

Influence of HPV16 E2 and its localisation on the expression of matrix metalloproteinase-9

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Received November 25, 2009; Accepted February 4, 2010

DOI: 10.3892/ijo_00000682

Abstract. Infection with the high-risk HPV types 16 and 18 is the major cause of cervical cancer and plays a role in the development of certain head and neck and skin cancers. We have previously demonstrated that the Early Protein 2 of the Cottontail Rabbit papillomavirus (CRPV), required for skin carcinogenesis in a rabbit model, is able to induce the expression of a matrix metalloproteinase (MMP-9); a protease known to play a key role in invasion and metastasis. However, as of now we do not understand the underlying mechanism of activation nor relevance for the human system. Here, we report that high-risk human papillomavirus HPV16 E2 similar to our previously reported results on CRPV E2 activates the human MMP-9 promoter predominantly via the MEK1-ERK1/2-AP-1-signaling pathway. In addition this activation is associated with a nuclear sub-localisation of HPV16-E2 suggesting a nuclear protein-protein or protein-DNA interaction of E2 as the underlying mechanism of activation.

Introduction

Human papillomaviruses have been shown to be the cause for cervical cancer (1) and are associated with other malignancies such as of the head and neck (2), and skin (3). The serotype most frequently found in HPV-positive cervical and head and neck tumours is HPV16 (1,2).

The papillomavirus E2 protein is a phosphoprotein of approximately 48 kDa and functions as a regulator of virus

genome replication. It is composed of two domains which are separated by a flexible hinge region (4). The N-terminal part of the protein contains the transactivation domain while the C-terminus is occupied by the DNA-binding domain (DBD) (5) and mediates the interaction of E2 with E1 (6). Dimerized, the DBD recognizes E2-binding sites (E2BSs) consisting of the palindromic sequence 5'-AAC(N)₆GTT-3' (7). E2 is suggested to be the major regulator of E6 and E7 expression and loss of oncogene regulation can be detected in late stages of cervical cancer due to integration of the viral DNA into the host genome and resulting disruption of the E2 ORF (8). In contrast, in HNSCCs viral DNA is found not integrated in the host genome in approximately 35% of the cases or both episomal and integrated in 17% of tumours (9). Overexpression of HPV16 and -18 E2 proteins has been shown to induce cell cycle arrest and apoptosis in cervical carcinoma cell lines (10). Further studies led to the discovery that HPV16 E2 interacts with p53 (11) and may induce apoptosis in a p53-dependent manner (12). High-risk HPV E2 proteins can be detected within both, the nucleus and the cytoplasm due to exportin-1 receptor (CRM1)-dependent nucleo-cytoplasmic shuttling, while low-risk HPV E2 is strictly nuclear. These differences are felt to explain why high- but not low-risk HPV E2 induces apoptosis through caspase-8 *in vitro* (13).

The E2 protein of the Cottontail rabbit papilloma virus (CRPV) has previously been demonstrated to play a major role in the development of CRPV dependent skin carcinogenesis (14). This is particularly interesting in light of our recently reported finding that the E2 protein of CRPV (15) has the ability to activate the *in vitro* expression of matrix metalloproteinase 9 (MMP-9), a protein involved in the degradation and remodelling of the extracellular matrix (ECM). Overexpression of MMP-9 has been detected in many different epithelial cancers including of the cervix (16), head and neck (17), and skin (18). MMP-9 plays an important role in the degradation of cell-cell and cell-matrix molecules, induction of cell migration, and the release and activation of growth factors (19).

We also previously reported that the CRPV-E2-induced expression of MMP-9 is regulated via the transcription factor complex AP-1 (20). AP-1 is a homo- or heterodimeric transcription factor consisting of members of the jun, fos and/or fra family (21) and is a main target of MAPKs (22). MAPKs are components of signal-transduction pathways that consist of protein kinases of which the extracellular regulated kinase

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Key words: human papillomavirus 16, E2, matrix metalloproteinase-9, AP-1, extracellular regulated kinase

(ERK), the c-Jun N-terminal kinase (JNK) and the p38 pathway represent the most extensively studied (23). The ERK pathway consists of Raf-, MEK, and ERK-proteins that are sequentially activated through both extrinsic (i.e. EGF, FGF, PDGF and NGF) (23) and intrinsic (cytoplasmic) (i.e. Ras associated with Golgi, PARP-1, p53R2) (2426) signals.

As of now we do not understand the relevance of CRPV-E2-dependent activation of MMP-9 for the human system, i.e. whether high-risk HPV-E2 has the same effect on MMP-9. Although we have previously shown that CRPV-E2 requires an activation of MEK-ERK but not p38 and JNK to induce rabbit MMP-9 promoter activity (20), the underlying mechanism of activation remains obscure. Given that nuclear and cytoplasmic proteins activate the MEK-ERK signalling pathway and moreover that the sub-cellular localisation of HPV16 E2 is critical for the execution of E2 dependent phenotypes, i.e. apoptosis (13), we hypothesized that the same may hold true for the induction of MMP-9 expression.

Towards the end of this study we provide evidence that similar to CRPV-E2 human high-risk HPV16 E2 induces MMP-9 expression via MEK1-ERK1/2, AP-1. Moreover, our data suggest that the activation of MMP-9 expression is associated with the intracellular localisation of the viral protein. Our finding that depleting the nucleus from HPV16 E2 significantly reduced MMP-9 promoter activation suggests that a nuclear protein-protein or protein-DNA interaction is relevant for MMP-9 expression.

Materials and methods

Plasmids. Human MMP-9 promoter (2305 bp) were amplified using PCR to introduce restriction sites *KpnI* and *BglII* to allow for cloning of the promoter fragment into the chloramphenicol acetyltransferase reporter-gene vector pCAT3 Basic (Promega). The mammalian expression vector pCMV-HA (Clontech) was used to construct pCMV-HA HPV16 E2 and pCMV-HA HPV16 E2 eGFP fusion protein plasmids. In both cases the full length HPV16 E2 DNA fragment was amplified by PCR and introduced into the vector. For localisation studies, the HPV16 E2 gene introduced into pCMV-HA was mutated using QuikChange (Stratagene) according to the manufacturer's recommendations and confirming the desired mutations by sequencing. The TAM67 construct has been described elsewhere (27), and the dominant negative ERK constructs ERK1 (K71R) and ERK2 (K52R) which are mutated within their ATP-binding pockets were cloned into pcDNA3 (Invitrogen).

Cell culture, and transient transfections, and preparation of cell lysates. C33A human cervical keratinocytes (ATCC) and NIH3T3 mouse fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) with glutamine (PAA, Linz, Austria) supplemented with 10% fetal bovine serum (PAA) and 5% AntibioticAntimycotic (Invitrogen). Cells were grown at 37°C and 5% CO₂. Transfection of cells with plasmids was carried out using Lipofectamine™-2000 (Invitrogen) according to the supplied protocol. Forty-eight hours after transient transfection, cells were harvested and the protein content determined using BCA (BCA kit, Pierce). The lysates were equalized for protein content and used for reporter-gene assays, immunoprecipitation or separated by SDS/PAGE.

RNA isolation with subsequent RT-PCR. The RNA isolation was carried out using the RNeasy kit (Qiagen) according to the manufacturer's protocol. On-column DNA digestion was performed using the RNase free DNA kit from Qiagen. After RNA isolation 1 µg RNA was used in a reverse transcription reaction with the high-capacity cDNA kit (Applied Biosystems) as per manual. Primer sequences are as follows: MMP9PhumKpnF: 5'-CAGGTACCTACCCACTTCTATAC CTGGG-3' (*KpnI*); MMP9PhumKpnR: 5'-GGTACCAGAG GCTCATGGTGAGGG-3' (*KpnI*); hMP-E2mut-for: 5'-GCA GCTTAGAGCCCAATAACATGGTTTTGTGATTCCAA G-3'; hMP-E2mut-rev: 5'-CTTGGAATCACAAAACCAT GTTATTGGGCTCTAAGCTGC-3'; NLS1 (107/108): 5'-GT GTATTTAACTGCACCAAAACGATGTATAAAAAAAC ATGGATATACAGTGGGAAGTGC-3'; NLS1 (107/108) rev: 5'-GCACTTCCACTGTATATCCATGTTTTTTTATACAT CGTTTTGGTGCAGTTAAATACAC-3'; NLS2 (241/244): 5'-CACAGACGACTATCCAGCGAAAAAGATCACGGC CAGACACCGGAAACCCC-3'; NLS2 (241/244)rev: 5'-GG GGTTCGCGGTGTCTGGCCGTGATCTTTTTTCGCTGGAT AGTCGTCTGTG-3'; NLSDBDmt: 5'-GATGCTAATACTT TAAAATGTTTtaggATATGGATTACAAAGCATTGTA CATTGTATACT-3'; NLSDBDmt rev: 5'-AGTATACAA TGTACAATGCTTTGTAAATCCATATCCTAAACATTT TAAAGTATTAGCATC-3'; QPCRMM9hFor 5'-CGACGT CTTCCAGTACCGA-3'; QPCRMM9hRev 5'-CTCAGGG CACTGCAGGAT-3'; QPCRACtBhFor 5'-GCACAGAGC CTCGCCTT-3'; QPCRACtBhRev 5'-CCTTGCACATGCCG GAG-3'; QPCRGA PDHhFor 5'-CTCTGCTCCTCCTGTTC GAC-3'; QPCRGA PDHhRev 5'-TTAAAAGCAGCCCTGG TGAC-3'; qPCRHPV16E2 5'-ATGGAGGACTCTTTGCC ACG-3'; qPCRHPV16E2rev 5'-CCTGTTGGTGCAGTTAA ATACAC-3'.

Semi-quantitative PCR. Semi-quantitative PCR was carried out in a BioRad thermocycler (BioRad, Munich, Germany) using 10 ng template cDNA and amplifying for 33 cycles at 60°C.

Reporter-gene assays. The cell lysates normalized for protein content were subjected to CAT-ELISA (Roche) according to the manufacturer's manual.

Western blotting and immunoprecipitation. After SDS/PAGE proteins were transferred to PVDF membrane for 1 h at 100 V. Membranes were blocked either in 4% BSA/TBST overnight. Anti-ERK primary antibody was used at 1:2000 and anti-p-ERK at 1:1000 (both CST). Primary antibody incubation was conducted at 4°C overnight in 5% BSA/TBST on a rotary shaker. An HRP-conjugated secondary antibody was used at 1:5000 for 1 h at RT followed by ECL plus (GE Healthcare, Chalfont St. Giles, UK) based chemiluminescence detection. p-ELK was detected after immunoprecipitation overnight and subsequent kinase assay according to the manufacturer's protocol using the p44/42 MAP Kinase Assay kit (CST).

DNA staining and immunofluorescent detection of proteins. For nuclear staining prior to microscopic investigation or fixation for immunofluorescence, cells were incubated with either 0.2 µg/ml Hoechst-33342 Dye (Sigma-Aldrich, Munich, Germany) or 1 µg/ml DAPI (Carl Roth GmbH, Karlsruhe, Germany) for 30 min to 1 h at 37°C, 5% CO₂.

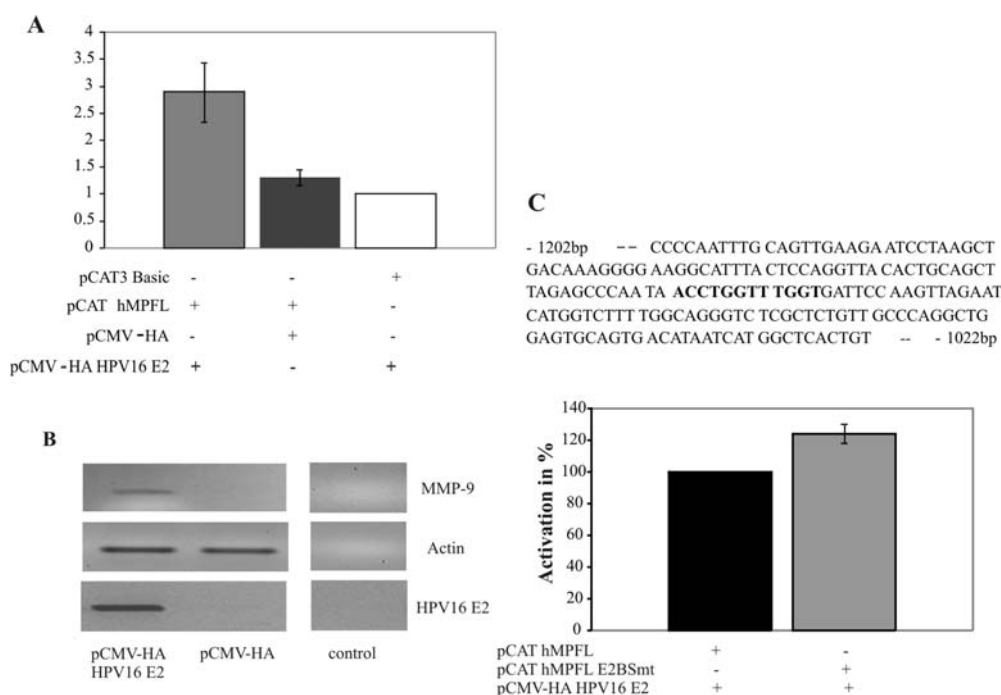


Figure 1. Activation of the MMP-9 promoter and MMP-9 transcript upregulation by HPV16 E2. (A) Reporter gene assay of C33A cells transiently transfected with the full length MMP-9 promoter reporter-gene vector, empty control vector, empty expression vector, and HPV16 E2. (B) Relative amount of MMP-9, actin, and HPV16 E2 mRNA transcripts at cycle 33 after transient transfection with indicated constructs, with H₂O as negative control. (C) Schematic representation of the MMP-9 promoter construct used with the sequence of the E2BS indicated in bold and effect of the E2BS mutation on HPV16 E2-mediated activation as compared to the wild-type promoter. *P≤0.05, **P≤0.01 and ***P≤0.005 as obtained by Student's t-test. Results show mean value out of three experiments performed in triplicate.

For direct immunofluorescence of the HPV16 E2-fusion proteins, the transfected cells were grown on glass coverslips for 24 h, fixed with 4% paraformaldehyde in phosphate-buffered saline for 15 min at room temperature. Following fixation, the coverslips were blocked in 1% BSA in PBST for 30 min. The anti-actin antibody (sc-1616; Santa Cruz Biotechnology, Heidelberg, Germany) was diluted 1:100 in 1% BSA in PBST and cells were incubated for 1 h. After incubation with the secondary antibody (sc-2313; Santa Cruz Biotechnology) for 1 h, coverslips were washed with PBS and rinsed in water before transferring them onto slides using VectaShield mounting medium (Linaris, Wertheim, Germany).

Correlation of sub-cellular fluorescence. Imaging software ImageJ was used to determine the correlation between whole cell and nuclear fluorescence after imaging of immunofluorescences. Relative fluorescence ratios were thus determined for an average of 10 transfected cells.

Results

Firstly, the capacity of HPV16 E2 to activate the MMP-9 promoter was investigated using a reporter-gene vector containing 2305 bp of the human MMP-9 promoter (termed full length) in front of the gene encoding chloramphenicol acetyltransferase (CAT). Cell lysates of C33A cells were obtained to examine the effect of HPV16 E2 on the transcription of MMP-9 from cells transiently co-transfected with HPV16 E2 and the reporter-gene vector were analysed for CAT-expression by ELISA. It could be observed that HPV16 E2 was able to significantly activate the human MMP-9

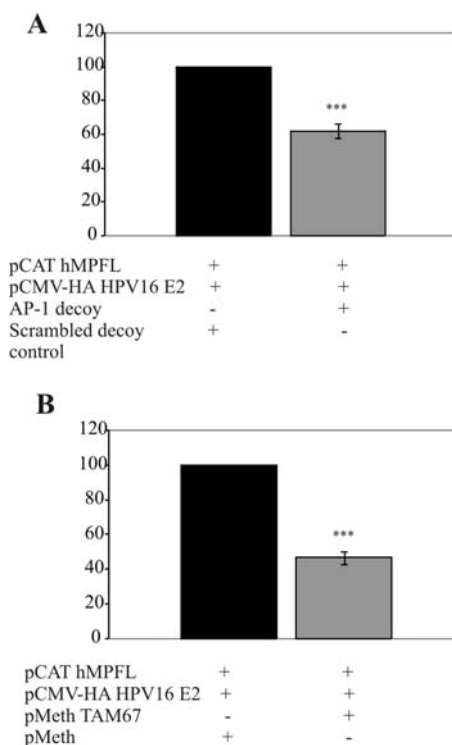


Figure 2. Involvement of AP-1 in the activation of the MMP-9 promoter by HPV16 E2. (A) Effect of transient co-transfection of an AP-1 decoy in reporter-gene assays as compared to the scrambled decoy control, and (B) effect of co-transfection of the transactivation-deficient c-Jun mutant TAM67 along with HPV16 E2 expression plasmid as compared to the empty vector control. *P≤0.05, **P≤0.01 and ***P≤0.005 as obtained by Student's t-test. Results show mean value out of three experiments performed in triplicate.

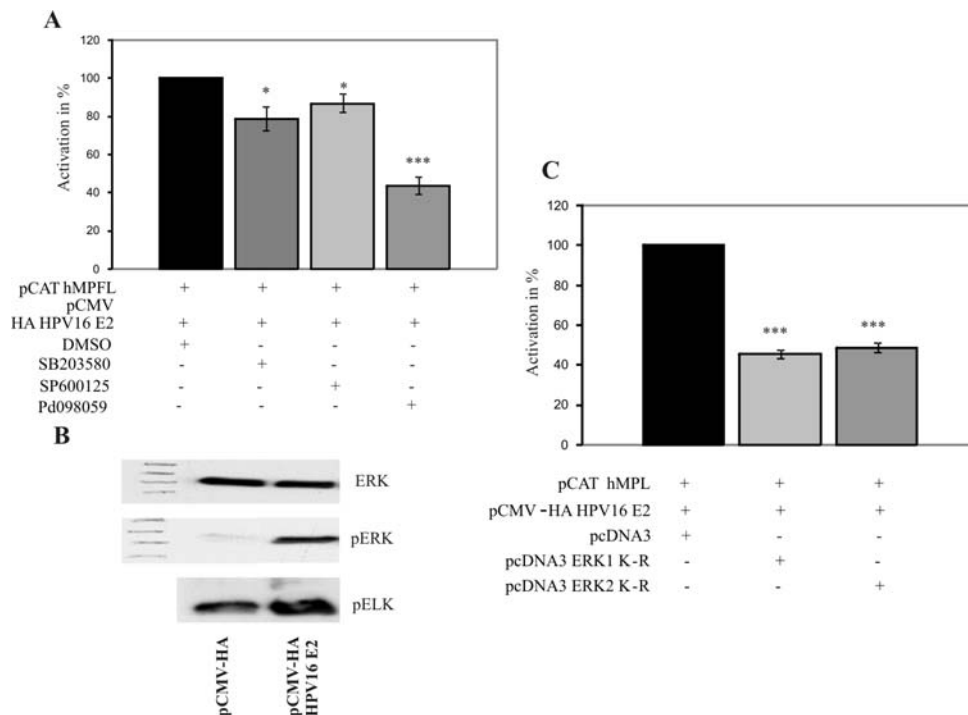


Figure 3. Involvement of MAP-kinases in the HPV16 E2-mediated activation of the MMP-9 promoter. (A) Effect of MAP-kinase inhibitor treatment on the activation of the MMP-9 promoter by HPV16 E2, and (B) phosphorylation state determination of ERK and its downstream target ELK after transient transfection of C33A cells with HPV16 E2. (C) The effect of ERK activation on E2-mediated promoter activation as determined by co-transfection with dominant-negative ERK 1 or 2. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.005$ as obtained by Students' t-test. Results show mean value out of three experiments performed in triplicate.

promoter by 3 ± 0.8 -fold (Fig. 1A). To address the question of up-regulation of the endogenous MMP-9 transcripts, human cervical keratinocytes were transiently transfected with HPV16 E2. After isolation of RNA, RT-PCR was performed. The obtained cDNA was subjected to semi-quantitative PCR where a significant amount of MMP-9 mRNA in pCMV-HA HPV16 E2 transfected cells could be detected (Fig. 1B). These results suggest a relevance of E2-dependent MMP-9 expression in the human system.

Examining the promoter sequence of the human MMP-9 construct, we observed the presence of a binding site for E2 (E2BS; Fig. 1C) at -1120 to -1109 bp in the MMP-9 promoter which is not present in the rabbit MMP-9 promoter recently described by us (20). We hence investigated whether direct binding of HPV16 E2 plays a role in the activation of the promoter by deleting the consensus sequence using site-directed mutagenesis. It could be seen from the results of subsequent reporter-gene assays with the mutant promoter that this E2BS has no influence on the activation of the human MMP-9 promoter by HPV16 E2 (Fig. 1C).

It has been previously described that the binding sites for the transcription factor complex AP-1 are of importance in the activation of the rabbit MMP-9 promoter by CRPV E2 and that the proximal AP-1 binding site in the human promoter is of importance in E2-mediated activation (15,20). Hence, an AP-1 decoy was used in this study to confirm the involvement of the AP-1 complex in promoter activation by HPV16 E2. A decoy is a short synthetic oligonucleotide that resembles the transcription factor consensus sequences and hence competes for binding of the transcription factor with consensus sequences

in target genes. Co-transfection of the AP-1 decoy with the human MMP-9 promoter and HPV16 E2 resulted in a decrease in activation by $40 \pm 4\%$ as compared to the negative control decoy (Fig. 2A).

The transcription factor AP-1 is a dimeric complex which consists of members of the jun-, fos-, and fra-families (21). To determine the importance of the AP-1 complex for activation of the MMP-9 promoter, the transactivation-deficient c-Jun mutant TAM67 (27) was used for co-transfection with the MMP-9 promoter and HPV16 E2. In subsequent reporter-gene assays, the activation of the MMP-9 promoter was shown to be decreased to $46 \pm 4\%$ when compared to the empty vector control (Fig. 2B).

Expression of the components of the transcription factor complex AP-1 and its activation through phosphorylation is largely regulated by the members of the mitogen-activated protein kinase (MAPK) pathway. In order to investigate the role that MAP-kinases play in the activation of E2-mediated MMP-9 promoter activation, chemical inhibitors of the three main MAPK/SAPK pathways JNK, ERK, and p38 were used in reporter-gene assays. It could be observed that use of the MEK1 inhibitor PD098059 led to a highly significant decrease in the activation potential of the HPV16 E2 protein of about 60% (Fig. 3A). The p38 inhibitor SB203580 led to a moderate decrease in promoter activation of about 20%, while the JNK inhibitor SP600125 had little effect on the E2-mediated activation of the promoter. Looking at the effect that the ERK inhibitor has on the activation of the MMP-9 promoter, ERK must be phosphorylated in response to E2 expression. Hence, the phosphorylation status of ERK in transfected cells was

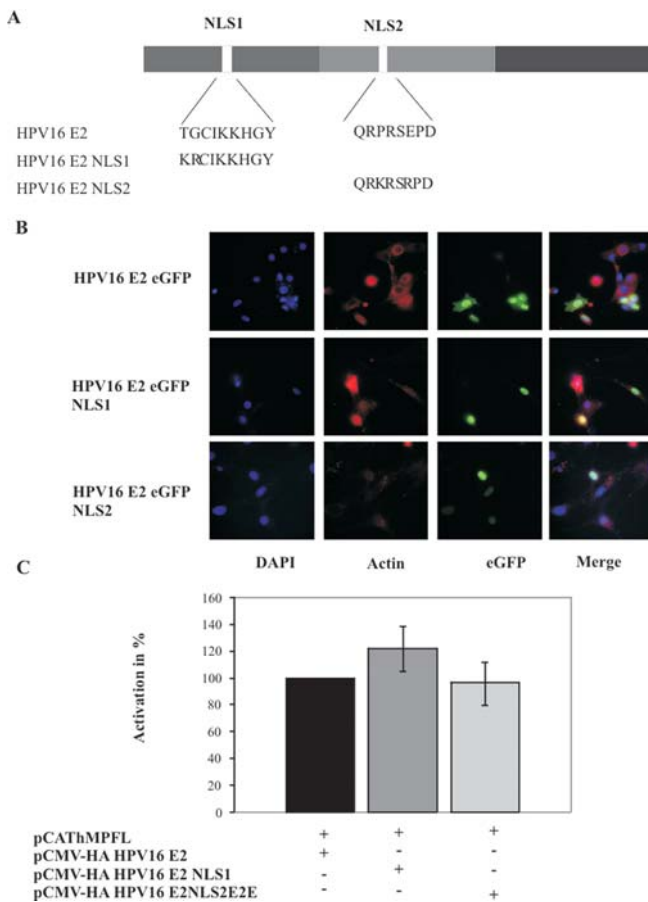


Figure 4. Effect of the nuclear localisation signals of low risk papillomavirus E2 proteins introduced into HPV16 E2. (A) Schematic representation of the mutations introduced into the E2 ORF, (B) and determination of the subcellular localisation of the resulting mutant-eGFP fusion proteins in NIH3T3 cells (blue, DAPI; red, actin; green, eGFP). Pictures were taken as mentioned in Fig. 4. (C) Effect of the mutated HPV16 E2 on the activation of the MMP-9 promoter as compared to wild-type E2 referred to as 100%. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.005$ as obtained by Students' t-test. Results show mean value out of three experiments performed in triplicate.

investigated more closely by immunoprecipitation and Western blotting. As was expected, expression of E2 led to an elevated level of fully functional pERK able to activate an exogenously added downstream-target ELK as shown by *in vitro* kinase assay as compared to expression of the empty vector control (Fig. 3B).

Further, human cells were transiently co-transfected with the reporter-gene vector, HPV16 E2, and dominant negative mutants of ERK1 and ERK2. Subsequent reporter-gene assays led to the observation that co-expression of either of the two dominant negative mutants had a similar effect which resulted in a reduction of activation of about 50% as compared to the empty vector control (Fig. 3C).

It has been reported by Blachon *et al* (13) that high-risk human papillomavirus E2 as opposed to low-risk HPV is able to shuttle between nucleus and cytoplasm. The study related the cytoplasmic localisation of the E2 protein with the induction of apoptosis through a caspase-8 dependent mechanism by high-risk HPV. Moreover, the MEK1-ERK1/2 signalling pathway can be induced via various activators that can be cytoplasmic proteins (i.e. PARP, p53R2) through direct protein-protein interaction (26,25) or *de novo* synthesized

growth factors (i.e. EGF, FGF) through protein-receptor interaction (28,29). It was hence of interest for the present study whether the localisation of the E2 protein has an impact on its induction of MMP-9 expression.

In order to carry out localisation studies, HPV16 E2 was tagged with eGFP to allow for easy visualisation. The protein also obtained an HA-tag at the N- and a KT3-tag at the C-terminus to determine the integrity of the protein.

Immunofluorescence revealed that eGFP-HPV16 E2 was present in the nucleus as well as within the cytoplasm (Fig. 4B). It has been described that HPV16 E2 contains only one nuclear localisation sequence (NLS) as compared to HPV6b E2 which has three (13). However, HPV16 E2 has a nuclear export sequence (NES) which is not found in the low-risk type. Hence, different approaches were used to investigate the potential role of E2 localisation in MMP-9 promoter activation. Firstly, in order to increase the amount of nuclear HPV16 E2 either one of the two additional NLS found in HPV6b E2 were introduced to HPV16 E2, resulting in the mutant constructs HPV16 E2 NLS1 and HPV16 E2 NLS2 (Fig. 4A). We decided not to delete the NES found in HPV16 E2, because the position in the N-terminal domain is within the transactivation domain of the protein with the consecutive risk of false-positive results (14). Using immunofluorescence and the eGFP-tagged E2 mutants, the localisation of the mutant proteins was determined.

Immunofluorescence of the NLS mutant HPV16 E2-eGFP fusion proteins revealed that both mutants containing an additional NLS site were localised solely within the nucleus (Fig. 4B). In reporter-gene assays, the ability of these mutant proteins to activate the MMP-9 promoter was investigated. Here, the NLS mutant constructs showed no significant decrease in their activation potential of the MMP-9 promoter (Fig. 4C) suggesting that a cytoplasmic localisation of E2 is not critical for the induction of MMP-9 promoter activation.

To further investigate this hypothesis, a second mutant was created in which the only NLS found in HPV16 E2 is rendered non-functional (Fig. 5B) resulting in a mainly cytoplasmic localisation of the protein (Fig. 5A and C). When this mutant was used in reporter-gene assays with the human MMP-9 promoter, a 60% reduction of CAT-expression compared with wild-type HPV16 E2 was observed (Fig. 5D) indicating that a nuclear localisation of HPV16 E2 is significantly elevating MMP-9 promoter activity.

Discussion

Recently published data indicate that CRPV-E2 is required for the development of skin cancers in a rabbit model and moreover activates the expression of a matrix metalloproteinase highly relevant for epithelial cancer invasion. However, we currently do not know, if this finding translates equally into the human system nor do we understand the underlying mechanism of activation. In this report we demonstrate that similar to CRPV-E2 the human high-risk HPV16 E2 protein induces the expression of MMP-9 through the activation of MEK1-ERK1/2 and AP-1. Moreover, our data suggest that this mechanism relies on a predominantly nuclear sublocalisation of HPV16.

Aberrant expression of c-Jun has been shown to be involved in several human cancers such as myeloid leukaemia, Hodgkin lymphoma, and prostate cancer (30,31). In head and neck

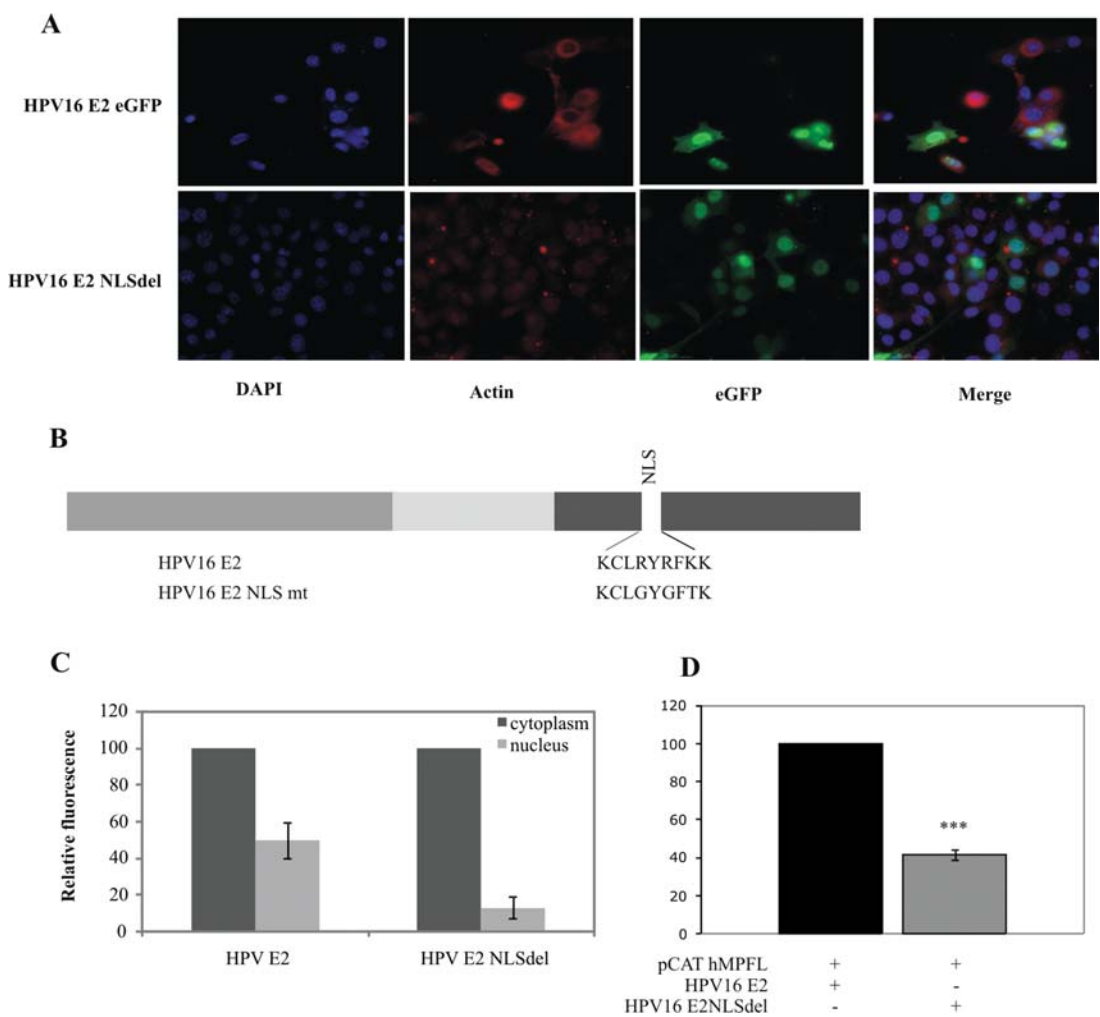


Figure 5. Effect of the deletion of the nuclear localisation signal present in HPV16 E2. (A) The localisation of the mutant as determined using an eGFP-fusion protein and immunofluorescence (blue, DAPI; red, actin; green, eGFP). Pictures were taken using a Zeiss AxioImager PlanAPOCHROMAT microscope (63x,1.4 oil), an AxioCam MRm R3, and AxioVision 4.7 software. (B) Schematic representation of the mutation introduced in order to eliminate the NLS. (C) ImageJ software was used to determine the correlation between whole cell fluorescence and nuclear fluorescence. Relative fluorescence ratios between nucleus and whole cells of an average of 10 transfected cells. (D) Effect of the NES mutant on the activation of the MMP-9 promoter as compared to the wild-type E2. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.005$ as obtained by Students' t-test. Results show mean value out of three experiments performed in triplicate.

tumors, c-Jun expression has been shown to correlate with the expression of the matrix metalloproteinase-1 and -9 (32). c-Jun negative ras-transformed cells lack many characteristics of Ras transformation including the ability to induce tumours in nude mice (33). Furthermore, ectopical expression of c-Jun in breast cancer cells resulted in a change in AP-1 composition and subsequent conversion into a more aggressive cell line and additionally also an increase in MMP-9 (34). Co-transfection of cells with the transactivation-deficient c-Jun mutant TAM67 leads to the integration of the TAM67 into newly forming AP-1 complexes which compete with functional AP-1 complexes for binding to the AP-1 binding motifs. Upon binding of a complex containing TAM67, transcription of the respective gene cannot progress and is abolished (35). TAM67 expression has already shown the importance of c-Jun in the growth of non-small cell lung cancer cells (36). Furthermore, TAM67 can abrogate an oncogenic phenotype in Epstein-Barr Virus (EBV) late-membrane protein-1 (LMP-1) positive nasopharyngeal carcinoma (37) and inhibits breast cancer growth *in vivo* and *in vitro* (38). Most interestingly for this

study, TAM67 has been shown to block HPV16 E7-enhanced tumour progression in mice (39). Here, using co-transfection of TAM67, we were able to observe a highly significant inhibition of MMP-9 promoter activation by HPV16 E2. It can hence be concluded that c-Jun is a member of the transcription-factor complex binding to the MMP-9 promoters which is also supported by the above mentioned finding by Smith *et al* (34) that an overexpression of c-Jun results in an increase in MMP-9 expression. Furthermore, the highly significant inhibition detected could probably be due to the fact that TAM67 may also inhibit NF- κ B (40).

An enhanced activation of ERK has been described in prostate (41), ovarian (42), and cervical cancers (43) among others. Its up-regulation is further known to be of importance in the phosphorylation and regulation of c-Jun (44). It has often been described that the human MMP-9 promoter is regulated by the activity of the mitogen-activated protein kinases. Most often this regulation is a result of the ability of MAP-kinases to induce transcription of members of the transcription factor complex AP-1 (22,45). To investigate the

role of the MAP-kinase members JNK, p38, and ERK, in the papillomavirus E2-mediated activation of the MMP-9 promoter, chemical inhibitors were added to the cells. In subsequent reporter-gene assays, a highly significant decrease in promoter activation could be detected in cells treated with PD098059, a MEK1-inhibitor (46). As it has previously been shown by our group (15,20), this inhibitor was able to block CRPV E2-induced invasion in rabbit epithelial cells. Western blot analysis of the phosphorylation state of ERK in this study yielded evidence for phosphorylation of ERK and its downstream target ELK in human cells after transfection with the HPV16 E2 proteins. It has been shown that several viral proteins are involved in the regulation of MAPK pathways. The E5 protein of HPV16 is commonly known to activate ERK through its upregulation of EGF-receptor signalling (28,47,48). The Kaposi Sarcoma-Associated Herpesvirus (KSHV) induces AP-1 by activation of primarily ERK and JNK (49) similar to the hepatitis B Virus HBx protein, which also initiates AP-1 activity via ERK and JNK (50). The LMP-1 protein of EBV, which is also known to upregulate MMP-9, has been shown to influence a variety of cellular transcription factors such as NF- κ B and AP-1 (51).

In a study by Blachon *et al* (13) it was shown that E2 of high-risk HPV-types is located within the nucleus and cytoplasm of an infected cell. It is able to move freely between the two compartments due to the presence of a nuclear export signal (NES) and a nuclear localisation sequence (NLS). Low-risk HPV E2 on the other hand is present solely within the nucleus. As opposed to high-risk E2, low-risk E2 proteins contain three NLSs but no NES. In their study, Blachon *et al* associated the localisation of the proteins with the induction of apoptosis as can be detected in high-risk HPV infection. Using HPV16 E2 mutants, we set out to investigate the role of E2 localisation on the protein's ability to activate the human MMP-9 promoter through the MEK1-ERK1/2/AP-1 signalling pathway. To confirm the localisation of the constructed mutants within the cell, the E2 protein and mutants were tagged with enhanced green fluorescent protein (eGFP). Introduction of either of the two additional NLS sites identified in low-risk HPV E2 into the HPV16 E2 sequence resulted in a mainly nuclear localisation of the protein. Activation of the human MMP-9 promoter as detected by reporter-gene assays was similar to that achieved by wild-type HPV16 E2.

As the predominantly nuclear localised protein was still able to activate the MMP-9 promoter, it could be hypothesised that the E2 protein induced MMP-9 expression relies on the localisation of the protein within the nucleus. Two methods were available in order to prove this theory. It is known that the nuclear import of the E2 protein occurs via the exportin-1 receptor (CRM1) which can be inhibited by addition of actinomycin D. However, the import of MAP-kinases into the nucleus occurs by a similar mechanism and hence inhibition of nuclear import may lead to non-interpretative results in the reporter-gene assays. The second option consisted of a deletion mutant of the NLS region of the E2 protein. Immunofluorescence of the created mutant confirmed a mainly cytoplasmic localisation of the protein. Reporter-gene assays showed a significant decrease in the activation potential of the mainly cytoplasmic E2 mutant. As the mutation introduced is not located within the transactivation domain of the

E2 protein, this decrease in activation cannot be accounted for by a loss in transactivation efficiency. Furthermore, as activation of the promoter cannot be due to direct binding of the E2 protein to the MMP-9 promoter, as no effect upon deletion of the E2 consensus sequence in the MMP-9 promoter was detected, this decrease in activation must be a result of the change in E2 localisation.

These results are interesting since it appears less likely that a cytoplasmic protein-protein interaction of E2 with protein kinases or phosphatases belonging to the ERK or p38-signal transduction pathway accounts for the activation of MMP-9 expression. It seems more conceivable that a protein-protein or protein-DNA interaction within the nucleus and consecutive *de novo* synthesis of a protein yet to be identified is critical for the activation of MMP-9 through MEK1-ERK1/2/AP-1.

We have shown in this study that high-risk HPV16 E2 activates ERK1/2, AP-1 and MMP-9, all major regulators during cancer progression (22). MMP-9 has been demonstrated to play a role during the process of local cancer invasion, extracellular matrix remodelling, and is associated with poor outcome in various epithelial cancers (52). AP-1 is a major regulator of several proteases that are involved in cancer invasion (19). Given that integration of viral DNA is less common in HNSCCs as compared with cervical cancers, we may speculate that also loss of E2 expression is less common in these tumours. This considered, E2 may have a role in cancer progression in HNSCCs. This idea is further supported by the fact that episomal localisation of viral DNA is more commonly found in advanced stages of HNSCCs (9).

In summary, in this report we demonstrate that HPV16 E2 activates targets such as ERK1/2, AP-1 and MMP-9 but more importantly that this process is greatly enhanced by a nuclear localisation of the E2 protein. The mechanism of inducing MMP-9 expression does not rely on direct binding of E2 to the MMP-9 promoter but depends on the integrity of the transactivation domain of the viral protein (15). It is therefore less likely that a cytoplasmic protein-protein interaction between protein kinase/phosphatase members of the MEK1-ERK1/2 pathway or p38 and HPV16-E2 accounts for the activation of MMP-9 expression. We hypothesise that the interaction with other proteins in the nucleus culminating in the transcriptional activation of a promoter and expression of a yet unknown protein activates the ERK signal transduction cascade, AP-1, and consequently MMP-9.

Acknowledgements

We would like to thank S. Schinkel and Dr S. Poppelreuther for their excellent technical support with the imaging. Special thanks go to K. Feldbauer. This study was supported by a DFG grant (Si 634-4/1, Si 634-5/1), and a Deutsche Krebshilfe e.V. grant (107689) to C. Simon and a DFG grant (IF 6/1-1/2) to T. Iftner

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