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FAST TRACK

Human Papillomavirus-16 E7 Protein Inhibits the DNA Interaction of the TATA Binding Transcription Factor

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Abstract Previous studies have shown that the HPV-16 E7 protein interacts with TBP. This interaction was found to take place through residues in the carboxy terminal half of E7, mutation of which resulted in weaker transforming activity. In addition, binding of E7 to TBP was found to be increased following protein kinase CK2 (casein kinase II) phosphorylation of E7, and mutation of this CK2 site also reduces E7's transforming activity. To date, however, there is no information on the effects of E7 upon TBP function. In order to address this we have performed a series of assays to investigate the effects of E7 upon the ability of human and *S. pombe* TBP to bind DNA. We show that HPV-16 E7 is indeed a potent inhibitor of TBP DNA binding activity. Further, this activity of E7 is increased following CK2 phosphorylation of E7, consistent with it having an increased affinity for TBP. Finally, a mutant E7 protein defective in its ability to bind TBP, has no effect upon TBP binding to DNA. These results demonstrate that one consequence of the E7–TBP interaction is abolition of TBP DNA binding activity, and may provide an explanation for the transcriptional inhibitory effects of E7. J. Cell. Biochem. 85: 663–669, 2002. © 2002 Wiley-Liss, Inc.

Key words: protein kinase CK2; casein kinase II; E7 mutants; DNA gel retardation assay; transcription initiation

Infection with human papillomaviruses (HPVs) is intimately associated with the development of cervical cancer [reviewed by zur Hausen, 1991], which is one of the most serious threats to the health of women throughout the world [Pisani et al., 1999]. It is now well established that the oncogenicity of a subset of HPV types, in particular HPV-16 and -18, is due to the action of two viral oncoproteins, E6 and E7. Both of these viral proteins continue to be retained and expressed in cervical tumours and derived cell lines, many years after the initial immortalising events [Schwarz et al., 1985; Smotkin and Wettstein, 1986; Androphy et al., 1987; Banks et al., 1987]. Indeed, numerous studies have shown that both proteins are essential for continued cell proliferation and maintenance of the transformed phenotype [von Knebel Doeberitz et al., 1988; Wang-Johanning et al., 1998; Nauenberg et al., 2001]. The transforming capacity of these viral proteins is linked to their abilities to deregulate the function of essential cellular tumour suppressor proteins. Thus, E6 binds and targets p53 for ubiquitin mediated degradation [reviewed by Thomas et al., 1999] and, likewise, E7 targets pRb for proteasome mediated degradation [reviewed by Munger et al., 2001]. Although both functions appear to be essential for the transforming activity of the viral proteins, it is becoming clear that several other activities also contribute.

In the case of E7, a large number of studies have shown that it has the capacity to interact with a variety of cellular proteins involved in the regulation of cell cycle and transcriptional control. Thus, in addition to pRb, it also interacts with the two other members of the pocket protein family, p130 and p107 [Dyson et al., 1992; Davies et al., 1993]. E7 has also been shown to possess important transcriptional activities, including binding to c-*fos* and upregulating AP-1 activity [Antinore et al., 1996], as well as having more general promoter activation and

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repression capabilities [Zwerschke et al., 1996; Massimi and Banks, 1997; Luscher-Firzlaff et al., 1999; Rey et al., 2000; Prathapam et al., 2001]. Although the underlying mechanisms behind these functions are, in many cases, still unknown, it is clear that E7 can interact with some of the basic elements of the transcriptional machinery, including TAF110 [Mazzerilli et al., 1995] and TATA box binding protein (TBP) [Massimi et al., 1996]. Recent studies have shown that the ability to bind TBP correlates with the ability of E7 to bring about cell transformation [Massimi et al., 1997a]. In addition, the interaction with TBP appears to be stimulated following CK2 phosphorylation of the E7 protein on serines 31 and 32 [Massimi et al., 1996]. Interestingly, the phosphorylation pattern of E7 has been found to change during the cell cycle, thereby implying that the interaction with TBP may be subject to cell cycle regulation [Massimi and Banks, 2000]. Although there is considerable biological data supporting a role for CK2 phosphorylation of E7 in contributing to its ability to bring about cell transformation [Barbosa et al., 1990; Firzlaff et al., 1991], there is as yet no biochemical explanation of the possible effects of a E7-TBP interaction upon TBP function. In this study we have investigated further the E7-TBP interaction and showed that E7 is a potent inhibitor of TBP DNA binding activity. This is augmented following CK2 phosphorylation of E7, consistent with its having an increased affinity for TBP. In contrast, a mutant of E7 incapable of binding TBP has no effect on TBP's DNA binding activity. These results thus may provide a significant biochemical explanation for the effects of E7 upon TBP function.

MATERIALS AND METHODS

Purification of Recombinant Proteins

Recombinant *Xenopus laevis* CK2α and CK2β were expressed and purified by NTA-agarose chromatography according to published procedures [Hinrichs et al., 1993; Cosmelli et al., 1997]. Recombinant TFIIA was expressed and purified according to Sun et al. [1994]. Recombinant human TBP (from a clone kindly donated by Dr. Arnold Berk [Kao et al., 1990]) was purified by chromatography on a DEAE-52 column followed by chromatography on a phosphocellulose column. Recombinant GST-E7, GST-E7Δ4 (deleted in amino acids 79–83) and GST were purified by glutathione–agarose chromatography as previously described [Massimi et al., 1997a]. Gel polyacrylamide electrophoresis of the recombinant GST-E7 and GST-E7Δ4 showed that these proteins were highly purified. Recombinant *Schizosaccaromyces pombe* TBP was purified by a phosphocellulose column, followed by a DEAE-52 and a S-Sepharose column [Maldonado E, unpublished procedure].

Gel Retardation

The gel retardation experiments were performed in a 20 μl reaction volume using: 0.1–1 ng of a 3′-end-labelled [^{32}P] DNA fragment containing the adeno-major late promoter(Ad-MLP) TATA motif (approximately 5,000 cpm) extending from −40 to + 20 nucleotides from the start of transcription. The reactions mixtures contained 10 mM HEPES, pH 7.8, 4 mM $MgCl_2$, 4 mM ammonium sulfate, 60 mM KCl, 5 mM 2-mercaptoethanol, 8% (v/v) glycerol, 2% (w/v) polyethylene glycol 20,000, 0.2 mM EDTA, 10 ng TBP, 10 ng TFIIA and 100 ng of poly (dG-dC)-poly (dG-dC). The reactions were incubated at 30°C for 30 min. The complexes were analysed in on 5% polyacrylamide gel containing 3% (v/v) glycerol and 0.5 × TBE (45 mM Tris-borate, pH 8.0, 1 mM EDTA). The same buffer and concentration were used to run the gels. The gels were run until the bromophenol blue was at 1 cm from the bottom of the gels. The gels were dried and the complexes visualised by autoradiography. Gel retardation experiments performed with AP2 were done with the Gel Shift Assay System from Promega and according to the manufacturers' instructions.

Phosphorylation Assays

The phosphorylation assays were performed incubating 200 ng of GST-E7 with 200 ng of wild type recombinant *Xenopus laevis* CK2 holoenzyme tretramer ($\alpha_2\beta_2$) or a complex generated with the catalytically inactive mutant CK2α (A156) [Cosmelli et al., 1997] together with CK2β. The reaction buffer contained 50 mM HEPES, pH 7.8, 100 mM KCl, 7 mM $MgCl_2$, 0.5 mM DTT and 1 mM ATP. The reaction mixtures were incubated for 20 min at 30°C. The proteins were purified in a 1 ml column of P10 (Bio Rad) equilibrated in 20 mM Tris-HCl, 10% (v/v) glycerol, 10 mM 2-mercaptoethanol, 0.5 mM EDTA, 0.1 mM PMSF and 100 mM KCL. After purification, the proteins were used in the gel retardation assays.

RESULTS

Previous studies have shown that the HPV-16 E7 oncoprotein binds strongly to TBP [Massimi et al., 1996, 1997a]. However there is no information as to the possible effects of this interaction upon TBP function. It is known that the c-terminal domain of TBP is sufficient for the binding of the TATA DNA sequence and basal transcription initiation by RNA polymerase II [Hoffmann et al., 1990; Peterson et al., 1990]. This is also the region of the TBP protein bound by E7 [Massimi et al., 1997a]. Therefore we proceeded to investigate the effects of E7 upon TBP DNA binding activity. In order to do this, the HPV-16 E7 protein was purified as a GST fusion protein and increasing amounts added to TBP in a gel retardation assay. The results obtained are shown in Figure 1A. As can be seen, increasing quantities of GST-E7 has a profound inhibitory effect upon TBP DNA binding activity whilst the GST alone has no effect. This effect of E7 is very potent since inhibition of TBP DNA binding activity can be detected with as little as 10 ng of the GST-E7 protein. Complete inhibition of TBP DNA binding activity is obtained with only 20 ng of the E7 fusion protein whilst no effects are obtained with 80 ng of GST protein alone, thereby demonstrating that the effects of E7 are highly specific.

In order to determine whether this activity of E7 was specific for TBP or represented a generalised ability to inhibit DNA–protein interactions, we repeated the assay using purified AP2 transcription factor for comparison. The results obtained are shown in Figure 1B. As can be seen, HPV-16 E7 again strongly inhibits TBP DNA binding activity (lanes 2–4) but has a much smaller effect upon AP2 DNA binding activity (lanes 7–9). These results suggest that the ability of E7 to inhibit TBP DNA binding activity is specific to TBP and does not represent a generalised inhibition of protein–DNA interactions.

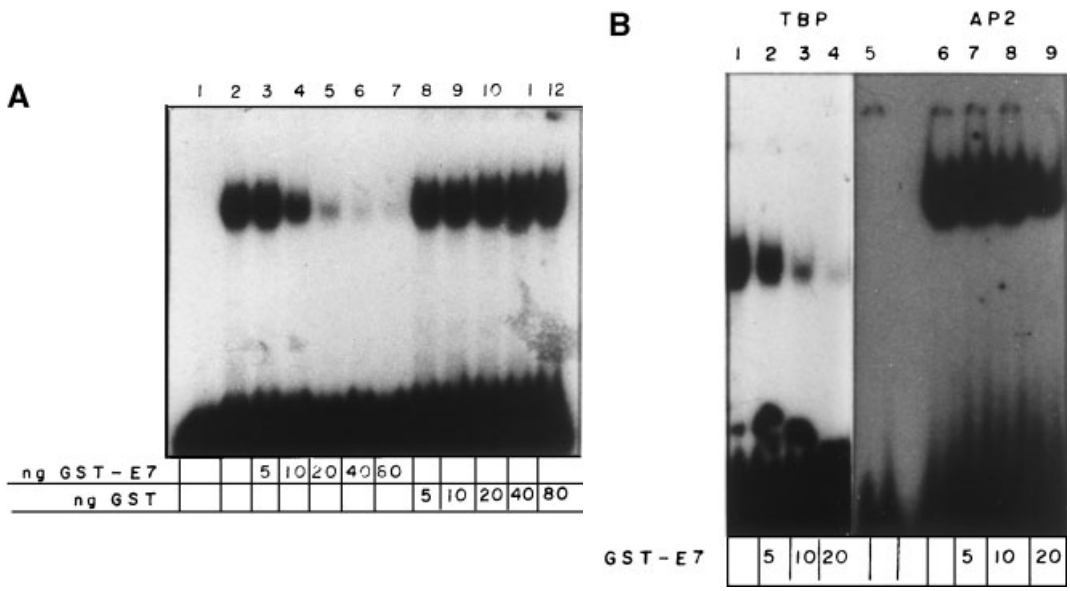

Fig. 1. Recombinant GST-E7 inhibits the binding of human TBP to the TATA box, but does not inhibit the binding of AP2. **A**: Labelled DNA containing the Adeno major late promotor containing a TATA box was incubated with 10 ng of recombinant human TBP and 10 ng of recombinant human TFIIA. Different amounts, of recombinant GST-E7 (**lanes 2–7**) and GST (**lanes 8–12**) were added to gel retardation assay, as indicated at the bottom of the figure. In **lane 1**, the labelled probe was incubated with no protein. In lane 2, the labelled probe was incubated with TBP and TFIIA. The products of the gel retardation assays were analysed on 5% polyacrylamide gels that contained 0.5 × TBE buffer (Materials and Methods). The gels were dried and exposed overnight to X-ray films. **B**: A labelled fragment containing the Ad-MLP TATA box was incubated with 10 ng of human TBP and 10 ng of human TFIIA and different amounts, as indicated at the bottom of the figure, of GST-E7 were added (**lanes 2–4**). In **lane 1**, the labelled probe was incubated with TBP and TFIIA without GST-E7. Also, a labelled oligonucleotide containing the binding site for AP2 was incubated with 1 μl of an extract containing recombinant AP2 protein (Promega Corp.), and different amounts of GST-E7 were added to the binding reactions (**lanes 7–9**). **Lane 5** contains the labelled AP2 probe without protein, and **lane 6** contains the probe with AP2 protein. The products of the gel retardation assays were analysed on 5% polyacrylamide gels that contained 0.5× TBE buffer (Materials and Methods). The gels were dried and exposed overnight to X-ray films.

We were next interested in determining whether this inhibitory effect of E7 was directly related to its ability to bind TBP. We had previously shown that the Δ4 mutant of HPV-16 E7 (deleted in amino acids 79–83) was defective in its ability to bind TBP [Massimi et al., 1997a]. Therefore we purified wild type and the Δ4 mutant of E7 as GST fusion protein and then assessed their ability to inhibit TBP DNA binding activity in a gel retardation assay. The results obtained are shown in Figure 2, where it is clear that wild type E7 again strongly inhibits TBP DNA binding. In contrast the mutant E7, defective in its ability to bind TBP, is also completely defective in its ability to inhibit TBP DNA binding activity. These results demonstrate that the ability of E7 to inhibit TBP binding to DNA is a direct consequence of E7's ability to bind TBP. Moreover, the inhibition of the binding of TBP to the DNA is not due to the effect of E7 on the activity of TFIIA, which is present in the assay. This is suggested by the fact that while TBP binding to GST-E7 can easily be demonstrated by a GST pull down assay, while no interaction is observed between GST-E7 and TFIIA in a similar experiment (data not shown).

We had previously shown that phosphorylation of HPV-16 E7 by CK2 greatly enhanced the affinity of the viral protein for TBP [Massimi et al., 1996]. Therefore we proceeded to investigate whether phosphorylation of the GST-E7 fusion protein by CK2 could also increase its ability to inhibit TBP DNA binding activity. In order to do this, the purified GST-E7 fusion protein was incubated with a CK2 holoenzyme tetramer containing either a wild type active catalytic subunit CK2α or a kinase defective subunit CK2α (A156), [Cosmelli et al., 1997] together with ATP. Following the kinase reaction the GST-E7 fusion proteins were then added to TBP in a gel retardation assay, and the results obtained are shown in Figure 3. As can be seen the ability of E7 to inhibit TBP DNA binding activity is greatly enhanced following CK2 phosphorylation, with approximately a fivefold increase in its inhibitory activity when compared with the non-phosphorylated E7 protein. These results demonstrate that the increased affinity of E7 for TBP following phosphorylation by CK2, results in an increased ability to inhibit TBP DNA binding activity.

The C-terminal domain of TBP is highly conserved through evolution whilst the amino terminal region is highly divergent. Since we had already shown that E7 was capable of

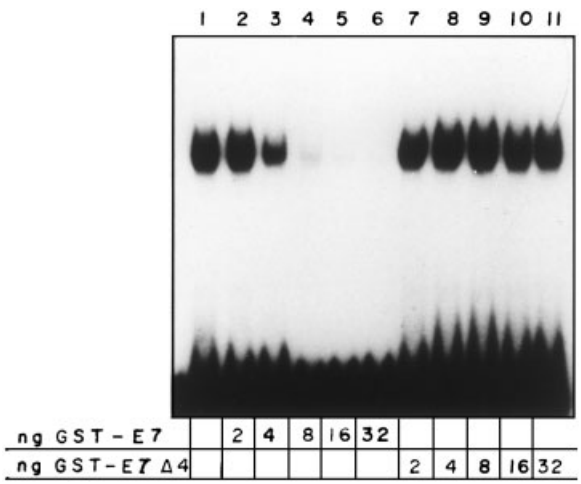

Fig. 2. An E7 deletion mutant that does not bind TBP has no effect on the binding of human TBP to the TATA box. A labelled probe containing the Ad-MLP TATA box was incubated with 10 ng of human TBP and 10 ng of human TFIIA. As indicated in the bottom of the figure, different amounts of wild type GST-E7 (**lanes 2–6**), or the deletion mutant GST-E7Δ4 (**lanes 7–11**), were added to the binding reactions. In **lane 1**, the labelled probe was incubated with TBP and TFIIA. The products of the gel retardation assays were analysed as described in Figure 1 Materials and Methods.

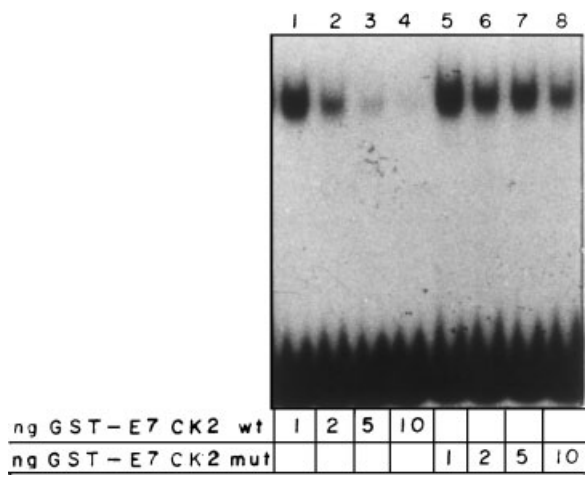

Fig. 3. Phosphorylation of E7 augments the inhibitory effects on the binding of TBP to the TATA box. Binding reactions, which contained 10 ng of TBP, 10 ng of TFIIA, and a labelled fragment containing the TATA box of the Ad-MLP promoter, were incubated with GST-E7 phosphorylated by recombinant *Xenopus laevis* CK2α (**lanes 1–4**) or with GST-E7 similarly treated with an inactive CK2α (**lanes 5–8**) (as described in Materials and Methods). The products of the gel retardation assays were analysed as described in Materials and Methods.

binding this conserved carboxy terminal region of TBP [Massimi et al., 1997a], we were next interested in determining whether E7 could likewise inhibit the DNA binding activity of TBP derived from *S. pombe*, which is phosphorylated by CK2 [Maldonado and Allende, 1999]. To do this, recombinant *S. pombe* TBP was purified and incubated with the GST-E7 fusion protein and then analysed in a gel retardation assay. The results obtained and shown in Figure 4 demonstrate that the HPV-16 E7 protein can also inhibit *S. pombe* TBP DNA binding activity. Interestingly this occurs with very similar kinetics to that observed with human TBP. These results confirm that the interaction of HPV-16 E7 with TBP occurs through an evolutionarily conserved domain of the protein, further supporting the notion that a consequence of E7 binding to TBP is inhibition of the highly conserved DNA binding function of the protein.

DISCUSSION

Although several studies have previously reported HPV E7 interaction with TBP [Massimi et al., 1996, 1997a], there is no information as to the biochemical consequences of this interaction with respect to TBP function. In this study we have shown that the HPV-16 E7 protein is a potent inhibitor of TBP DNA binding activity on specific TATA sequences found in many RNA polymerase II promotors. This suggests that a function of E7 with respect to TBP may be to block its ability to activate transcription of TATA containing promotors. Mutational analysis of E7 demonstrates that this activity of E7 requires the ability to bind TBP and interestingly this activity is greatly enhanced following CK2 phosphorylation of the E7 protein, consistent with it having an increased affinity for TBP [Massimi et al., 1996].

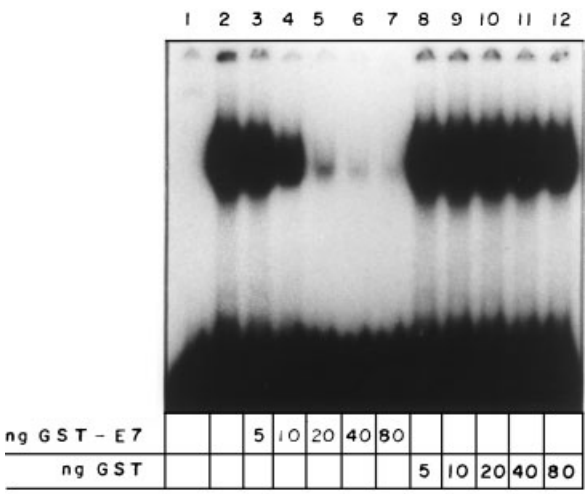

Fig. 4. GST-E7 inhibits the binding of *S. pombe* TBP to the TATA box. A labelled probe containing the TATA box of the Ad-MLP was incubated with 10 ng of *S. pombe* TBP and 10 ng of human TFIIA. Different amounts of GST-E7 (**lanes 3–7**) or GST (**lanes 8–12**) were added to the binding reactions, as indicated at the bottom of the figure. In **lane 1**, the probe was incubated with no protein, and **lane 2** contains *S. pombe* TBP and TFIIA without GST-E7. The products of the gel retardation assays were analysed as described in Figure 1 Materials and Methods.

At present the biological relevance of the E7-TBP interaction is unclear. Previous studies have shown that it would appear to be important for the ability of E7 to transform cells [Massimi et al., 1997a]. However, it is unlikely that E7 will globally inhibit TBP DNA binding activity on all pol II promoters, and it seems more probable that this activity of E7 will be restricted to a subset of cellular and/or viral promoters. In addition, TBP has been found to play also an important role in RNA polymerases I and III transcription. These polymerases have many promoters that do not contain TATA sequences [Rigby, 1993; White, 1998]. Nonetheless, it is interesting that E7 has been reported to possess a range of transcriptional inhibitory activities. These include inhibition of Skip co-activator function [Prathapam et al., 2001], suppression of the fibronectin promoter [Rey et al., 2000] as well as suppression of p53 transcriptional activity [Massimi and Banks, 1997]. Interestingly, both the p53 and Skip suppression functions of E7 appear to require an intact CK2 recognition site [Massimi and Banks, 1997; Prathapam et al., 2001], suggesting that these activities may represent a manifestation of E7's ability to interact with TBP.

It is also interesting to note that the region of TBP bound by E7 lies within the evolutionarily conserved carboxy terminal region of the protein, which has been reported previously to be sufficient for TBP DNA binding activity [Hoffmann et al., 1990; Peterson et al., 1990]. This is consistent with the studies shown here, where clear inhibition of *S. pombe* TBP DNA binding activity was also obtained with HPV-16 E7. This suggests that the region of TBP bound by E7 most likely corresponds to the functional domain of TBP required for its DNA binding activity. This also indicates that the mechanism by which E7 blocks TBP DNA binding is probably by steric hindrance. It may be relevant that phosphorylation of *S. pombe* and

S. cerevisiae (TBP) by CK2 also inhibits TBP binding of DNA. However human TBP cannot be phosphorylated by CK2 [Maldonado and Allende, 1999].

The effects of CK2 phosphorylation of E7 upon its ability to inhibit TBP DNA binding activity is also striking. Although unphosphorylated E7 is quite capable of inhibiting TBP binding to DNA, phosphorylation by CK2 increases that activity of E7 by at least fivefold. This is highly consistent with previous studies showing that E7 binding to TBP was stimulated following CK2 phosphorylation [Massimi et al., 1996]. This also raises an interesting aspect as to when in the cell cycle E7 may be inhibiting TBP function. Recent studies have shown that E7 would appear to be predominantly phosphorylated by CK2 during the G1/S phase transition [Massimi and Banks, 2000] which, interestingly, corresponds to the period of the cell cycle when E7 possesses optimal activity. This suggests therefore, that it is also at this point of the cell cycle when E7 will preferentially inhibit TBP function.

In summary, we have shown that the biochemical consequence of the E7-TBP interaction is inhibition of TBP DNA binding activity. This requires the direct binding of E7 to TBP, and most likely occurs by steric hindrance.

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