Control of viral replication and transcription by the papillomavirus E8È2 protein

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ABSTRACT

Human papillomaviruses have adjusted their replication levels to the differentiation state of the infected keratinocyte. PV genomes replicate in undifferentiated cells at low levels and to high levels in differentiated cells. Genome replication requires the viral E1 helicase and the viral E2 transcription/replication activator. The limited replication in undifferentiated cells is predominantly due to the expression of the highly conserved E8È2 viral repressor protein, which is a fusion between E8 and the C-terminal half of the E2 protein. E8È2 is a sequence-specific DNA binding protein that inhibits viral gene expression and viral genome replication. The E8 domain is required for repression activities, which are mainly due to the interaction with cellular NCOR/SMRT corepressor complexes. In the case of HPV16, the most carcinogenic HPV type, E8È2 not only limits genome replication in undifferentiated cells but also productive replication in differentiated epithelium. E8È2 is expressed from a separate promoter that is controlled by unknown cellular factors and the viral transcription and replication regulators E1, E2 and E8È2. In summary, E8È2 is an important negative regulator whose levels may be critical for the outcome of HPV infections.

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1. Introduction

Papillomaviruses are non-enveloped, double-stranded DNA viruses with currently more than 200 different sequenced human genotypes. Infections with human papillomavirus (HPV) may cause different kinds of warts or intraepithelial neoplasias on cutaneous or mucosal epithelia. Importantly, infections with high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 are the major risk factor for the development of cancer of the cervix uteri and anus and contribute to a fraction of cancers of the oro-pharynx, penis, vagina and vulva (Parkin and Bray, 2006).

HPV infect keratinocytes in the basal layer of cutaneous or mucosal skin where limited replication of the viral genomes occurs
and only early transcripts are expressed. Virus capsid protein expression and amplification of viral genomes to high levels occurs only in the upper layers of the epithelium and is preceded by a reentry of the infected cells into the cell cycle (Moody and Laimins, 2010). Viral gene expression and replication are tightly controlled in the different phases of the viral replication cycle by both host cell and viral proteins.

2. The E2 Protein

The E2 protein is a key regulator protein that is highly conserved among papillomaviruses. E2 consists of a ∼200aa N-terminal domain which is connected by a flexible hinge region of varying length to a C-terminal domain of ∼100aa. The C-terminal domain is responsible for the specific recognition of DNA sequences (ACCN6GGT; E2 binding sites (E2BS)) and also for the dimerization of E2 proteins (see for a review: McBride, 2013). E2 is essential for the replication of PV genomes (Sankovskii et al., 2014; Stubenrauch et al., 1998b; Ustav and Stenuid, 1991). This is mainly due to the interaction of E2 with the viral E1 helicase (Bergvall et al., 2013; McBride, 2013). The papillomavirus origin of replication is composed of overlapping E1 binding sites flanked by E2BS (Fig. 1). The E1-E2 protein complex recognizes E2BS with high affinity and thus E2 acts as a helicase loader. E1 unwinds the viral DNA and then recruits cellular replication proteins which replicate the viral genomes (see for a review: Bergvall et al., 2013)). E2 can also activate transcription and this activity is crucial for bovine papillomavirus 1 (BPV1) gene expression (McBride, 2013). In contrast, activation of transcription by HPV31 E2 is not required for viral genome replication in undifferentiated human keratinocytes but may contribute to the differentiation-dependent replication (Klymenko et al., 2016; Sakakibara et al., 2013; Stubenrauch et al., 1998a). E2 can also act as a repressor of transcription from promoter-proximal E2BS which has been suggested to be important to limit expression of the viral E6 and E7 oncoproteins in carcinogenic HPV. Consistent with this, in the context of hybrid HPV16 genomes that contain the Epstein-Barr virus oriP sequence and an EBNA1 expression cassette, mutations in the N-terminal domain or in the DNA-binding domain (DBD) of E2 increased early viral gene expression (Soeda et al., 2006). However, in the context of full-length HPV16 genomes only mutations in the DBD but not in the N-terminal domain of E2 increased viral early gene expression which supports the idea that E2 repressor proteins that only share the C-terminal but not the N-terminal domain with E2 are mainly responsible for the repression of viral transcription (Lace et al., 2008). Thus, the physiological relevance of E2’s repression activity for the replication of high-risk HPV is still controversial. In addition, E2 has been implicated in the nuclear retention of viral genomes after cell division by attaching the viral genomes to mitotic chromosomes (McBride et al., 2012).

3. E2 repressor proteins

3.1. Transcripts for PV repressor proteins

Studies with BPV1 indicated that additional proteins are derived from the E2 gene that are called E2TR (or E2C) and E8/E2 (Choe et al., 1989; Hubbert et al., 1988; Lambert et al., 1987). E2TR is derived from a RNA initiating at the P3080 promoter within the E2 gene and gives rise to an N-terminally truncated E2 that starts at residue 162 (Lambert et al., 1987). The E8/E2 mRNA is derived from the P890 promoter within the E1 gene and is spliced from nt. 1235 to nt. 3225 and the corresponding protein consists of residues 1-11 of E8 fused to residues 207-410 of E2 (Choe et al., 1989). The analysis of RNA from HPV1, 5, 11, 16, 18, 31 and 33-positive cells and cottointeral rabbit PV (CRPV)-induced papillomas indicated that HPV and CRPV express an RNA homologous to the BPV1 E8/E2 mRNA (Fig. 2) (Doorbar et al., 1990; Ferty et al., 2011; Isok-Paas et al., 2015; Jeckel et al., 2003; Lace et al., 2008; Palermo-Dilts et al., 1990; Rotenberg et al., 1989; Snijders et al., 1992; Stubenrauch et al., 2000; Wang et al., 2011). The respective HPV gene products have been labeled E2C, E8E2C or E8E2. Experts in the field (A. Abroi, T.H. Haugen, P.M. Howley, A.A. McBride, P.F. Lambert, F. Stubenrauch, Z.M. Zheng) have now agreed on E8E2 as the official designation. Comparable to BPV1, the HPV E8E2 mRNA is generated from a separate promoter within the E1 gene with transcriptional start sites located ∼70-150 nt upstream of the E8 ATG start codon (Fig. 2) (Chen et al., 2014; Isok-Paas et al., 2015; Lace et al., 2008; Milligan et al., 2007; Sankovskii et al., 2014; Straub et al., 2015; Toots et al., 2014; Wang et al., 2011). Quantitative transcript analyses also indicated that the major transcript expressed by this promoter is the E8E2-encoding transcript (Straub et al., 2015). The HPV16 E8 promoter displays basal activity in HPV-negative keratinocytes. In contrast to the major viral early promoter P97, the basal activity of the E8 Promoter is not modulated by enhancer elements in the URR (Straub et al., 2015). Instead, constitutive activity of the HPV16 E8 promoter requires two conserved elements (CE2 and CE3, Fig. 2) close to the transcription start site which bind to unknown cellular proteins (Straub et al., 2015). In addition, the E8 promoter is regulated by viral proteins. E8E2 inhibits its own promoter and E2 weakly activates it (Straub et al., 2015). Interestingly, the combination of E1 and E2 activates the E8 promoter to higher levels than the P97 promoter (Straub et al., 2015). Since the E8 promoter is both positively and negatively regulated by viral replication proteins, it might act as a sensor and modulator of viral copy number.

Similar to BPV1 E8/E2, HPV E8E2 consists of 12-16 residues encoded by the E8 exon fused to the hinge/DBD/dimerization domains of E2 (Fig. 2). Bioinformatic analyses suggest that the potential to generate E8E2 transcripts and the corresponding fusion proteins is highly conserved as E8 exons can be found in more than 300 mammalian PV genomes, including all HPV types in the alpha, beta, gamma and mu genera (Ferty et al., 2011; Puustusmaa and Abroi, 2016), Both E2TR and E8E2 (E8/E2) share the hinge and dimerization/DNA binding domain with E2 and thus are able to form homo- and heterodimers and interact with E2BS in the viral genome (Kurg et al., 2006; McBride et al., 1989). In BPV1 infected cells E2TR is the predominant species followed by E8/E2, whereas E2 is the least abundant form (Hubbert et al., 1988; Kurg et al., 2006; Lambert et al., 1989).

3.2. Phenotypes of E2 repressor knock-out genomes

The analysis of BPV1 E2TR- genomes revealed that E2TR limits BPV1 replication in C127 cells (Table 1) (Lambert et al., 1987; Riese et al., 1990). In contrast, the knock-out of E8/E2 had no discernible phenotype (Table 1) (Lambert et al., 1990). Surprisingly, BPV1 E8/E2-/E2TR- genomes showed both a decreased replication...
activity and transformation efficiency in long-term assays (Table 1) (Lambert et al., 1990). When BPV1 genomes were retrieved from E8/E2- or E8TR-transformed cells and sequenced, a frequent reversion of the E8 ATG mutation but not of the E8TR ATG mutation was observed (Zemlo et al., 1994) indicating that E8/E2 is crucial for the transformation process. CRPV E8E2 (originally labeled E9E2C) knock-out genomes were tested in domestic rabbits and did not show a different rate of tumor induction, tumor size, viral transcription or viral copy number in the tumors (Table 1) (Jekel et al., 2003). However, it should be kept in mind that the natural host for CRPV are cottontail rabbits (Sylvilagus floridanus) where virus progeny is produced whereas experimental infections of domestic rabbits (Oryctolagus cuniculus) are abortive infections (Shope and Hurst, 1933). Thus, a role for CRPV E8E2 may only be evident in productive infections. HPV1, 5, 8, 11, 16, 18 and 31 E8E2 knockout genomes replicate to 10-100-fold higher levels than wt genomes in short-term assays in immortalized human keratinocytes (HPV16, 31), normal human keratinocytes (HPV1, 8, 16, 31) or the U2OS osteosarcoma cell line (HPV5, 11, 18) (Table 1) (Dreer et al., 2016; Isok-Paas et al., 2015; Kurg et al., 2010; Lake et al., 2008; Sankowski et al., 2014; Straub et al., 2014; Stubenrauch et al., 2000; Zobel et al., 2003). In contrast to BPV1, no antibodies are currently available that allow the detection of HPV E8E2 proteins in infected cells, but evolutionary conservation, viral mutants and complementation assays strongly suggest that the loss of the E8E2 fusion protein is responsible for the phenotypes of HPV E8E2- mutants. Firstly, mutation of the E8 exon splice donor site in HPV16 or 31 has a similar replication phenotype as E8 ATG or translation termination linker (TTL) mutants in E8 (Lake et al., 2008; Stubenrauch et al., 2000) which indicates that the spliced E8E2 product is responsible. Secondly, TTL mutants in E8 or E2 downstream of the splice acceptor used to generate the E8E2 mRNA, but not in E2 upstream of the splice acceptor increase activity of the viral major early promoter P97 (Lake et al., 2008) which also shows that the spliced E8E2 is the main transcriptional repressor. Thirdly, complementation assays with HPV16 mutant genomes also indicate that E8E2 is the main trans-factor to limit genome amplification (Lake et al., 2008) and finally, co-transfection of E8E2 expression plasmids with HPV E8E2- genomes results in a concentration-dependent decrease in viral genome replication (Lake et al., 2008; Sankowski et al., 2014).

### Table 1

<table>
<thead>
<tr>
<th>PV type</th>
<th>knock-out</th>
<th>short term</th>
<th>long term</th>
<th>differentiation</th>
<th>in vivo</th>
<th>host</th>
<th>reference</th>
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<tbody>
<tr>
<td>BPV1</td>
<td>E8/E2</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>mouse (C127)</td>
<td>mouse (C127)</td>
<td>Lambert et al. (1990)</td>
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<td></td>
<td>E8TR</td>
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<tr>
<td>CRPV</td>
<td>E8E2</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>mouse (C127)</td>
<td>domestic rabbit</td>
<td>Lambert et al. (1990)</td>
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<td></td>
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<td>Sankowski et al. (2014)</td>
</tr>
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**Fig. 2.** Schematic structure of the HPV16 early region. E1BS (white dot) and E2BS (black dots) in the upstream regulatory region and the early region of HPV16 are shown. Viral transcription initiates at the major early promoter P97, at the differentiation-dependent P670 and at the E8 promoter and terminates at the early polyadenylation site (pAe). The E8E2 transcript is initiated at the E8 promoter (P8e) and is then spliced to the C-terminal part of the E2 gene via the splice donor at nt. 1302 (SD1302) and the splice acceptor at nt. 3358 (SA3358). The P8e is controlled by unknown cellular factors that bind to conserved elements (CE2 and 3) upstream of the transcription start site indicated by white boxes.
3. EZTR homodimers act as repressors of BPV1 replication and activation of transcription by E2. EZTR homodimers possess transactivation activity and guide E1 to the viral origin of replication to induce BPV replication (left side). E2/E2TR heterodimers are still able to transactivate and to interact with E1 to activate replication (right side). Transactivation activity of heterodimers is slightly reduced compared to E2 homodimers (dotted line). In contrast, EZTR homodimers act as repressors of BPV replication and E2-transactivation by binding site competition at E2BS.

Taken together, this indicates that, in contrast to BPV1, E8E2 is the main negative regulator of HPV replication.

While increased replication in short-term assays is a general phenotype for HPV E8E2 knock-out genomes, long-term cultivation revealed differences between HPV16 and 31. HPV16 E8E2 knock-out genomes can be maintained long-term with an elevated copy number as extrachromosomal elements, whereas HPV31 E8E2-genomes give rise to only integrated viral genomes (Table 1) (Lace et al., 2008; Straub et al., 2014; Stubenrauch et al., 2000). The underlying mechanism is currently not understood and cannot be easily explained as HPV16 and 31 are closely related viruses. Since E8E2 limits viral replication in undifferentiated cells in tissue culture which is thought to mimic initial infection, E8E2 represents a prime candidate to control the switch from the non-productive to the productive HPV16 replication cycle in differentiating keratinocytes. However, the comparison of HPV16 wt with E8E2- genomes in organotypic keratinocyte cultures revealed that E8E2- genomes also display higher levels of viral genomes, transcripts and the late viral proteins E4 and L1 in differentiated cells (Straub et al., 2014). Consistent with E8E2 being active upon differentiation, E8E2 transcript levels are higher in cells maintained in organotypic than in monolayer cultures (Straub et al., 2014; Straub et al., 2015). This indicates that HPV16 E8E2 not only limits replication in undifferentiated cells but is also present in differentiated cells to restrict productive replication. While it appears counter-intuitive that a virus actively limits productive replication, this might represent an immunoevasive mechanism to restrict the expression of highly antigenic viral proteins such as the L1 capsid protein.

3.3. Mechanism of E2 repressor protein activity

3.3.1. BPV1 E2 repressor proteins

The negative effect of EZTR on BPV1 replication is due to its interference with both E2’s transactivation and activation of DNA-replication functions (Lambert et al., 1987; Lim et al., 1998). This has been confirmed by BPV1 E2 single-chain heterodimers in which the E2 DBD has been fused to full-length E2 (Kurg et al., 2006; Kurg et al., 2009). These single-chain E2/E2DBD heterodimers also activate E2-dependent transcription albeit at lower levels than E2 homodimers (Kurg et al., 2006). EZTR homodimers inhibit E1/E2-dependent replication by binding site competition with E2 homodimers (Lim et al., 1998). Taken together, these data suggest that EZTR homodimers compete with E2 homodimers and E2/E2TR heterodimers at E2BS to inhibit viral replication (Fig. 3).

Similar to EZTR, overexpression of E8/E2 inhibits focus formation by wt BPV1 and also transactivation by E2 (Choe et al., 1989; Lambert et al., 1990). However, E8/E2 has E2-independent activities as it inhibits the constitutive promoter activity of the BPV1 upstream regulatory region (URR) (Choe et al., 1989; Sankovski et al., 2015). The E8 domain of E8/E2 acts as a nuclear matrix targeting signal and mutations that change the sub-nuclear localization of E8/E2 also loose transcriptional repression activity (Sankovski et al., 2015). Whether BPV1 E8/E2 acts mainly via competition with E2 comparable to EZTR or also by E2-independent transcriptional repression is currently unknown, but the observation that only E8/E2 and not EZTR expression is reconstituted in transformed cells derived from EZTR-/E8/E2- genomes, makes it likely that E8/E2 has different activities from EZTR.

3.3.2. HPV E8E2 repressor

HPV E8E2 inhibits transcription of the viral major early promoter far more efficiently than E2 and thus represses the expression of the HPV E6 and E7 oncoproteins as well as the E1 and E2 replication proteins (Stubenrauch et al., 2000). In contrast to E2, transcriptional repression by E8E2 also occurs from promoter-distal E2BS and acts over a distance of 1kbp (Straub et al., 2015; Stubenrauch et al., 2001). In addition, E8E2 inhibits the E1/E2-dependent replication of the viral origin (Dreer et al., 2016; Straub et al., 2014; Stubenrauch et al., 2000; Zobel et al., 2003). Thus, increased replication of E8E2 knock-out genomes is most likely due to a combined effect of both de-repressed viral transcription and de-repressed E1/E2-dependent replication (Fig. 4).

Transcriptional repression requires at least one E2BS and the DNA-binding domain of the E8E2 protein (Lace et al., 2008; Stubenrauch et al., 2007; Stubenrauch et al., 2001) which indicates that E8E2 acts mainly as a sequence-specific DNA binding transcription factor. Unexpectedly, it turned out that the E8 part is a major contributor to the transcriptional repression activity and the inhibition of E1/E2-dependent origin replication (Dreer et al., 2016; Straub et al., 2014; Stubenrauch et al., 2007; Stubenrauch et al., 2001; Zobel et al., 2003). Residues K5, W6 and K7, which are highly conserved among E8E2 proteins from alpha-PV (Fig. 5), are required for both the repression of transcription and E1/E2-dependent origin replication (Dreer et al., 2016; Straub et al., 2014; Stubenrauch et al., 2007; Stubenrauch et al., 2001; Zobel et al., 2003). HPV16 and 31 E8 KWK mt genomes replicate to similar levels as E8E2 knock-out genomes which strongly indicates that the E8 domain of E8E2 is crucial for its activity under physiological conditions (Straub et al., 2014; Zobel et al., 2003). A fusion protein between HPV31 E8E2 residues 1-37 and the DBD of the yeast transcription factor Gal4 acts as a transcription repressor and an inhibitor of E1/E2-dependent replication in a Gal4-binding site and E8 KWK- motif dependent manner (Ammermann et al., 2008; Zobel et al., 2003). This showed that the E8 part has repression activity that is independent from the E2 DBD/dimerization domain. This also suggested that the inhibition of E1/E2 dependent-origin replication does not mainly result from competition between E2 and E8E2 homodimers at E2BS in the viral origin.
3.3.3. Cellular interaction partners for HPV E8Æ2 proteins

Several host cell proteins have been identified as interactors of E8Æ2 proteins. In a yeast–2-hybrid approach the chromohel case domain 6 (CHD6) protein was found to interact with the DBD of E8Æ2 and this contributes to transcriptional repression (Feretey et al., 2010). A candidate approach suggested class I histone deacetylases (HDAC) 1, 2 and 3 and TRIM28/KAP1/transcription intermediary factor 1-beta as E8 domain-dependent interactors (Ammermann et al., 2008). Of those interactors, HDAC3 was shown to be involved in the E8Æ2 dependent transcriptional repression of the viral major early promoter in an independent study (Powell et al., 2010). Using an unbiased affinity-purification/mass spec (AP/MS) approach, additional six high-confidence interacting proteins of the E8 domain of HPV31 E8Æ2 were identified: ARG1, BLMH, CASP14, NCOR1, TBLR1 and TGM1 (Powell et al., 2010). Of those, NCOR1 contributed to transcriptional repression by E8Æ2 (Powell et al., 2010). Recent AP/MS analyses confirmed HDAC3, NCOR1 and TBLR1 and newly identified GPS2, SMRT and TBL1 as interacting proteins for HPV16 and 31 E8Æ2 (Fig. 4) (Dreer et al., 2016). GPS2, HDAC3, NCOR1, SMRT, TBL1 and TBLR1 are known to form the stable NCoR/SMRT co-repressor complexes which have been shown to mediate the transcriptional repression activity of several cellular transcription factors (Karagianni and Wong, 2007; Perissi et al., 2010). NCoR and SMRT (also known as NCoR2) are highly homologous proteins that serve as scaffolding proteins by interacting with GPS2, HDAC3, TBL1 and TBLR1 (Watson et al., 2012). TBL1 and TBLR1 are also homologous proteins which form homotetramers or heterotetramers that interact with two molecules of NCoR or SMRT (Watson et al., 2012). The interaction between NCoR/SMRT complexes and E8Æ2 is dependent upon the E8 KWK motif (Dreer et al., 2016). The E8Æ2 proteins of HPV1 and 8 also interact with NCoR/SMRT complex components in an E8-dependent manner (Dreer et al., 2016). Consistent with a functionally relevant interaction siRNA knock-down of NCoR/SMRT complex components or over-expression of a dominant-negative NCoR fragment relieved repression of transcription and replication by E8Æ2 in an E8 domain dependent manner (Dreer et al., 2016). This implicated for the first time NCoR/SMRT complexes in the negative control of viral replication. Co-localization experiments showed that NCoR/SMRT complexes can be recruited by E8Æ2 into E1/E2-positive replication foci which is consistent with the idea that E8Æ2 proteins recruit NCoR/SMRT complexes to the viral genome to inhibit viral promoter activity and the E1/E2-dependent replication (Dreer et al., 2016).

The interaction of NCoR/SMRT with HPV8 E8Æ2 requires KWK residues at position 2–4, which resembles the KWK motif in alphahp (Dreer et al., 2016). Since the E8 KWK motif is highly conserved among alpha-HPV and the E8 KWK motif is highly conserved among beta-, gamma- and mu-HPV (Fig. 5), it is very likely that the E8Æ2-NCoR/SMRT interaction may take place with all HPV E8Æ2 proteins. Thus, the interaction between E8Æ2 and NCoR/SMRT may be as conserved as the one between E2 and the cellular Brd4 protein (McBride, 2013).

4. Conclusion

E2 repressor proteins were first identified in BPV1 and E2TR was shown to be the main repressor of viral replication. In contrast, studies with HPV have indicated that the highly conserved E8Æ2 is the main inhibitor of viral replication. E8Æ2 not only counteracts E1 and E2 but has transcriptional repression activity on its own. E8Æ2 interacts via the E8 part with the cellular NCoR/SMRT co-repressor complexes to repress viral transcription and replication. Surprisingly, E8Æ2 not only limits the initial amplification of HPV but also the productive replication of HPV16. This may indicate that the outcome of HPV infections in vivo is critically dependent on E8Æ2 levels. Shortly after infection E8Æ2 levels may determine whether the infection becomes latent or productive and E8Æ2 lev-
els during the productive phase may determine how much virus is produced.

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