Journal of General Virology (2002), **83**, 829–833. Printed in Great Britain SHORT COMMUNICATION

Complementation of a p300/CBP defective-binding mutant of adenovirus E1a by human papillomavirus E6 proteins

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Previous studies have shown that the human papillomavirus type 16 (HPV-16) E6 protein binds to p300/CBP and abrogates its transcriptional co-activator function. However, there is little information on the biological consequences of this interaction and discrepancy as to whether the interaction is high-risk E6 specific or not. We performed a series of studies to compare the interactions of HPV-18 and HPV-11 E6 with p300, and showed that both high- and low-risk E6 proteins bind p300. In addition, using a transformation-deficient mutant of adenovirus E1a, which cannot interact with p300, we demonstrated that HPV-16, HPV-18 and, to a lesser extent, HPV-11 E6, can complement this mutant in cell transformation assays. In contrast, a mutant of HPV-16 E6 which does not bind p300 failed to rescue the E1a mutant. These results suggest that the E6–p300 interaction may be important for the ability of HPV E6 to contribute towards cell transformation.

The human papillomavirus (HPV) E6 protein is one of two major viral oncoproteins encoded by the virus (reviewed in Pim *et al.*, 2001). It is continually retained and expressed in cervical tumours and derived cell lines (Schwarz *et al.*, 1985; Androphy *et al.*, 1987; Banks *et al.*, 1987). It has also been shown to cooperate with the viral E7 protein in the immortalization of primary human keratinocytes (Barbosa & Schlegel, 1989; Hawley-Nelson *et al.*, 1989), the natural host cells of the virus *in vivo*.

Many studies have been performed to investigate the mechanisms of action of HPV E6. High-risk E6 proteins, derived from HPV-16 and HPV-18, interact with a large number of cellular proteins whose functions are associated with the regulation of cell growth and survival. One of the most important of these interactions is with the p53 tumour suppressor protein (Werness *et al.*, 1990). In this case, E6 targets p53 for ubiquitin-mediated degradation (Scheffner *et al.*, 1990; Huibregtse *et al.*, 1991) and thereby overcomes a vital checkpoint in the host cell's defence against incorporation of DNA damage and subsequent progression to malignancy (Lane, 1992; Wu & Levine, 1994; Foster *et al.*, 1994; Kessis *et al.*, 1993).

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Recent studies have demonstrated that the HPV-16 E6 and bovine papillomavirus type 1 (BPV-1) E6 can also interact with the p300/CBP co-activator protein (Patel *et al.*, 1999; Zimmermann *et al.*, 1999, 2000). As a result of this interaction, the transcriptional activity of p300 is greatly reduced, suggesting that E6 can interfere with the normal functioning of p300 (Patel *et al.*, 1999; Zimmermann *et al.*, 1999). This was a particularly important observation, since a number of other DNA tumour virus oncoproteins, including simian virus 40 LT and adenovirus E1a, have also been shown to require interaction with p300 in order to efficiently transform cells (Stein *et al.*, 1990; Smith & Ziff, 1988; Eckner *et al.*, 1996). Thus, the demonstration that E6 could likewise inhibit p300 activity suggested that this interaction may be important for the ability of HPV E6 to contribute toward cell transformation. At present, however, there is little biological evidence to support this hypothesis. In addition, conflicting results have been published as to whether the interaction between E6 and p300 is restricted only to the oncogenic HPV types (Patel *et al.*, 1999; Zimmerman *et al.*, 1999).

We therefore proceeded to investigate the ability of high- and low-risk HPV E6 proteins to interact with p300 in a series of *in vitro* GST pull-down assays. Different regions of p300 were expressed as GST fusion proteins (Fig. 1A). These proteins were induced and purified as described previously (Massimi *et al.*, 1996), and then incubated with *in vitro* translated, radiolabelled HPV-11 E6 and HPV-18 E6. The results from representative binding assays are shown in Fig. 1(B). As can be seen, both E6 proteins show significant levels of interaction with p300. Interestingly, the fragments of p300 bound by both E6 proteins are the same, and overlap with the CH1, CH2 and CH3 domains of p300. No significant binding is seen with fragments of p300 spanning residues 672–1193 and 1893–2414. This is largely in agreement with earlier observations where the CH1 and CH3 domains were bound by HPV-16 E6, although binding to CH2 was not assessed in

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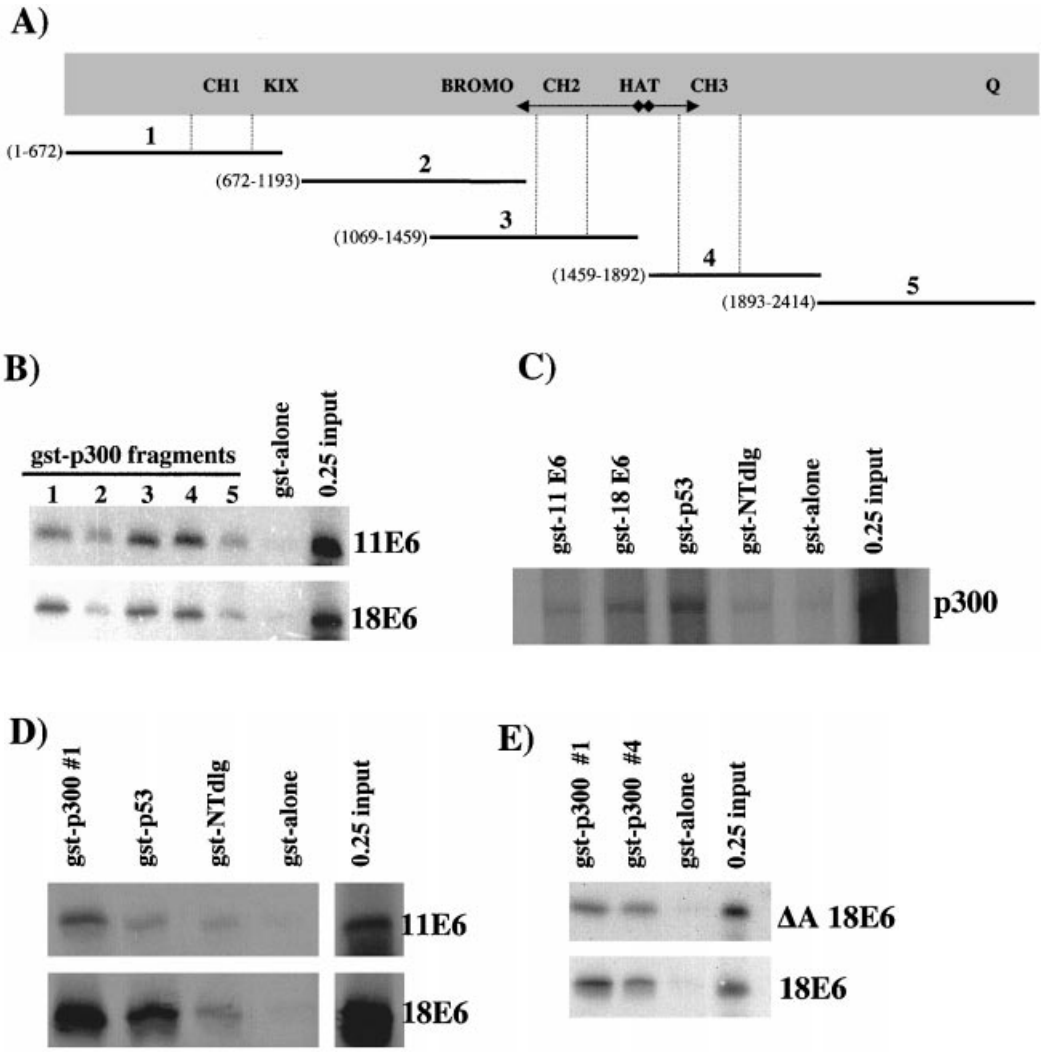

Fig. 1. (A) Schematic representation of the GST–p300 fusion clones used for *in vitro* binding assays. The most conserved domains of p300 are indicated (CH1–3, cysteine/histidine-rich motifs/zinc-finger domains; Q, glutamine-rich domain; HAT, functional histone acetyltransferase domain; Bromo, bromodomain; KIX, kinase induced domain). (B) Interaction between the HPV-11 and HPV-18 E6 oncoproteins with p300. Major sites of interaction encompass p300 fragments 1, 3 and 4 which encompass the CH1, CH2 and CH3 domains. (C) Interaction between *in vitro* translated p300 and HPV-11 E6, HPV-18 E6, p53 and amino-terminal dlg (amino acids 1–222) GST fusion proteins. (D). HPV-11 E6 and HPV-18 E6 binding to a p300 fragment encompassing amino acids1–672 (fragment no. 1) and to p53 and amino-terminal dlg for comparison. (E) Comparison of the binding of wild-type HPV-18 E6 and the HPV-18 E6ΔA mutant to p300.

those studies (Patel *et al.*, 1999; Zimmermann *et al.*, 1999). To further verify the specificity of these interaction assays, we then compared the relative ability of the HPV-11 and HPV-18 E6 proteins to bind p300 in comparison with their abilities to interact with p53 and the amino-terminal region of the Discs Large (Dlg) protein (NTdlg), as positive and negative control respectively (Werness *et al.*, 1990; Gardiol *et al.*, 1999). As can be seen, HPV-18 E6 binds strongly to p300 and, interestingly, this is similar to the level of interaction seen between p53 and p300 (Fig. 1C). HPV-11 E6 also shows a significant degree of interaction with p300 when compared with the NTdlg negative control and p53 (Fig. 1D), although the levels of interaction between HPV-11 E6 and p300 are considerably weaker than that observed with HPV-18 E6 (see Fig. 1C and 1D). Taken together, these results demonstrate that both HPV-18 and HPV-11 E6 also interact with p300, with stronger levels of association being observed with the high-risk E6 protein compared with the low-risk E6 protein.

Having confirmed that the interaction between E6 and p300 is common to both high- and low-risk E6 proteins, we next focused our attention on investigating the potential biological consequences of this association. To do this, we used an amino-terminal deletion mutant of adenovirus E1a (Δ2–36). This mutant had previously been shown to be defective both in its ability to bind p300 and to cooperate with EJ-ras in the transformation of primary baby rat kidney cells (BRKs) (Stein *et*

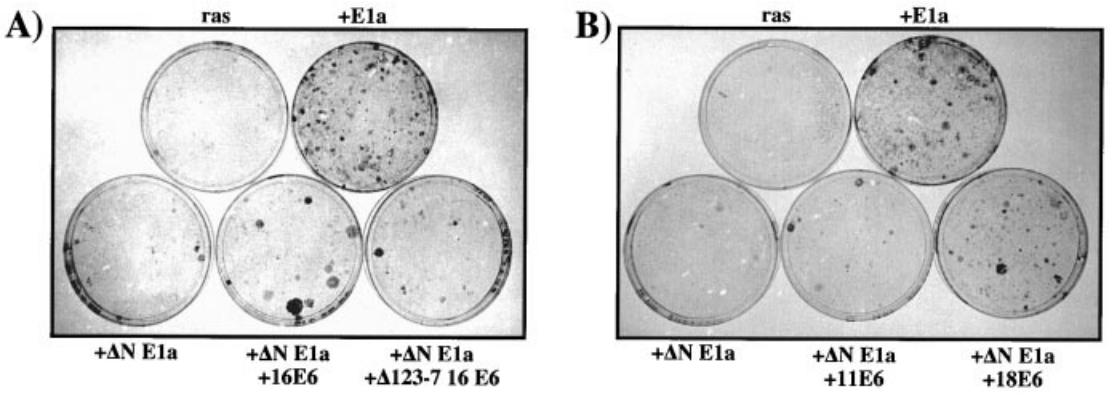

Fig. 2. Focus formation assay in BRK cells. Representative plates from two separate transformation assays co-transfected with the indicated oncogenes are shown.

Table 1. Complementation of a transformation-defective adenovirus E1a mutant by HPV E6

Primary BRK cells were transfected with the indicated plasmid combinations together with a neomycin-selectable marker. Numbers represent the number of colonies obtained after 2 weeks of selection in 200 μg/ml G418. The mutants used are adenovirus E1a ΔN (Δ2–36), HPV-16 E6 Δ123–7 and HPV-18 E6 ΔA (Δ47–49). ND, Not done.

	No. of colonies in experiment:					
Plasmid	1	2	3	4	5	6
ras	0	0	2	2	0	1
ras + E1A	> 200	> 200	> 200	> 200	> 200	> 200
ras + ΔN	4	14	13	6	6	8
ras + ΔN + 11E6	23	47	32	ND	ND	ND
ras + ΔN + 16E6	35	57	48	ND	ND	ND
ras + ΔN + Δ123–7	4	10	10	ND	ND	ND
ras + ΔN + 18E6	58	112	69	47	48	65
ras + ΔN + ΔA	ND	ND	ND	41	48	52
ras + 11E6	2	0	8	ND	ND	ND
ras + 16E6	4	0	6	ND	ND	ND
ras + 18E6	0	0	2	4	2	3

al., 1990; Whyte *et al.*, 1988). We reasoned that if the E6–p300 interaction was important for the ability of E6 to contribute towards cell transformation then E6 should be able to complement the p300 binding- and transformation-deficient mutant of adenovirus E1a in a BRK transformation assay. To examine this possibility, primary BRK cells were obtained from 9-day-old Wistar rats, and co-transfected with the adenovirus E1a mutant together with different combinations of the E6 expression vectors, in the presence of EJ-ras as a cooperating, activated oncogene. After 2 weeks under G418 selection the cells were fixed, stained and the colonies were counted. The results obtained from two typical assays are shown in Fig. 2, and the results from a series of independent experiments are shown in Table 1. It is apparent from Fig. 2(A) that the Δ2–36 E1a mutant, when compared with E1a, shows greatly reduced ability to cooperate with EJ-ras, and this is consistent with previous reports (Stein *et al.*, 1990; Whyte *et al.*, 1988). Similarly, HPV-16 E6 shows very little co-transforming activity in the primary BRK cell transformation assays (Table 1), also in agreement with previous studies (Storey *et al.*, 1988). However, it is clear that HPV-16 E6 efficiently complements the defect in Δ2–36 adenovirus E1a protein when both proteins are co-expressed. In addition, the mutant of HPV-16 E6 (Δ123–127), defective in its ability to interact with p300 (Patel *et al.*, 1999), is likewise defective in its ability to complement the transformation-deficient E1a mutant, suggesting that the capacity of E6 to bind p300 is required for efficient transformation. We then investigated the ability of HPV-18 and HPV-11 E6 proteins to rescue the E1a mutant. The results obtained are shown in Fig. 2(B) and Table 1. It is clear that

HPV-18 E6 very efficiently complements the Δ2–36 mutant of adenovirus E1a. In contrast, the ability of HPV-11 E6 to complement this mutant is significantly reduced, with fewer and smaller colonies being obtained.

To exclude any indirect effects due to inactivation of p53 in these assays, we also included a mutant of HPV-18 E6 (ΔA), which we had previously shown to be defective in its ability to target p53 for degradation (Pim *et al*., 1994). As can be seen from the results presented in Fig. 1(E), this mutant binds to p300 as efficiently as the wild-type HPV-18 E6 protein and easily complements the adenovirus E1a mutant (Table 1). This suggests that the ability of E6 to complement a p300 binding- and transformation-defective mutant of E1a is unrelated to its capacity to target p53 for degradation.

In this study the interaction between E6 and p300 has been further analysed. We found that the E6 proteins derived from both high- and low-risk HPV types were capable of interacting with p300. Taken together with the recent observation that BPV-1 E6 also interacts with p300 (Zimmermann *et al*., 2000), it indicates that this activity is conserved across many PV types, suggesting that this interaction may be a common requirement for virus replication. In addition, the HPV-18 and HPV-11 E6 binding sites on p300 overlap with the CH1, CH2 and CH3 domains of the protein, although at present we do not know whether these three regions of p300 are bound directly by E6, or whether any of these interactions are mediated via an unknown intermediate protein present within the reticulocyte lysate. However, previous studies would indicate that HPV-16 E6 binding to CH1 and CH3 was indeed direct (Patel *et al*., 1999). Interestingly, the CH3 region of p300 is also the contact point for other viral transforming proteins such as adenovirus E1a, simian virus 40 LT and human immunodeficiency virus Tat (Eckner *et al*., 1994, 1996; Hottiger & Nabel, 1998). In addition, as we have seen with HPV E6, adenovirus E1a also interacts with multiple sites on p300/CBP (Kurokawa *et al*., 1998; Korzus *et al*., 1998), implying that diverse viral transforming proteins may have evolved common strategies for interfering with the normal function of p300.

We then performed a series of studies to investigate directly the potential biological significance of the E6–p300 interaction. Using a mutant of adenovirus E1a unable to bind p300 and, as a consequence, defective in transformation of primary BRK cells, we were able to efficiently rescue this defect by co-transfecting HPV E6. In contrast, a mutant of E6 that was unable to bind p300 failed to complement the defect of the adenovirus E1a mutant. This activity of E6 appears to be unrelated to its ability to degrade p53 since a mutant of E6 unable to degrade p53 nonetheless retained the ability to complement the E1a mutant. Interestingly, low-risk E6 was not as effective as high-risk E6 protein in these assays which is consistent with the weaker interaction with p300 demonstrated here, and supports previous studies where HPV-6 E6 had only marginal effects on p300 transcriptional activity (Patel *et al*., 1999).

Taken together, these results demonstrate that the interaction between E6 and p300 is biologically relevant and suggests that this may contribute towards E6-induced cellular transformation.

We are most grateful to Thomas Dobner for the Δ2–36 mutant of adenovirus E1a and to Dennis McCance for the HPV-16 E6 mutant Δ123–127. This work was supported in part by a research grant from the Associazione Italiana per la Ricerca sul Cancro.

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Received 24 September 2001; Accepted 5 December 2001