Characterization of an RNA Aptamer Against HPV-16 L1 Virus-Like Particles

Ana Gabriela Leija-Montoya,¹ María Luisa Benítez-Hess,¹
Julia Dolores Toscano-Garibay,² and Luis Marat Alvarez-Salas¹

The human papillomavirus (HPV) capsid is mainly composed of the L1 protein that can self-assemble into virus-like particles (VLPs) that are structurally and immunologically similar to the infectious virions. We report here the characterization of RNA aptamers that recognize baculovirus-produced HPV-16 L1 VLPs. Interaction and slot-blot binding assays showed that all isolated aptamers efficiently bound HPV-16 VLPs, although the Sc5-c3 aptamer showed the highest specificity and affinity ($K_d = 0.05$ pM). Sc5-c3 secondary structure consisted of a hairpin with a symmetric bubble and an unstructured 3' end. Biochemical and genetic analyses showed that the Sc5-c3 main loop is directly involved on VLPs binding. In particular, binding specificity appeared mediated by five non-consecutive nucleotide positions. Experiments using bacterial-produced HPV-16 L1 resulted in low Sc5-c3 binding, suggesting that recognition of HPV-16 L1 VLPs relies on quaternary structure features not present in bacteria-produced L1 protein. Sc5-c3 produced specific and stable binding to HPV-16 L1 VLPs even in biofluid protein mixes and thus it may provide a potential diagnostic tool for active HPV infection.

Introduction

APTAMERS ARE SMALL LIGAND moieties capable of adopting three-dimensional structures that bind tightly and specifically to a wide diversity of molecules [1]. DNA and RNA aptamers are *in vitro* selected by the iterative application of the SELEX (systematic evolution of ligands by exponential enrichment) method over large combinatorial oligonucleotide libraries with vast structural diversity [2,3]. Aptamers have been compared to antibodies due to their high affinity and specificity [4]. Moreover, aptamers are easier and more economical to synthesize than antibodies and can be modified to render highly stable moieties in biofluids. Several aptamers have been selected against many types of different targets, ranging from small inorganic molecules to proteins and even whole cells [5,6].

Human papillomaviruses (HPVs) are nonenveloped DNA viruses that have tropism for cutaneous and mucosal epithelium [7]. High-risk HPVs, particularly HPV type 16 (HPV-16), are the primary etiologic agent for anogenital tumors, including cervical carcinoma [8]. The small (50–60 nm) icosahedral HPV-16 capsids are composed of two late gene products, the L1 (the major component of the capsid) and the L2 proteins. The L1 protein is arranged in 72 capsomers in a T=7 icosahedral surface lattice, each capsomer containing

five monomers of the 55-kDa L1 protein [9] and a single molecule of the 74-kDa L2 protein in a 5:1 ratio of L1 to L2 [10]. Although the L1 gene sequence is the most conserved gene within the HPV genome, it still can be used to identify different HPV types (>10% variation), subtypes (2% to 10% variation) and variants (<2% variation) [11], suggesting that HPV typing and risk assessment require of highly specific and sensitive techniques.

The L1 protein can self-assemble forming virus-like particles (VLPs) that are structurally and immunologically similar to the infectious virions [12]. VLPs lack genetic material and can be produced by the L1 protein alone (L1 VLPs) or complexed with the L2 protein (L1/L2 VLPs). HPV-16 L1 VLPs have been broadly used in HPV virology research [13,14], as delivery agents for epitopes or genes [15–18] and to successfully produce prophylactic vaccines against HPV infection [19].

Aptamers have been successfully applied for molecular diagnostics and target isolation and also as therapeutic alternatives to some diseases [20–22]. Recently, RNA aptamers have been isolated against the transforming HPV-16 E7 protein [23,24], and tested as apoptosis-inducing agents in cultured cells [25]. Also, DNA aptamers isolated by cell-SELEX can distinguish cell surface differences between HPV-transformed cervical carcinoma cancer cells and isogenic, nontumorigenic,

¹Laboratorio de Terapia Génica, Departamento de Genética y Biología Molecular, Centro de Investigación y de Estudios Avanzados del I.P.N., México D.F., México.

²Laboratorio de Medicina Regenerativa, Dirección de Investigación, Hospital Juárez de México, México D.F., México.

revertant cell lines [26]. Therefore, aptamers have great potential as diagnostic and therapeutic tools for cervical cancer.

In this work, we describe the characterization of RNA aptamers that bind HPV-16 L1 VLPs. The highest affinity aptamer (Sc5-c3) presented a hairpin structure that binds HPV-16 L1 VLPs through a single-stranded loop while specificity appeared mediated by five nonconsecutive nucleotide positions within the loop. Interestingly, aptamer-VLP binding appeared dependent on the VLP quaternary structure as binding to bacteria-produced L1 was significantly reduced. Sc5-c3 can specifically and stably bind HPV-16 L1 VLPs even in complex protein mixes suggesting that, with further refinement, it may develop into a potential cost-effective diagnostic tool for HPV infection.

Materials and Methods

Oligonucleotides and plasmids

All oligonucleotides were purchased from Sigma-Aldrich Co. (St. Louis, MO) and T4 oligo (Irapuato Gto., México) and are listed in Table 1. Plasmid pGST-FSL1 contains a wild-type HPV-16 L1 variant derived from p16L1h plasmid

(kindly donated by Dr. John T. Schiller, Center for Cancer Research-National Cancer Institute/National Institutes of Health) [27] cloned in the pGEX-2TK expression vector (GE Healthcare Life Sciences, Piscataway, NJ). Plasmid pGST-GFP contains the green fluorescent protein (GFP) gene in a *NcoI/EcoRI*-restricted pGEX-2TK-MCS vector [28].

Cell culture

Invitrogen™ *Spodoptera frugiperda* Sf-21 cells (Life Technologies Corporation, Carlsbad, CA) were cultured in 25 cm² flasks (Corning Incorporated Life Sciences, Tewksbury, MA) containing Sf900™II serum-free medium (Life Technologies) at 26°C.

HPV-16 L1 VLP production

HPV-16 L1 recombinant baculovirus were produced using Invitrogen™ Bac-to-Bac® Baculovirus Expression System (Life Technologies) with the shuttle construct pFB1-L1 (derived from the p16L1h plasmid) and *Escherichia coli* DH10Bac™ cells as described by the manufacturer. Production of HPV-16 L1 VLPs was made by infecting Sf21

TABLE 1. OLIGONUCLEOTIDES FOR LIBRARY AMPLIFICATION, MUTAGENESIS, AND DELETIONS

Name	Sequence*
Aptlib	5'-TAATACGACTCACTATAGGGAGACCCAAGC(N ₁₅)AGCTTTTGTTCCTTTAGTGAG GGTTAATT-3'
Fwd	5'-TAATACGACTCACTATAGGGAGACCCAAGC-3'
Rev	5'-AATTAACCCCTCACTAAAGGGAACAAAAGCT-3'
Fwd(-6)	5'-GACTCACTATAGGGAGACCCAAGCG-3'
Fwd(-12)	5'-CTATAGGGAGACCCAAGCGGGGG-3'
Fwd(-18)	5'-GGAGACCCAAGCGGGGGTAAAGTG-3'
Fwd(-24)	5'-CCAAGCGGGGGTAAAGTGTTGG-3'
Fwd(-30)	5'-TTAATTGGGAGTGATTTCCCTTGTTTTCGAGGTGTGAAATGGGGG-3'
Rev(-30)	5'-CCCCATTTACACCTCGAAAACAAGGGAAATCACTCCCAATTAA-3'
Fwd(-36)	5'-TTAATTGGGAGTGATTTCCCTTGTTTTCGAGGTGTGAAA-3'
Rev(-36)	5'-TTTCACACCTCGAAAACAAGGGAAATCACTCCCAATTAA-3'
Fwd(-42)	5'-TTAATTGGGAGTGATTTCCCTTGTTTTCGAGGT-3'
Rev(-42)	5'-ACCTCGAAAACAAGGGAAATCACTCCCAATTAA-3'
Rev(-6)	5'-AAAGGGAGCTCCACACTTTAC-3'
Rev(-12)	5'-AAAGGGACACTTTACCCCGC-3'
Rev(-24)	5'-AAAGGGCGCTTGGGTCTCC-3'
T3 R	5'-CGGAATTAACCCCTCACTAAAGGG-3'
Mut 1	5'-ATTAACCCCTCACTAAAGGGAACAAAAGCTGCCAGGATACCCCGCTTGGGT CTCCCTATAGTGAGTCGTATTA-3'
Mut 2	5'-AATTAACCCCTCACTAAAGGGAACAAAAGCTGCACCGGATACCCCGCTTGGGT CTCCCTATAGTGAGTCGTATTA-3'
Mut 3	5'-AATTAACCCCTCACTAAAGGGAACAAAAGCTGCACAGGATACCCCGCTTGGGT CTCCCTATAGTGAGTCGTATTA-3'
Mut 4	5'-AATTAACCCCTCACTAAAGGGAACAAAAGCTGCACAGGATACCCACGCTTGGGT CTCCCTATAGTGAGTCGTATTA-3'
Mut 5	5'-AATTAACCCCTCACTAAAGGGAACAAAAGCTGCACAGGATACCCAGCTTGGG TCTCCCTATAGTGAGTCGTATTA-3'
Mut 6	5'-AATTAACCCCTCACTAAAGGGAACAAAAGCTTCACATTCTACCCCGCTTGGGT CTCCCTATAGTGAGTCGTATTA-3'
Mut 7	5'-AATTAACCCCTCACTAAAGGGAACAAAAGCTGCAAAGGATACCCCGCTTGGG TCTCCCTATAGTGAGTCGTATTA-3'
Mut 8	5'-AATTAACCCCTCACTAAAGGGAACAAAAGCTGAAAATTCGAAAACCGCTTGGG TCTCCCTATAGTGAGTCGTATTA-3'

N corresponds to any nucleotide (A, G, C, T) at a randomized position.

*Underlined positions on Rev correspond to T3 promoter.

Aptlib, aptamer library; Fwd, library forward primer; Mut, mutant; Rev, library reverse primer; T3 R, T3 promoter reverse primer.

cells essentially as described [27]. Briefly, 1×10^{10} pfu recombinant baculovirus particles were infected in proliferating Sf21 cells for 48 hours. Infected cells were harvested in lysis buffer (0.5 M NaCl, 1% octylphenoxypolyethoxyethanol (IGEPAL CA-630) dissolved in $1 \times$ Dulbecco's phosphate-buffered saline (D-PBS) (2.67 mM KCl, 1.47 mM KH_2PO_4 , 137.93 mM NaCl mM, and 8.06 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ pH 7.2–7.7)) and lysed by sonication in a GEX 130 PB ultrasonic processor (Cole-Parmer, Vernon Hills, IL) at 100% cycle duty. VLPs were purified through CsCl isopycnic centrifugation and extensively dialyzed against $1 \times$ D-PBS at 4°C. VLP production was verified by electron microscopy and immunoblotting (data not shown) and stored in $1 \times$ D-PBS at 4°C until use.

Bacterial protein expression and purification

Recombinant GST-L1 protein was produced by transforming pGST-FSL1 in *E. coli* BL21(DE3) (Life Technologies). Exponential bacterial cultures were induced with 100 mM IPTG (Sigma-Aldrich) at 30°C for 2 hours and harvested by centrifugation at 1,000 g. Bacterial pellets were resuspended in 500 μL bacterial lysis buffer [20 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% (v/v) NP40, 0.5 mM 4-(2-Aminoethyl)benzenesulfonyl fluoride] and lysed in a ultrasonic processor GEX 130 PB (Cole-Parmer) at 80% cycle duty. GST-L1 protein was purified by incubating bacterial lysates with glutathione-agarose beads (Sigma Aldrich) for 1 hour at 4°C and washed three times with $1 \times$ D-PBS. Purified L1 protein was released by digestion with 1 U bovine thrombin (Sigma-Aldrich) 16 hours at 4°C and confirmed by immunoblotting.

DNA and RNA libraries

The aptamer library (Aptlib) single-stranded DNA library has been previously described [23] consisting of a central 15-nt randomized domain ($\sim 10^9$ variants) with no fixed positions (data not shown) flanked by two anchor regions for polymerase chain reaction (PCR) amplification and the T3 and T7 RNA polymerase promoters at each end (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/nat). The Aptlib library was synthesized at a 200-nmol scale and purified as reported [29]. Library DNA was resuspended in 50 μL deionized water and PCR-amplified by 15 cycles using the FWD and REV primers (denaturing at 94°C for 1 minute, hybridization at 55°C for 45 seconds, and polymerization at 72°C for 2 minutes), using 1 U Invitrogen™ Taq DNA polymerase (Life Technologies). The resulting double-stranded DNA library was *in vitro* transcribed using the Riboprobe® System T3 kit (Promega Corporation, Madison, WI) to produce a combinatorial RNA pool.

SELEX procedure

Initially, the RNA pool (500 μg) was negatively selected to discard sequences that may bind the components of the selection system. Briefly, three initial preselection cycles were performed to remove unspecific RNA binding by incubating the RNA pool with activated 0.5 cm^2 polyvinylidene fluoride (PVDF) membranes (EMD Millipore Corporation, Billerica, MA) and 20 U RNasin® ribonuclease inhibitor (Promega) in 100 μL $1 \times$ D-PBS for 1 hour at room temperature. After incubation, PVDF membranes were removed and unbound

RNA was amplified by RT-PCR using Invitrogen™ SuperScript® 3 One-Step RT-PCR system (Life Technologies) under the following conditions: first-strand synthesis step at 55°C for 30 minutes, and 15 cycles of denaturing step at 94°C for 1 minute, hybridization step at 55°C for 45 seconds, and polymerization step at 72°C for 2 minutes.

Positive selection was performed by incubating the pre-selected RNA pool with PVDF-immobilized HPV-16 L1 VLPs (60 ng VLP protein/0.5 cm^2 PVDF) and 20 U RNasin® inhibitor (Promega) in 100 μL $1 \times$ D-PBS for 1 hour at room temperature. After incubation, unbound RNA was removed by three $1 \times$ D-PBS washes and membranes were placed in 50 μL $1 \times$ D-PBS at room temperature. Aptamers were recovered from the supernatant after heating the membranes at 65°C for 8 minutes. After each selection cycle, the recovered aptamers were amplified by RT-PCR and transcribed to regenerate a RNA pool and start a new selection cycle. After the last selection cycle, aptamers were cloned using the Invitrogen™ TOPO® TA Cloning® Kit (Life Technologies), and six clones from a pool of over 100 clones were sequenced and selected for further analysis (Supplementary Fig. S1).

VLP interaction assays

Initial binding characterization was set with 500 ng of HPV-16 L1 VLPs or bovine serum albumin (BSA) immobilized on 0.5 cm^2 PVDF membranes. The immobilized proteins were incubated with α -[^{32}P]-UTP labeled aptamer RNA (30 pM) in 100 μL $1 \times$ D-PBS for 1 hour at 37°C. After incubation, all of the membranes were washed three times with 100 μL $1 \times$ D-PBS to eliminate unbound RNA. Bound aptamer RNA was recovered by heating the membranes at 65°C for 8 minutes and resolved by denaturing electrophoresis in 7 M urea 8% (w/v) polyacrylamide (19:1 acrylamide: N,N'-Methylene-bis-acrylamide) gels. Gels were dried quantified using a Typhoon 8600 fluorographic scanner (GE Healthcare Life Sciences).

Binding analysis

Dissociation constant (K_d) determination was performed by triplicate as previously described using a two-membrane "sandwich" slot-blot assay [30]. Briefly, labeled aptamers (30 pM) were incubated with increasing protein concentrations in 100 μL $1 \times$ D-PBS for 1 hour at room temperature. After incubation, binding mixtures were loaded into a slot blot apparatus and filtrated through a nitrocellulose (NC) membrane (GE Life Sciences) layered over a positively charged nylon (NY) membrane (GE Life Sciences) at constant pressure. Aptamer-protein complexes were retained on the NC membrane, while unbound RNA remained on the positively charged NY membrane. Residual radioactivity on each membrane was quantified using a Typhoon 8600 fluorographic scanner (GE Life Sciences) and fractional saturation (θ) was calculated using the following formula [$\theta = A_B / (A_B + A_F)$] [31].

Where A_B is the NC-bound RNA and A_F is the NY-bound RNA. The K_d was directly obtained from Scatchard plots [32].

Ribonuclease mapping

In vitro transcribed RNA was dephosphorylated with calf intestine alkaline phosphatase (Thermo Fisher Scientific

Biosciences, Lafayette, CO) and 5' end-labeled using γ -[32 P]-ATP 3000 Ci/mmol (Perkin Elmer) and T4 polynucleotide kinase (Thermo Fisher). End-labeled RNA (1,000 cpm) was digested with Ambion[®] 0.1 U ribonuclease T1 (RNaseT1) (Life Technologies), 0.001 U RNaseA (Sigma-Aldrich), or Ambion[®] 0.0005 U RNaseV1 (Life Technologies) containing 2 μ L of 10 $\times$ RNase Buffer (100 mM Tris-HCl pH7.0, 1 M KCl, 10 mM MgCl₂) in 20- μ L reaction mixtures at 37°C for 5 minutes. Digestions were stopped by addition of 10 μ L RNA loading buffer [80% (v/v) formamide, 10 mM EDTA, 0.01% (w/v) bromophenol blue, 0.01% (w/v) xylene cyanol] and heated at 65°C for 5 minutes before electrophoresis in 20% polyacrylamide 7 M urea denaturing gels. Gels were dried and analyzed in a Typhoon 8600 fluorographic scanner (GE Life Sciences).

Ribonuclease footprinting assays

Aptamer RNA was 5' end-labeled and incubated with increasing concentrations (0.3 to 4.8 μ g) of HPV-16 L1 VLPs or BSA in 100 μ L selection buffer for 1 hour at room temperature and then digested with RNaseA (0.001 U) and RNaseT1 (0.1 U) or RNaseV1 (0.0005 U). After digestion, reactions were stopped by incubation with 1 U Proteinase K (Thermo Fisher) for 10 minutes at 37°C and purified with Trizol[®] reagent extraction and 2-propanol precipitation. Recovered RNA was resuspended in 10 μ L RNA loading dye and incubated 5 minutes at 65°C before denaturing electrophoresis in denaturing 20% polyacrylamide 7 M urea gels. Gels were dried and analyzed in a Typhoon 8600 fluorographic scanner (GE Life Sciences).

Deletion analysis

Progressive 3'-deleted aptamer variants were obtained by PCR using the REV primer as 5' end anchor and several 3' end forward primers (Table 1) complementary to consecutive 6-nt-long positions within the full-length template and 32 P-labeled by *in vitro* transcription. Inversely, 5' deletions were made by using the FWD primer as 3' end anchor and three different 5' end reverse primers including the T3 promoter by PCR reactions using FWD and T3 R primers (Table 1). Labeled RNAs were incubated with HPV-16 L1 VLPs in binding buffer for 1 hour at room temperature as described above.

Mice cervical wash preparation

Ten FvB female mice (2–3 months of age) at different estrous cycle were used to obtain a protein pool from cervical washes. Each cervical wash was performed using 20 μ L sterile 1 $\times$ D-PBS per mouse to produce a 200 μ L pool. Pools were cleared by centrifugation and supernatants were collected in sterile 1.5 mL microcentrifuge tubes and stored at –70°C until use. Protein integrity of the cervical washes was verified by Coomassie-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (Supplementary Fig. S2).

Results

Aptamer specificity to HPV-16 L1 VLPs

The isolation of RNA aptamers that specifically bind HPV-16 L1 VLPs was performed by an adaptation of a previously reported SELEX procedure [23,29]. The modifica-

tion consisted in using HPV-16 L1 VLPs as immobilized targets on PVDF membranes for a RNA pool with a randomized core of 15 nt ($\sim 10^9$ variants). Before incubation with the VLPs, the RNA pool was negatively selected against blank PVDF membranes to discard unspecific RNA binding to the selection system. Several aptamers were obtained after five positive selection cycles against the immobilized VLPs (Supplementary Fig. S1).

VLP interaction assays were performed to test aptamer binding specificity to HPV-16 L1 VLPs. Three isolated aptamers (Sc5-c3, Sc5-c2, and Sc5-7) were radioactively labeled and incubated with PVDF-immobilized HPV-16 L1 VLPs. BSA-saturated and blank PVDF membranes were used as negative controls under the same binding conditions. Bound RNA on the membranes was recovered and electrophoresed in denaturing polyacrylamide gels to verify integrity and quantification. Aptamer RNAs were recovered with a high degree of integrity as suggested by the presence of a single band in the gels although reproducible quantitative differences were noted amongst the different aptamers (Fig. 1A). Aptamer Sc5-c3 showed the strongest interaction with VLPs (>80% of the input RNA) and had minimal interaction with BSA (<10% of the input) and blank PVDF (<5% of the input). Sc5-c2 aptamer showed high interaction with VLPs (50% of the input) but also significant binding to BSA (30% of the input) and PVDF (20% of the input) suggesting unspecific binding. Finally, aptamer Sc5-c7 aptamer also had high interaction with VLPs ($\sim 60\%$ of the input), but showed significant unspecific binding to BSA ($\sim 25\%$ of the input) and PVDF ($\sim 10\%$ of the input) although to a lesser degree than Sc5-c2 (Fig. 1B). Because aptamer Sc5-c3 showed the highest specificity, it was selected for further analysis.

Because the relative abundance of positively charged amino acids on the surface of HPV-16 L1 VLPs may cause unspecific electrostatic binding with nucleic acids [33], a “sandwich” slot-blot binding assay was made to confirm specific Sc5-c3-VLP binding after SELEX. Labeled RNAs from the unselected RNA pool and Sc5-c3 were incubated with HPV-16 L1 VLPs (200 ng), BSA (200 ng) or binding buffer alone in binding reactions. RNA-protein complexes retained on the nitrocellulose (NC) membrane from the unselected pool showed a small background binding to VLPs similar to that of the controls. However, the Sc5-c3 aptamer showed higher binding (3-fold) to VLPs than the RNA pool suggesting that interaction to VLPs was specific (Fig. 1C).

Sc5-c3 aptamer binds tightly to HPV-16 L1 VLPs

To characterize the molecular binding between Sc5-c3 and HPV-16 L1 VLPs, we determined the dissociation constant (K_d) using the “sandwich” slot-blot approach with a NC membrane (protein retainer) layered on a NY membrane (RNA retainer). Labeled Sc5-c3 aptamer (30 pM) was incubated with increasing amounts (0, 0.25, 0.5, 1, 2, 4, and 8 μ g) of HPV-16 L1 VLPs in binding reactions and loaded on the slot-blot set up. An increase on protein-aptamer complexes retained on the NC membrane correlated with a decrease on the free aptamer RNA on the NY membrane again showing Sc5-c3 binding to the VLPs. A control experiment using the same increasing amounts of BSA showed no significant residual label on the NC membrane, thus suggesting specific Sc5-c3 binding to HPV-16 L1 VLPs (Fig. 2A).

FIG. 1. Binding of RNA aptamers to human papillomavirus (HPV)-16 L1 virus-like particles (VLPs). **(A)** Interaction assays. Radioactively labeled aptamers obtained by systematic evolution of ligands by exponential enrichment (SELEX) (Sc5-c2, Sc5-c3 and Sc5-c7) were incubated with 500 ng of polyvinylidene fluoride (PVDF)-immobilized bovine serum albumin (BSA), 500 ng HPV-16 L1 VLP (VLPs) or PVDF membranes alone (PVDF) in binding reactions. Membranes were thoroughly washed, and bound aptamer RNA was recovered and resolved by denaturing electrophoresis in 8% polyacrylamide/7 M urea gels. Arrows indicate migration of bound aptamer RNA. **(B)** Interaction assay digital analysis. The plot shows the average percentage of bound RNA relative to the input expressed as the mean and standard deviation of three independent experiments. **(C)** Sc5-c3 specificity control. A “sandwich” slot-blot binding assay was made to confirm specific Sc5-c3-VLP complex formation after SELEX. Binding assays were performed incubating the 5′ end-labeled unselected RNA pool (Aptlib) and the Sc5-c3 aptamer with 200 ng HPV-16 L1 VLPs (closed bars), 200 ng BSA (open bars), or binding buffer only (slotted bars). Aptamer–protein interactions were represented as relative to Sc5-c3 binding to VLPs. The bars represent the mean and standard deviation of three independent experiments.

The residual radioactivity on both membranes was quantified and used to elaborate fractional saturation plots (θ vs. [VLP]) that showed a classic bimolecular interaction saturation curve for one interaction site (Fig. 2B, left panel). Data were fit on a linear regression analysis through Scatchard plots ($\theta/[VLP]$ vs. [VLP]) resulting in a K_d value of 0.05 pM (Fig. 2B, right panel), indicating that Sc5-c3 binds tightly to HPV-16 L1 VLPs.

Sc5-c3 secondary structure

To map Sc5-c3 secondary structure, end-labeled Sc5-c3 RNA was partially digested with RNaseA (which cleaves in single-stranded C and U residues), RNaseT1 (targeting single-stranded G residues) or RNaseV1 (specific for double-stranded RNA). Digested fragments were visualized through 20% polyacrylamide 7 M urea denaturing gels. RNaseA cleaved at positions U13, C15, U22, C24, C26, C28, U42, and U44 while RNaseT1 cleaved at positions G19, G20, G33, G34, G46, G48, G50, and G53. Therefore, most of these positions corresponded to single stranded regions within Sc5-c3 structure (Fig. 3A). RNaseT1 cleavage at position 7 is inconsistent with sequence data (no G residue) and was not considered for the structural analysis. A minor cleavage site at position 11 is also

visible although co-migration with V1 residues suggests that Sc5-c3 general structure might be relatively unstable, switching single strands for double-stranded regions. Cleavage by RNaseV1 at positions 1–6, 9–12, 29–32, 35–36, and 38–41 suggested double stranded regions, although some cleavage was also observable at positions G19–G20 and C26–C27, adding to the notion of an unstable overall structure with double strands may be forming between G19–C27 and G20–C26. The mapping results best fitted with an adjustment to the *in silico* secondary structure produced by Mfold [34], showing that Sc5-c3 structure contained a 6-nt stem (S1) and a mismatch at the 3′ strand of the stem. S1 is followed by a small symmetric bubble (B1) and a second stem of 4 nt (S2). The main loop (ML) is 16 nt long and includes the entire Aptlib randomized region. Sc5-c3 aptamer structure also contained a 17-nt single-stranded unstructured region (UR) at 3′ end (Fig. 3B). Nevertheless, further structural analysis by other methods (i.e., nuclear magnetic resonance, X-ray diffraction, etc.) would be required to absolutely resolve Sc5-c3 structure.

Sc5-c3 interaction regions with HPV-16 L1 VLPs

To further characterize Sc5-c3 interaction with HPV-16 L1 VLPs, 5′ end-labeled Sc5-c3–VLP complexes were digested

FIG. 2. Dissociation constant (K_d) determination of Sc5-c3 binding to HPV-16 L1 VLPs. (A) K_d determination was performed on “sandwich” slot blot binding assays using α -[32 P]-UTP labeled aptamer RNA (30 pM) incubated with increasing amounts of the HPV-16 L1 VLPs (left panel) and BSA (right panel). Protein–aptamer complexes were retained on the nitrocellulose membrane (NC), whereas the free aptamer is bound to the positively charged nylon membrane (NY). (B) The K_d for Sc5-c3 binding with HPV-16 L1 VLP were obtained using fractional saturation [defined as the bound RNA relative to the total RNA in a binding reaction vs. VLP protein concentration (pM)] (left panel) and Scatchard [fractional saturation vs. fractional saturation/VLP protein concentration (pM)] (right panel) plots. The plots represent the mean and standard deviation from three experiments.

FIG. 3. Ribonuclease mapping of Sc5-c3 secondary structure. (A) 5′-end labeled Sc5-c3 RNA was partially digested with ribonuclease A (RNaseA), RNaseV1, and RNaseT1. Cleavage products were resolved in 20% polyacrylamide/7M urea denaturing gels. Molecular weight markers (MWM) were obtained from diverse radioactively labeled transcripts. IVT, *in vitro* transcribed Sc5-c3 RNA. IVT 37, Sc5-c3 transcript incubated with RNase reaction buffer at 37°C in the absence of ribonuclease. Brackets indicate the boundaries of the structural domains stem 1 (S1), bubble (B1), stem 2 (S2), main loop (ML) and an unstructured region (UR). (B) Schematic representation of Sc5-c3 secondary structure. *In silico* secondary structure analysis of Sc5-c3 ERNA showed five well-defined structural domains: stem 1 (S1), bubble (B1), stem 2 (S2), main loop (ML), and an unstructured region (UR) which were adjusted according to ribonuclease mapping data. Ribonuclease cleavage sites are indicated as follows: RNaseA (squares), RNaseV1 (triangles), and RNaseT1 (circles). ΔG , Gibbs’ free energy.

with ribonucleases V1 in footprinting assays. Footprints showed that VLP binding protected Sc5-c3 nucleotide positions 2–6, 9, and 10 from RNaseV1 cleavage in a concentration-dependent manner. Also, a faint footprint, suggesting an unstable double strand, was observable at G19–C27 and G20–C26 (Fig. 4A, right panel). No footprint was observed with Sc5-c3 and increasing amounts of BSA (Fig. 4A, left panel).

Additional RNaseA and RNaseT1 footprints were performed to further refine Sc5-c3 interaction sequences. Changes in sensitivity to RNaseT1 were not observed even with high VLP amounts (Fig. 4B, left panel), suggesting that most G positions in single-stranded regions were not directly involved with VLP interaction. Also, the data showed that VLP binding protected positions 22–28 from RNaseA cleavage (Fig. 4B, right panel), suggesting that the ML region is relevant for interaction with VLPs. Footprinting results were summarized in Fig. 4C.

Sc5-c3-VLP binding sequence requirements

Nucleotide-specific Sc5-c3 binding interactions was initially analyzed using aptamers Sc5-c1 and Sc5-c8 (Fig. 5A). *In silico* sequence analysis showed that although nucleotide differences were present at positions G14 and G20 for Sc5-c1 and G14, G19, G20, U22, C24, and C25 for Sc5-c8 all aptamers shared the same hairpin structure with an internal loop and unstructured 3′ end found for Sc5-c3 (Fig. 5A and data not shown).

Binding assays using both VLPs and bacteria-produced L1 confirmed that Sc5-c1, Sc5-c3, and Sc5-c8 aptamers have minimal interaction with NC membranes, bacteria-produced L1, BSA, and GFP. Nevertheless, although Sc5-c1 showed VLP binding similar to Sc5-c3, aptamer Sc5-c8 only retained

FIG. 4. Ribonuclease footprinting assay. (A) Footprinting assay of the Sc5-c3-VLP complex using increasing amounts (0.3 to 4.8 μ g) of BSA (left panel) or HPV-16 L1 VLP protein (right panel), in the presence of RNaseV1. Cleavage products were resolved in 20% polyacrylamide/7 M urea denaturing gels. Brackets indicate the protected sequences. MWM, molecular weight marker. Lane 1 contains the undigested Sc5-c3 RNA and lane 2 includes the Sc5-c3 transcript incubated with the ribonuclease reaction buffer but no ribonuclease. Arrows indicate G residues (G*) within in the ML region. (B) Footprinting of the Sc5-c3-VLP complex using RNaseT1 (left panel) and RNaseA (right panel) showing a higher resolution view of the protected nucleotides. Arrows indicate G residues (G*) within in the ML region. (C) Schematic representation of Sc5-c3 contact region with HPV-16 L1 VLPs. Positions protected from ribonuclease cleavage by VLP binding are indicated by boxes. Sc5-c3 structural features are shown as reference (B1, bubble 1; ML, main loop; S1, stem 1; S2, stem 2; UR, unstructured region).

about the half of VLP binding compared to Sc5-c3 (Fig. 5B). The lower Sc5-c3 interaction with bacteria-produced L1 suggested that Sc5-c3 binding may rely on VLP quaternary structure features not present in bacteria-produced L1 as it has been reported that bacteria produced HPV-16 L1 is found mainly in pentameric form [9,35]. In addition, these results showed that changes at positions 14 (G $\rightarrow$ C) and 20 (G $\rightarrow$ U)

did not significantly affect binding to VLPs. However, changes at positions 22 (U $\rightarrow$ C), 24 (C $\rightarrow$ U), and 25 (C $\rightarrow$ G) significantly (> 50%) reduced binding to VLPs, suggesting that U22, C24, and C25 are relevant for efficient VLP binding (Fig. 5B).

Because all isolated aptamers against HPV-16 L1 VLPs presented five conserved nucleotides at positions A16, A18, A23, C27, and C28 (Supplemental Fig. S1) we introduced the

FIG. 5. Sc5-c3 binding sequence requirements. (A) Multiple alignment of co-isolated aptamers against HPV-16 L1 VLPs (Sc5-c1, Sc5-c3, and Sc5-c8). Differences in nucleotide positions are indicated. Nucleotide numbering is shown. (B) Binding analysis of co-isolated aptamers against HPV-16 L1 VLPs. Labeled RNA (30 pM) from Sc5-c1 (open bars), Sc5-c3 (closed bars), and Sc5-c8 (slashed bars) aptamers were incubated with 500 ng of HPV-16 L1 VLPs (VLP), bacteria-produced HPV-16 L1 (L1), bovine serum albumin (BSA), or green fluorescent protein (GFP) using a sandwich slot-blot binding assay. A negative control of the background binding to the nitrocellulose membrane was included (Ctrl). The plot displays the mean and standard deviation of three independent experiments.

FIG. 6. Mutational analysis of Sc5-c3 binding to HPV-16 L1 VLPs. (A) Several Sc5-c3 mutants (M1 to M8) were produced by site-directed mutagenesis. Underlined positions correspond to constant nucleotides presented in all isolated aptamers against HPV-16 L1 VLPs and mutations on specific Sc5-c3 nucleotide positions are indicated by squares. Nucleotide numbering is indicated. (B) Sc5-c3 mutants binding to HPV-16 L1 VLPs. Binding assays were performed using end labeled aptamer RNA incubated with 100 ng of BSA protein (open bars), HPV-16 L1 VLPs (closed bars), or binding buffer alone (slotted bars). The plots represent the mean and standard deviation of relative binding from three independent experiments.

site-specific mutations A16→C (M1), A18→C (M2), A23→C (M3), C27→A (M4), and C28→A (M5) determine their relevance for VLP binding (Fig. 6A).

Binding of Sc5-c3 mutants to VLPs, BSA, and NC was analyzed through “sandwich” slot blot binding assays (Fig. 6B). M1 and M5 did not significantly affect VLP binding but unspecific BSA and NC binding significantly increased compared to Sc5-c3. VLP binding of M2, M3, and M4 mutants showed a significant increase relative to wild-type Sc5-c3, but also presented higher unspecific binding to BSA (14-, 7-, and 3-fold, respectively) and NC binding (3-, 7-, and 2-fold, respectively). A control mutation introduced on the nonconserved position C17→A (M7) did not significantly affect VLP binding or specificity (Fig. 6B).

The triple mutant G19→U, G20→U, and U21→C (M6) lacking the G19–G20 and C26–C27 unstable double strand, showed increased VLP, BSA, and NC binding (2-, 6-, and 2-fold, respectively) compared with Sc5-c3, suggesting that the single-stranded ML region could be required for a more efficient binding.

The M8 mutant, containing changes in non-conserved positions C15→A, C17→A, G19→U, G20→U, U21→C, U22→G, C24→A, C25→A, and C26→A did not significantly affect VLP binding but showed increased unspecific BSA and NC binding. Thus, this experiment showed that modification of any of the five conserved positions is sufficient to decrease Sc5-c3 specificity to VLPs, although the results also suggested that the conserved positions A18, A23, and C27 have an important role on VLPs binding.

FIG. 7. Deletion analysis of Sc5-c3 binding to HPV-16 L1 VLPs. Binding assays were performed using α -[32 P]-UTP labeled RNA from Sc5-c3 and 5' or 3'-deleted derivatives (30 pM) incubated with BSA protein (open bars), HPV-16 L1 VLPs (closed bars), and binding buffer alone (slotted bars). The deleted Sc5-c3 schematic maps and name are included as reference (B1, bubble 1; ML, main loop; S1, stem 1; S2, stem2; UR, unstructured region). Initial and end nucleotide positions are indicated for each deleted derivative. The bars show the mean fractional saturation ($n=3$) and standard deviation.

FIG. 8. Sc5-c3 binding to HPV-16 L1 VLPs in murine cervical washes. (A) Murine cervical washes do not interact with Sc5-c3. Binding assays were performed incubating labeled Sc5-c3 (30 pM) with increasing concentrations (0, 50, 100, 500 and 1,000 ng) of HPV-16 L1 VLPs (VLPs) or cervical wash protein (CP). The plot represent the mean and standard deviation of bound RNA relative to the input ($n=3$). (B) Specific Sc5-c3 binding to HPV-16 L1 VLPs in murine cervical washes. Five hundred nanograms of cervical wash protein were mixed with increasing concentrations of HPV-16 L1 VLPs (triangles) and 500 ng of HPV-16 L1 VLPs were added to increasing concentrations of cervical wash protein (squares). The plot represent the mean and standard deviation of bound RNA relative to the input ($n=3$).

Minimal binding motif of Sc5-c3 for HPV-16 L1 VLPs

Deletion analysis on Sc5-c3 was performed to establish a minimal binding moiety for HPV-16 L1 VLPs. Deleted Sc5-c3 constructs were labeled and incubated with VLPs in binding experiments using NC and BSA as negative controls (Fig. 7). Removal of 6 nt and 12 nt from the 3' end reduced binding by 50% compared to that of full-length Sc5-c3. Nevertheless, further 3' nucleotide removals (18 nt and 24 nt) substantially incremented VLP binding (>40%) relative to full-length Sc5-c3. Longer deletions (of 24 nt to 42 nt) presented decreased specificity to VLP, probably due to the loss of the ML region, and significantly reduced VLP binding (Fig. 7). Removal of 6 nt from 5' end did not affected binding but removal of 12 nt slightly reduced binding (<10%). Also, deletion of 24 nt reduced binding by one-half compared with full-length Sc5-c3 (Fig. 7). Thus, Sc5-c3 40-nt core (–18 nt) appeared as the minimal binding moiety able to bind HPV-16. Moreover, the deletion analysis also suggested that the 3' UR may participate but is not required for VLP binding, while the 5' end (B1 and S2 regions) is not required for binding.

Taken together, the data from footprinting, mutational, and deletion analyses strongly suggest that the ML region, located at the center of the aptamer, is the primary binding region for Sc5-c3 with the HPV-16 L1 VLPs.

Sc5-c3 binding to HPV-16 L1 VLPs in biofluids

To test Sc5-c3 specificity in complex protein mixes, increasing amounts of HPV-16 L1 VLPs or cervical washes from FvB mice (0, 50, 100, 500, and 1,000 ng) were incubated with labeled Sc5-c3 aptamer RNA in binding assays. Increments on VLP concentration correlated with increasing bound RNA quantities reaching up to 20% binding at the highest VLP concentration used. Identical amounts of cervical wash protein resulted in approximately 1% binding in all cases suggesting a low unspecific Sc5-c3 binding, thus suggesting specific Sc5-c3-VLP binding (Fig. 8A).

Further binding experiments now mixing increasing concentrations (50, 100, 500, and 1,000 ng) of HPV-16 L1 VLPs with a fixed amount (500 ng) of cervical wash proteins were incubated with labeled Sc5-c3. A significant increase in

bound RNA was observed from 500 ng of VLPs (4%) up to 22% bound RNA at 1000 ng VLPs. Reciprocal Sc5-c3 binding assays were also made using increasing concentrations of cervical wash protein (50, 100, 500, and 1,000 ng) mixed with a saturating amount (500 ng) of VLPs. In this set up, the VLPs saturation hindered any significant cervical wash protein binding up to the highest concentration used (1,000 ng) (Fig. 8B), suggesting a stable Sc5-c3-VLP binding that correlated with the low K_d observed (Fig. 2B). Therefore, Sc5-c3 aptamer can specifically and stably bind HPV-16 L1 VLPs even within a complex protein mix.

Discussion

We report here the characterization of an RNA aptamer (Sc5-c3) against HPV-16 L1 VLPs. The isolated Sc5-c3 aptamer showed a very high affinity ($K_d=0.05$ pM) for its intended target, far superior to that of aptamers isolated against other HPV proteins [23,24], but within the range of RNA aptamers isolated against other viruses or VLPs [36–40]. It is likely that such high affinity is owed to the 360 copies of L1 protein conforming HPV-16 VLPs [9]. Interestingly, Sc5-c3 interaction with HPV-16 VLPs appears more stable than the described HPV-33 interaction with the cell surface ($K_d=100$ pM) which is mainly governed by electrostatic interactions and probably conserved for high-risk HPVs [14,41]. This provides an insight to the potential Sc5-c3 applications as a competitor to block HPV infection, as previously shown for aptamers against human influenza virus [42,43], hepatitis C virus [44], and cytomegalovirus [36] infections.

Our binding and structural data suggested that Sc5-c3-VLP interaction involved the ML region. This single-stranded ML region may provide the molecular flexibility required for the adaptive recognition characteristic of aptamer binding [1]. In this regard, RNaseV1 mapping suggested an alternative ML structure including a double strand between G19–G20 and C26–C27. This alternative double-stranded structure is observed in lesser proportion respect to the single stranded form (Fig. 3A). Moreover, as observed in footprinting analysis, the double-stranded ML structure is further decreased in the presence of VLPs in favor of the more stable single-stranded form, suggesting that the double-stranded sequence within the ML may be involved with HPV-16

L1 VLP binding. Furthermore, M6 mutant, incapable of forming double strand, showed a 6-fold increase in binding relative to Sc5-c3. Therefore, it is feasible that the unstable double-stranded ML form results in adaptive binding of Sc5-c3 to VLPs where positions G19 and G20 remain in a stable single-stranded form. This is further supported by the observation that five conserved positions (A16, A18, A23, C27, and C28) in all isolated aptamers were located within the ML region and that nucleotide changes in these five positions affect binding and specificity. In a previous report, it was shown that single nucleotide changes have different effects on binding activity of RNA aptamers raised against HPV-16 E7 protein [24]. Interestingly, positions U22, C25, and C24 (located at the ML from high binding aptamers Sc5-c1 and Sc5-c3, but not in the low binding aptamer Sc5-c8), appeared relevant but not necessary for VLP binding, further suggesting that the Sc5-c3 ML region is mainly involved on VLP binding.

Although the 3' deletion analysis suggested the additional participation of the 3' UR in VLP binding, the footprinting results showed no VLP interaction with the 3' end. Interestingly, the footprinting analysis also showed two VLP interaction sites (ML and one strand of S1) within Sc5-c3 but further analysis showed that the 5' end strand of S1 is not absolutely necessary for VLP binding. However, the binding curve obtained for Sc5-c3 and VLPs showed a classical bimolecular single-site noncooperative interaction suggesting that the VLPs have only one contact region with Sc5-c3. Therefore, additional structural changes should follow the initial Sc5-c3-VLP interaction to allow two separate aptamer domains interact at one point within a VLP. In this regard, it has been reported that small changes in aptamer structure affect binding or function and that secondary structures do not fully represent the structure of aptamer–target complexes [1,24,45].

Sc5-c3 is able to bind VLPs but has low interaction with purified bacteria-produced L1 protein (pentameric form [9,35]). The cause of this low binding may be a consequence of the L1 conformational changes on structured VLPs that are not present on pentameric L1. Previous studies have demonstrated that VLP formation does not require any L1 post-translational modification [46,47], but they do exhibit N-terminal acetylation [48]. In this terminus lies the inter-pentamer contacts on VLPs and according to that, it has been reported that the L1 C-terminal arm (helix 4) act as a flexible hinge to mediate pentamer–pentamer interactions within the VLP which is exposed on the virion surface near to the outer rim of the L1 pentamer [49]. Helix 4 remains folded back on L1 pentamers like those formed when L1 are expressed in bacteria [50]. These observations suggest that helix 4, a characteristic and relatively accessible structure on VLP surface not present in bacteria-produced L1 pentamers, may be a possible target region for Sc5-c3.

Although L1 is the most conserved gene within papillomavirus genome [11], it has been demonstrated that the virion surface contain sites of sequence variation among HPV types [50], thus reducing the possibility of Sc5-c3 cross-reactivity with other HPV types. Nevertheless, it has been reported that helix 4 (a possible contact point for Sc5-c3) has a highly conserved structure and sequence in HPV-11, HPV-16, and HPV-18 [50], which would allow the use of Sc5-c3 between different HPVs. In addition, it has been described that the superficial residues Lys²⁷⁸, Lys³⁵⁶, and Lys³⁶¹ (located at the

FG and HI loops joining the respective β -sheets within the L1 monomer [49]) are required to interact with cell membrane receptors [51]. Through the interaction with these positions, Sc5-c3 may be used as a potential prophylactic agent by competing with cellular receptors for the HPV virion, thus blocking infection or as a therapeutic agent by interfering with capsid assembly. Further studies on Sc5-c3 binding with mutant L1, L1/L2 VLPs, and other HPV types will be necessary to elucidate the potential application of Sc5-c3 in the gynecological clinic.

Although Sc5-c3 binding to HPV-16 L1 VLPs was demonstrated even in the complex and diverse protein mixture present in murine cervical washes, interaction remains to be tested in actual human cervical samples and other HPV types. In conclusion, Sc5-c3 is the first RNA aptamer isolated and characterized against HPV-16 L1 VLPs. Although further structural and binding specificity issues remain to be solved, the stable and specific Sc5-c3 binding to HPV-16 VLPs even in complex protein mixtures may provide the basis for the development of a novel diagnostic for active HPV infections.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Luis Marat Alvarez-Salas, PhD
Genética y Biología Molecular
Avenida Instituto Politecnico Nacional 2508
Col. San Pedro Zacatenco
Mexico City 07360
Mexico

E-mail: lalvarez@cinvestav.mx

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