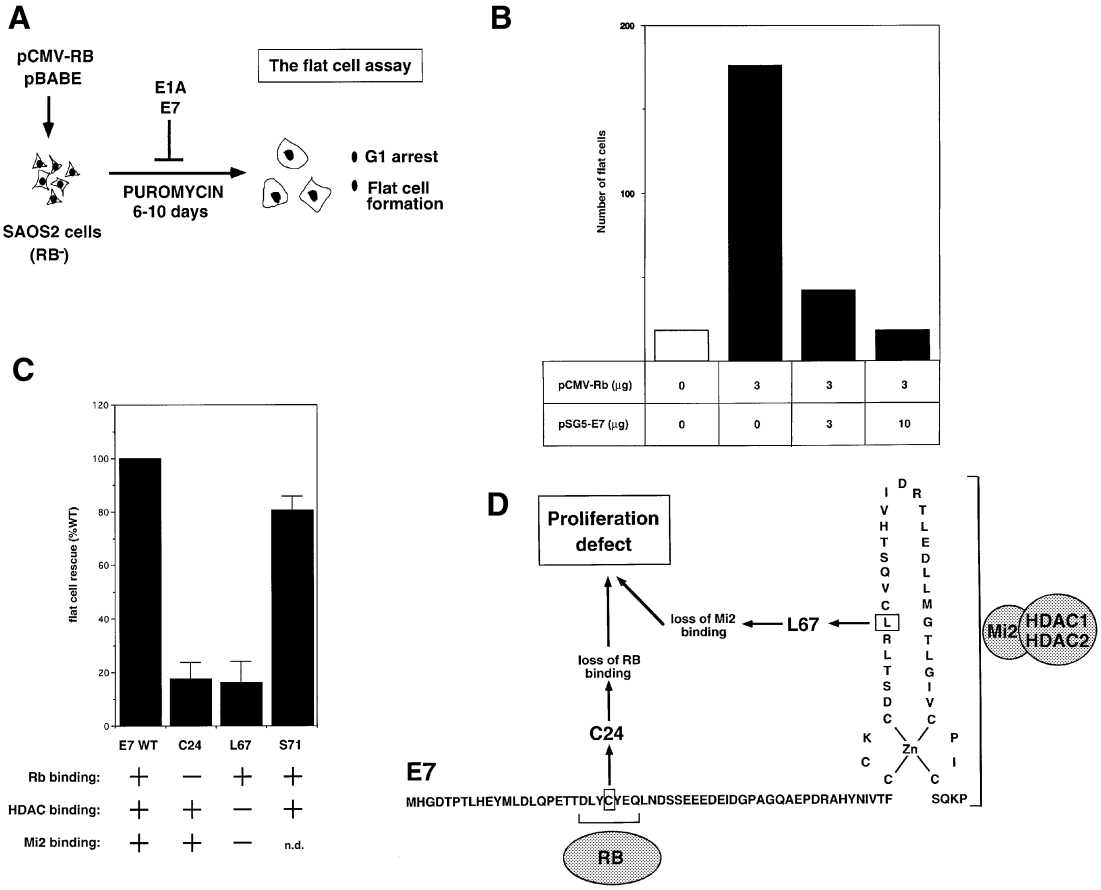


Fig. 8. Histone deacetylase-binding mutants fail to overcome flat cell formation efficiently in Saos2 cells. (**A**) Schematic diagram of the flat cell assay. The flat cell assay was carried out essentially as described previously (Hinds *et al.*, 1992). Saos2 cells were transfected with an Rb expression vector and a vector conferring resistance to puromycin. After 6–10 days under puromycin selection, cells were fixed, stained and morphological changes were assessed. Co-expression of E1A (Hinds *et al.*, 1992) or E7 (this work) can inhibit cell cycle arrest and flat cell formation. (**B**) HPV16 E7 counteracts Rb-induced flat cell formation. Saos2 cells were transfected with Rb and E7 expression vectors as indicated. The total amount of transfected DNA was kept constant by including empty expression vectors. The numbers of flat cells scored in seven fields of vision (40× magnification) are plotted. (**C**) Rescue from flat cell formation by E7 was assessed by comparing the number of flat cells in a dish transfected with E7 expression vector with the number of flat cells in a dish transfected with empty expression vector. The reduction in the number of flat cells produced by wild-type E7 was set to 100% and the activity of E7 mutants was determined relative to that. The graph shows the average of three independent experiments. The ability of the GST–E7 proteins to bind Mi2β, Rb and histone deacetylase (HDAC), respectively, is denoted below the graph. +, wild-type binding activity; –, weak or no binding activity. (**D**) Rb binding and Mi2β/HDAC binding are required for the transformation functions of E7. Schematic representation of Rb- and Mi2β/HDAC-binding regions on E7. The positions of the C24G and L67R point mutations are indicated.

The results presented here suggest that E7 also targets chromatin remodelling histone deacetylase complexes through its C-terminal zinc finger (Figure 8D). This would enable E7 to alter expression of cellular genes by modulating their chromatin structure.

Our results provide the first indication that viral oncoproteins might manipulate gene expression by interfering with chromatin remodelling and histone deacetylation. Interestingly, the binding of the opposite enzymatic activity, in the form of CBP/p300 acetyltransferase (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996), is required for the immortalization capacity of the viral E1A oncoprotein (Lundblad *et al.*, 1995). These observations highlight the importance of acetylation pathways in the regulation of cell proliferation, since distinct viral oncoproteins from different viruses have each evolved to regulate cell proliferation, at least partly by binding either acetylase or deacetylase activity.

Materials and methods

Constructs

pGEX-E7, pGEX-E7 H2G, pGEX-E7 Δ6-10, pGEX-E7 SS31/32DD, pGEX-E7 Δ35-37, pGEX-E7 39–98, pGEX-E6, pGEX-Rb 379–928, pGEX-E2F 380–437, pCMV-HA-HDAC1 and pCMV-Rb have all been described (Chesters *et al.*, 1990; Hagemeier *et al.*, 1993; Demers *et al.*, 1996; Brehm *et al.*, 1998). pGEX-E7 L67R, pGEX-E7 S71C, pGEX-E7 LL82/83RR, pcDNA3-E7 L67R and pcDNA3-E7 S71C were created by site-directed mutagenesis using the Quickchange kit (Stratagene) according to the manufacturer's instructions. pGEX-E7 62–98, pGEX-E7 84–98, pQE30-E7, pQE30-E7 C24G, pQE30-E7 L67R, pGEX-Mi2β 1015–1118, pET30-HDAC1, pBTM116-E7 39–98 and pcDNA3-E7-F were cloned by PCR using appropriate sets of primers. All constructs were verified by DNA sequencing.

GST pull-downs from nuclear extract

GST fusion proteins pre-bound to glutathione–Sepharose beads were incubated with 30 μl of HeLa nuclear extract (Computer Cell Culture Centre, Moens, Belgium) in 250 μl of IPH buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40) at 4°C for 1–2 h. Beads were washed three times in 1 ml of IPH buffer and resuspended

in loading buffer (for SDS–PAGE) or 100 µl of IPH buffer (for histone deacetylase assays). Peptide competition experiments were carried out in the presence of 20 µg/ml LXCXE or HA peptide (Brehm *et al.*, 1998).

Histone deacetylase assays

Histone deacetylase assays were carried out essentially as described previously (Taunton *et al.*, 1996; Brehm *et al.*, 1998) in a volume of 100 µl of IPH buffer containing 250 000 c.p.m. of a tritium-labelled acetylated histone H4 peptide. For inhibition experiments, TSA was added to the reaction prior to H4 peptide.

SDS–PAGE and Western analysis

SDS–PAGE and Western blotting were performed according to standard procedures (Martin *et al.*, 1995).

Protein expression

GST and GST fusion proteins were expressed in *E.coli* XA90 using the pGEX (Pharmacia) vector system. Purification of GST and GST fusions from crude bacterial lysates was performed as in Bannister and Kouzarides (1996). Histidine-tagged proteins were expressed in *E.coli* BL21 DE3 using the pQE (Qiagen) or pET (Novagen) vector systems. Histidine-tagged proteins were purified according to standard procedures. Bacterial pellets were resuspended and lysed by sonication in Tris-buffered saline (TBS) containing 1% Triton X-100 and 10 mM imidazole. Debris was removed by centrifugation. Ni^{2+}-NTA–agarose beads (Qiagen) were added and incubated with the lysate for 30 min. Beads were washed extensively and protein was eluted with TBS containing 150 mM imidazole.

Cell culture

Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum at 37°C and 5% CO_2. Cells were transfected using the $Ca_3(PO_4)_2$ technique as described (Hagemeier *et al.*, 1993).

Flat cell assay

The flat cell assay was performed essentially as described in Hinds *et al.* (1992). Saos2 cells in culture dishes (10 cm diameter) were transfected with expression plasmids and 1 µg of pBABE (encoding puromycin resistance). At 36 h post-transfection, cells were subjected to puromycin selection (2.5 µg/ml). Puromycin selection was continued for up to 6 days. Cells were then fixed and stained with crystal violet. Flat cells were easily identifiable and were counted using a light microscope (40× magnification).

Immunoprecipitations

U2OS cells in culture dishes (15 cm diameter) were transfected with up to 20 µg of expression vectors and harvested 36 h post-transfection. Cells were lysed in 1 ml of IPH buffer, debris was removed by centrifugation and the cleared lysate was subjected to immunoprecipitation with 2 µg of αFlag antibody (M2, Kodak) pre-bound to agarose beads. Antibody beads were washed three times with 1 ml of IPH buffer and resuspended in loading buffer for SDS–PAGE.

Yeast two-hybrid screen

E7 39–98 was cloned into pBTM116 as a LexA fusion. This construct was transformed into L40 yeast together with a mouse 9.5–10.5 d.p.c. cDNA library ligated into pVP16 as described (Lavender *et al.*, 1997). An estimated 5×10^6 transformants were screened. Proteins interacting with E7 39–98 were identified by growth on –THULL plates in the presence of 5 mM 3-aminotriazole (3-AT) and confirmed by assaying for β-galactosidase activity. Plasmids rescued from positive yeast colonies were retransformed into L40 together with either pBTM116-E7 39–98 or pBTM116-lamin to assess the specificity of the interaction.

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