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Disruption of pocket protein dream complexes by E7 proteins of different types of human papillomaviruses

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Summary. – It has been shown that the E7 protein of the high-risk HPV-16 transforms cells *in vitro* and binds pRB, p107 and p130, so called pocket proteins associated in cells with DREAM proteins, while that of the low-risk HPV-6 does not transform cells and binds p130 but not pRB or p107. These facts may indicate that p130 is essential for the HPV life cycle. To gain further insight into the relationship between HPV E7 proteins and pocket protein-DREAM complexes, E7 proteins of HPVs of various risk categories were expressed via appropriate vectors in T98G cells and the levels of various pocket proteins either total or associated with DREAM were analyzed. The obtained results demonstrated that high-risk HPV-16, HPV-18 and HPV-33, low-risk HPV-1 and HPV-11, and cutaneous HPV-48 disrupted pocket protein-DREAM complexes in T98G cells to a similar extent.

Keywords: human papillomavirus; E7 protein; pocket proteins; DREAM complex

Introduction

The human papillomaviruses (HPV) are responsible for some of the most common sexually transmitted infections in women. The HPVs belong to the *Papovaviridae* family, which are non-enveloped viruses that contain a covalently closed-double stranded DNA genome. The 8 kbp HPV DNA molecule contains the early and late genes clustered in separate regions of the HPV genome. Early genes (E1, E2, E4, E5, E6, and E7) code for proteins involved in viral DNA replication, transcription control and cellular transformation, whereas late genes encode the major viral capsid protein (L1) and a minor capsid protein (L2) (Harald zur H., 2002).

HPV types that are associated with genital infections have been classified into two types according to their risk of causing cervical cancers; that is, high risk types (e.g.HPV-16 and HPV-18) and low risk types (e.g.HPV-6 and HPV-11) (Harald zur H, 2002). It appears that both high and low risk HPV-encoded E6 and E7 proteins show some ability to disrupt the normal control of cell proliferation, however, only E6 and E7 proteins encoded by high risk types are able to contribute to cell transformation (Hwang *et al*., 2002). It is well-established that cell division follows a series of complex events which constitute the cell cycle. Actively dividing cells pass through distinct stages of DNA synthesis (S-phase) and mitotic division (M-phase), and these activities are separated by gaps (G1 and G2). The main control of the cell cycle involves a family of kinases, known as cyclin-dependent kinases which control passing through both S and M phases (Johnson and Walker, 1999).

The E6 and E7 genes of high risk HPV types are sufficient to immortalize human keratinocytes in tissue culture (Arroyo *et al*., 1993), and this activity requires inactivation of the cellular tumor suppressors p53 and pRB. Whilst E6 interacts with p53, the E7 proteins from high risk HPVs bind the retinoblastoma (pRB) family of proteins. pRB was identified as an important target of oncoproteins that are expressed by DNA tumor viruses, including the E7 protein

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Abbreviations: dE2F and Myb-interacting proteins; DREAM = *Drosophila* RBF; HPV = human papillomavirus; LIN complex = stable core complex of five Muv-B like proteins; pRB = retinoblastoma (phospho) protein

of HPV (DeFilippis *al*., 2003). These findings indicated that pRB is a regulator of cell proliferation. pRB is closely related to two genes in mice and humans (p107 and p130), which are not commonly mutated in tumors and these proteins are referred to as the pocket proteins because their main sequence similarity resides in a domain that mediates interaction with the viral oncoproteins (Classon and Harlow, 2002). Interaction of E7 with the pocket proteins results, in the constitutive activation of E2F transcription factors (Classon and Harlow, 2002).

Recently, it has been discovered that the pocket proteins are constituents of mammalian DREAM complexes. DREAM was first discovered in Drosophila embryonal cells and was named from its composition of *Drosophila* RBF, dE2F, and Myb-interacting proteins (Korenjak *et al.,* 2004). Mammalian DREAM consists of a core LIN complex (LINC: comprised of Lin-9, Lin-37, Lin-52, Lin-54, and RBBP4) that associates with p130/E2F4 in G0/G1 to repress E2F-regulated genes (Litovchick *et al*., 2007) or with B-Myb in S/G2 to activate genes required for mitosis (Pilkinton *et al*., 2007; Schmit *et al*., 2007). Since we have previously shown (Nor Rashid *et al*., 2011) that the E7 protein of high-risk HPV-16 disrupts the p130-DREAM complex, in this study we investigated the above phenomenon extended to various pocket proteins in relation to high-risk and low-risk HPVs.

Materials and Methods

Cells and vector. Human glioblastoma (T98G) cell lines were maintained in Dulbecco′s modified Eagle′s medium (Gibco) supplemented with 10% FCS, 100 U/ml of penicillin and 100 µg/ml of streptomycin. Incubation was carried out at 37°C in 10% CO_2 and 95% humidity. The pMSCV puro (Clontech) plasmid was used to express the different types of human papillomaviruses. The constructs were confirmed by DNA sequencing.

Antibodies. Polyclonal Lin-9 antibody (ab46415) used in western blot was from Abcam; B-myb (sc-724), p107 (sc-318), and p130 (sc-317) antibodies were from Santa Cruz; β-actin (A-2066) antibody was from Sigma Aldrich and HA (MMS-101R) antibody was from Covance (USA). The B-myb LX015.1 monoclonal antibody was described previously (Tavner *et al*., 2007). The Lin-9 rabbit polyclonal antibody (Knight *et al*., 2009) used for immunoprecipitation of DREAM complexes was purified by using a protein A-sepharose column.

Transfection of cells. Transient transfections were carried out with calcium phosphate precipitation. T98G cells (1 x 10^6) were plated in 10 cm dishes and incubated at 37°C with 10% CO_2. The next day, T98G cells were transiently transfected with 30 µg of the respective pMSCV puro plasmids (pMSCV puro, pMSCV puro HPV-1 E7HA, HPV-11 E7HA, HPV-16 E7HA, HPV-18 E7HA, HPV-33 E7HA, and HPV-48 E7HA). Following transfection, the culture medium was changed and 2 µg/ml of puromycin was supplemented and the cells incubated for a further 24 hrs. Finally, cells were harvested 48 hrs after puromycin selection for RNA and protein extractions.

Immunoprecipitation and Western blot analysis. Immunoprecipitation was performed as described previously (Nor Rashid *et al*., 2011). Proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and subjected to Western blot analysis using standard procedure.

Results

E7 proteins from a number of different HPV types were expressed ectopically in T98G cells, which have well characterized DREAM complexes. Six different E7 proteins were assayed in this system, from the high risk types HPV-16, HPV-18 and HPV-33, the low risk type HPV-11, and the cutaneous types HPV-1 and HPV-48 (Table 1). T98G cells were transiently transfected with HA-tagged E7 genes cloned in the pMSCVpuro vector using the calcium phosphate co-precipitation method. Transfected cells expressing the E7 types were selected with puromycin. To test whether the E7 proteins were expressed at comparable levels, 100 µg of total proteins of nuclear lysates were immunoprecipitated with

Table 1. Characteristics of HPVs compared in this study

HPV			E7 protein	
Type	Risk category	Pathogenicity	LXCXE motif	Affinity to pocket proteins
1		Plantar warts	**LYCYE**	High
11	Low	Genital warts	**LHCYE**	Low
16			**LYCYE**	High
18	High	Intraepithelial neoplasia & carcinoma	**LLCHE**	High
33			**LYCYE**	High
48	Cutaneous	Non-melanoma skin carcinoma	**LISDE**	Nil

HPV types that have frequently been associated with malignant progression are in bold. The L, C, and E residues presented within the putative LXCXE motif of each HPV type are also shown in bold. It is important to note that the classification of each E7 type as having low or high pocket protein affinity is a broad categorisation. Information was taken from Munger *et al*. (1989), Ciccolini *et al*. (1994), Caldeira *et al*. (2000), Dong *et al*. (2001).

HA antibody, resolved in a 15% SDS-PAGE gel, transferred to the membrane and detected by an anti-HA mouse monoclonal antibody. It was found to be necessary to transfer the E7 proteins onto a PVDF membrane, as the low expression levels made them more difficult to detect on a nitrocellulose membrane. The resultant Western blot (Fig. 1) showed expression of each E7 type at comparable levels, albeit with some minor variations.

Nuclear lysates from various E7 proteins transfected in T98G cells were assessed by immunoprecipitation with Lin-9 antibody and detected by p130, p107, and B-myb antibodies to determine whether the p130/DREAM complex was also disrupted in the various types of HPV. The results obtained with the input controls indicated that expression of p130 was reduced in cells expressing high risk HPV-16 E7, HPV-18 E7, HPV-33 E7, and low risk HPV-11 E7 (Fig. 2). This is also reflected in the p130/DREAM complexes, which were greatly disrupted by HPV-16 E7, HPV-18 E7, and HPV-33 E7 because they can bind to pocket proteins at high efficiency and thereby disrupt the interaction with the DREAM complex (Tommasino and Crawford, 1995). Even though HPV-11 E7 reduced p130 expression, it did not reduce p130/DREAM complex formation to the same extent as the high risk E7 proteins (Fig. 2). On the other hand, expression of HPV-1 E7 from a cutaneous HPV type did not decrease p130 levels to the same extent, as indicated by the input control. However, it did result in diminished p130/DREAM complex formation compared to control cells (Fig. 2). The results obtained with HPV-48 E7, which is encoded by another cutaneous HPV type and lacks a LXCXE motif, were quite unexpected, as it slightly reduced the p130 levels and significantly disrupted

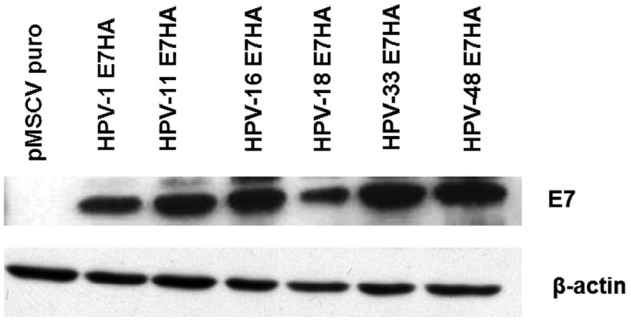

Fig. 1

Expression of the E7 protein of HPVs of various types in T98G cells

The cells were transfected with vectors expressing the HA-tagged E7 protein of various type of HPV, harvested after 48 hrs, lysed, immunoprecipitated with a HA antiserum and subjected to Western blot analysis. The figure shows the E7 protein and β-actin as control.

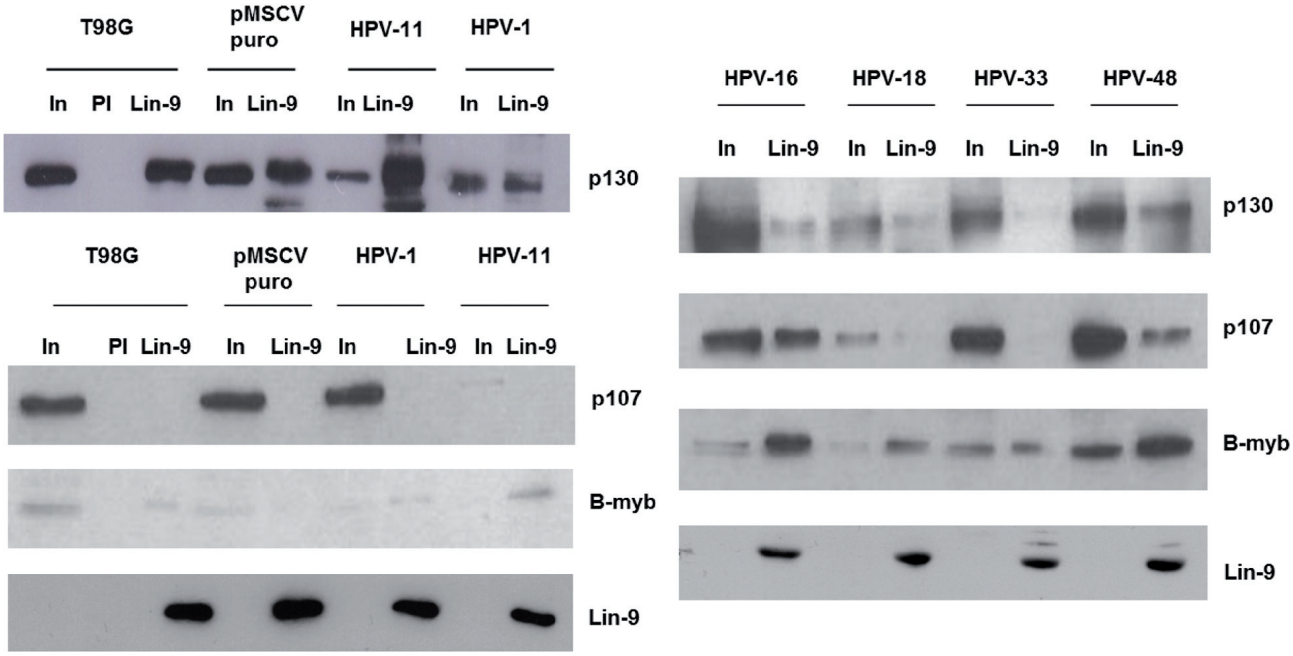

Fig. 2

Levels of various pocket proteins and their complexes with DREAM in T98G cells expressing the E7 protein of HPVs of various types

The cells were transfected with vectors expressing HA-tagged E7 protein with various type of HPV, harvested after 48 hrs, lysed, immunoprecipitated with a Lin-9 antiserum and subjected to Western blot analysis. Individual pocket proteins were detected using specific antisera/antibodies (p130, p107, and B-myb; (In) input controls; (PI) pre-immune serum.

the p130/DREAM complex even though HPV-48 E7 binds to pocket proteins with extremely low affinity (Caldeira *et al.*, 2000; Dong *et al.*, 2001). However, our previous study (data not shown) has shown that HPV-48 E7 protein was competent to bind to the cdki p21 *in vitro*. It is known that p21 is involved in E2F/pocket protein-cyclinE/A-CDK2 pathways in G1 and S phase. By binding to p21, HPV-48 E7 will abrogate its inhibition of cyclin/CDK activity and therefore lead to p130 hyperphosphorylation. This is predicted to result in impairment of p130-DREAM complex formation and may also affect p130 stability.

Expression of p107 is usually low when compared to p130 in T98G cells and p107 is a minor constituent of DREAM complexes. However, p107 expression is induced following the G1/S transition. There was no detection of the p107/DREAM complex in T98G cells transfected with the empty vector or in cells transfected with most E7 types, however, p107/DREAM was detectable in cells transfected with HPV-16 E7 and HPV-48 E7 (Fig. 2). The formation of p107/DREAM complexes upon HPV-16 E7 and HPV-48 E7 expression presumably reflects their ability to disrupt the p130/DREAM complex (Fig. 2), either through direct interaction of HPV-16 E7 with p130 or possible effects of both HPV-16 E7 and HPV-48 E7 on Cdk2 phosphorylation activity and promotion of the cell cycle into S phase entry. It is notable that HPV-16 E7 and HPV-48 E7 expression also induced the most pronounced increase on B-myb/DREAM complex formation (Fig. 2). Whereas HPV-18 E7, HPV-33 E7 and to a lesser extent HPV-11 E7 also induced this complex to an extent, when compared to the non-transfected cells and empty vector (Fig. 2), HPV-16 E7 and HPV-48 E7 induced equivalently the highest B-myb/DREAM complex expression.

Discussion

High risk and low risk E7 proteins share the ability to target the p130 protein for degradation (Zhang *et al.*, 2006) and this property is significant with other studies that demonstrated that the S phase is induced in cells infected with different E7 types which were targeting p130 protein (Genovese *et al.*, 2008). Recently, p130 has been found as one of the constituents of a transcriptionally repressive complex termed DREAM. Therefore, we would like to associate the correlation of E7 proteins of HPVs of various types with p130/DREAM complex. Our work showed that the expression of p130 was reduced in cells expressing high risk and low risk HPVs, which is probably due to E7-mediated degradation (Roman *et al.*, 2006). It is notable that strong affinity does not necessarily correlate with the ability to induce cellular transformation (Ciccoloni *et al.*, 1994; Caldeira *et al.*, 2000), since the E7 protein from the low risk HPV-1 can associate strongly with p130, but fails to induce degradation and transformation (Alunni-Fabronni *et al.*, 2000). The cutaneous HPV-48 E7 and the low risk HPV-11 E7 disrupted p130/DREAM complexes less efficiently than these other E7 types. HPV-11 E7 expression nonetheless reduced p130 expression quite dramatically, and the lesser effect on complex formation probably relates to its lower affinity binding to pocket proteins. Comparison of the pocket protein binding domains of the high risk and low risk E7 proteins reveals a difference of one amino acid, aspartic acid 21 in HPV-16 versus glycine 22 in HPV-11. Substitution of glycine 22 with an aspartic acid in HPV-11 E7 confers greater affinity in binding to pocket proteins. However the correlation between the efficiency of binding to pocket protein does not hold for every HPV E7 types. In fact HPV-1, which has never been associated with cancer, has an aspartic acid in the pocket protein binding site, like the high risk HPV E7s and binds to pocket protein with approximately the same affinity as HPV-16 E7.

The results with HPV-48 E7 were unexpected. Firstly, the HPV-48 E7 has shown the ability to disrupt p130/DREAM complexes quite dramatically; in fact HPV-48 E7 binds to pocket protein with a very low affinity (Caldeira *et al.*, 2000; Dong *et al.*, 2001). However, HPV-48 E7 has an ability to bind to p21 and inactivate the E2F/p130-cyclin/cdk pathway which indirectly inhibits p130 to associate with E2F. This may explain how HPV-48 E7 disrupted the p130/DREAM complex. Secondly, it is also apparent from the results that only T98G transfected with cutaneous HPV-48 E7 and high risk HPV-16 E7 expressed p107/DREAM complexes and they also expressed the B-myb/DREAM complex at the highest level compared to other HPV types. Schmit *et al.* (2007), have shown that p107 associates with the B-myb/DREAM complex in T98G cells. Therefore, the presence of p107 in DREAM complexes in HPV-16 E7 and HPV-48 E7 transfected cells may relate to this association rather than the formation of repressive complexes.

Based on our results, we suggest that the binding of E7 protein to p130 protein is the most important step for HPVs to induce the host cell cycle into S phase. Our data also demonstrate that both high risk and low risk HPV E7 proteins have the ability to dissociate p130/DREAM complex even though the low risk HPV E7 types bind at lesser affinity to the p130. Indeed, the cutaneous HPV-48 E7 had similar activity to the high risk HPV-16 E7 in this study.

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