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APOBEC3A and 3C decrease human papillomavirus 16 pseudovirion infectivity

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ABSTRACT

Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) proteins are cellular DNA/RNA-editing enzymes that play pivotal roles in the innate immune response to viral infection. APOBEC3 (A3) proteins were reported to hypermutate the genome of human papillomavirus 16 (HPV16), the causative agent of cervical cancer. However, hypermutation did not affect viral DNA maintenance, leaving the exact role of A3 against HPV infection elusive. Here we examine whether A3 proteins affect the virion assembly using an HPV16 pseudovirion (PsV) production system, in which PsVs are assembled from its capsid proteins L1/L2 encapsidating a reporter plasmid in 293FT cells. We found that co-expression of A3A or A3C in 293FT cells greatly reduced the infectivity of PsV. The reduced infectivity of PsV assembled in the presence of A3A, but not A3C, was attributed to the decreased copy number of the encapsidated reporter plasmid. On the other hand, A3C, but not A3A, efficiently bound to L1 in co-immunoprecipitation assays, which suggests that this physical interaction may lead to reduced infectivity of PsV assembled in the presence of A3C. These results provide mechanistic insights into A3s' inhibitory effects on the assembly phase of the HPV16 virion.

1. Introduction

Apolipoprotein B mRNA-editing catalytic polypeptide (APOBEC) deaminases are a family of proteins, which include AID, APOBEC1, 2, 3A, 3B, 3C, 3DE, 3F, 3G, 3H, and 4 [1–4]. APOBEC3 (A3) proteins are antiviral factors that inhibit viruses and transposable elements, both of which use reverse transcription during their life cycle [1–4]. The antiviral functions of A3 proteins have been extensively studied in human immunodeficiency virus 1 (HIV-1) and hepatitis B virus (HBV) infections. In the case of HIV-1, A3G-induced hypermutation of viral DNA inhibits HIV-1 replication either by base excision repair (BER)-mediated DNA cleavage or accumulation of destructive mutations in the viral genome [1–4]. Furthermore, deaminase-independent antiviral activities are reported; A3G is proposed to block elongation of HIV-1 and HBV DNA by reverse transcription through a deaminase-independent mechanism [5,6]. In addition, binding of A3G with viral RNA is proposed to be important for inhibiting reverse transcription of retroviral genomes [7].

Human papillomaviruses (HPVs) are small double-stranded DNA viruses, and a subset of HPVs are recognized as causative agents of anogenital and oropharyngeal cancers [8–10], where HPV16 accounts for at least 50% of cervical cancer cases worldwide [11]. The HPV16 genome is a 7.9-kb closed circular DNA comprising at least eight open reading frames (ORFs) (*E1*, *E2*, *E4*, *E5*, *E6*, *E7*, *L1*,

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and *L2*) and a noncoding long control region (LCR). The LCR contains viral replication origin and an early promoter responsible for transcription of *E6* and *E7* oncogenes required for cellular transformation, while *L1* and *L2* encode the viral capsid proteins [8,9,11]. HPV16 infects the basal cell in cervical epithelia and establishes its genome as extrachromosomal episomes. Viral replication and capsid expression are induced in synchrony with host cell differentiation, and virions are assembled in the upper layer of epithelia and released into cell surroundings once the host cell is exofoliated after terminal differentiation [9].

We have recently demonstrated that the HPV16 *E2* gene is hypermutated by endogenous A3A and A3G induced by interferon β (IFN-β) in W12 cells [12], human cervical keratinocytes derived from a CIN1 biopsy that maintains HPV16 episomes [13]. Despite the observed hypermutation, the levels of HPV16 episomal genomes were not affected under the condition of A3s up-regulation. Thus, the pathophysiological relevance of A3 proteins in HPV infection remains unknown.

To examine the antiviral role for A3 proteins, particularly in the virion assembly, we utilized an HPV16 pseudovirion (PsV) system [14]. Our results suggest that A3A and A3C exert their antiviral activities during the assembly phase of the HPV16 virion.

2. Material and methods

2.1. Cell culture

293FT cells were purchased from Life Technologies and maintained in DMEM (10% FBS, 0.1 mM Non-Essential Amino Acids, 6 mM L-glutamine, 1 mM Sodium Pyruvate, and Penicillin/Streptomycin), as instructed by the supplier. HeLa cells were maintained in DMEM (10% FBS, Penicillin/Streptomycin).

2.2. Plasmid construction

To create pEF-nLuc, the NanoLuc coding sequence from the pNL1.1 vector (Promega, N1001) was subcloned into the pEF-BOS-EX vector [15]. pHPV16-L1/L2 was previously described [16]. FLAG-tagged green fluorescent protein (GFP), A3A (Acc. No. XM_005261387), A3F (Acc No. NM_145298) and A3G (Acc No. NM_021822) expression vectors were previously described [12,17]. For the FLAG-A3C expression vector, an ORF of human A3C (NM_014508) was amplified by RT-PCR with forward (5′-AAAGAATTCATGAATCCACAGATCAGAA-3′) and reverse (5′-AAAACTCGAGTCACTGGAGACTCCCGTAG-3′) primers using cDNA derived from HepG2 cells. The fragment was then cloned into pCMV3TAG1A (Invitrogen). For the FLAG-A3H expression vector, an ORF of human A3H (FJ376616) was amplified by RT-PCR with forward (5′-AAAGAATTCATGGCTCTGTTAACAGCCGAA-3′) and reverse (5′-AATAGTCGACTCAGACCTCAGCATCACACA-3′) primers using cDNA derived from CaSki (human keratinocyte cell line) cells. The fragment was subsequently cloned into pCMV3TAG1A. Successful construction of plasmids was confirmed by DNA sequencing.

2.3. PsV preparation and infectivity assay

We closely followed the protocol provided by Buck et al. [14]. In brief, 293FT cells were cotransfected using Lipofectamine 2000 (Life technologies) with pEF-nLuc, pHPV16-L1/L2, and pFLAG-A3 proteins, as per the manufacturer's instruction. Two days after transfection, the cells were harvested and lysed with phosphate-buffered saline (PBS) containing 0.5% Triton-X 100, 1 mM ATP, 25 mM ammonium sulfate, 1 mg/mL RNase A (Sigma), 50 μg/ml DNase I grade II (Roche), and 0.1% Plasmid-Safe (Epicentre). The lysates were incubated for at least 16 h at 37 °C to induce maturation of PsVs, followed by addition of sodium chloride at a final concentration of 0.85 M. The lysates were incubated on ice for 10 min and centrifuged at 4 °C at 5000 × *g* for 5 min. 2×10^4 HeLa cells per well of a 24 well plate were incubated with the supernatant (high salt extract, HSE) with a 2000-fold dilution of the culture media, unless noted. Two days later, the cells were harvested by trypsin digest, and the luciferase activity was measured using the Nano-Glo Luciferase Assay system (Promega), as per the manufacturer's instruction. Challenge of HSEs from the GFP control to a well without HeLa cells yielded a luciferase activity of ~300–400 relative luciferase units (data not shown), comparable to the value of the empty wells. Thus, the residual luciferase activity in HSEs was considered to be negligible. In addition, Buck et al. demonstrated that challenging HSEs allowed 293H cells to express the reporter gene in a L1 or L2 dependent manner [18]. Hence, we justified challenging HSE as a method to assess infectivity of PsVs.

2.4. Western blotting

Western blotting was performed as previously described [12,17]. The antibodies used in this study were: rabbit anti-GAPDH (G9545, Sigma), horseradish peroxidase (HRP)-conjugated antirabbit IgG (GE Healthcare), mouse anti-FLAG (M2, Sigma), mouse anti-Myc (9E10, sc-40, Santa-Cruz), mouse anti-HPV16 L1 (ab69, Abcam), and antimouse IgG–HRP (GE Healthcare). Signal development was performed using ECL Western Blotting Detection Reagents (Amersham) and signal detection was achieved using the LAS1000 imager system (FujiFilm).

2.5. Copy number determination of pEF-nLuc by qPCR

Total DNA from PsV-producing 293FT cells was extracted as described [12]. To prepare nuclease-resistant DNA, 2 μl of HSEs were added to 10 μl of extraction buffer (10 mM Tris pH 8.0, 150 mM NaCl, 25 mM EDTA, 1% NP-40, and 200 μg/ml proteinase K) at 50 °C overnight to degrade PsV capsid proteins. The resulting extracts were diluted 10-fold in double-distilled water, incubated at 95 °C for 7 min to heat-inactivate proteinase K, and subjected to qPCR. For quantification of the pEF-nLuc copy number, forward (5′-TCCTTGAACAGGGAGGTGTGT-3′) and reverse (5′-CGATCTTCAGCCCATTTTCAC-3′) primers were used. Specificity and linearity of the quantification were confirmed using pEF-nLuc as a template (data not shown). Copy numbers of pEF-nLuc in total cell lysates or HSEs were determined by qPCR. Serially diluted pEF-nLuc was used to obtain the standard curve for calculating the copy numbers of each sample.

2.6. Immunoprecipitation

Cells were lysed with PBS containing 1% Triton-X 100, 0.5% Tween 20, and a complete protease inhibitor cocktail tablet (Roche). Immune complexes were collected using an Anti-FLAG M2 Affinity Gel or an Anti-c-Myc Agarose Affinity Gel (Sigma, A2220 and A7470, respectively), as per the manufacturer's instructions. Precipitated complexes were purified using Micro Bio-Spin Chromatography Columns (BioRad, #732–6304) and eluted with buffer containing 6% SDS, 50 mM Tris–HCl, and 150 mM NaCl.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software). The two-tailed unpaired *t*-test was used for determining significance by qPCR and luciferase assay. *P* values of <0.05 obtained between experimental groups were considered statistically significant. In all graphs displayed in this study, error