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Papillomavirus E2 protein is regulated by specific fibroblast growth factor receptors

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ABSTRACT

The papillomavirus (PV) E2 protein activates transcription and replication by recruiting cellular proteins and the E1 DNA helicase to their binding sites in the viral genome. We recently demonstrated that phosphorylation of tyrosine 102 in the bovine papillomavirus (BPV-1) E2 protein restricts these activities and that fibroblast growth factor receptor-3 (FGFR3) tyrosine kinase binds PV E2. Expression of FGFR3 decreased viral replication with both wild-type and the phenylalanine substitution at position 102, inferring that another kinase targets Y102. Here we tested FGFR- 1, −2 and −4 for association with PV E2 proteins. FGFR2 but not FGFR1 or FGFR4 co-immunoprecipitated with BPV-1 E2. We found that FGFR2 suppressed replication but did not depend on phosphorylation of BPV-1 Y102. HPV-16 and −31 E2 interacted with FGFR1, −2, and −4. These results imply that the expression and activity of FGF receptors in epithelial cells can regulate the function of E2 in viral replication.

1. Introduction

Papillomaviruses infect stratifying epithelia that are programmed to undergo terminal differentiation. Upon entry into basal cells, a single closed circular double stranded viral episome replicates to perhaps 10–50 copies per cell. This stage depends on host replicative factors; cellular and viral proteins are not packaged within the virion. Despite this initial amplification, PVs only express trace amounts of viral proteins and do not cause lytic infections. The E1 and E2 proteins are required for PV replication and mutations that render these inactive eventuate in either integration into stochastic chromosomal locations or loss of viral DNA [(Schiller et al., 1989), reviewed in (Kadaja et al., 2009)].

The E2 protein binds directly to and recruits the E1 DNA helicase to their recognition motifs that form the viral *origin* of replication (Androphy et al., 1987; Mohr et al., 1990). E1 monomers assemble into active double hexamers, which requires release from the E2 protein (Sanders and Stenlund, 1998). Several post-translational modifications of E2 protein are known to regulate its activities. We recently reported the acetylation of the bovine papillomavirus (BPV-1) E2 protein at lysine (K) 111 and K112 (Quinlan et al., 2013) and HPV-31 E2 at K111, and that this was necessary for unwinding of the replication fork (Thomas and Androphy, 2018). Phosphorylation of E2 on specific serine and threonine residues are known to affect its stability, chromatin binding and protein-protein interactions [(Chang et al., 2014), reviewed in (McBride, 2013)]. Recently, we detected tyrosine (Y) phosphorylation of BPV-1 E2 at amino acid 102 and that the phosphomimetic glutamate substitution reduced E2 transcription and replication activity (Culleton et al., 2017). Subsequently, fibroblast growth factor receptor-3 (FGFR3) was found to induce phosphorylation of tyrosine that restricts PV genome replication, although this was not mediated through Y102 (Xie et al., 2017).

Fibroblast growth factor receptors (FGFRs) are a group of four transmembrane tyrosine kinase receptors with multiple isoforms (Gong, 2014). The FGF signaling pathway regulates multiple biological processes such as angiogenesis, and tissue development and regeneration (Touat et al., 2015). FGFRs are involved in varying stages of viral infections. For example, FGFR1 may be a co-receptor for adeno-associated virus (AAV) 2 (Qing et al., 1999) and AAV-3 (Blackburn et al., 2006). FGFR1 suppresses influenza virus replication (Liu et al., 2015) and is activated by Epstein Barr Virus protein latent membrane protein 1 (LMP1) facilitating epithelial cell transformation (Lo et al., 2015).

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FGFR4 is involved in infectivity of a modified Influenza virus (Konig et al., 2010). FGFR1 and FGFR4 expression were increased in long-term Kaposi's sarcoma-associated herpesvirus (KSHV) infected telomerase-immortalized human umbilical vein endothelial cells (An et al., 2006). HPV-16 E5 protein targeting of FGFR2 inhibits autophagy, possibly affecting the early stages of HPV infection (Belleudi et al., 2015).

In a search for tyrosine kinases in complex with the E2 protein, an activated FGFR3 mutant was shown to suppress transient viral DNA replication (Xie et al., 2017). However, this did not require phosphorylation of E2 at Y102, inferring that another tyrosine kinase might target this residue and that other phosphotyrosines could be mediating the inhibitory effect of FGFR3. Our next goal was to determine if another FGFR family member complexes with and regulates E2 function. We found that only FGFR2 interacted with BPV-1 E2 while FGFR-1, −2, and −4 complex with HPV-16 and HPV-31 E2. However only endogenous FGFR-2 could be co-immunoprecipitated (co-ip'd) with HPV-16 E2.

2. Materials and methods

2.1. Plasmids and antibodies

Codon optimized FLAG HPV-31 E2 (DeSmet et al., 2016) and the ori-luciferase plasmids for the PV transient replication assay (Fradet-Turcotte et al., 2010) were used as previously reported (DeSmet et al., 2016). FGFR-1, 2, and 4 constructs were provided by L. Thompson (UC Irvine). A Myc tag was added to the C terminus of FGFRs by PCR amplification using the following primers Bam-FGFR1-F: GATCGGATCCATGTGGAGCT, FGFR1-myc-Not-R: GTACGCGGCCGCTCACAGATCCTCTTCTGAGATGAGTTTTTGTTCGCGGCGTTT, Bam-FGFR2-F: GATCGGATCCATGGTCAGCT, FGFR2-myc-Not-R: GTACGCGGCCGCTCACAGATCCTCTTCTGAGATGAGTTTTTGTTCTGTTTTAACACTG, Bam-FGFR3-F: GATCGGATCCATGGGCGCCCCT, FGFR3-myc-Not-R: GTACGCGGCCGCTCACAGATCCTCTTCTGAGATGAGTTTTTGTTCCGTCCGCGA, Kpn-FGFR4-F: GATCGGTACCATGCGGCTGC, FGFR4-myc-Not-R: GTACGCGGCCGCTCACAGATCCTCTTCTGAGATGAGTTTTTGTTCTGTCTGCAC, and inserted into pcDNA3. The following antibodies were used: mouse anti-FLAG M2, phospho-Tyrosine specific PY-99 (Santa Cruz) and PY-100 (Cell Signaling), rabbit anti-MYC (Cell Signaling), anti-FGFR1 (Abcam), anti-FGFR2 (Santa Cruz), and anti-FGFR4 (Santa Cruz). BPV-1 E2 was identified with B201, a mouse monoclonal antibody with an epitope between amino acids (aa) 160–220 (Breiding et al., 1996). Mouse-anti HPV-16-E2 (TVG-261) and HPV-16 E2 sheep-antiserum (Siddiqa et al., 2015) were used to identify HPV E2 proteins.

2.2. Cell Culture

All cell lines were maintained at 37 °C and 5% CO_2. HEK293TT (from J. Schiller and C. Buck) and C33A (from D. Lowy) were cultured in Dulbecco's Modified Eagle Medium (Life Technologies) with 10% fetal bovine serum (Peak Serum) and penicillin/streptomycin (100 U/ml; Life Technologies). CIN612-9E (from Laimonis Laimins) a clonal cell line that maintains HPV-31 genomes were grown in E-medium with J23T3 fibroblast feeders (from Howard Green). W12 (from M. Stanley and P. Lambert) that maintain HPV-16 episomes were grown in F medium with J23T3 fibroblast feeders. NOKS (from I. Morgan) were grown in Keratinocyte-SFM containing human recombinant Epidermal Growth Factor 1–53, Bovine Pituitary Extract (BPE) and penicillin/streptomycin (100 U/ml; Life Technologies).

2.3. In-situ proximity ligation assay (PLA)

In-situ PLA was performed using the PLA Red kit following Olink Biosciences' instructions. CIN612 cells were transfected with FLAG-HPV-31 E2. NOKS cells were transfected with FLAG-HPV-31 E2 and FGFR2. 24 h later, cells were fixed in 4% paraformaldehyde for 10 min, permeabilized for 15 min in 0.5% Triton-X 100/PBS, washed in PBS, blocked with 5% goat serum in 0.2% Triton-X 100/PBS, then incubated overnight with primary antibody combinations (mouse M2 FLAG-HPV-31 E2 or rabbit FGFR2) at 4 °C.

2.4. Co-immunoprecipitations and Immunoblotting

Cells were transfected using Lipofectamine 2000 (Life Technologies) according to manufacturers' instructions or with polyethylenimine (PEI) (2 mg/ml) (1 μg DNA: 2 mg PEI). After 24–48 h., cells were lysed in 0.5% NP-40 containing 150 mM NaCl, 20 mM HEPES, pH 7.4, 1 mM Na_3VO_4 and protease inhibitor cocktail (Sigma). To each reaction, 30 μl of 50% protein A/G slurry (Invitrogen) along with 1 μg of antibody or FLAG epitope specific M2 beads (Sigma) were added and rotated overnight at 4 °C. Beads were washed 3 times in lysis buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto PVDF membranes (Millipore), blocked in 5% Tris buffered saline (TBS)-Tween (0.1%) BSA, and probed with specific antibodies and corresponding secondary antibodies. Chemiluminescence substrates (Thermo Scientific) were used to detect horse raddish peroxidase conjugated secondary antibody signal. For endogenous HPV-16 E2 co-immunoprecipitation, W12 cells were grown to 80% confluence. Cells were lysed in 50 mM HEPES [pH 7.4], 150 mM NaCl, 1 mM Na_3VO_4, 0.5% NP-40, 1 mM DTT, and 1 × protease inhibitor cocktail. Sheep-anti-HPV16-E2 polyclonal sera or sheep non-specific IgG (Santa Cruz) were added to the lysates and rotated overnight at 4 °C. Immunoprecipitates were collected using protein A/G slurry for 3 h at 4 °C. Beads were washed with lysis buffer and with high salt buffer (50 mM HEPES [pH 7.4], 500 mM NaCl, 1 mM Na_3VO_4, 0.5% NP-40, 1 mM DTT, and 1× protease inhibitor cocktail). Proteins were removed from beads with 2× SDS lysis buffer, separated by 4–12% SDS-PAGE, and detected by western blot using anti-FGFR and anti-HPV16 E2 (TVG-261) antibodies.

2.5. Luciferase DNA replication assays

C33A cells (0.5% FBS DMEM) were seeded into a 96 well plate and each well was transfected with 100 or 200 ng FGFR constructs, 0.5 ng pRL (Rluc), 2.5 ng pFLORIBPV-1 or pFLORI31, 10 ng pCG-BPV-1 Eag1235 E1 or codon optimized triple FLAG-HPV-31-E1 (Fradet-Turcotte et al., 2010) and 10 ng pCG-E2 or pSG5-HA-HPV-31 E2 using Lipofectamine 2000. 72 h later cells were lysed and luciferase activity was measured using Dual Glo (Promega). Firefly luciferase levels were normalized to renilla luciferase levels.

2.6. Luciferase PV transcriptional assays

C33A cells (0.5% FBS DMEM) were seeded into a 96 well plate and each well was transfected with 100 ng FGFR2, 10 ng pCG-BPV-1 E2 and 75 ng pGL2-E2BS-Luc (Kumar et al., 2007) using Lipofectamine 2000. 48 h later cells were lysed and Firefly luciferase activity was measured using Dual Glo (Promega).

2.7. Mass spectrometry

293TT cells in 6 × 15 cm plates were transfected with BPV-1 E2 or FGFR2 constructs using PEI for 48hrs. Cells were washed in HBSS buffer (10 mM HEPES pH 7.5, 140 mM NaCl, 0.5 mM $CaCl_2$, 0.8 mM $MgCl_2$), and incubated with the irreversible tyrosine phosphatase inhibitor pervanadate (30 μM) for 1 h at 37 °C. Cells were lysed in 20 mM HEPES pH 7.5, 150 mM NaCl, 30 μM pervanadate, 7.5 μM Trichostatin A (TSA), and 0.525 mg/ml NaF and immunoprecipitated overnight with B201 antibody. Bands were excised from Coomassie stained polyacrylamide gels for tandem mass spectrometry. The gel bands were subjected to reduction (10 mM DTT) and alkylation (55 mM iodoacetamide) and digested with trypsin (Promega) overnight at 37 °C and injected into a