

# The human papillomavirus type 11 and 16 E6 proteins modulate the cell-cycle regulator and transcription cofactor TRIP-Br1

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## Abstract

The genital human papillomaviruses (HPVs) are a taxonomic group including HPV types that preferentially cause genital and laryngeal warts (“low-risk types”), such as HPV-6 and HPV-11, or cancer of the cervix and its precursor lesions (“high-risk types”), such as HPV-16. The transforming processes induced by these viruses depend on the proteins E5, E6, and E7. Among these oncoproteins, the E6 protein stands out because it supports a particularly large number of functions and interactions with cellular proteins, some of which are specific for the carcinogenic HPVs, while others are shared among low- and high-risk HPVs. Here we report yeast two-hybrid screens with HPV-6 and -11 E6 proteins that identified TRIP-Br1 as a novel cellular target. TRIP-Br1 was recently detected by two research groups, which described two separate functions, namely that of a transcriptional integrator of the E2F1/DP1/RB cell-cycle regulatory pathway (and then named TRIP-Br1), and that of an antagonist of the cyclin-dependent kinase suppression of p16INK4a (and then named p34SEI-1). We observed that TRIP-Br1 interacts with low- and high-risk HPV E6 proteins in yeast, *in vitro* and in mammalian cell cultures. Transcription activation of a complex consisting of E2F1, DP1, and TRIP-Br1 was efficiently stimulated by both E6 proteins. TRIP-Br1 has an LLG E6 interaction motif, which contributed to the binding of E6 proteins. Apparently, E6 does not promote degradation of TRIP-Br1. Our observations imply that the cell-cycle promoting transcription factor E2F1/DP1 is dually targeted by HPV oncoproteins, namely (i) by interference of the E7 protein with repression by RB, and (ii) by the transcriptional cofactor function of the E6 protein. Our data reveal the natural context of the transcription activator function of E6, which has been predicted without knowledge of the E2F1/DP1/TRIP-Br1/E6 complex by studying chimeric constructs, and add a function to the limited number of transforming properties shared by low- and high-risk HPVs.

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## Introduction

Among the three transforming genes of papillomaviruses (Howley, 1996; IARC, 1995; zur Hausen, 1996), E6 has attracted much interest due to the surprising diversity of the functions of its product. The E6 oncoprotein has—in the

case of human papillomavirus 16 (HPV-16)—a size of 151 amino acids, and the positional conservation of eight cysteine residues and the protein’s ability to bind zinc ions suggest a structure with two zinc fingers, with their large size of 29 amino acids unique among all known zinc finger proteins (Ullman et al., 1996). For several years, there was only a single paradigm for the function of the E6 protein, namely binding and degrading, together with the ubiquitin ligase E6AP, the transcription factor, and cell-cycle regulator p53 (Scheffner et al., 1990, 1993; Werness et al., 1990), an obviously useful function of a virus with the strategy to overcome cell-cycle repression and apoptosis. However, more recently a surprising number of additional functions

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could be attributed to E6 proteins. Interactions of E6 proteins with hDLG and paxillin may influence signaling from the extracellular matrix to the nucleus and the cell shape (Das et al., 2000; Kiyono et al., 1997; Lee et al., 1997; Vande Poll et al., 1998), with E6BP calcium metabolism (Chen et al., 1998), with IRF-3 the immuneresponse against papillomavirus lesions (Ronco et al., 1998), with CBP transcriptional regulation (Patel et al., 1999; Zimmermann et al., 1999, 2000), and with a transcription factor regulating the telomerase gene expression, the immortalization process (Gewin and Galloway; 2001). These and other functions of E6 proteins and their presence or absence in high-risk or low-risk HPV types or the bovine papillomavirus-1 have been reviewed (Degenhardt and Silverstein, 2001a,b; Kuhne and Banks, 1998; Mantovani and Banks, 2001; Rapp and Chen, 1998). Similarly, more than a dozen interactions with cellular proteins have been reported for the second HPV oncoprotein, E7 (reviewed in Munger et al., 2001).

Cancer of the cervix, caused by chronic infection of the transformation zone of the cervix with the “high-risk” HPV types HPV-16, HPV-18, and their relatives, is a more important public health problem than genital and laryngeal warts, which are caused by HPV-6, HPV-11, and related types (Chan et al., 1995). Many of the interactions between the E6 and E7 oncoproteins and cellular targets are specific for oncoproteins of the high-risk HPV types, while a subset of these proteins also interacts with low-risk HPVs. Recently, a cellular protein was observed for the first time that can specifically bind the low-risk HPV-6 E6 but not several other E6 proteins including those of high-risk types (Degenhardt and Silverstein, 2001a). As one compares the conspicuous neoplasia caused by HPV-6 or HPV-11 with the often barely detectable precursors of cervical malignant lesions, cervical intraepithelial neoplasia (CIN), one has to conclude that the oncoproteins of low-risk viruses must have substantial molecular functions.

Against this background, we initiated this research in the hope that screening for cellular proteins that interact with the E6 oncoproteins of low-risk viruses would identify novel targets that may be important during the etiology of warts as opposed to CIN-like precursor lesions. In this screen we found several interesting cellular candidates for being HPV-6 and HPV-11 E6 targets, and in this article, we describe the detection and functional analysis of one of them, TRIP-Br1. While our data show how HPV E6 protein chose yet another target among cell-cycle regulators, our original goal, to find a target specific for wart viruses, was not achieved, since TRIP-Br1 was similarly targeted by HPV-16 E6. TRIP-Br1 has been independently described by two different research groups (Hsu et al., 2001; Sugimoto et al., 1999), which followed different experimental strategies and consequently observed two different molecular properties of this protein. Sugimoto and colleagues (1999) detected it in a yeast two-hybrid screen with INK4A as a bait and termed it p34SEI-1 (SEI, selected with INK4). They could show that it forms a quaternary complex with CDK4,

cyclin D, and INK4. In this complex, the p34SEI annihilates the kinase inhibitory function of INK4, without displacing it from CDK4. As a consequence, CDK4 can still phosphorylate the retinoblastoma tumor suppressor (RB) protein, relieving its transcriptional repression function on E2F1/DP1, which is an important transcription factor at the G<sub>1</sub>/S checkpoint of the cell cycle (reviewed in Trimarchi and Lees, 2002). Hsu and colleagues (2001) found the same protein in a screen originally aiming at proteins interacting with the PHD-bromodomain of the transcriptional cofactor KRIP-1 and termed it TRIP-Br1 (transcriptional regulator interacting with the PHD-bromodomain, “1” indicating that it was one member in a family of two related genes). Subsequently, it became clear that TRIP-Br1 makes physical contact with the DP1 subunit of the E2F1/DP1 heteromer, where it acts as a transcriptional coactivator. In summary, TRIP-Br1 stimulates transcription of cell-cycle promoting and E2F1/DP1 regulated genes by two mechanisms, release of repression of E2F1 by RB and as a transcriptional coactivator of E2F1/DP1.

In this article, we report that HPV E6 oncoproteins bind to and further increase the transcription stimulatory function of TRIP-Br1. Our findings add another facet of knowledge about the alterations of the E2F1/DP1/RB cell-cycle regulatory pathway by both papillomavirus oncoproteins, report one of the few cellular proteins targeted both by high- and low-risk HPVs, and point to the natural context of a previously predicted transcription factor function of E6 (Desaintes et al., 1992; Lamberti et al., 1990; Ned et al., 1997).

## Results

### *A yeast two-hybrid screen identifies the TRIP-Br1 cell-cycle regulator as a target of the HPV-6 E6 protein*

We cloned the HPV-16 E6 protein into the bait vector, pAS2-1 (Clontech), and selected His<sup>+</sup>/LacZ<sup>+</sup> positive clones of a human keratinocyte cDNA library with  $5 \times 10^6$  independent inserts in pGAD10 (Clontech) vector. Among 100 arbitrarily chosen clones, nine encoded the E6AP ubiquitin ligase, one of the best characterized HPV-16 E6 interacting proteins (Scheffner et al., 1990, 1993; Werness et al., 1990). Most of the remaining clones encoded frequently encountered unspecifically binding proteins (e.g., heat shock proteins). We transformed E6AP containing pGAD10 vectors into yeast expressing HPV-6 E6 protein from pAS2-1 constructs and confirmed that they also led to a His<sup>+</sup> and LacZ<sup>+</sup> phenotype, although the blue color in the X-Gal test did not develop as strongly as with HPV-16 E6 clones. This was expected as the E6 proteins of low-risk HPV-type E6 proteins are known to have less affinity to E6AP than those of high-risk HPV types (Huibregtse et al., 1993).

In a similar experiment with the HPV-6 E6 protein as bait, we obtained only nine clones that were both His<sup>+</sup> and

scored positive in the X-gal test. None of these clones contained an insert that had previously been reported as an E6 target. Six of these clones unspecifically encoded binding cellular proteins, while three clones represented three different genes that did not fall into this category. One of these clones represented a cDNA with a length of 1.2 kb containing a 711-bp open reading frame, whose sequence was 100% identical to a gene in the data base that had been previously characterized by two different groups and published under the names p34SEI-1 (Sugimoto et al., 1999) and TRIP-Br1 (Hsu et al., 2001) (GenBank Accession Nos. NM013376 and AF366402). This clone was designated pGAD10-TRIP-Br1. By using HPV-11 E6 as bait, we confirmed that this new interaction partner leads to similar positively reacting clones as HPV-6 E6. This article deals with the analysis of this new interaction, while the remaining two proteins are still under investigation.

#### *Low-risk and high-risk HPV E6 proteins interact with the TRIP-Br1 protein*

We had begun this project in the hope of detecting cellular proteins that had functions preferentially in the context of HPV types that generate genital warts, as opposed to cervical cancer precursors. To investigate this possibility, we transformed pGAD10-TRIP-Br1 into Y190 cells containing pAS2-1 HPV-16 E6 vectors. Contrary to our hopes, these experiments showed that Y190/pGAD10-TRIP-Br1 cells grew equally well and gave color reactions of similar intensity in X-Gal test in the presence of HPV-6, HPV-11, or HPV-16 E6 protein. We conclude that in yeast all three oncoproteins react similarly with TRIP-Br1.

#### *Binding of TRIP-Br1 and HPV E6 proteins in vitro*

To examine whether HPV E6 and TRIP-Br1 proteins interact in vitro, we prepared GST fusion proteins of the full-length HPV-16 and HPV-11 E6 protein, both being efficiently expressed at similar levels in *Escherichia coli* BL21, as shown in Fig. 1A. Since the expression level of HPV-11 E6 was significantly higher than the expression level of HPV-6 E6, we performed this and the following experiments with HPV-11 E6. HPV-6 and HPV-11 are among the two most closely related HPV types, the amino acid sequence of HPV-11 differing from that of HPV-6 E6 in 28 of 150 residues. Most of these exchanges are conservative, and HPV-6 and HPV-11 are very close relatives with indistinguishable biological and pathological properties (Chan et al., 1995; Myers, 1994) (for an exception see Degenhardt and Silverstein, 2001a). Equal amounts of the HPV-11 and HPV-16 E6 GST fusion proteins were immobilized on GSH beads, followed by the addition of [<sup>35</sup>S]methionine-labeled in vitro translated (IVT) TRIP-Br1 and IVT C-terminal hMCM7 (residues 563–719). Fig. 1B shows that the IVT TRIP-Br1 bound with almost the same affinity

to both E6 proteins, while we detected no binding of TRIP-Br1 to GST alone.

As a reference to the binding efficiency of TRIP-Br1 protein to HPV-11 and 16 E6 proteins, we chose a previously identified cellular interacting partner, namely the human minichromosome maintenance 7 (hMCM7) protein, which is involved in the control of the cellular replication process (Kukimoto et al., 1998). The C-terminal region (residues 563 to 719) of hMCM7 has been demonstrated to bind HPV-11 and -16 E6 (Zimmermann et al., 2000). The bottom of Fig. 1B shows that IVT hMCM7 binds with equal affinity to both E6 proteins, but with only 15% of the affinity of TRIP-Br1, confirming the validity of this interaction.

#### *Binding between TRIP-Br1 and HPV-11 and HPV-16 E6 proteins in vivo*

To demonstrate that HPV E6 proteins bind to TRIP-Br1 in vivo, 293T cells were transfected with plasmids expressing the FLAG-tagged TRIP-Br1 together with GST-tagged HPV-11 E6, HPV-16 E6, or GST alone. Twenty-four hours after transfection, the cells were lysed and TRIP-Br1-E6 complexes were captured on GSH beads. The samples were run on sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by blotting onto a nitrocellulose membrane and bound TRIP-Br1 protein visualized with an anti-FLAG antibody.

Fig. 2A shows that the TRIP-Br1 protein gave equally strong signals with lysates of cells cotransfected with the TRIP-Br1 and either HPV-11 or -16 E6 constructs, while it was undetectable in the lysate cotransfected with TRIP-Br1 and the GST expression constructs. Fig. 2B shows direct anti-FLAG Western blotting of whole cell lysates, indicating that approximately similar amounts of TRIP-Br1 proteins were expressed, while the panel on the right shows roughly equal amounts of GST E6 fusion proteins being expressed as well. These results confirm that TRIP-Br1 is able to bind to HPV-11 and -16 E6 proteins in vivo.

#### *An LLG motif of TRIP-Br1 potentially contributes to the interaction with HPV E6 proteins*

The E6 proteins of papillomaviruses interact with their multiple cellular targets by various mechanisms, most of which are not understood in much detail, in particular, since the three-dimensional structures of E6 proteins are not yet known. However, about half of all known cellular E6 targets possess an LLG motif (Kuhne and Banks, 1998; Mantovani and Banks, 2001), which contributes to the interaction with E6 (Chen et al., 1998). By inspecting the 236 amino acid sequence of TRIP-Br1, we observed such an LLG motif at positions 157–159. To study whether this LLG motif plays a role in the binding of TRIP-Br1, we altered the coding sequence in two ways, namely by replacing the LLG se-

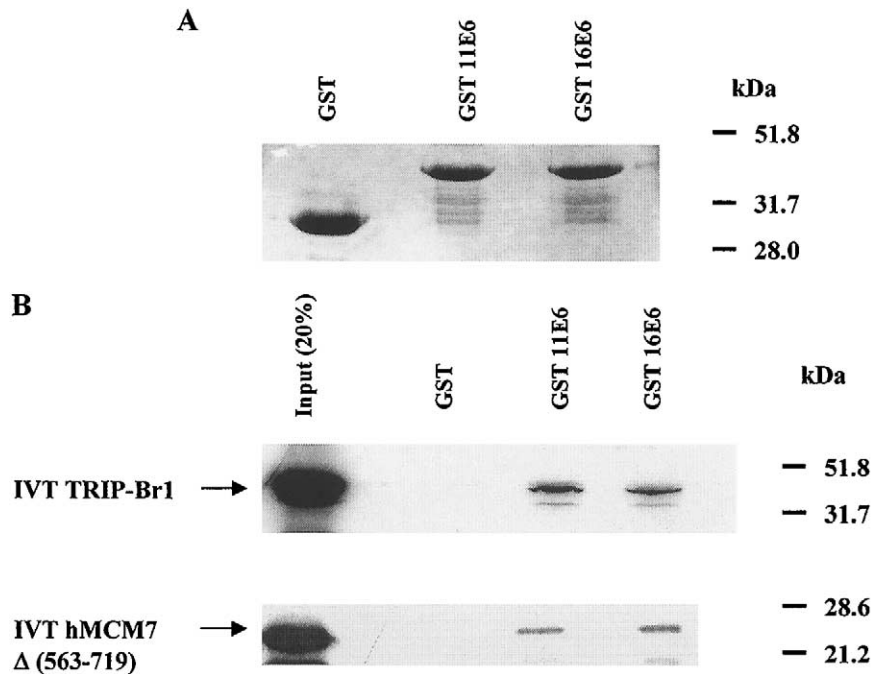


Fig. 1. Binding of TRIP-Br1 and HPV E6 proteins in vitro. (A) The GST, HPV-11, and HPV-16 GST fusion proteins are expressed at similar concentrations in *E. coli* BL21, as seen from the Coomassie blue stained SDS gel. (B) [<sup>35</sup>S]methionine-labeled in vitro translated (IVT) TRIP-Br1 binds to GST-linked and GSH column bound HPV-11 and HPV-16 E6 proteins with similar affinity. In comparison to the binding efficiency of TRIP-Br1, the lower panel indicates that the IVT C-terminal hMCM7 binds to both HPV-11 and HPV-16 E6 proteins with equal affinity as well but at a lower binding efficiency of about 15% as determined by the Bio-Rad densitometer software. The numbers on the right side of the figures refer to size markers not included in this figure. Total input of in vitro translated TRIP-Br1 and hMCM7 proteins in the experiment was at 20%.

quence with an AAA motif, and by deleting these three amino acids altogether. A GST pull-down experiment in Fig. 3 shows that the binding affinity of these two mutations between HPV-11 E6 and HPV-16 E6 is reduced. By comparing with the wild-type (wt) TRIP-Br1 protein, the reduction in binding efficiency was 30 to 35%, as determined from using the Bio-Rad densitometer software via integration of the bands. We conclude that the LLG motif contributes to but is not alone responsible for the interaction with E6.

#### *Stability of TRIP-Br1 in the presence of HPV E6 proteins*

The E6 proteins of papillomaviruses can catalyze the modulation of cellular target proteins by inducing proteolytic degradation via the ubiquitination pathway. To examine this potential mechanism, we mixed in vitro translated [<sup>35</sup>S]methionine-labeled p53 and TRIP-Br1 proteins with [<sup>35</sup>S]methionine-labeled HPV-11 and HPV-16 E6 proteins. Fig. 4A shows that neither HPV-11 nor HPV-16 E6 proteins affect the stability of TRIP-Br1 over 5 h, while Fig. 4B indicates strong degradation of p53 under the influence of HPV-16 E6, and a slight degradation by HPV-11 E6, as expected from published research (Scheffner et al., 1990, 1993). We conclude that the TRIP-Br1 protein is not a target for E6-induced degradation.

#### *HPV-11 and HPV-16 E6 proteins further increase the transcription stimulation of E2F1/DP1 by TRIP-Br1*

TRIP-Br1 is a transcriptional coactivator of E2F1/DP1. This function has been studied in the context of the myb promoter, which is solely dependent on this transcription factor, as shown by some of us (Hsu et al., 2001). Since both factors are present in many commonly used cell lines, it is difficult to study effects of externally added TRIP-Br1 on E2F1/DP1. We have determined, however, that such effects can be monitored with a novel construct, pGL3-TATA6xE2F, which has six E2F1/DP1 binding sites upstream of a TATA box and the luciferase reporter gene (Hsu et al., 2001).

To examine whether HPV E6 proteins could influence TRIP-Br1 transactivation ability, we cotransfected COS-7 cells with pCMV-E2F1, pCMV-DP1, the firefly luciferase expression vector pGL3-TATA6xE2F, pXJ40-TRIP-Br1 (FLAG tagged), and the control Renilla luciferase reporter plasmid, pRL-CMV, in the presence or absence of expression vectors expressing FLAG-tagged HPV-11 and -16 E6 proteins.

Columns 1 and 2 of Fig. 5 show the background level of expression of the reporter construct (pGL3-TATA6xE2F) and the approximately threefold stimulation after transfection of the TRIP-Br1 expression vector. Transfection of the

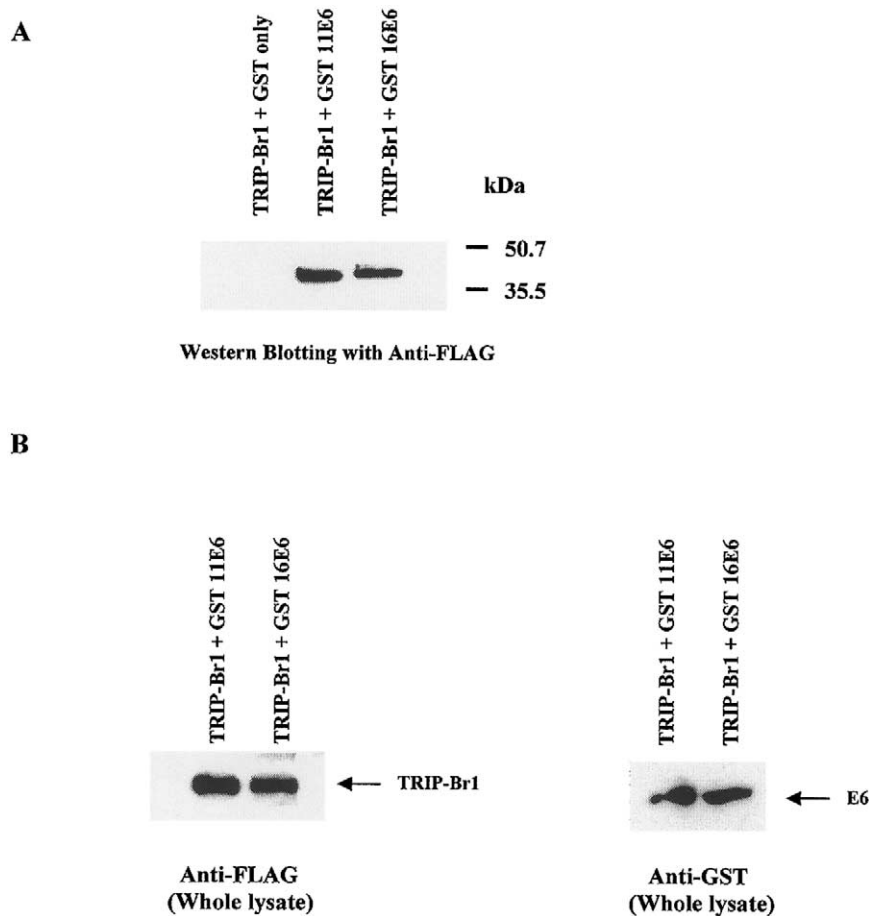


Fig. 2. Binding of TRIP-Br1 to HPV-E6 proteins in vivo. (A) 293T cells were transfected with 2.5  $\mu$ g each of pXJ-derived vectors expressing FLAG-tagged TRIP-Br1 together with GST, GST-tagged HPV-11, or 16 E6 as indicated. Twenty-four hours after transfection, cells were lysed and incubated with GSH beads, followed by Western blotting with anti-FLAG antibody. TRIP-Br1 + GST, TRIP-Br1 + GST 11E6, or TRIP-Br1 + GST 16E6 refer to constructs transfected together. The blot shows TRIP-Br1 binding to both HPV-11 and 16 E6 fusion proteins with similar affinity, while there was no binding detected with the GST protein alone. (B) Direct anti-FLAG and anti-GST Western blotting of whole cell lysate indicates equal expression of FLAG-tagged TRIP-Br1 and GST-tagged HPV-11 or HPV-16 E6, respectively, in the transfected cells.

HPV-11 and HPV-16 E6 constructs alone stimulates luciferase activity similarly due to endogenous E2F1/DP1 and TRIP-Br1 present in the cell line (columns 3 and 4). Co-transfection of TRIP-Br1 and HPV-11 E6 or -16 E6 leads to an approximately 4-fold stimulation of the promoter activity over the level obtained with only one of these proteins and about 12-fold over the basal level, respectively (columns 5 and 6). The transfection of E2F1/DP1 alone stimulates the luciferase expression level again due to endogenous TRIP-Br1 (column 7), which can be further stimulated by cotransfection with TRIP-Br1 (column 8). Cotransfection of E2F1/DP1 and either one of the two HPV E6 vectors increases luciferase expression about threefold over the level reached with E2F1/DP1 alone (columns 9 and 10). This expression level again doubles if additional TRIP-Br1 is cotransfected (columns 11 and 12). The combined effects of E2F1/DP1, TRIP-Br1, and either of the E6 expression vectors lead to more than 30-fold stimulation over the luciferase levels achieved in the absence of any of these expression vectors. We conclude that HPV-11 and -16 E6 proteins have coac-

tivator function in E2F1/DP1/TRIP-Br1-mediated transcription. A similar result was observed when we used 293T cells in place of COS-7 cells (data not shown).

## Discussion

High- and low-risk HPVs are infectious agents that alter the physiology of the infected cell in a way favorable to viral replication. To permit viral replication in the mitotically incompetent suprabasal epithelial cells, HPVs have to exercise strategies that permit passage through cell-cycle check points. Several of these properties are known and discussed below, and this article describes yet another one of these strategies, a function of the E6 oncoprotein to up-regulate the activity of E2F1/DP1, which is independently stimulated by the second HPV oncoprotein, E7, through its interference with the repressor function of the RB protein.

The heterodimeric transcription factor E2F1/DP1 plays a

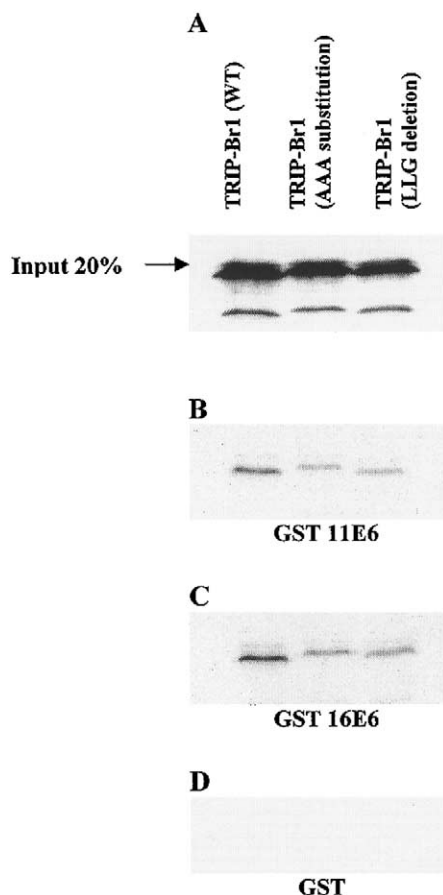


Fig. 3. An LLG motif potentially contributes to the binding between E6 and TRIP-Br1. (A) Identical input of 20% in each of the three lanes; the central panels (B,C) show the differential binding affinity of the wild-type (wt) TRIP-Br1 protein, *LLG* to *AAA* substitution, and *LLG* deleted mutant TRIP-Br1 proteins to HPV-11 E6 and -16 E6. Both mutant IVT TRIP-Br1 proteins show a 30 to 35% reduction in binding affinity. (D) The lack of binding of all three types of IVT TRIP-Br1 to GST alone. IVT-translated TRIP-Br1 frequently appeared in our experiments with a weaker faster migrating band due to unknown translational/posttranslational effects.

crucial role at the  $G_1/S$  check point of the cell cycle, as it controls the expression of a number of rate-limiting factors for cellular DNA synthesis. The activation function of E2F1, can be repressed by formation of a complex with RB and several related proteins, which mask the transcription activation domain of E2F1 and form additional complexes with transcriptional repressors such as histone acetyl transferases (HDACs). The formation of the E2F1–RB complex is inhibited by cyclin-dependent kinases, which on their part are stimulated by cyclins and repressed by inhibitors such as p16INK4a. This well-established model may require further modifications, as it recently became clear that several related factors within the E2F family have in part antagonistic functions (for references and reviews see Dyson, 1998; Khleif et al., 1996; Trimarchi and Lees, 2002). The HPV E7 proteins and unrelated proteins of polyoma- and adenoviruses alter this fine-tuned mechanism by abrogating the interaction between RB and E2F1 (Munger et al., 2001).

While this mechanism alone is sufficient to induce certain carcinogenic processes in transgenic mice (Song et al., 2000), the most frequent consequence of this disturbance of the cell cycle is the induction of apoptosis under the influence of p53 (Mantovani and Banks, 2001). Consequently, degradation of p53 by the E6 oncoprotein does not only serve to deregulate a second cell-cycle check point and thereby favor the carcinogenic progression, but is also a necessity to avoid that the E7-affected cell proceeds into apoptosis.

TRIP-Br1 binds the DP1 subunit of E2F1/DP1 and functions as transcriptional cofactor and integrator of regulatory signals by recruiting PHD-zinc finger and bromodomain-containing factors such as KRIP-1 and p300/CBP (for details see Fig. 9 in Hsu et al., 2001). In other words, TRIP-Br1 modulates transcription of genes relevant for  $G_1/S$  transition in the same direction as the oncoprotein E7 does. Here we report that the E6 oncoprotein further stimulates this TRIP-Br1 function and that the E2F1/DP1 factor is dually influenced by both oncoproteins. Fig. 6 schematically visualizes these mechanisms. This finding is of particular interest, as this mechanism is also employed by low-risk HPV types, which have been reported to use only a subset of the molecular transformation mechanisms used by the E6 and E7 proteins of high-risk HPVs (Mantovani and Banks,

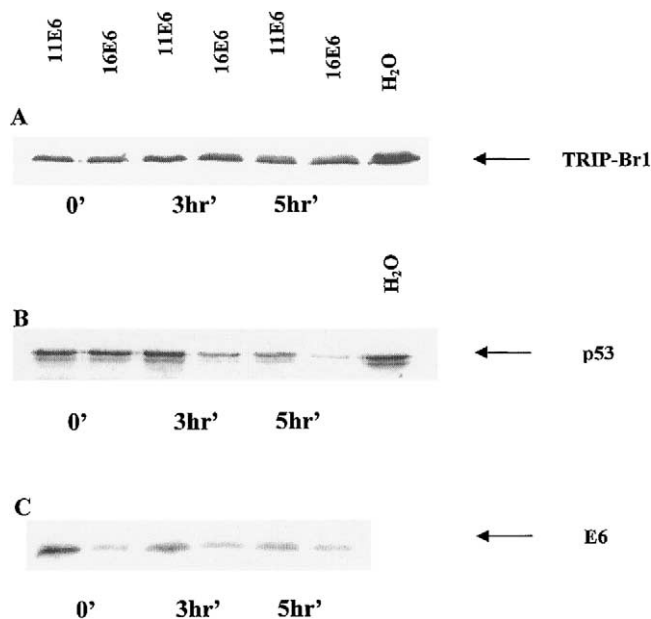


Fig. 4. E6 does not lead to degradation of TRIP-Br1. Approximately equal amounts of [ $^{35}$ S]methionine-labeled TRIP-Br1 or p53 were mixed with [ $^{35}$ S]methionine-labeled HPV-11 or -16 E6 proteins and analyzed for degradation of up to 5 h. As a negative control, water was added to both [ $^{35}$ S]methionine-labeled TRIP-Br1 and p53 in a total volume equal to that of the test reactions and incubated for 5 h as well. (A) shows that neither HPV-11 nor HPV-16 E6 affect the stability of TRIP-Br1 over 5 h, while (B) shows strong degradation of p53 under the influence of HPV-16 E6, and a slight effect by HPV-11 E6. As a control, (C) shows the concentrations of the E6 proteins were largely unaffected over the time course of the experiment.

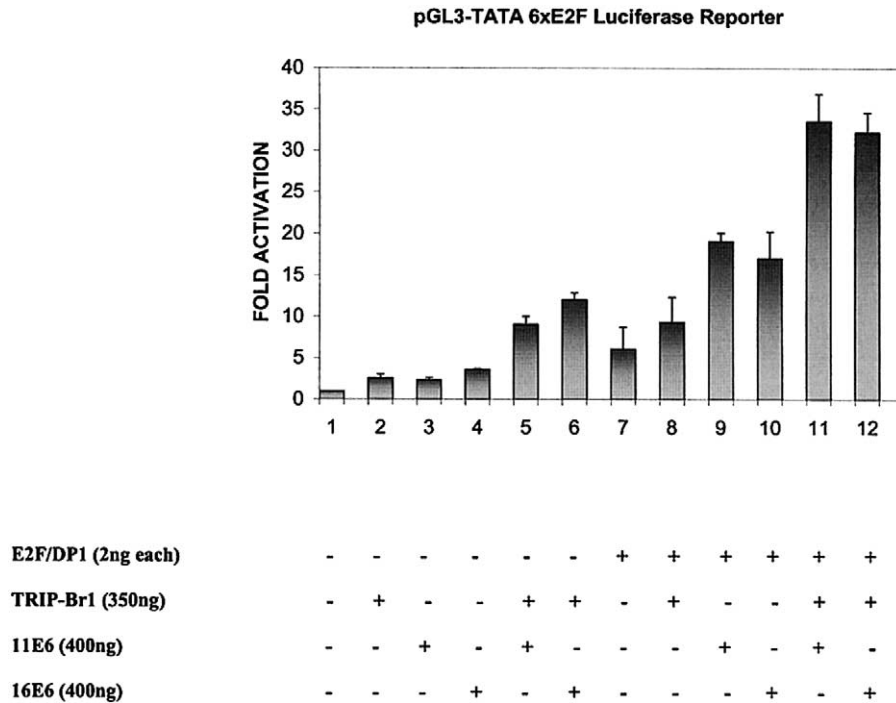


Fig. 5. Cooperative stimulation of E2F1/DP1 responsive luciferase expression by TRIP-Br1 and HPV 11/16 E6 proteins. COS-7 cells were transiently transfected with the indicated expression constructs, 200 ng of the pGL3-TATA6XE2F luciferase reporter plasmid, 2 ng of E2F/DP1 each, 350 ng of TRIP-Br1, 400 ng HPV-11 E6 or -16 E6. As an internal standard 10 ng of the Renilla luciferase plasmid pRL-CMV was added. Values represent the average of three independent experiments. Fold activation refers to luciferase activity normalized to Renilla luciferase activity and is expressed relative to the activity observed with the expression of the experimental reporter (pGL-3TATA6XE2F) and the control reporter (pRL-CMV) alone.

2001; Munger et al., 2001). It is particularly notable that we have detected a natural transcription activation scenario induced by papillomavirus E6 proteins. Transcription activation functions of the E6 proteins of the bovine papillomavirus-1 and of HPV-16 have been observed and extensively studied in the context of artificial vector systems, but could not yet address the question of a natural transcription activation context stimulated by E6 (Chan et al., 1995; Desaintes et al., 1992; Lamberti et al., 1990).

## Materials and methods

### Yeast two-hybrid techniques

Yeast two-hybrid screens were done with the Clontech Matchmaker Gal4 System (Clontech Laboratories, Palo Alto, CA) by cloning the E6 genes of HPV-6, HPV-11, and HPV-16 as polymerase chain reaction (PCR) generated fragments into the *EcoRI* and *BamHI* sites of the bait plasmid, pAS2-1, which were expressed in *Saccharomyces cerevisiae* Y190 with N-terminal Gal4 DNA-binding domains. The pAS2-1 HPV-6 and 16 E6 vectors were tested against a human foreskin cDNA library with  $5.0 \times 10^6$  primary recombinants with an average insert size of 1 kb from Clontech Laboratories. The cDNA was cloned into the *EcoRI* site of pGAD10 with an N-terminal Gal4 activation domain.

We established the pAS2-1 E6 constructs in Y190 cells by selection of a Trp marker and confirmation of stable maintenance of the E6 inserts by PCR and transformed cultures of individual clones with the pGAD10 library DNA. Y190 is histidine auxotroph and does not express the *E. coli LacZ*. The His<sup>+</sup> and LacZ<sup>+</sup> markers can be expressed from chromosomally integrated genes, depending on the establishment of Gal4 transcription factors dimerized by HPV E6 domains and putative E6 interacting cellular proteins expressed from the cDNA library. The Y190 cells were by an order of magnitude more transformation competent than CG1945 and used for this project, although it was necessary to inhibit the leaky His3 marker with 25 mM 3-AT (3-amino-1,2,4-triazole). Transformants were selected for Leu<sup>+</sup> and Trp<sup>+</sup> prototrophy (to select for the two vectors) and for His<sup>+</sup> prototrophy, to identify vectors with E6 interacting inserts. His<sup>+</sup> colonies were lifted on filter papers and examined for *LacZ* expression by X-Gal (5-bromo-4-chloro-3-indoyl-*b*-D-galactopyranoside) assays.

### In vitro binding assay

HPV-11 and HPV-16 E6 proteins were expressed in *E. coli* BL21 in the form of glutathione-S-transferase (GST) fusions after cloning the E6 genes into modified pGEX2TKP vectors (a derivative of the Pharmacia vector pGEX2TK with a new polylinker) as described (Zimmer-

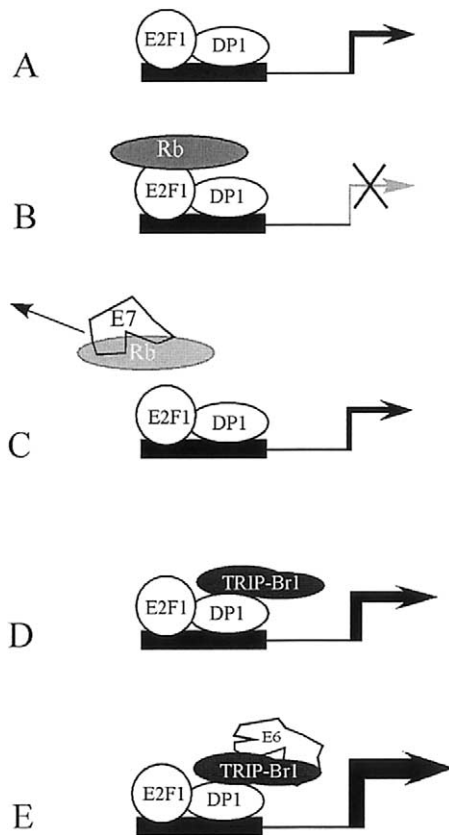


Fig. 6. Schematic representation of the effects of the HPV E6 and E7 oncoproteins on the function of the transcription factor E2F1/DP1. In the absence of coactivators, E2F1/DP1 has an inherent transcription activation function due to the activation domains of these two subunits (A). This activation function is suppressed by forming a complex with hypophosphorylated RB protein (B). The HPV oncoprotein E7 interferes with binding of RB and restores the activation function of E2F1/DP1 (C). The coactivator TRIP-Br1, possibly in cooperation with additional factors such as CBP/p300, hyperstimulates E2F1/DP1 activity (D). The HPV oncoprotein E6 has transcription coactivator function, as it further stimulates this function of TRIP-Br1 (E).

mann et al., 1999). The proteins were extracted with lysis buffer [50 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol (DTT), 150 mM KCl, 0.1% Triton X-100, and 10% glycerol] and, after sonication and centrifugation, stored at  $-80^{\circ}\text{C}$ . The lysates were incubated under rotation with glutathione-Sepharose (GSH) beads (Pharmacia) for about 3 h at  $4^{\circ}\text{C}$  in  $1\times$  NENT buffer (100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, Tris-HCl (pH 8.0)). After centrifugation and two washes with 1 ml of  $1\times$  NENT, the beads were loaded into a pipette tip containing a glass bead (BDH; catalog no. 332134Y) to create a  $40\ \mu\text{l}$  GST microaffinity column.

In vitro transcription and translation of [ $^{35}\text{S}$ ]methionine-labeled (Perkin-Elmer) TRIP-Br1, which was subcloned from the cDNA obtained in the pGAD10 vector in the form of a *Bam*HI and *Hind*III restriction fragment into the pXJ40-FLAG vector (Xiao et al., 1991), was based on TNT T7 rabbit reticulocyte kits (Promega). The C-terminal hMCM7

(residues 563 to 719) construct was subcloned in the form of *Bam*HI and *Hind*III restriction fragments into the pXJ40-FLAG vector (Zimmermann et al., 2000) and underwent the same procedure of in vitro transcription and translation as mentioned above. Briefly,  $40\ \mu\text{l}$  of a  $50\ \mu\text{l}$  IVT reaction mixture was diluted with  $360\ \mu\text{l}$  of binding buffer [50 mM KCl, 40 mM HEPES (pH 7.5), 1 mM DTT, 0.1% Tween 20, 0.5% milk powder] and passed through the GST microaffinity column. After the column was washed twice with  $200\ \mu\text{l}$  of wash buffer (binding buffer with 150 mM instead of 50 mM KCl), the GSH beads were resuspended with wash buffer, transferred to a fresh microcentrifuge tube, and pelleted by gentle centrifugation. Bound  $^{35}\text{S}$ -labeled proteins were dislodged from the beads with  $40\ \mu\text{l}$  of an SDS-containing loading buffer, heated to  $95^{\circ}\text{C}$  for 5 min, and centrifuged at 14,000 rpm for 30 s; the supernatant was resolved on a SDS-12% PAGE and visualized by autoradiography. The resulting bands after autoradiography were integrated by using the Bio-Rad densitometer software.

#### *In vivo binding assay*

The HPV-11 and -16 E6 genes were subcloned in the form of *Bam*HI and *Hind*III restriction fragments into the vector pXJ40-GST (Zimmermann et al., 1999) for expression as GST-tagged proteins. FLAG-tagged TRIP-Br1 proteins were expressed from the pXJ40-FLAG vector, as described above. 293T cells were grown in 10-cm dishes and transfected with  $2.5\ \mu\text{g}$  each of pXJ40 encoding the FLAG-tagged TRIP-Br1 and together with either pXJ40 encoding the GST-tagged HPV-11, 16 E6 genes, or GST alone, by using Superfect transfection reagent, in accordance with the manufacturer's recommendations (Qiagen, Hilden, Germany). Twenty-four hours after transfection, the cells were washed in phosphate-buffered saline (PBS) before being harvested in 0.5 ml lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 0.5% NP-40, 1 mM DTT, and "Complete EDTA-free" protease inhibitor cocktail (Roche)]. The extracts were rotated at  $4^{\circ}\text{C}$  for 30 min and centrifuged at 12,000 rpm for 10 min. The resulting lysates were incubated with  $50\ \mu\text{l}$  of GSH beads (washed twice with lysis buffer first) for 3 h at  $4^{\circ}\text{C}$  with rotation. After which, the samples were washed twice at  $4^{\circ}\text{C}$  in 0.5 ml lysis buffer, boiled at  $95^{\circ}\text{C}$  for 5 min in  $40\ \mu\text{l}$  SDS loading buffer, resolved by SDS-12% PAGE, blotted onto a nitrocellulose membrane, processed with mouse anti-FLAG monoclonal antibody (Upstate Biotech, US), and detected with the enhanced chemiluminescence (ECL) detection system (Amersham, Piscataway, NJ).

#### *Mutation of the LLG motif of TRIP-Br1 and in vitro interaction between TRIP-Br1 mutants and E6 proteins*

To examine the function of a leucine-leucine-glycine (LLG) motif in TRIP-Br1, we (i) substituted this motif by a trimeric alanine repeat (AAA) and (ii) deleted this sequence



completely. We obtained commercially 56 nucleotide primers encoding centrally the LLG to AAA substitution or deletion of LLG completely and targeted them to the TRIP-Br1 gene cloned in the pXJ40 FLAG vector. Products were generated by PCR with the ExSite Kit (Stratagene) using the Pfu DNA polymerase and the template DNA removed with *DpnI* before transformation into *E. coli* as described by the manufacturer. In vitro translation of wild-type TRIP-Br1, both mutant TRIP-Br1 proteins, and GST pull-down with GST, GST-tagged HPV-11, and 16 E6 proteins were performed as described earlier. After washing and elution of bound proteins, the samples were boiled at 95°C for 5 min with 40  $\mu$ l SDS loading buffer and centrifuged at 14,000 rpm for 30 s and the supernatant was loaded on a SDS–12% PAGE gel. The resulting bands after autoradiography were integrated by using the Bio-Rad densitometer software.

#### *Stability of TRIP-Br1 in the presence of E6 proteins*

TRIP-Br1, p53 and HPV-11 and 16 E6 proteins were translated in vitro as described above. To achieve equimolar concentrations, 5  $\mu$ l of [<sup>35</sup>S]methionine-labeled IVT TRIP-Br1 or p53 was mixed with 25  $\mu$ l of [<sup>35</sup>S]methionine-labeled IVT HPV-11 or -16 E6 protein and incubated at 30°C for up to 5 h. Five microliters of the reaction mixture was extracted at the start, at 3 h and at the end of 5 h of the reaction, stopped by adding 40  $\mu$ l of SDS loading buffer, and boiled at 95°C for 5 minutes. Twenty microliters of the mixture was then resolved by SDS–12% PAGE and analyzed by autoradiography.

#### *Transient transfection studies and luciferase assays*

The transcription activation function of TRP-Br1 and its stimulation by HPV E6 proteins was measured with the Dual-Luciferase Reporter Assay system (Promega) by transfecting COS-7 cells with various amounts of the indicated expression plasmids, i.e., pCMV-E2F1, pCMV-DP1, the firefly luciferase expression vector pGL3-TATA6x E2F, pXJ40-TRIP-Br1 (FLAG Tagged), and the control Renilla luciferase reporter plasmid, pRL-CMV, in the presence or absence of FLAG-tagged HPV-11 and -16 E6 expression vectors. pGL3-TATA6x E2F had been constructed by two of us (Yang, C.M., and Hsu, S.I., unpublished data). It contains six repeated E2F/DP1 binding sites.  $1 \times 10^4$  cells/well were seeded into 24-well plates 24 h before transfection, in DMEM medium supplemented with 10% fetal bovine serum and antibiotics, and transfected with the Polyfect reagent (Qiagen) in accordance with the manufacturer's instructions. The total amount of DNA transfected was kept constant at 1  $\mu$ g per well. The cells were harvested after 24 h by removal of the medium, washing with PBS, and adding 100  $\mu$ l of 1 $\times$  passive lysis buffer (Promega). The plates were rocked at 200 rpm for 30 min at room temperature, followed by a cycle of freeze/thaw lysis after 1 h at

–80°C. The activity of the firefly luciferase was measured using a Turner TD-20/20 luminometer as instructed by the manufacturer (Promega), i.e., 20  $\mu$ l of cell lysate was mixed with 100  $\mu$ l of luciferase assay substrate (Promega), and the luciferase activity was recorded as the mean of three independent transfections. The luciferase activity was standardized against the activity of the control Renilla luciferase reporter, pRL-CMV, which serves as the baseline response.

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