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Human papillomavirus (HPV)-18 E6 oncoprotein interferes with the epithelial cell polarity Par3 protein

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ABSTRACT

High-risk human papillomavirus (HPV) infection is the principal risk factor for the development of cervical cancer. The HPV E6 oncoprotein has the ability to target and interfere with several PSD-95/DLG/ZO-1 (PDZ) domain-containing proteins that are involved in the control of cell polarity. This function can be significant for E6 oncogenic activity because a deficiency in cell polarisation is a marker of tumour progression. The establishment and control of polarity in epithelial cells depend on the correct asymmetrical distribution of proteins and lipids at the cell borders and on specialised cell junctions. In this report, we have investigated the effects of HPV E6 protein on the polarity machinery, with a focus on the PDZ partitioning defective 3 (Par3) protein, which is a key component of tight junctions (TJ) and the polarity network. We demonstrate that E6 is able to bind and induce the mislocalisation of Par3 protein in a PDZ-dependent manner without significant reduction in Par3 protein levels. In addition, the high-risk HPV-18 E6 protein promotes a delay in TJ formation when analysed by calcium switch assays. Taken together, the data presented in this study contribute to our understanding of the molecular mechanism by which HPVs induce the loss of cell polarity, with potential implications for the development and progression of HPV-associated tumours.

1. Introduction

Persistent infection with high-risk HPVs, such as HPV-16 and HPV-18, is the principal risk factor for the development of cervical cancer, which is the third most common cancer in women, with over 500,000 cases being reported globally each year (zur Hausen, 2002; Lowy and Schiller, 2012). In addition, HPV infection has been associated with the development of other malignancies, including: vulvar, vaginal, penile, anal, and oropharyngeal tumours (Lowy and Schiller, 2012).

Abbreviations: DLG1, human Disc large; E6AP, E6 associated protein; GFP, green fluorescent protein; HA, Influenza Virus Hemagglutinin epitope; HPV, Human Papillomavirus; MAGUKs, membrane-associated guanylate kinase homologues; Par, Partitioning defective; PATJ, PALS1 associated tight junction protein; PBM, PDZ-binding motif; PDZ, PSD-95/DLG/ZO-1 domains; RhPV, Rhesus papillomavirus; Scrib, Scribble; TJ, tight junction; ZO-1, zonula occludens 1; ZO-2, zonula occludens 2.

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Oncogenic HPV functions depend on the combined and complementary action of both HPV E6 and E7 oncoproteins, whose continuous expression is required for the maintenance of the transformed phenotype in carcinoma-derived cell lines (Yoshinouchi et al., 2003; Jonson et al., 2008). High-risk HPV E6 is a multifunctional protein that has the ability to bind and interfere with key cellular proteins, and these interactions are required for E6 to demonstrate full transforming activity. An interesting characteristic of HPV E6 oncoproteins is the presence, in the carboxy terminal region, of a conserved PDZ-binding motif (PBM) that is able to recognise and bind PDZ interaction domains (Thomas et al., 2008; Pim et al., 2012). PDZ-containing proteins are involved in diverse biological processes and many of them are scaffolding proteins that allow the assembly of multiprotein complexes at the cell membrane (Humbert et al., 2003, 2008). There is a growing list of PDZ proteins that are targeted by E6, including human Disc Large 1 (DLG1), Scribble (Scrib), PALS1-associated tight junction protein (PATJ), and membrane-associated guanylate kinase with inverted domain structure 1 (Gardiol et al., 1999; Nakagawa and Huibregtse, 2000; Storrs and Silverstein, 2007; Kranjec and Banks, 2011). Most of these proteins are involved in cell junction assembly, control of cell signalling and establishment of apicobasal polarity, and have been characterised as potential tumour suppressor proteins (Facciuto et al., 2012). Interestingly, the interaction of E6 with certain of these PDZ proteins can result in their degradation and/or mislocalisation, which has implications for polarity deregulation and HPV carcinogenesis (Thomas et al., 2008; Pim et al., 2012). Moreover, this activity is restricted to E6 derived from high-risk HPV because the PBM is absent in low-risk HPV-derived E6 proteins (which are associated with benign lesions), highlighting the fact that disruption of cell polarity is potentially a key event in the progression toward malignancy (Banks et al., 2012).

Epithelial cell polarity is defined by the interplay of three protein complexes: the Scribble, the Crumbs, and the Par complexes, which determine the basolateral domain, apical domain, and apical-lateral cell border, respectively (Assemat et al., 2008). The mammalian Par complex comprises the Par3, Par6, and atypical protein kinase C (aPKCs) proteins and is required for the establishment and maintenance of cell polarity (Goldstein and Macara, 2007). Among these components, Par3 is the central organiser for complex assembly; it contains three PDZ domains and is necessary for both TJ formation and spatial regulation of important signalling pathways (Feng et al., 2007; Goldstein and Macara, 2007; Pieczynski and Margolis, 2011). Par3 is a multi-modular scaffold protein that interacts with diverse cell polarity regulators and these specific interactions ensure that Par3 is localized at specific membrane domains (Chen and Zhang, 2013).

Furthermore, recent findings emphasise the importance of Par3 in cancer development (Facciuto et al., 2012). Reduced Par3 expression, in association with tumour progression and poor prognosis, was observed in several human cancers, including primary oesophagus tumours, glioblastomas, breast carcinomas, and skin cancer (Zen et al., 2009). Recently, two studies have reported that Par3 protein is an important suppressor of tumourigenesis and metastasis, highlighting its significant role in human breast cancer progression (McCaffrey et al., 2012; Xue et al., 2012).

Additionally, it is important to note that Rhesus papillomavirus (RhPV), which causes anogenital malignancy in Rhesus Macaque monkeys, presents a PBM in the C- terminus of the E7 protein instead of E6, as it is for HPV. This motif confers PDZ-binding activity and directs the interaction of RhPV E7 with Par3, suggesting that the targeting of cell polarity components is evolutionary conserved among PVs (Tomaic et al., 2008).

Considering *i*) that Par3 is critical for the establishment of TJs and apicobasal polarity and appears to be an oncosuppressor, *ii*) that HPV E6 is able to target and interfere with PDZ proteins involved in polarity machinery and, specifically, to members of the polarity protein complexes (e.g., Scrib, DLG1, and PATJ), and *iii*) that a finely tuned interplay among the different components of such complexes has been established, we initiated a series of studies to investigate the effect of HPV E6 on the Par polarity complex.

We show that the expression of high-risk HPV E6 results in a dramatic change in Par3 cellular distribution in a PBM dependent manner. We observe that HPV-18 E6 oncoprotein and Par3 interact *in vivo* and that this protein binding does not result in a significant reduction in Par3 protein level. Moreover, HPV E6 interferes with TJ formation in calcium switch assays. Overall, the data presented in this study contribute to the understanding of HPV E6 activities as they relate to interference of cell polarity during HPV-mediated cell transformation.

2. Materials and methods

2.1. *Cell culture and transfection*

HEK293, HaCaT and HeLa cells were grown in Dulbecco's modified Eagle's medium DMEM (Gibco, NY, USA) supplemented with 10% fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria). HEK293 and HaCaT cells were transfected using calcium phosphate precipitation (Matlashewski et al., 1987) or EcoTransfect reagent, respectively (OZ Biosciences, Marseille, France). To generate stable cell lines expressing HA-E6 fusion proteins (Influenza Virus Hemagglutinin epitope [HA] tagged-HPV E6 proteins), HaCaT cells were transfected with pcDNA3-HA-E6 and selected with G418 (Sigma Aldrich, Saint Louis, USA, 500 μg/ml). Single colonies were analysed for HA-E6 expression by RT-PCR and immunofluorescence (IF) analysis. Parallel transfections and selections were performed using an empty expression vector as a control. For 3D Matrigel culture, HaCaT cells were grown using Matrigel Basement Membrane Matrix (BD Biosciences, San Jose, USA). Briefly, cells were trypsinised and suspended in complete medium containing 2% Matrigel to a concentration of 1.2×10^5 cells/dish. Cell suspensions were seeded into 35 mm plastic tissue culture plates containing coverslips pre-coated with Matrigel. The cells were then covered with complete medium and grown at 37 °C under 5% CO_2 for 72 h (Debnath et al., 2003).

For the delivery of all siRNAs (Dharmacon, Thermo Fisher Scientific, Rockford, USA), the cells were seeded on six well dishes at a confluence of 1.2×10^5 and were transfected using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) with siRNA against either luciferase, HPV-18 E6/E7 (5′-