厚生労働科学研究委託費

肝炎等克服緊急対策研究事業

B型肝炎ウイルスcccDNAを標的とした宿主因子の解析

平成２６年度　委託業務成果報告書

業務主任者　喜多村　晃一

平成２７（２０１５）年　３月

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本報告書は、厚生労働省の厚生労働科学研究委託事業による委託業務として、国立大学法人金沢大学学長 山崎 光悦が実施した平成２６年度「B型肝炎ウイルスcccDNAを標的とした宿主因子の解析」の成果を取りまとめたものです。

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厚生労働科学研究委託費（肝炎等克服緊急対策研究事業）
委託業務成果報告

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業務主任者　喜多村　晃一　金沢大学　医薬保健研究域　医学系　助教

研究要旨：本研究は、B 型肝炎ウイルス（HBV）持続感染に必須の閉環状ウイルス DNA である cccDNA の形成維持に関わる宿主因子について解析し、分子機構解明への知見を得ることを目的とした。まず先行研究を踏まえ、抗ウイルス因子 APOBEC3G が HBV cccDNA へ変異を導入するか、またその変異除去に DNA 修復因子 UNG が関わるかについて培養細胞系を用いて検討した。その結果、インターフェロンγによって肝細胞での発現が誘導される APOBEC3G が cccDNA へ変異を導入すること、その変異を UNG が除去していることを明らかにした。HBV 感染実験では UNG を阻害する条件下で変異の蓄積とウイルス複製能の低下が確認された。また、別の修復因子 FEN1 が cccDNA の形成に関与するか解析を行ない、HBV 産生細胞への FEN1 阻害剤の添加によって cccDNA 量が低下することを明らかにした。以上の結果は、これまで解明の進んでいない HBV cccDNA 形成と維持に関わる宿主因子のメカニズムの一端を明らかにするものであり、これを標的とした B 型肝炎創薬実用化に向けて有用な知見を提供するものと考える。

A. 研究目的

HBV cccDNA はウイルス DNA 存在様式の一つで、肝細胞感染後に核内で形成されウイルス複製の鋳型となる。現行の治療法では除去困難で肝炎再燃や薬剤耐性株出現の原因となりうる。しかし cccDNA に作用する宿主因子についての研究はこれまでほとんど報告がなかった。そこで本研究では宿主細胞の持つ複数の DNA 修復因子について、cccDNA の量と変異頻度及びウイルス複製活性への寄与を明らかし、HBV 完全排除のための新たな標的分子を提示することを目的とした。研究代表者はこれまでの研究で cccDNA 産生量の高い Duck HBV（DHBV）を用いて、cccDNA へ変異導入や分解を引き起こす抗ウイルス因子と、その分子機序に関わる DNA 修復因子を明らかにしてきた。これを踏まえて本研究では、HBV 産生細胞、HBV 感染細胞を用いた in vitro 実験で、変異導入因子 APOBEC3G と修復因子 UNG の HBV cccDNA への関与を解析した。またその作用機序から cccDNA 形成過程に関与すると推測される DNA 修復因子 FEN1 についても併せて解析を行い、cccDNA 形成・維持に関わる分子メカニズムの解明を目指した。

B. 研究方法

(1) HBV を安定的に産生する培養細胞 HepG2.2.15.7 細胞を使用した。先行研究より内在性 APOBEC3G を発現誘導すると考えられるインターフェロンγで 4 日間細胞を刺戟すると

同時に、UNG 活性を阻害する UGI タンパク質を導入することで cccDNA への変異率に変化が生じるか検討した。インターフェロンγ刺戟による APOBEC3 ファミリーの発現プロファイルを定量 RT-PCR により評価した。予想通り APOBEC3G が最もよく発現上昇したので、これを抑えるために siRNA により APOBEC3G ノックダウンを試みた。また、cccDNA 特異的な解析を行うために閉環状 DNA を特異的に増幅する rolling circle amplification 法（RCA 法）を用いた。この増幅産物をクローニングし配列決定を行った。

(2) 近年明らかにされた HBV 感染受容体である NTCP を安定発現する HepG2-hNTCP-C4 細胞を用いて HBV 感染実験を行なった。インターフェロンγ刺戟と UNG 阻害によって HBV の複製活性がどのように影響するか検討した。HBV 産生量の解析では培養上清中及び細胞質ウイルス粒子の HBV DNA コピー数を定量 PCR によって検出した。

(3) FEN1 の解析。上記の HepG2.2.15.7 細胞及び HBV 感染系において、FEN1 阻害剤を培養液中に添加してその効果を検討した。shRNA による FEN1 ノックダウン細胞を樹立し、cccDNA 形成能を RCA 法及び Taqman probe による cccDNA qPCR で解析した。

（倫理面への配慮）

遺伝子組換え実験は、遺伝子組換え生物等の使用等の規制による生物多様性の確保に関する法律において機関実験として承認されており、「金沢大学遺伝子組換え実験安全管理規定」に基づき第二種使用等に該当する拡散防止措置を執って実験管理を行っている。HBV 実験における大臣確認も行なっている。臨床検体は本研究では扱わない。ヒト由来培養細胞は実験株としてすでに確立されたものおよびその派生物のみを扱う。

C. 研究結果

(1) 肝細胞においてインターフェロンγによって導入される cccDNA 突然変異の頻度が APOBEC3G に対する shRNA ノックダウンで低下することが明らかになった。さらに UGI で DNA 修復因子 UNG を阻害することで cccDNA 高頻度突然変異の頻度が上昇した。cccDNA 変異頻度は同じ細胞サンプルから回収したウイルス粒子内 DNA の変異頻度よりも高いという結果が得られ、ウイルス粒子 DNA 変異の持ち込みではなく新たに cccDNA に対して変異が導入されていることが示唆された。

(2) HBV in vitro 感染実験において、UNG の阻害による cccDNA への変異の蓄積と相関してウイルス産生量が減少することが、細胞質ウイルス粒子 DNA 及び培養上清中 HBV DNA の定量によって観察された。

(3) cccDNA 形成に関与すると想定される DNA 修復因子 FEN1 の阻害剤添加あるいは shRNA ノックダウンによって、cccDNA 量の低下及びウイルス産生量の低下が見られた。

D. 考察

以上のように、変異導入因子 APOBEC3G 及び DNA 修復因子 UNG の、HBV cccDNA に対する作用を培養細胞の系を用いて解析した。その結果、インターフェロンγによって発現誘導される APOBEC3G が cccDNA に変異を導入し、その変異を UNG が修復していることが示唆された。また、別の DNA 修復因子 FEN1 の阻害により cccDNA 量が減少した。FEN1 は DNA のフラップ構造を除去する因子として知

られており、cccDNA の前駆体である rcDNA からフラップ様の構造を除去するステップに関与する cccDNA 形成因子と考えられる。

E. 結論

本研究では、HBV cccDNA に作用してその変異頻度や形成に関わる宿主因子を明らかにした。cccDNA 形成維持に関わる因子群とその作用機序の理解が進めば、抗 HBV 薬開発に向けての標的となりうる。また、ウイルスの変異率増減に関わる宿主因子の作用がウイルス複製活性や病原性変化の原因となるという結果が得られれば、これらの因子はリスクマーカーとなりうるため、今後さらなる分子メカニズムの解明が期待される。

F. 健康危険情報

該当なし

G. 研究発表

1. 論文発表

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2. 学会発表

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H. 知的財産権の出願・登録状況

該当なし

学 会 等 発 表 実 績

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１．学会等における口頭・ポスター発表

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APOBEC deaminases as mutators in oncogenic DNA viruses／口頭発表	喜多村 晃一	金沢大学がん進展制御研究所	2014年11月
Role of host DNA repair factor in the maintenance of hepatitis B virus cccDNA／ポスター発表	喜多村 晃一、島津 美幸、小浦 美樹、村松 正道	第37回日本分子生物学会年会	2014年11月

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期
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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

bars indicate the standard error of the mean from duplicate or triplicate samples.

3. Results

3.1. A3A and A3C reduce infectivity of HPV16 PsV

We previously reported that A3s are abundantly expressed in W12 cells, compared to *AID* or *A1* [12]. Furthermore, our RT-qPCR analysis revealed that both HPV16 (−) and (+) cervical tissues express A3s (data not shown). These findings motivated us to explore the possibility that A3 proteins influence the assembly of the HPV16 virion by utilizing the production system of PsV [14]. Each expression vector producing A3 proteins or GFP was co-transfected with an HPV16 capsid expression vector (pHPV16-L1/L2) and a luciferase reporter plasmid (pEF-nLuc). Two days later, the cell lysates containing PsV were prepared under high salt conditions. These lysates (called HSEs) were further treated with a nuclease to digest DNA not encapsidated into the PsV, while leaving encapsidated reporter plasmids intact.

To determine infectivity, HeLa cells seeded in a well of 24-well plate were challenged with 0.25 μl of HSEs prepared from 293FT cells overexpressing each A3, and the luciferase activity was determined 48 h after infection (Fig. 1A). As shown in Fig. 1A, HSEs prepared from A3A, A3C, A3F, and A3G were less infectious than that prepared from GFP. The reduced infectivity of PsV in the presence of A3G, A3F and A3H could be partly attributed to decreased capsid protein (L1) levels in HSEs of A3 transfected cells compared with that of GFP transfected cells (Fig. 1B). It is currently unknown why L1 protein levels decreased in A3 transfected cells compared with GFP transfected cells.

To compare infectivity of PsVs produced from A3 transfected cells that contained varying amounts of the L1 capsid protein, we titrated GFP HSE by 2-fold serial dilution, and determined relation between luciferase activity and L1 protein levels, by plotting the L1 level against the luciferase activity for both GFP and each A3 protein (Fig. 1C). As expected, the amount of L1 protein and luciferase activity correlated well across the serially diluted GFP HSE. Plots of A3A, A3C, and A3H were positioned below the curve of GFP (Fig. 1C). This result indicates that the infectivity of A3A, A3C, and A3H HSEs was lower than the GFP HSE, even after normalizing for the amount of L1. A3F and A3G plotted to the same line as GFP (Fig. 1C), indicating that A3F and A3G reduced L1 protein levels but did not reduce the infectivity of the PsV. A3H expression slightly reduces infectivity even after normalization (Fig. 1C right side); however, decrease of L1 protein level was significant (Fig. 1B right side). Therefore, it is not easy to determine whether A3H decreases PsV infectivity. Taken together, we concluded that A3A and A3C attenuate HPV16 PsV infectivity.

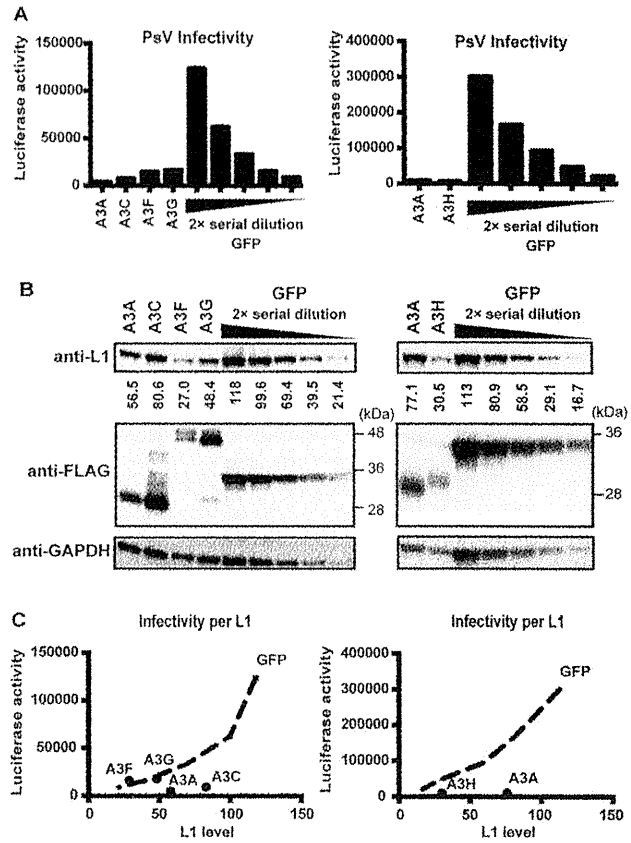

Fig. 1. A3 protein expression decreased infectivity of HPV16 PsV. (A) 293FT cells were cotransfected with an L1/L2 expression vector (pHPV16-L1/L2), reporter plasmid (pEF-nLuc), and an expression vector producing each A3 protein or GFP. Transfected 293FT cells were harvested at 48 h after transfection and HSE-containing PsVs were prepared. 100 μl of HSE was prepared from a well of a 6-well plate of transfected 293FT cells. 0.25 μl of each HSE (0.25% of the HSE fraction) was used to challenge HeLa cells seeded in a well of a 24-well plate. Cells were harvested and luciferase activity was determined 48 h post infection. For control cells transfected with pFLAG-GFP, 0.25, 0.125, 0.0625, and 0.03125 μl of HSEs were used to challenge to HeLa cells to obtain a standard curve of L1 protein level and infectivity. (B) Protein levels of HPV16-L1, FLAG-A3 proteins, and GAPDH in 2 μl (2% of a 6-well plate) of each HSE were determined by immunoblotting followed by densitometry. For control cells transfected with pFLAG-GFP, L1 protein levels in 2.0, 1.0, 0.5, and 0.25 μl of HSEs were determined. (C) Each HSE was plotted according to its L1 protein level and luciferase activity. Serially diluted HSEs from GFP transfected cells were plotted to compare infectivity of HSEs containing different amounts of L1. Errors bars in (A) and (C) represent the standard deviations of three independent experiments.

3.2. Expression of A3A, but not A3C, affects encapsidation of PsV DNA

We then investigated how A3A and A3C act to decrease PsV infectivity. First, total DNA was purified from the producer cells and reporter plasmid levels were determined. As shown in Fig. 2A, the copy numbers of the reporter plasmid in total DNA were comparable between each sample. Because A3A is thought to hypermutate foreign DNA [19], DNA sequencing of the reporter plasmid was performed. A reporter gene fragment (560 bp) was amplified from the total DNA of A3A-expressing producer cells and the fragment was cloned into the pGEM-T Easy Vector (Promega). This vector was transformed into *Escherichia coli* and 24 clones were randomly selected. DNA sequencing of these clones ($24 \times 560 = 13440$ bp) revealed an absence of C-to-T and G-to-A mutations (data not shown). These results suggest that neither differences in transfection efficiency or foreign DNA hypermutation can explain the reduction of infectivity by A3A (Figs. 1C and 2A).

Furthermore, we determined reporter plasmid levels in the HSEs. Because HSEs were obtained following nuclease digestion, reporter plasmid levels in HSEs should reflect the level of encapsidated PsV DNA. As expected from the decreased L1 protein levels in the HSEs compared to the GFP HSE, all A3 samples exhibited a lower copy number of the reporter plasmid than the GFP control (Fig. 2B). To evaluate the copy number of the reporter plasmid per single PsV, copy numbers of serially diluted HSE from GFP transfected cells were determined and plotted along with A3 proteins against L1 levels (Fig. 2C). Only A3A exhibited a reduced reporter plasmid copy number as measured per L1 level.

Other groups compared the infectivity of PsVs by adjusting the amount of reporter plasmid [20, 21]. To evaluate infectivity of A3

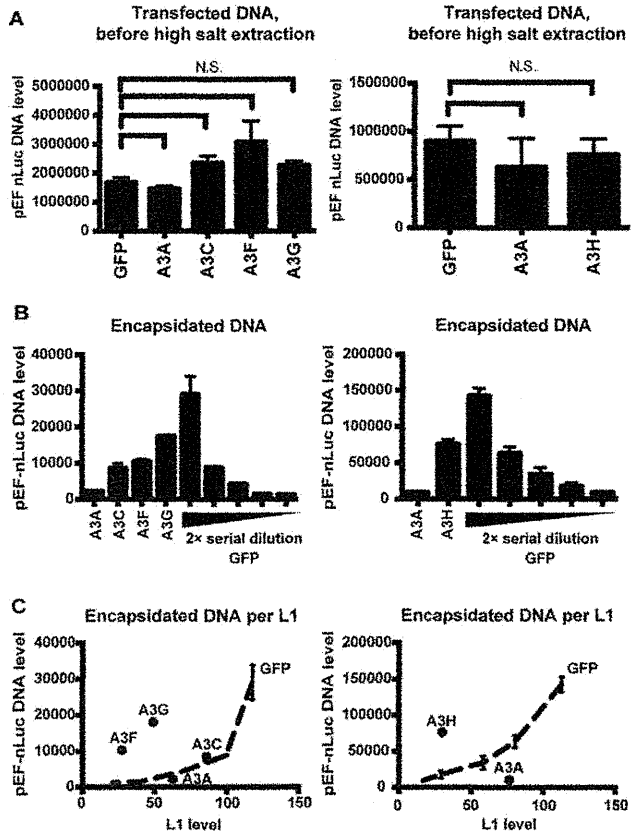

Fig. 2. A3A expression, but not that of other A3s, decreased the amount of encapsidated DNA. (A, B) Total DNA from 293FT cells (A) or corresponding HSEs (B) were subjected to qPCR to measure copy numbers of the reporter plasmid (pEF-nLuc). Reporter plasmid levels in HSEs were equivalent to encapsidated reporter plasmid levels, as HSEs were prepared after DNaseI treatment. Reporter plasmid levels from 0.167 μl of each HSE were determined. For the GFP control, reporter plasmid levels from 0.167 μl of the HSE and its serial dilution were determined. N.S. = not statistically significant. (C) HSEs from each transfected cell were plotted according to L1 protein level and copy number of reporter plasmid. Serially diluted HSEs from GFP transfected cells were plotted to compare the copy numbers between HSEs containing different amount of L1. Errors bars represent standard deviations of two independent experiments.

HSEs per pseudogenome, the same data used in Figs. 1 and 2 were plotted according to the copy number of the reporter plasmid. (Supplementary Fig. 1). Infectivity of A3A and A3C per pseudogenome was lower than that of GFP, however, L1 levels per psuedogenome were higher for A3A HSE and equivalent for A3C to that of GFP, while in A3F, A3G, and A3H HSEs, both infectivity and L1 protein levels per pseudogenome were lower than those of GFP (Supplementary Fig. 1, lower). Even after conducting different analyses, we obtained the same conclusion; the decreased infectivity of HSEs from A3F, A3G, and partly A3H, could be attributed to the decreased L1 expression, while A3A reduce infectivity by reducing pseudogenome per PsV.

Taken together, these results suggests that A3A interferes with encapsidation of the reporter plasmid, whereas A3C decreases PsV infectivity by a mechanism other than encapsidation of the pseudogenome.

3.3. Binding of A3 proteins with L1 capsid protein in vitro

Because the encapsidation process failed to explain the decreased infectivity of PsVs in A3C HSE, we next explored whether A3C affects PsV infectivity by interactions with the capsid proteins. To this end, we expressed Myc-tagged L1 and FLAG-tagged A3 proteins in 293FT cells. Co-immunoprecipitation (IP) experiments revealed that FLAG-A3C, -A3F, -A3G, and -A3H, co-precipitated Myc-L1, while almost no GAPDH, FLAG-GFP and negligible FLAG-A3A proteins were precipitated with Myc-L1 (Fig. 3 middle). Consistent with this result, IP complexes of Myc-L1 exhibit detectable levels of FLAG-A3C, 3F, 3G, and 3H, but not GFP and A3A (Fig. 3 bottom). Of note, FLAG-A3C most efficiently pulled down Myc-L1 among these four (Fig. 3 middle). The distinguished binding property of A3C for L1 was also demonstrated by another co-IP experiment, using total lysates from 293FT cells transfected with pHPV16-L1/L2, pEF-nLuc, and FLAG-A3s, from which we obtained HSEs (Supplementary Fig. 2). Collectively, these data indicate that A3C efficiently (while A3F and A3G modestly) binds to the L1 capsid protein *in vitro*.

4. Discussion

In this study, we investigated the role of APOBEC3 proteins during the assembly phase of the HPV16 virion. Using the PsV production system, which recapitulates the assembly phase of the HPV16 virion, we demonstrated that A3A and A3C reduce the PsV infectivity when co-expressed in 293FT cells (Fig. 1). The levels of the encapsidated pseudogenome were decreased in PsVs prepared from A3A transfected cells, but not other A3s transfected cells (Fig. 2). To clarify the mechanism underling the reduced infectivity, we examined whether A3 proteins bind to the HPV16 L1 capsid. A3C efficiently bound to L1, but A3A did not (Fig. 3, Supplementary Fig. 2), implying that the A3C binding to L1 may impair PsV infectivity. Notably, A3A and A3C localize both in the nucleus and cytoplasm, whereas A3F and A3G are distributed to the cytoplasm [22].

Unexpectedly, co-transfection of pHPV16-L1/L2 with pFLAG-A3s resulted in decreased L1 expression (Fig. 1B, Supplementary Fig. 2, top). Meanwhile, Myc-L1 was not obviously decreased by

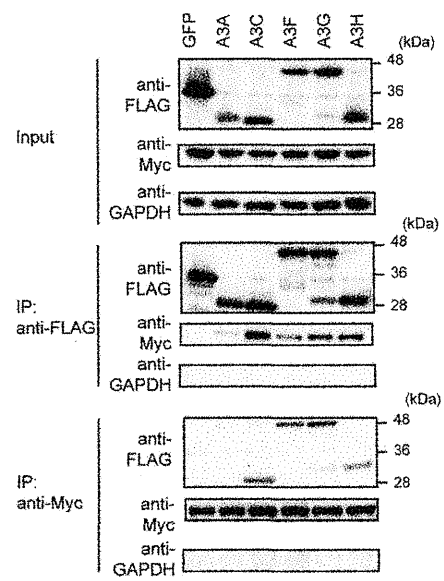

Fig. 3. Physical interaction of A3 proteins with HPV16 L1. 293FT cells were transfected with FLAG-tagged A3 proteins and Myc-tagged L1 and immunoprecipitated by anti-FLAG or anti-Myc antibody. Immunoprecipitated samples as well as the input were immunoblotted by anti-FLAG and anti-Myc antibodies. GAPDH blot was used as a control. Displayed is representative of two independent experiments.

co-expression with FLAG-A3s (Fig. 3, top). We do not deny the possibility that A3s are involved in the degradation of L1 protein or mRNA, which does not target Myc-tagged L1.

We have demonstrated that A3C binds to HPV16 L1 *in vitro*, which implies the mechanism how A3C reduces the infectivity. Binding capacity of L1 with heparan sulfate proteoglycans (HSPGs) is proposed to be important for the primary attachment on the cell surface, internalization, and uncoating of the capsid, to allow the pseudogenome to enter the nucleus [23]. Therefore, it is intriguing to speculate that A3C blocks either of these steps, by binding with L1, to deprive HSPGs of its access to the PsVs.

During preparing this manuscript, Warren et al. reported that A3A could decrease the infectivity of HPV16 PsV, which is consistent with this study [21]. However, they concluded that A3C does not affect HPV16 PsV infectivity. In their study, recombinant HPV16 genomic DNA with a GFP reporter gene, driven by SV40 promoter, was used as a pseudogenome and infectivity of PsVs prepared from A3C-expressing cells was determined by GFP expression in infected cells. In this study, a vector encoding a luciferase reporter gene driven by EF1*a* promoter was used, therefore, the discrepancy between that study and the present work can be attributed to the differences in reporter gene and cell lines used.

In summary, this study provides for the first time the evidence for the involvement of A3 proteins in interference with HPV16 virion assembly. Further studies are required to clarify the specific mechanism of how A3 proteins mediate this infectivity defect.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2014.12.103.

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