Virology 396 (2010) 47–58

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

HPV16 E6 augments Wnt signaling in an E6AP-dependent manner

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ARTICLE INFO

Article history:
Received 5 July 2009
Returned to author for revision
5 August 2009
Accepted 6 October 2009
Available online 6 November 2009

Keywords:
HPV
E6
E6AP
Wnt signaling
β-Catenin
TCF
GSK3β

ABSTRACT

In this study we investigated the effect of HPV16 E6 on the Wnt/β-catenin oncogenic signaling pathway. Luciferase reporter assays indicated that ectopically expressed E6 significantly augmented the Wnt/β-catenin/TCF-dependent signaling response in a dose-dependent manner. This activity was independent of the ability of E6 to target p53 for degradation or bind to the PDZ-containing E6 targets. Epistasis experiments suggested that the stimulatory effect is independent of GSK3β or APC. Coexpression, half-life determination, cell fractionation and immunofluorescence analyses indicated that E6 did not alter the expression levels, stability or cellular distribution of β-catenin. Further experiments using E6 mutants defective for E6AP binding and E6AP knockdown cells indicated the absolute requirement of the ubiquitin ligase E6AP for enhancement of the Wnt signal by E6. Thus, this study suggests a role for the E6/E6AP complex in augmentation of the Wnt signaling pathway which may contribute to HPV induced carcinogenesis.

Introduction

Infection of the anogenital mucosa with papillomavirus types referred to a "high risk" or oncogenic such as HPV16 and HPV18 is a major factor for the subsequent development of cancer (Zur-Hausen, 1996, 2002). HPV infection of the basal epithelium perturbs cell differentiation and enhances cell proliferation. Two oncoproteins are encoded by the oncogenic HPVs, E6 and E7, which are invariably expressed in HPV-positive cervical cancers and cancer-derived cell lines (Zur-Hausen, 1996, 2002; Narisawa-Saito and Kiyono, 2007). E6 and E7 contribute to the oncogenic process, at least in part, through their ability to interact with cellular tumor suppressor proteins and inactivate their functions. E7 associates with the tumor suppressor pRb and other cell cycle regulatory proteins that control cell cycle progression, whereas the E6 oncoprotein primarily associates with proteins participating in the control of genome stability or signaling pathways that involve cell–cell interactions and cytoskeleton organization (reviewed in Narisawa-Saito and Kiyono, 2007; Mammas et al., 2008; McLaughlin-Drubin and Munger, 2009; Howie et al., 2009). E6 is also responsible for transcriptional activation of the telomerase reverse transcriptase (TERT) gene which is the catalytic subunit of the enzyme telomerase (reviewed in Howie et al., 2009).

The Wnt signaling pathway plays an important role in the development of several types of cancer, particularly colorectal cancer (CRC; reviewed in Polakis, 2000; Lustig and Behrens, 2003; Giles et al., 2003; Kikuchi, 2003; Clevers, 2006). Abnormalities in the canonical Wnt/β-catenin pathway lead to tumor formation. The canonical Wnt signal pathway is controlled by two major regulatory multiprotein complexes; the β-catenin "destruction complex" that controls the level of β-catenin, a key effector protein of the canonical Wnt pathway, and the TCF transcription complex, comprised of various corepressors and coactivators, which controls the level of the TCF transcriptional activity (reviewed in Polakis, 2000; Lustig and Behrens, 2003; Giles et al., 2003; Kikuchi, 2003; Clevers, 2006; Mosimann et al., 2009; Willert and Jones, 2006). In the absence of Wnt signaling, intracellular β-catenin levels are regulated by a multiprotein complex encompassing kinases, such as the glycogen synthase kinase-3β (GSK3β) and casein kinase 1 and 2 (CK1 and 2), and the scaffolding proteins adenomatous polyposis coli (APC), Axin1 and Axin2 (conductin). This "destruction complex" binds and phosphorylates β-catenin at serine and threonine residues, thus targeting it for ubiquitination and proteolytic degradation. In the presence of Wnt ligands, coactivation of the Frizzled and LRP (low-density lipoprotein receptor-related proteins) receptors leads to inhibition of the destruction complex and the consequent stabilization of β-catenin. Intracellular β-catenin accumulation eventually results in its nuclear translocation where it binds to members of the TCF/LEF family of transcription factors, alters the association of TCF with corepressors such as Groucho/TLE and CtBP, interacts with coactivators such as the histone acetylase CBP and chromatin remodeling proteins such as Brg1, and activates transcription of various genes including cyclin D1, MYC, TCF-1 and others.

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doi:10.1016/j.virol.2009.10.011

Among various molecules involved in Wnt pathway, alterations in the β-catenin, APC, and Axin genes have been frequently found in several human cancers leading to stabilization and accumulation of β-catenin and increase of TCF-mediated gene expression (Lustig and Behrens, 2003; Giles et al., 2003; Kikuchi, 2003; Clevers, 2006). In addition to the canonical Wnt signal-mediated β-catenin stabilization, β-catenin expression is regulated also by other pathways via GSK3β-dependent or -independent mechanism (Liu et al., 2001; Matsuzawa and Reed, 2001; Sadot et al., 2001; Xiao et al., 2003; Nastasi et al., 2004; Sharma et al., 2004; Bachar-Dahan et al., 2006).

Several of the cellular targets of HPV E6 oncoproteins were associated with pathways acting in the control of β-catenin stability or activity including hDlg, hScrib, p53 and p300/CBP (reviewed in Mammas et al., 2008; Howie et al., 2009). Dlg binds to the carboxy-end of APC in a region shown previously to be required for the tumor suppressing activity of APC (Matsumine et al., 1996) and APC–hDlg complexes were shown to be important for the negative regulation of cell cycle progression (Ishidate et al., 2000; Matsumine et al., 1996). Human Scribble also interacts with APC (Takizawa et al., 2006). The tumor suppressor p53 was linked to both phosphorylation-dependent and -independent pathways that mediate β-catenin degradation (Liu et al., 2001; Matsuzawa and Reed 2001; Sadot et al., 2001), apparently through its ability to activate transcription of APC and Siah-1, whereas CBP/p300 were shown to act as bimodal regulators of Wnt signaling (Li et al., 2007).

The role of Wnt pathway in the development of cervical cancer is unknown, although recent reports provide evidence for a possible link. Mutations in β-catenin gene were found to be uncommon in cervical carcinoma (Ueda et al., 2001; Shinohara, 2001; Su et al., 2003), however abnormal expression of β-catenin was reported in several studies. Immunochemistry analysis of cervical tumors showed the accumulation of β-catenin within the cytoplasm and/or nuclei in a high percentage of these tumors (Shinohara et al., 2001; Pereira-Suarez et al., 2002; Rodriguez-Sastre et al., 2005; Uren et al., 2005). Abnormal β-catenin immunoreactivity was associated with advanced pathologic stages of adenocarcinoma (Imura et al., 2001) and changes in cadherins and β-catenin expression were shown to be associated with progression of squamous intraepithelial lesions in the cervical uterine (Rodriguez-Sastre et al., 2005; Uren et al., 2005). Microarray expression studies conducted on cervical cancer tissues identified alterations in the expression of positive and negative regulators of the Wnt signaling pathway (Shim et al., 1998; Perez-Pereira et al., 2007).

As HPV E6 oncoprotein interacts with several components acting in the Wnt–β-catenin pathway we hypothesized that E6 might affect Wnt signaling. To this end, we investigated the effect of HPV16 E6 expression on a β-catenin/TCF-dependent luciferase-based reporter. We present evidence that HPV16 E6 is able to augment β-catenin/TCF-dependent transcription induced by Wnt3a or β-catenin expression. We show that E6 mediated enhancement of β-catenin/TCF signaling is independent of GSK3β activity and phosphorylation of β-catenin and is not associated with major alterations in the levels, stability or cellular distribution of β-catenin. We also demonstrate that augmentation of TCF/β-catenin transcription by E6 is independent of the ability of E6 to target p53 and PDZ-containing proteins. However, it depends on the expression of the E3-ubiquitin ligase E6AP and ability of E6 to associate with E6AP, as well as on the activity of the proteasome system.

Results

Wnt-induced β-catenin/TCF-dependent transcription is enhanced by the E6 oncoprotein

The canonical Wnt signaling results in the stabilization of β-catenin, which forms active transcriptional regulation complexes with members of the TCF/LEF transcription factors. A well-established functional assay of Wnt signaling makes use of TCF-dependent luciferase reporter constructs containing several copies of the TCF-binding sites (TOPFLASH) or mutated TCF-binding sites (FOPFLASH) that is used as control (Korinek et al., 1997). The effect of HPV16 E6 on the canonical Wnt pathway was first examined in transient co-expression assays using HEK293T cells. Ectopic expression of E6 alone failed to induce a TCF-dependent signaling response (data not shown). However, addition of Wnt3a condition medium (CM; Fig. 1A) or coexpression of E6 with Wnt3a and HFz1 (human Frizzled 1; Fig. 1B–D) resulted in a dose-dependent enhancement of the Wnt3a-induced response reaching up to 5-fold. The stimulatory effect of E6 was also evident on a native TCF-responsive promoter, the cyclin D1 promoter, a Wnt target gene containing the TCF-binding domain within its transcriptional unit (Shtutman et al., 1999; Fig. 1B). E6 also caused a moderate elevation of the endogenous levels of cyclin D1 protein in addition to the elevation in the reporter activity (data not shown). Both, the native, wild-type HPV16 E6 and the AU1 epitope-tagged E6, stimulated the β-catenin/TCF-dependent transcription whereas HPV16 E7 caused no enhancement and even reduced β-catenin/TCF transcription (Fig. 1C). The specificity of the E6 effect on TCF-dependent transcription was demonstrated by the minimal signal readouts induced by the control plasmid superFOPFLASH (0.001–0.01 of superTOPFLASH; data not shown) and further confirmed by coexpression of a dominant-negative TCF4 protein that resulted in downregulation of the Wnt induced signal both in the vector and E6 transfected cells (Fig. 1D). Induction of Wnt signaling and augmentation by HPV16 E6 could be also demonstrated in other cells, besides HEK293T, including HEK293 that lack the SV40 T antigen, Madin-Darby, canine kidney (MDCK) cells (data not shown) and human C33A cervical carcinoma cells (Fig. 1E). Luciferase readouts in extracts of C33A cells were lower than in HEK293T, however, either transient transfection of E6 (Fig. 1E) or stable transduction of E6 (data not shown) augmented the TCF-dependent luciferase reporter activity. Consistent with the effect of HPV16 E6 on TCF-dependent transcription, elevated cyclin D levels were detected in several cervical carcinoma cell lines containing HPV including SiHa, CaSki and HeLa as compared to C33A that lack HPV (Fig. 2).

To determine whether E6 proteins of low-risk HPV types also enhance β-catenin/TCF transcription, we examined the activities of E6 proteins from high risk, HPV16 and HPV18, versus low risk, HPV11, in the luciferase reporter assay. The results (Fig. 1F) indicated that HPV16 E6 and to a lower extent, HPV18 E6, were capable of augmenting the TCF-luciferase reporter activity whereas HPV11 E6 only minimally enhanced luciferase activity. Hence, these studies demonstrate distinction in the ability of high and low risk HPV types to augment the Wnt signal as well as difference among high risk types, with HPV16 E6 being more potent than HPV18.

HPV16 E6 enhancement of β-catenin/TCF transcription is independent of p53 and the PDZ containing E6 targets

A well-known target of the high-risk E6s is p53. E6 could possibly mediate its stimulatory activity through p53 inactivation. Activity of the tumor suppressor p53 was linked to both pathways that regulate β-catenin destruction, the phosphorylation-dependent GSK 3β-mediated pathway (Sadot et al., 2001) and the phosphorylation-independent pathway mediated by Siah-1 (Liu et al., 2001; Matsuzawa and Reed, 2001). Elimination of p53 by E6 could theoretically lead to inhibition of β-catenin degradation resulting in increased cytoplasmic pools of β-catenin leading to activation of TCF/LEF-mediated transcription. To evaluate whether E6 effect is mediated by inactivation of p53, we compared the ability of a series of HPV16 E6 mutants to enhance TCF-dependent transcription and degrade p53. In addition, we tested the ability of HPV16 E6 to augment TCF/LEF activity in cells that lack p53. Analysis of a set of previously characterized E6 mutants (Sherman et al., 2002) indicated that a truncated E6 protein lacking 10 amino acids

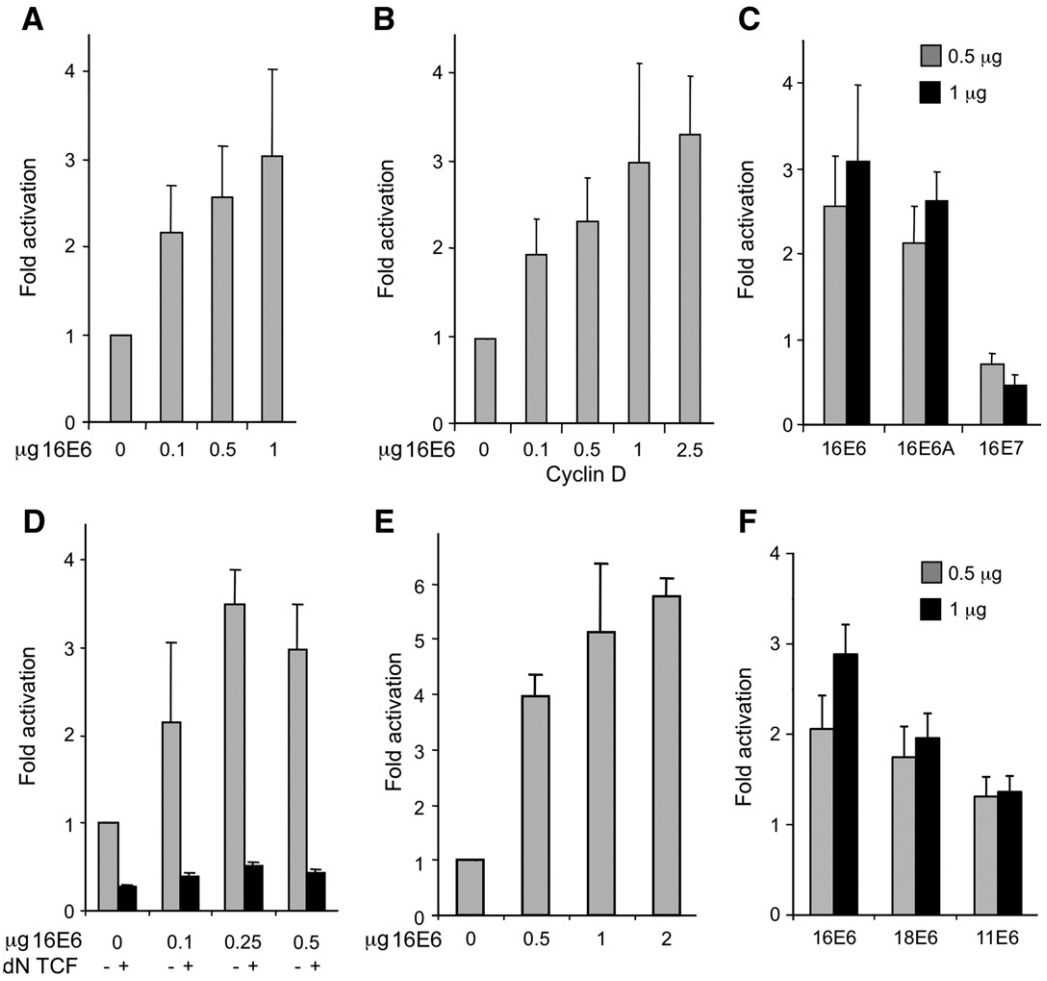

Fig. 1. β-Catenin/TCF-dependent transcriptional response is enhanced by the E6 oncoprotein. (A) HEK293T cells were transfected with the TCF-luciferase reporter superTOPFLASH (TOP) or the mutated TCF reporter superFOPFLASH (FOP), β-gal, and increasing amounts of the HPV16 E6 DNA as indicated. Wnt signal was induced by treating the cells with Wnt3a CM 24 h after transfection. (B) HEK293T cells were cotransfected with the Cyclin D luciferase reporter and β-gal, Wnt signal was induced by cotransfection of the HFz1 and Wnt3a plasmids. (C) HEK293T cells were cotransfected with Wnt3a and HFz1, the TOP/FOP luciferase reporter, β-gal and increasing amounts of the HPV16 E6, E6A or E7 DNA as indicated. (D) HEK293T cells were cotransfected as in C and in addition with a dominant negative TCF encoding plasmid (0.1 μg). (E) C33A cells were cotransfected with Wnt3a and HFz1, the TOP/FOP and Renilla luciferase reporters, and increasing amounts of the 16E6 DNA. (F) HEK293T cells were transfected with the TOP/FOP luciferase reporter, β-gal and two amounts of the 16E6, 18E6 or 11E6 plasmid DNA. Cells were treated with Wnt3a CM to induce the signal. In all reporter assays luciferase activity was determined 48 h after transfection The readouts obtained with the mutated TCF luciferase reporter (FOP; not shown, extremely low) were subtracted from the readouts obtained with the TOP reporter. Data are presented as fold activation (readouts obtained in the E6 expressing cells relative to the readouts in the vector expressing cells which were presented as 1). Fold activation values obtained by E6 or E7 from three to five independent experiments (mean ± SD) are shown. The increases in signal (fold activation) induced by E6 (0.1–2.5 μg) are statistically significant ($P<0.05$).

from the carboxy-terminus (M141) was active in stimulation of transcription and even induced higher levels of transcription, whereas the E6 truncation mutant lacking 19 amino acids (M132) failed to stimulate Wnt3a-induced β-catenin/TCF transcription (Fig. 3A).

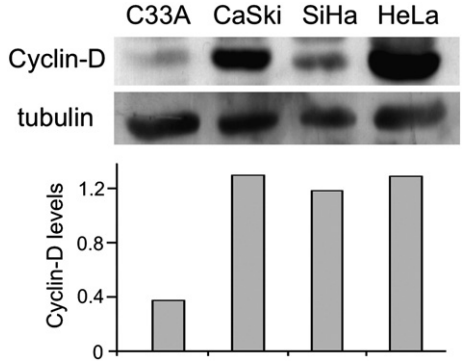

Fig. 2. Cyclin D protein levels in different cervical carcinoma cell lines. Western blot analysis of cyclin D protein levels in cell lysates from the indicated cervical carcinoma cell lines (100 μg). Tubulin was used as a loading control.

Similarly, mutants containing small deletions within the putative carboxy-terminal zinc-finger failed to enhance Wnt/TCF transcription whereas mutant G136C containing substitution of glycine in place of cysteine in amino acid 136 exhibited reduced activity (Fig. 3A). These results suggested that integrity of the zinc-finger structure may possibly be necessary for the E6 stimulatory function in Wnt/TCF transcription. The results also indicated that the ability of E6 to augment Wnt/TCF signaling is independent of its p53 degradation activity (Fig. 3B). Supporting this conclusion is the fact that mutant d118-122, previously demonstrated (Sherman et al., 2002) and in this study (Fig. 3B) to be active in p53 degradation, was unable to stimulate TCF transcription. Furthermore, E6 also enhanced TCF-dependent transcription in the lung carcinoma H1299 cell line lacking p53 (Fig. 3C). These findings were further confirmed by the enhancing effect of E6 on the Wnt signal induced in C33A cervical cancer cells (Fig. 1E and data not shown), SW480 and colo320 colon carcinoma cell lines (described below), all containing mutated p53, as well as in HEK293T cells which lack a functional p53 protein. These results also indicated that enhancement of the β-catenin/TCF transcription is not mediated via binding of E6 to the PDZ motives in the PDZ-containing partners (reviewed in Mammas et al., 2008; Howie et al., 2009), as

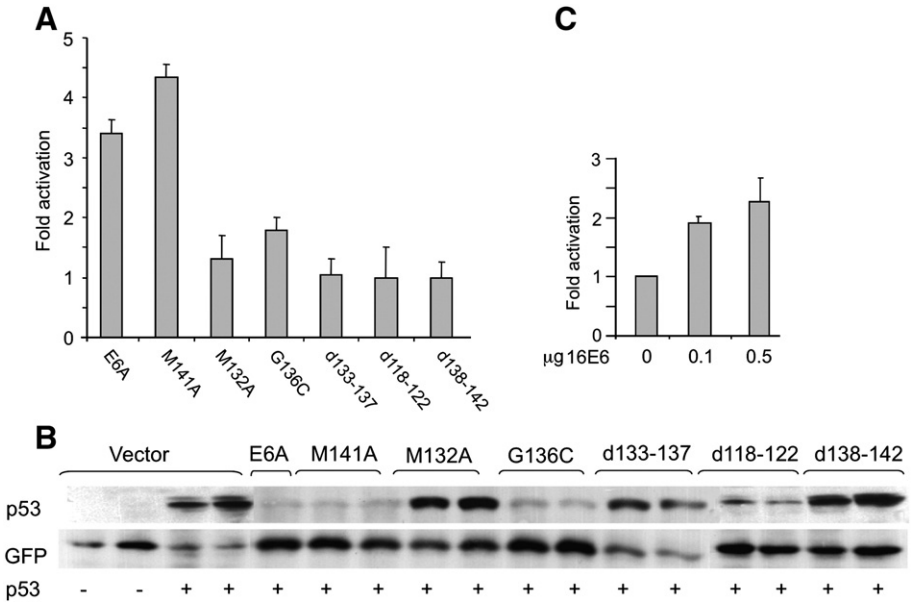

Fig. 3. Abilities of HPV16 E6 mutants to enhance TCF/β-catenin-dependent transcription and induce p53 degradation. (A) HEK293T cells were cotransfected with the TOP/FOP luciferase reporter, β-gal and HPV16 E6 or mutant plasmid (0.5 µg), as indicated. Wnt signal was induced by cotransfection of HFz1 and Wnt3a. Luciferase activity was determined 48 h posttransfection. Fold activity, average and SD, from at least three assays are shown. (B) H1299 cells were transfected with the plasmids encoding p53 (1 µg), HPV16 E6 or mutant E6 (0.5 µg) and GFP (0.5 µg). Total cell lysates were prepared 48 h after transfection and levels of residual p53 were determined in a Western blot assay. (C) H1299 cells were transfected with DNA of the TOP/FOP and Renilla luciferase reporters together with the indicated amounts of the 16E6 DNA. Wnt signal was induced by addition of Wnt3a CM to the cells 24 h after transfection. Data of fold activation from three assays (mean ± SD) are shown.

mutant M141 lacking the PDZ binding motif was capable of enhancing β-catenin/TCF transcription. This effect of M141 was exhibited in assays where Wnt signal was induced by Wnt3a and HFz1, Wnt3a CM or wild-type β-catenin (Fig. 3A and data not shown).

Enhancement of the TCF/β-catenin signal by HPV16 E6 does not depend on the APC/Axin/GSK3β complex

Cellular β-catenin levels are tightly controlled mainly by the APC/GSK3 β/Axin complex (the β-catenin degradation complex). E6 protein could possibly enhance the Wnt signal by interfering with the action of the β-catenin degradation complex. To test this possibility, we evaluated the effect of HPV16 E6 in cells where the activity of GSK3β was inhibited and in cells containing a mutated APC or β-catenin protein in which the Wnt signal is constitutively activated. Treatment of HEK293T cells with LiCl, an inhibitor of GSK3β, induced high signaling response that was further enhanced by ectopic expression of E6 in a dose-dependent manner. Fold enhancement by E6 was comparable to that obtained in cells where Wnt signaling was induced by transfection of the Wnt3a/HFz-1 plasmids (Fig. 4A). E6 also enhanced TCF transcription in SW480 cells containing mutant APC protein that is deficient in its ability to promote β-catenin degradation (Munemitsu et al., 1995; Fig. 4B). Similar results were obtained in coexpression assays using colo320 colon carcinoma cells that contain a different APC mutant (Rosin-Arbesfeld et al., 2003; Fig. 4C). These results indicated that E6 enhancement of TCF/β-catenin-dependent transcription is independent of GSK3β or APC.

Furthermore, we tested the effect of HPV16 E6 on the transcriptional response induced by a mutant β-catenin, S33Y, that is relatively refractory to GSK3β mediated phosphorylation and hence to β-TrCP degradation (Hart et al., 1999). Results showed that the signal induced by the mutant β-catenin was higher than the signal induced by the wild type β-catenin, however, the enhancement of transcription by E6 was at comparable levels (Fig. 5A). Consistent with this result, expression of E6 in a colon carcinoma cell line containing mutant β-catenin with deletion of serine 45 (HCT116; Morin et al., 1997) enhanced β-catenin/TCF transcription (Fig. 5B). Taken together, these results indicate that E6-mediated enhancement of β-catenin/TCF-dependent transcription is independent of the APC/GSK3β/Axin complex activity.

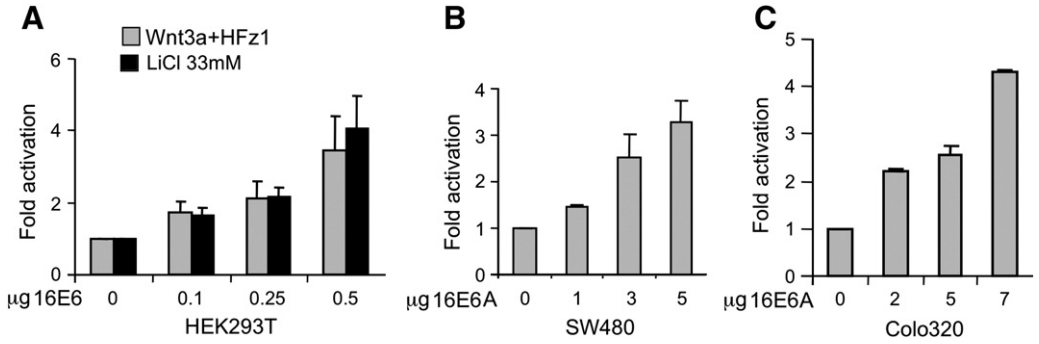

Fig. 4. E6 enhancement of the Wnt signal is independent from the β-catenin degradation complex. (A) HEK293T cells were transfected with increasing amounts of HPV16 E6 DNA and DNA of the TOP/FOP and β-gal reporters. Cells were either treated with LiCl or cotransfected with Wnt3a and HFz1 to induce the Wnt signal. Luciferase activity was measured 48 h after transfection. Average and SD of fold activation from three experiments are shown. (C, D) The indicated cells were transfected with increasing amounts of HPV16 E6 DNA and DNA of the TOP/FOP and Renilla reporter plasmids. Luciferase activity was measured 48 h after transfection. Data of fold activation from at least three assays (mean ± SD) are shown.

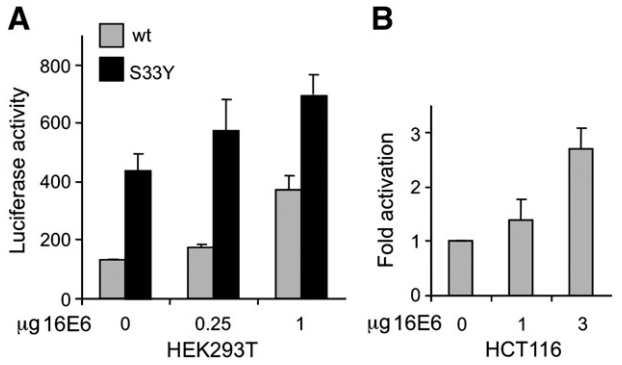

Fig. 5. TCF-dependent transcriptional response induced by mutant β-catenin is enhanced by HPV16 E6. (A) HEK293T cells were cotransfected with wt or mutant (S33Y) β-catenin DNA (1 μg), increasing amounts of the 16E6 DNA and DNAs of the TOP/FOP luciferase and β-gal reporters. Luciferase activities were measured 48 h after transfection. Average and SD of luciferase activity from three experiments are shown. (B) HCT116 cells were transfected with the TOP/FOP and Renilla luciferase reporters together with the indicated amounts of HPV16 E6. Luciferase activity was evaluated 48 h after transfection. Average and SD of fold activation from three experiments are shown.

HPV16 E6 does not significantly alter the expression levels or stability of β-catenin

Cellular β-catenin levels are also controlled by other mechanisms, via GSK3β-dependent or -independent mechanism (Liu et al., 2001; Matsuzawa and Reed, 2001; Sadot et al., 2001; Xiao et al., 2003; Nastasi et al., 2004; Sharma et al., 2004; Bachar-Dahan et al., 2006) and therefore we examined whether the activity of E6 is mediated by changes in β-catenin expression levels or stability. Initially, we determined the effect of ectopic expression of E6 on levels of β-catenin in HEK293T cells. As HEK293T cells are efficiently transfected, we expected to be able to detect the effect of the transfected E6 on the endogenous levels of β-catenin. In these experiments we used antibodies detecting all forms of β-catenin or a specific antibody which binds to the unphosphorylated N-terminus of β-catenin, thus recognizing the active pool of the protein (Hendriksen et al., 2005). Western blot analyses revealed no significant changes in levels of the total or active cellular β-catenin in cells where β-catenin/TCF transcription was enhanced by HPV16 E6 in a dose-dependent manner (Fig. 6A, B). Similar results were obtained in additional experiments where levels of endogenous or exogenous β-catenin were examined including HEK293T cells transfected with the GFP-16E6 expression vector, C33A cells stably transduced with 16E6 or HEK293T cells cotransfected with E6 and a GFP-β-catenin expression plasmid (data not shown).

Next, the ability of HPV16 E6 to alter β-catenin stability was assessed using half-life determination assays. The degradation rate of β-catenin following cycloheximide (80 μg/ml) addition was determined in E6/vector transfected HEK293T cells. The results indicated that levels of β-catenin gradually decreased following cycloheximide addition with similar rates in E6 and vector cells. After 4 h, β-catenin levels were reduced to about 70% both in E6 and vector transfected cells (Fig. 6C). Similar results were obtained in experiments where ectopically expressed GFP-β-catenin was coexpressed with E6 or vector. At 6 h, there was a 50% reduction in the ectopically expressed GFP-β-catenin with no significant variation between the E6 and vector transfected cells (data not shown). These data suggested that HPV16 E6 protein does not significantly alter the expression levels or stability of β-catenin. Consistent with these data, no major differences in the levels of β-catenin were observed between HPV-positive and HPV-negative carcinoma cell lines (Fig. 6D).

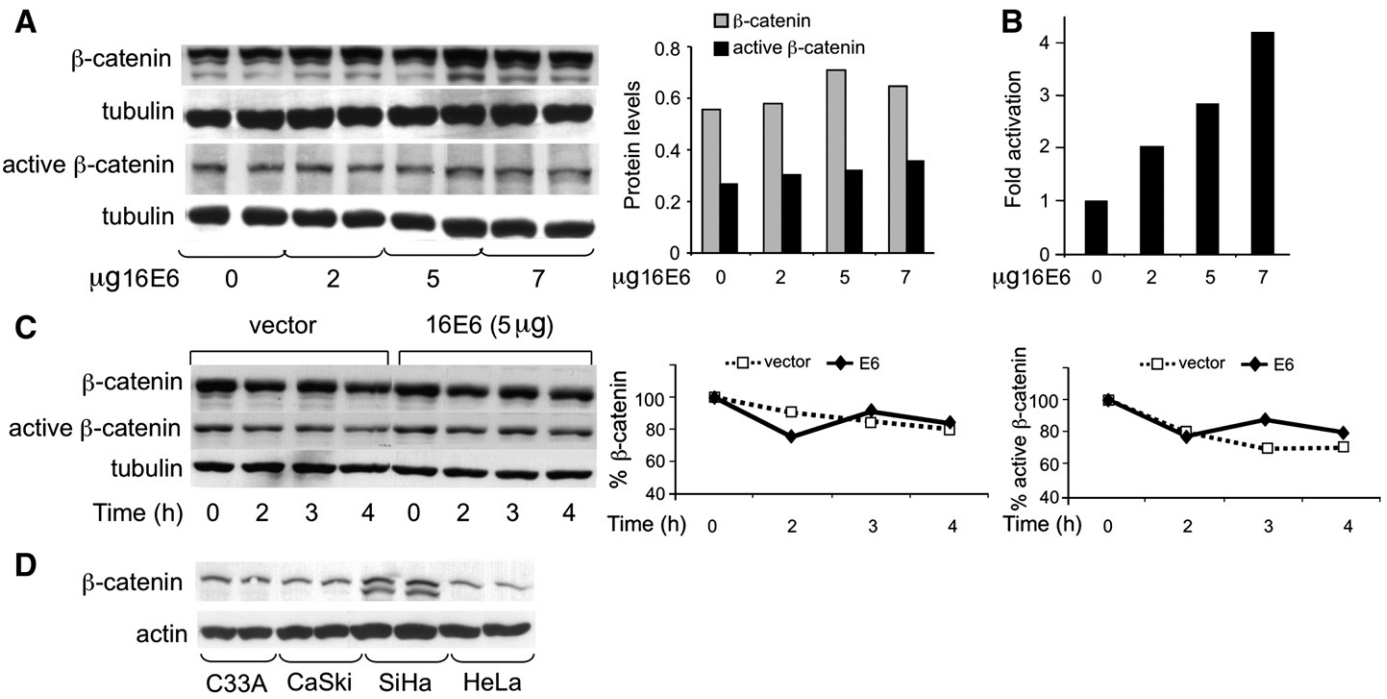

Fig. 6. HPV16 E6 does not alter the level or stability of the total or active pool of endogenic β-catenin. (A) HEK293T cells were cotransfected with Wnt3a and HFz1 and increasing amounts of HPV16 E6. At 48 h after transfection cells were harvested and β-catenin levels were measured in the total cell lysates by Western blot assay using specific antibodies against β-catenin and unphosphorylated (active) β-catenin. Tubulin was used for loading control. (B) Luciferase reporter activity was measured in the same cells by transfection of the TOP/FOP luciferase plasmids. (C) HEK293T cells were transfected with the 16E6 expression plasmid (5 μg) or vector. At 48 h after transfection, cycloheximide (80 μg /ml) was added. Cells were harvested at the indicated time points. Equal protein samples of total cell lysates (100 μg) were analyzed by SDS-PAGE and immunoblotting. A representative blot is shown. (D) Western blot analysis of β-catenin protein levels in the indicated cervical carcinoma cell lines. 100 μg protein from total cell lysates was analyzed. Actin was used as a loading control.

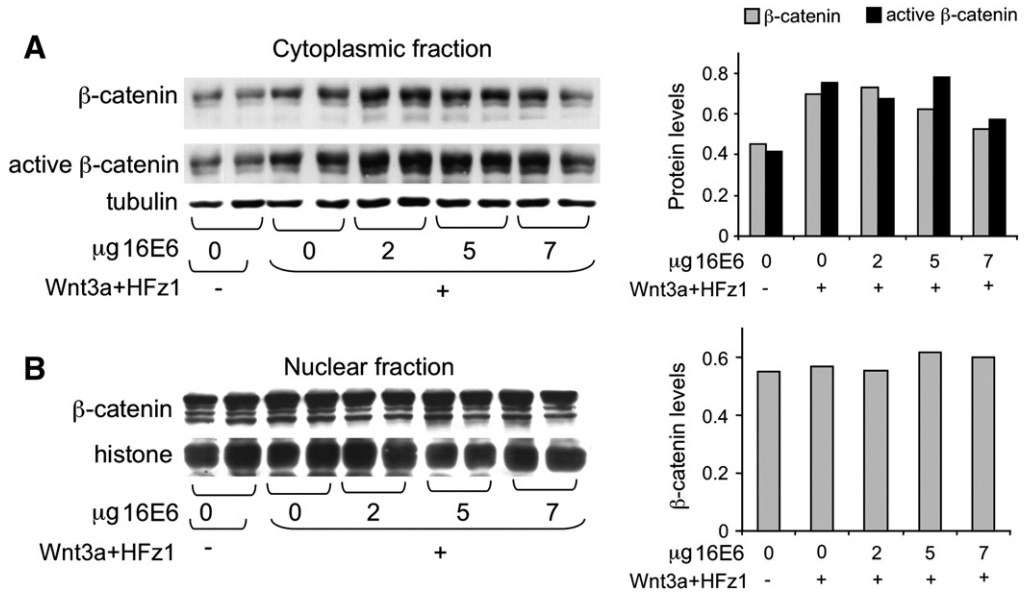

Fig. 7. HPV16 E6 does not alter the cytoplasmic or nuclear distribution of β-catenin. HEK293T cells were transfected with increasing amounts of E6. Wnt signal was induced by transfection of Wnt3a and HFz1. At 48 h after transfection the soluble cytoplasmic (A) or nuclear (B) fractions were isolated and levels of β-catenin or active β-catenin were evaluated by Western blot analysis. Representative blots and densitometric scans from the cytoplasmic (A) and nuclear (B) fractions are shown.

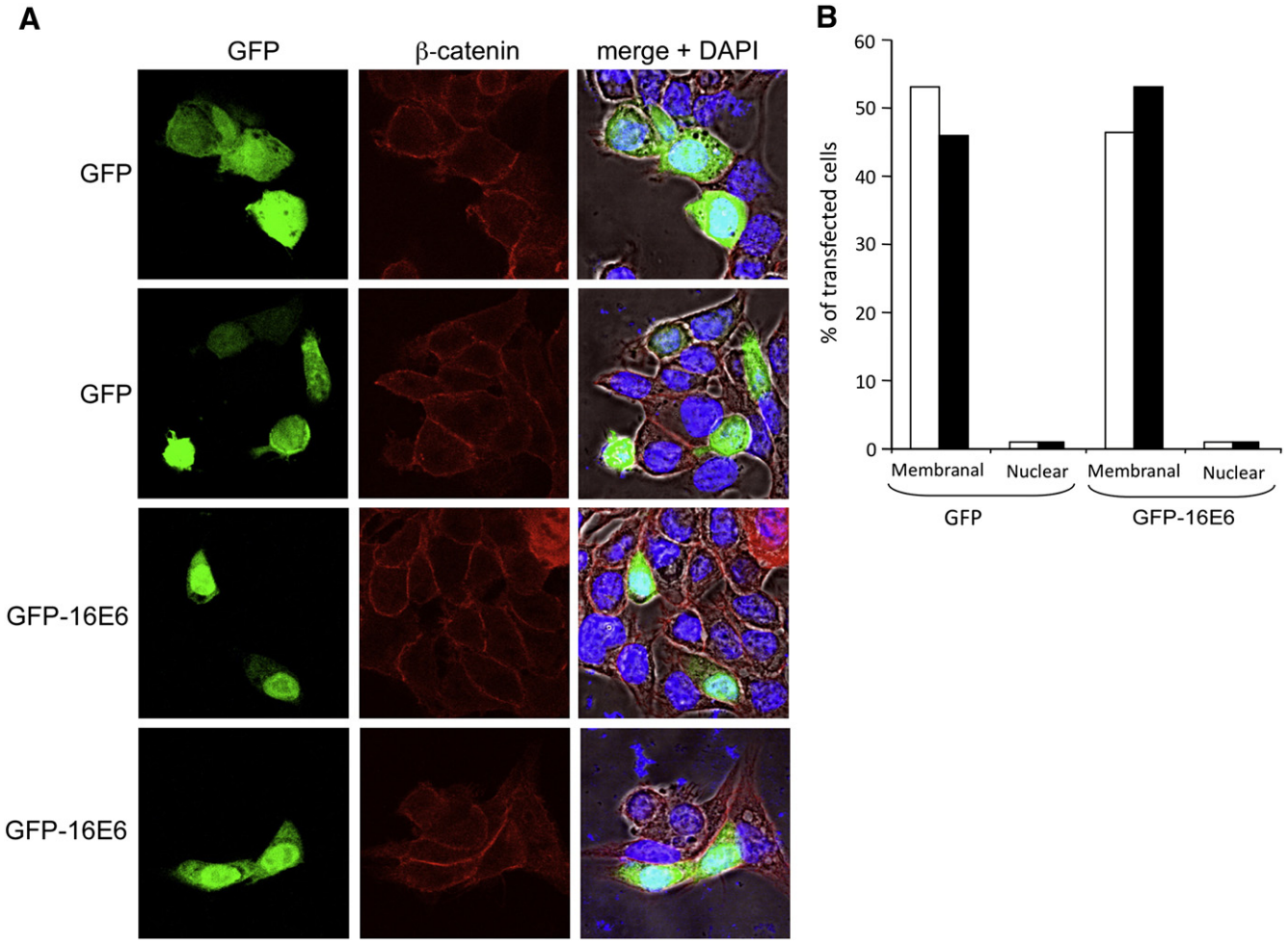

Fig. 8. β-Catenin localization in HPV16 GFP-E6 expressing cells. HCT116 cells were transiently transfected with plasmids encoding the GFP-16E6 or GFP using the jetPEI transfection reagent. At 48 h posttransfection, cells were subjected to immunofluorescence analysis with the anti-β-catenin antibody. The cells were also stained with DAPI to visualize nuclei (blue color in overlay image, right panels). Cells were visualized by confocal microscopy (A). The percentage of cells showing week or strong staining of β-catenin in the membrane or nuclei is shown in B (at least 30 transfected cells were analyzed in each slide).

Effect of HPV16 E6 on subcellular localization of β-catenin

The canonical Wnt signal is induced when unphosphorylated β-catenin accumulates in the cytoplasm and translocates to the nucleus. Thus, E6 could have possibly enhanced transcription by specifically increasing the soluble cytoplasmic or nuclear pool of β-catenin. The effect of E6 on cellular distribution of β-catenin was evaluated by cell fractionation and immunofluoresence. HPV16 E6 was transfected into HEK293T and 48 h posttransfection cells were fractionated and subcellular distribution of the endogenous β-catenin was analyzed by Western blot. Levels of cytoplasmic or nuclear β-catenin were determined relative to compartment specific protein markers, tubulin and histone, respectively (Fig. 7A, B). Transfection of the Wnt3a and HFz1 encoding plasmids caused moderate increase in β-catenin levels. However, no difference in the levels of β-catenin could be observed in the soluble (phosphorylated and nonphosphorylated; Fig. 7A) or nuclear (Fig. 7B) fractions between cells transfected with E6 or empty vector. In the same cells, enhancement of the Wnt signal by E6 was clearly indicated in the reporter assay (data not shown). Similar results were obtained in cells treated with the Wnt3a CM (data no shown). The effect of E6 on cellular localization of β-catenin was further investigated employing immunofluorescence analyses using the HPV16 GFP-tagged E6 protein (GFP-16E6) and the GFP expression vector as a control. The endogenous β-catenin was detected by immunohistochemistry. HCT116 cells transfected with GFP-16E6 showed strong nuclear staining of E6 with less intensity in the cytoplasm, unlike cells transfected with the GFP vector that showed strong GFP staining throughout the cell (Fig. 8A). These results indicated that E6 promoted the nuclear localization of GFP. In both cells, β-catenin staining was membrane associated. Nuclear expression of β-catenin was occasionally detected. The number of cells with membrane or nuclear staining in the GFP-E6 and controls was similar (Fig. 8B). Thus, consistent with the fractionation studies, E6 did not cause major changes in the subcellular localization of β-catenin.

HPV16 E6 enhancement of TCF/β-catenin transcription depends on E6AP and activity of the proteasome system

Previous studies indicated that E6AP is involved in mediating many E6 functions that impact on the global transcriptional program of HPV-positive cell lines (Kelley et al., 2005; Beaudenon and Huibregtse, 2008). Activation of telomerase by HPV16 E6 was reported to depend on E6AP (Gewin et al., 2004; Liu et al., 2005; James et al., 2006). To evaluate the role of E6AP in the E6 mediated augmentation of the TCF-dependent transcription, we tested the ability of E6 to enhance TCF reporter activity in U2OS cells expressing inducible E6AP shRNA (Fig. 9). The shRNA in these cells was induced by pretreatment with 0.2 μg/ml doxycycline for 72 h. Luciferase reporter assays indicated the ability of E6 to augment Wnt induced luciferase activity in doxycycline-untreated U2OS cells. However, the enhancement was significantly reduced upon depletion of E6AP in cells treated with doxycycline (Fig. 9A), indicating that the E6 enhancing activity depends on E6AP expression. Consistent with these results, cotransfection of the E6AP expression vector together with E6 into HEK293T cells increased the signal induced by Wnt3a and E6 alone in an E6 and E6AP dose-dependent manner (Fig. 9B, C). Moreover, reporter assays carried out with primary human keratinocytes stably transduced with HPV16 E6HA or vector indicated that

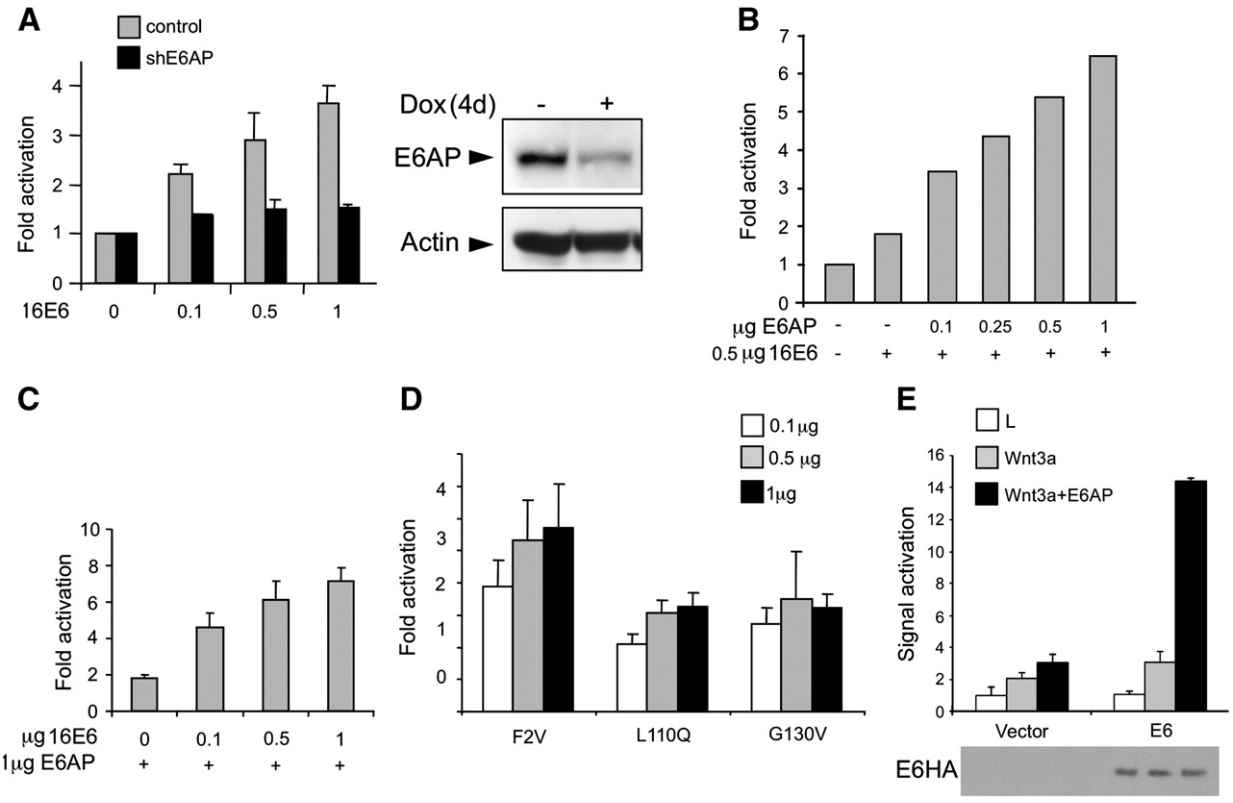

Fig. 9. Enhancement of the Wnt signal by HPV16 E6 is E6AP dependent. (A) U2OS cells expressing inducible shE6AP were either treated or not treated with doxycycline for 72 h. Cells were cotransfected with Wnt3A, HFz1, the TOP/FOP and Renilla luciferase reporters, and increasing amounts of E6. Luciferase activity was measured after 48 h. The levels of E6AP protein were analyzed by Western blot assay. (B–D) HEK293T cells were transfected with DNA of Wnt3a, HFz1, the TOP/FOP luciferase reporter, β-gal and the indicated amounts of HPV16 E6 and E6AP (B, C) or E6 mutant (D). (E) Primary human keratinocytes stably transduced with 16E6HA or vector were transfected using the jetPEI transfection reagent with the TOP/FOP and Renilla luciferase reporters, E6AP or pcDNA (1 μg). Wnt3a CM or control L medium was added 24 h after transfection. Luciferase reporter activity was evaluated 48 h posttransfection. Expression of E6HA was analyzed by Western blot. Data representing average and SD of fold activation (C, D) or luciferase activity (signal activation) (E) from at least three experiments are shown. A representative assay out of two is shown in B.

treatment with Wnt3a CM induced moderate signaling response that was about 2-fold higher in the E6 expressing cells. Cotransfection of E6AP caused marked elevation of the reporter signal in the E6 expressing cells with only small increase in the vector control, thus indicating the requirement of the E6/E6AP complex for augmentation of the Wnt signal in human keratinocytes (Fig. 9E).

In further assays using HEK293T cells, we tested the activity of E6 mutants. Functional analysis of E6 mutants previously characterized for their ability to interact with E6AP (Liu et al., 1999; Sekaric et al., 2008) indicated that mutant F2V, identified as capable of association with E6AP, was able to enhance Wnt3a induced transcription, whereas mutants L110Q and G130V, identified as defective in E6AP binding capacity, showed significantly reduced ability to enhance TCF/β-catenin transcription (Fig. 9D). These results further support the requirement of E6AP in the E6 mediated enhancement of TCF/β-catenin transcription.

As E6/E6AP complex acts in the ubiquitination of certain cellular proteins that are subsequently targeted to proteasomal degradation, we investigated whether E6/E6AP mediated enhancement of the Wnt signal depends on the activity of the proteasome. HEK293T cells were transfected with plasmids encoding the E6 and E6AP proteins. Cells were treated with Wnt3a CM and 2 μM MG132 or DMSO (Fig. 10). In the DMSO-treated control cells Wnt3a induced significant elevation in the reporter activity which was further augmented by E6, E6AP, and most prominently, E6 and E6AP. An increase in the transcription signal, up to 20-fold above the signal induced by Wnt, was demonstrated (Fig. 10A). Wnt3a also increased the protein levels of β-catenin with no further amplification upon addition of E6, E6A or both (Fig. 10B). Treatment of cells with the proteasome inhibitor MG132 induced high Wnt signal response that was slightly elevated in cells treated with the Wnt3a CM (Fig. 10A). Signal induction by MG132 was associated with an elevation in the levels of the β-catenin protein, likely due to inhibition of its proteasomal degradation (Fig. 10B). Expression of E6, E6AP or both did not further increase the luciferase reporter activity (Fig. 10A) or the levels of β-catenin (Fig. 10B). These results indicated that E6/E6AP function in Wnt signal elevation depends on the proteasome activity and is not mediated through alteration of β-catenin levels.

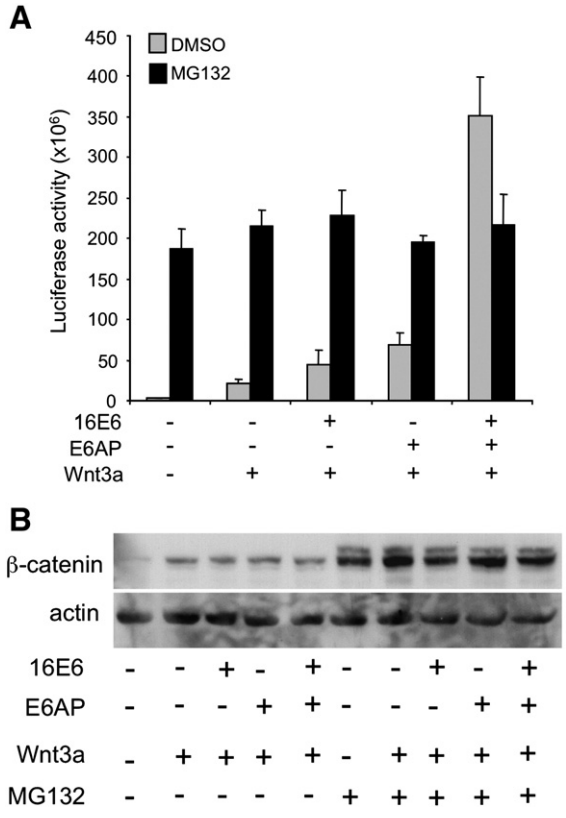

Fig. 10. Enhancement of the TCF/β-catenin-dependent transcription by HPV16 E6 depends on the proteasome activity. (A) HEK293T cells were transfected with the TCF-luciferase reporter, β-gal, and E6 (0.5 μg) or/and E6AP (1 μg) as indicated. At 24 h after transfection cells were treated with Wnt3a CM. At the same time MG132 (2 μM) or DMSO was added to the medium. Luciferase reporter activity was measured 48 h posttransfection. Average and SD of at least three experiments are shown. (B) 100 μg from the total cell lysates were analyzed by Western blot. β-Catenin levels were detected with the anti-β-catenin antibody. Actin was used as a loading control.

Discussion

The oncogenes E6 and E7 encoded by the high-risk HPVs directly contribute to cancer progression. A number of cellular pathways with which these proteins interact have been elucidated already; however, it is clear that others have yet to be identified (Zur-Hausen, 2002; Narisawa-Saito and Kiyono, 2007). The role of the Wnt–TCF signaling pathway in the development of cervical cancer has not been elucidated although recent reports provide evidence on deregulation of this pathway in cervical cancers which are associated in the majority with HPVs (Imura et al., 2001; Shinohara et al., 2001; Pereira-Suarez et al., 2002; Rodriguez-Sastre et al., 2005; Uren et al., 2005; Perez-Pereira et al., 2007).

The present study demonstrates for the first time that HPV16 E6 enhances the canonical Wnt–TCF signaling, thus providing a possible mechanistic link between HPV and the Wnt pathway in cervical cancers. Previous studies showed that activation of the canonical Wnt signal in HPV immortalized keratinocytes induced their malignant transformation while Wnt signal activation in cells absent of HPV did not (Uren et al., 2005). E6 ability to augment Wnt signaling could be one of the possible ways by which HPV oncoproteins may contribute to promote transformation of human keratinocytes.

Reporter assays described herein indicated that HPV16 E6 by itself could not induce TCF-β-catenin-dependent transcription, however, expression of E6 in cells where Wnt signal was induced by treatment with Wnt3a CM, transfection of HFz1 and Wnt3a or transfection of β-catenin, resulted in a dose-dependent enhancement of the Wnt-induced signal. This activity was exhibited by the high-risk HPV16 E6 and to a lower extent, by HPV18 E6, but only minimally by the low-risk HPV11 E6. The enhancement was specific to the TCF-dependent promoter as indicated by the minimal signal values induced upon transfection of the mutated TCF-luciferase plasmid (FOP) and by the inhibitory effect of the plasmid encoding a TCF-dominant negative protein, whose expression abolished signal activation as well as enhancement by E6. Induction of the Wnt signal and its augmentation by HPV16 E6 was reproduced in various cell types, including human epithelial cells from the cervix (C33A cervical carcinoma cell line) and primary foreskin keratinocytes the target cells of HPV infection. E6 activity was evidenced using artificial as well as native (cyclin D1) TCF-dependent promoter constructs. HPV16 E6 also increased cyclin D1 protein levels in cells where Wnt signal was induced (data not shown). Consistent with this finding high levels of cyclin D1 expression were observed in cervical carcinoma cell lines containing HPV (Fig. 2). These results are in agreement with a recent report demonstrating an association between cytoplasmic and nuclear expression of cyclin D1 and presence of HPV in premalignant lesions and cervical cancer (Carreras et al., 2007).

Numerous studies have shown that the Wnt signaling pathway is activated in virally associated cancers, including cancers induced by herpes and hepatitis viruses (reviewed in Hayward et al., 2006; Levrero, 2006). Various viral oncoproteins were shown to activate Wnt signaling through stabilization of β-catenin using different mechanisms (Hayward et al., 2006; Levrero, 2006). Results of epitasis experiments presented herein, based on TCF-luciferase reporter assays, suggested that E6 acts downstream of the GSK3β/APC/Axin

degradation complex and that its enhancing effect may not be associated with β-catenin stabilization. HPV16 E6 induced a similar boost in Wnt signal in cells where GSK3β activity was inhibited by LiCl or in cells transfected with a mutant β-catenin resistant to GSK3β/APC complex induced phosphorylation. In addition, signal enhancement by E6 was exhibited in colon carcinoma cell lines containing mutated APC or β-catenin, refractory to GSK3β induced phosphorylation (Figs. 4 and 5). Additional results gathered from coexpression and half-life determination assays indicated that E6 also did not significantly alter the expression levels or stability of β-catenin (Fig. 6).

β-Catenin is presented in cells in two pools exerting different functions. The membrane E-cadherin-bound β-catenin is involved in cell–cell adhesion whereas the cytoplasmic β-catenin is transported to the nucleus to activate Wnt target genes (Lustig and Behrens, 2003; Giles et al., 2003; Kikuchi, 2003; Clevers, 2006). As HPV16 E6 was previously shown to reduce the levels of E-cadherin in primary keratinocytes (Matthews et al., 2003), we assumed that E6 may alter the cellular distribution of β-catenin. However, results from our fractionation experiments with transfected HEK293T cells (Fig. 7) and immunofluorescence assays using HCT116 colon carcinoma cells (Fig. 8) did not support this assumption. We observed no major alteration in the soluble cytoplasmic or nuclear levels of β-catenin (Fig. 7) nor did we detect alteration in the ratios of membrane/nuclear staining of β-catenin in E6 expressing cells (Fig. 8). Thus, our studies indicate that different from mechanisms attributed to herpes, polyoma and hepatitis virus oncoproteins (Hayward et al., 2006; Gan and Khalili, 2004; Levrero, 2006), upregulation of β-catenin and its relocation to the nucleus may not be involved in the HPV16 E6 mediated enhancement of TCF-β-catenin transcription.

The mechanism by which HPV16 E6 promotes Wnt signaling needs further investigation. However, data presented herein indicate that this mechanism is independent from p53 and from the PDZ-containing E6 targets (reviewed in Mammas et al., 2008; Howie et al., 2009). Augmentation of the Wnt signal was clearly demonstrated by an HPV16 E6 truncation mutant (M141), which lacks the PDZ binding domain and could be reproduced by E6 in cell lines lacking p53 (H1299) or in cells where p53 activity is compromised (C33A, SW480, Colo32). Results from analysis of a series of HPV16 E6 mutants (Fig. 3) further supported this notion, indicating that the ability of E6 to induce degradation of p53 is insufficient for signal activation.

Our data point, however, to the requirement of the E3 ubiquitin ligase E6AP and the activity of the proteasome system for the E6 transcriptional function. Augmentation by E6 could not be induced in U2OS cells where E6AP was depleted by shE6AP expression or by E6 mutants defective for the E6AP binding function (Fig. 9). Moreover, coexpression of E6AP and E6 induced much higher increase of the Wnt signal, activity which was completely abolished in cells where the proteasome system was inhibited by MG132 (Fig. 10). Difference in E6AP binding could also explain the differences observed among E6 proteins of various HPV types in augmentation of the Wnt signal (Fig. 1F). HPV16 E6 that exhibited the highest ability to augment Wnt signaling was shown to bind more strongly to E6AP as compared to HPV18, whereas HPV11 has only minimal levels of binding to E6AP (reviewed in Howie et al., 2009). E6AP is hijacked by E6 to target several proteins for ubiquitin-dependent degradation. Known substrates of the E6/E6AP complex include the tumor suppressor p53, several PDZ domain proteins, E6 TPi, MCM7, Bak and the transcriptional repressor of hTERT gene expression, NFX1-91 (reviewed in Beaudenon and Huibregtse, 2008; Howie et al., 2009). The level of NFX1-91 is reduced in HPV16 E6 expressing cells as well as its occupancy at the hTERT promoter (Gewin et al., 2004; Xu et al., 2008). Thus, E6/E6AP could activate the Wnt pathway by targeting a yet unidentified regulator that suppresses Wnt signaling in the cytoplasm or more likely, in the nucleus. Known transcriptional corepressors for Wnt transactivation include the C-terminal-binding protein (ctBP), Groucho/TLE1 and the 9-kDa inhibitor of β-catenin and Tcf-4 (ICAT; Willert and Jones, 2006). Other chromatin remodeling complexes counteracting Wnt signaling were also described (Mosimann et al., 2009). In addition, several central cytosolic Wnt regulators including, APC, Axin, GSK3β and Dsh also localize to the nucleus where they may directly or indirectly interact with β-catenin and modulate its transcriptional activity (Willert and Jones, 2006).

Additional studies are needed to determine whether E6/E6AP proteins alter the assembly of transcription complexes on β-catenin/TCF promoters, whether they modify acetylation of promoter-bound histones or whether they target a yet unidentified repressor to ubiquitin-mediated degradation.

Material and methods

Plasmids

PCEV29/HFz1 and Wnt3a cloned in pLNCX vector were described previously (Gazit et al., 1999). Cloning of the Myc-tagged β-catenin and Myc-tagged constitutively active S33Y mutant β-catenin (pCCB/β-catenin S33Y-Myc) was described previously (Golan et al., 2004). The Wnt responsive TCF-dependent luciferase construct, superTOPFLASH and its mutated version superFOPFLASH (also named Super8xTOPFlash and Super8xFOPflash) are based on the original TOPFLASH and FOPFLASH plasmids from Hans Clevers laboratory (Korinek et al., 1997). However, these plasmids contain eight TCF/ β-catenin binding sites (superTOPFLASH) or mutated TCF-binding sites (superFOPFLASH) upstream from the promoter (minimal TK) that drives the expression of the Firefly luciferase gene (Veeman et al., 2003). The plasmids pCMV-Renilla (Promega) and pCMV/β-galactosidase (β-gal; Clontech) were purchased from the indicated companies and used to evaluate the efficiency of transfection. The cyclin D1 luciferase reporter (Cyclin D1/Luc), harboring the β-catenin/TCF response element, kindly provided by Dr. C. Albanese (Georgetown University Medical Center), was described previously (Golan et al., 2004). Expression plasmids encoding the HPV16 E6, E7, epitope-tagged E6 and E6 carboxy-terminal deletion mutants, all cloned in the pJS55 vector, were described previously (Sherman and Schlegel, 1996; Sherman et al., 2002).These plasmids were used in most experiments described herein. DNAs encoding the native forms of HPV18 E6, HPV11 E6 and HPV16 E6 (kindly provided by Dr. M. Tommasino) were excised from the pSP64 vector and cloned into the EcoRI and HindIII sites of pcDNA 3.1. The HPV16 GFP-E6 expression plasmid was constructed by excision of the E6wt coding region from the pJS55 vector using the restriction enzymes XhoI and HindIII and insertion in frame into the pEGFP-C3 vector (Clontech) cut with the same enzymes. The pcDNA/ΔTCF-HA plasmid encoding a dominant-negative TCF was a gift from Dr. A. Gazit (Department of Human Microbiology, Tel Aviv University). The HPV16 E6 mutants G130V, L110Q, F2V, cloned in LXSN vector, were described previously (Sekaric et al., 2008). The mutant E6 genes were recloned into the pJS-55 vector in the EcoRI and BamHI sites. The pcDNA3-HA-E6AP vector was kindly provided by Dr. Scheffner (Department of Biology, University of Konstanz).

Cell cultures, transfections and retroviral infections

Human embryonic kidney cells, HEK293T, colon carcinoma cell lines, HCT116, SW480, Colo320, cervical carcinoma cell lines, C33A, CaSki, SiHa, HeLa, the lung adenocarcinoma cell line, H1299, the osteosarcoma cell line, U2OS, the Wnt3a-secreting L cells and control L cells (ATCC) were cultured in DMEM supplemented with 10% fetal calf serum. Primary human keratinocytes obtained from neonatal foreskin explants were grown in keratinocyte serum-free medium (KSFM) supplemented with 5 ng/ml epidermal growth factor (EGF) and 50 μg/ml bovine pituitary extract (BPE; Invitrogen). For preparation of Wnt3a conditioned medium (Wnt3a CM), Wnt3a-

secreting L cells were cultured for 4 days, then medium was removed and fresh medium was added for another 3 days. Medium was harvested and sterilized using a 0.22-μm filter. Medium from L cells was used as control. Cells were transfected by a modified calcium phosphate procedure as described previously (Sherman and Schlegel, 1996) or by using the jetPEI reagent (polyPlus Transfection). Stable transduction of C33A cervical cancer cells or primary keratinocytes (PHKs) with retroviruses carrying the HPV16 E6 gene and selection in G418 (Geneticin) was carried out as previously described (Alfandari et al., 1999). Expression of the transduced genes was confirmed by RT-PCR with the appropriate E6 primers or by Western blot.

Luciferase reporter activity assay

Cells growing in 5 cm or six-well dishes were transfected at 60–70% confluence with DNA of the superTOPFLASH or the control super-FOPFLASH (containing mutated TCF/β-catenin binding sites; 1 μg) plasmid, the β-gal (0.1 μg) or Renilla luciferase (0.5 μg) expression plasmid (used to evaluate the transfection efficiency) and various amounts of the E6 plasmid (as indicated). Wnt signaling was induced by either cotransfection of the plasmids encoding the Wnt ligand, pWnt3a (0.1 μg), and the Wnt receptor, human frizzled 1, pHFz1 (0.3 μg), transfection of a β-catenin encoding plasmid (1 μg), or treatment with Wnt3a CM for 24 h prior to cell extraction. LiCl induced signal was achieved by treating the cells with 33 mM LiCl for 24 h. Empty vector DNA was added to adjust for equal amounts of transfected DNA. Forty-eight hours after transfection, luciferase levels were measured using the Luciferase Assay System kit (Promega). The Firefly luciferase activity was normalized relative to the β-galactosidase or Renilla luciferase activity, as described previously (Sherman et al., 2002; Lichig et al., 2006). Luciferase readouts obtained with the superFOPFLASH control plasmid were exceedingly low (0.01–0.001 of the superTOPFLASH signal) and were subtracted from the readouts obtained with the superTOPFLASH plasmid. Data are presented as luciferase readouts (luciferase activity) or as relative luciferase readouts (fold activation) compared between E6 and vector expressing cells, where luciferase readouts in the vector cells were presented as 1. The collated results of fold activation are mean values and standard deviations (SD) from at least three independent experiments done in duplicates.

Subcellular fractionation

To prepare the soluble-cytoplasmic fraction, cells were lysed in a solution of 50 mM Hepes pH 7.0, 250 mM NaCl, 0.1% NP40 and 1% aprotonin. Extracts were clarified by centrifugation at 13,000 rpm for 2 min and the supernatant was used (Massimi et al., 2006). Nuclear fractions were prepared as described previously (Dignam et al., 1983). 293T cells were washed in ice-cold phosphate-buffered saline and resuspended in cold buffer containing 10 mM HEPES pH 7.9, 10 mM KCl, 1 mM dithiothreitol, 0.1 mM EGTA, 0.1 mM EDTA and protease inhibitor cocktail (Sigma). Resuspended cells were lysed in 0.5% Nonidet P-40 for 15 min followed by centrifugation at 1000 × *g* for 5 min. Following separation of the cytoplasmic fraction, the nuclei were harvested by resuspension in ice-cold buffer (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 1mM EGTA and protease inhibitor cocktail) followed by centrifugation at 13,000 rpm for 15 min at 4 °C.

Western blot analysis

Total cell lysates were prepared using the lysis buffer provided in the Luciferase Assay System kit (Promega). Electrophoresis on polyacrylamide gels, transfer to nitrocellulose membranes and reaction with antibodies was carried out as described previously (Alfandari et al., 1999; Lichig et al., 2006). Filters were cut into strips, blocked with 5% low fat milk and then incubated with the specific antibodies. Membranes were saline washed and incubated for 1 h with the secondary antibody at 4 °C. After washing in Tween/phosphate-buffered saline, membranes were subjected to enhanced chemiluminescence detection analysis (Biological Industries) using horseradish peroxidase-conjugated secondary antibodies. Primary antibodies used were as follows: anti-β-catenin (mouse, E-5, sc-7963), anti-cyclin D1 (mouse, 72-13G, sc-450), anti-p53 (mouse, DO-1, sc-126) and anti-histone (rabbit, FL-219, sc-10806) all from Santa Cruz Biotechnology; anti-actin (mouse, AC-15, A5441) and anti-tubulin (mouse, B-5-1-2, T5168) from Sigma; anti-HA (rat, 1 867 423) and anti-GFP (mouse, 11814460001) from Roche; anti-active-β-catenin (anti-ABC) clone 8E7 mouse monoclonal antibody (05-665) from Upstate Cell Signaling Solution. Secondary antibodies used: anti-mouse, anti-rat and anti-rabbit conjugated to horseradish peroxidase from Jackson Immuno Research Laboratories.

Immunofluorescence staining

HCT116 cells grown on glass coverslips were transfected with the GFP or the HPV16 GFP-E6 expression plasmid. At 48 h after transfection cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 20 min. After washing with PBS cells were permeabilized with 0.1% Triton X-100 for 30 min and incubated in 1% BSA solution for 1 h. Cells were stained with the primary anti-β-catenin antibody (E-5, sc-7963) diluted 1/100 for 2 h, followed by 1 h staining with the secondary antibody conjugated to rhodamine (mouse-Jackson Immuno Research Laboratories) diluted 1/500. Cells were also stained with DAPI (0.004%) and visualized using confocal microscopy system (LSM-510 Carl-Zeiss).

Generation of U2OS cells stably transduced with shE6AP

Plasmids for the lentivirus vector-mediated drug-inducible RNA interference system (pLVTHM, ptTR-KRAB-Red) and cloning of oligonucleotides for E6AP shRNA were described previously (Louria-Hayon et al., 2009). For the production of lentiviral particles, HEK293T cells were transfected with ptTR-KRAB-Red and pLVTHM-shE6AP plasmids (10 μg) together with pCMV-R8.91 (packaging construct; 6.5 μg) and pMD2.VSVG (envelop construct; 3.5 μg) in 10 cm dishes using 2mg/ml PEI (polyethylenimine, Sigma). Collection of the virus from cell supernatants was carried out 40 h after transfection. Virus containing medium from seven transfected HEK293T 10-cm dishes was concentrated to 1 ml by centrifugation at 70,000 × *g* and concentrated virus was used to infect U2OS cells in the presence of polybrene (8 μg/ml). Two days later, the cells were seeded and doxycycline (DOX) was added to half of the cells at a final concentration of 0.2 μg/ml. Four days later the cells were harvested and analyzed for E6AP protein expression by Western blot.

Statistical analysis

Data of activity in the various functional assays are presented in the figures as average (mean) values ± SD. Data were subjected to a one-way ANOVA. Significance was accepted at $P<0.05$.

Acknowledgments

We thank Drs. Arnona Gazit and Abraham Yaniv (Department of Human Microbiology, Sackler School of Medicine, Tel Aviv University) for the Wnt3a, HFz1, β-catenin and dNTCF expression plasmids, Dr. Randall T. Moon (Howard Hughes Medical Institute, Department of Pharmacology and the Institute for Stem Cell and Regenerative Medicine, University of Washington School of Medicine) for the superTOPFLASH/FOPFLASH plasmids, Dr. Chris Albanese (Departments of Oncology and Pathology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center) for the cyclin D1

luciferase reporter, Dr. Elliot J. Androphy (Department of Medicine, University of Massachusetts Medical School) for the HPV16 E6 mutant G130V, L110Q, F2V expression plasmids, Dr. Massimo Tommasino (International Agency for Research on Cancer, WHO) for the HPV11, HPV18 and HPV16 E6 plasmids and Dr. Martin Scheffner (Department of Biology, University of Konstanz) for the E6AP encoding plasmid. Dr. Rina Rosin-Arbesfeld provided valuable comments on the manuscript. This work was funded by grants from the Leo Minz Fund for Cancer Research, Tel-Aviv University and the Cooperation Program in Cancer Research of the Deutsches Krebsforschungszentrum (DKFZ) and Israel's Ministry of Sciences and Technology (MOST), Project No. 1841 awarded to L.S. H.L.K. performed this work in partial fulfillment of the requirements for the Ph.D. degree from the Sackler School of Medicine, Tel-Aviv University.

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