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Specific interaction between hnRNP H and HPV16 L1 proteins: Implications for late gene auto-regulation enabling rapid viral capsid protein production

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ABSTRACT

Heterogeneous nuclear ribonucleoproteins (hnRNPs), including hnRNP H, are RNA-binding proteins that function as splicing factors and are involved in downstream gene regulation. hnRNP H, which binds to G triplet regions in RNA, has been shown to play an important role in regulating the staged expression of late proteins in viral systems. Here, we report that the specific association between hnRNP H and a late viral capsid protein, human papillomavirus (HPV) L1 protein, leads to the suppressed function of hnRNP H in the presence of the L1 protein. The direct interaction between the L1 protein and hnRNP H was demonstrated by complex formation in solution and intracellularly using a variety of biochemical and immunochemical methods, including peptide mapping, specific co-immunoprecipitation and confocal fluorescence microscopy. These results support a working hypothesis that a late viral protein HPV16 L1, which is down regulated by hnRNP H early in the viral life cycle may provide an auto-regulatory positive feedback loop that allows the rapid production of HPV capsid proteins through suppression of the function of hnRNP H at the late stage of the viral life cycle. In this positive feedback loop, the late viral gene products that were down regulated earlier themselves disable their suppressors, and this feedback mechanism could facilitate the rapid production of capsid proteins, allowing staged and efficient viral capsid assembly.

1. Introduction

hnRNP H is a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family. hnRNP H has been shown to form heterodimers during the regulation of mRNA splicing [1,2]. During this process, hnRNP H binds to intronic G triplets or G3: for example, the six "GGG" repeats in RNA as shown in Fig. 1A [3,4]. hnRNP H enhances the binding of cleavage/polyadenylation specificity factor (CPSF) to RNA polyadenylation sites [5]. This binding event leads to further enhancement of pre-mRNA cleavage and polyadenylation [6–8]. As previously reported [3,9], in mRNA, the polyadenylation site sequence procedes (i.e., is 5′ to) the GU-rich sequence, which includes several intronic G triplets. The binding of CPSF to the polyadenylation site failed to stimulate cleavage and polyadenylation [10], demonstrating that the binding of hnRNP H is required to initiate cleavage and polyadenylation [3,9]. In several viruses, hnRNP H has been shown to play critical roles in the splicing regulation of several viral mRNAs, including the splicing of simian vacuolating virus at 40 pAL [11], human papillomavirus (HPV) at pAE [3], Rous sarcoma virus pre-mRNA [12] and HIV type 1 tat-specific exon 2 and tev-specific exon 6D [13,14].

Over 100 serotypes of HPV exist, with more than 50 serotypes being human pathogens [15]. The most studied serotype, HPV16, is a high-risk serotype and the most common serotype that causes cervical cancer in women. In all HPV serotypes, there are two late viral proteins (L1 and L2) that form the viral capsid, with L1 being the predominant protein. The HPV16 L2 sequence encodes a polyadenylation element, which encompasses multiple GGG (or G3) motifs in the RNA. In addition, hnRNP H has been shown to interact with the G3-containing motifs, and it is speculated that hnRNP H regulates polyadenylation at the HPV16 early polyadenylation signal, which suppresses the expression of the late proteins during the early stage of the viral life cycle [3]. Whether this regulatory protein interacts with late gene products (once expressed) at the protein level, providing a feedback loop, remains unclear.

In this study, we assessed the possible association between the HPV L1 capsid protein and hnRNP H, the suppressor of the late gene expression. To ensure that the reporter module pPG was suitable for this purpose, the tuning of hnRNP H activity using RNAi

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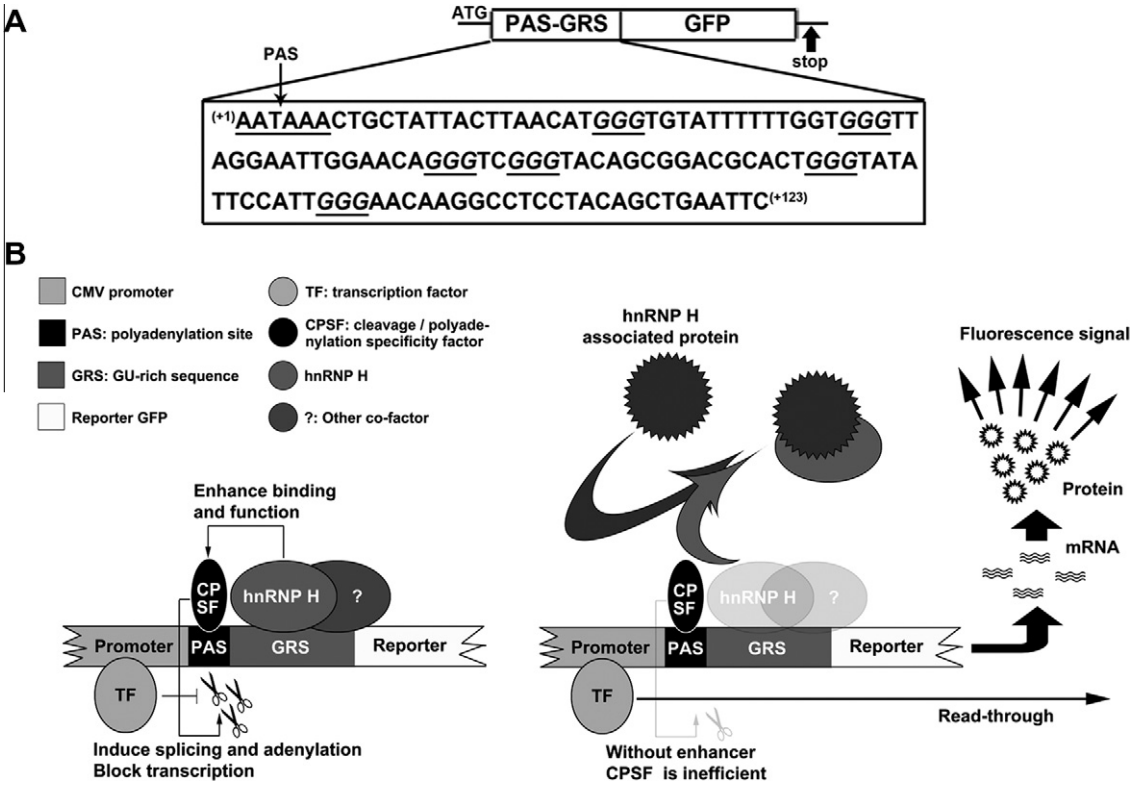

Fig. 1. Construction of the reporter plasmid pPG using GFP as a reporter (A) for functional analysis of hnRNP H activity and the envisioned mechanism of the RNA-binding proteins during gene regulation (B). The plasmid contains a strong CMV promoter, polyadenylation site (PAS), GU-rich sequence (GRS) and a green fluorescence protein (GFP) reporter, as shown in Fig. 1A. There are three differences between the pPG used in this study and the plasmid described by Oberg et al. [3]: (a) only the key sequence AAUAA was introduced instead of the full-length pAE sequence; (b) the HPV16 L1 and L2 genes were replaced by the GFP reporter gene; and (c) a synthetic GRS signal sequence was used in the absence of the L2 genes.

and other means was demonstrated with the varying level of the resultant green fluorescent protein (GFP) gene or protein. Once validated, the impact of HPV16 L1 co-expression with pPG was studied, and this co-expression demonstrated effective suppression of hnRNP H activity in the presence of the L1 protein. Moreover, the direct association between HPV16 L1 and hnRNP H was demonstrated on a molecular level by various techniques, including peptide mapping after specific pull-down experiments, co-immunoprecipitation and intracellular co-localization.

2. Materials and methods

2.1. Cell lines, proteins, and antibodies

HeLa cells (ATCC) were cultured with RPMI-1640 (GIBCO) containing 10% fetal calf serum at 37 °C with 5% CO_2. Recombinant HPV16 L1 self assembles into virus-like particles upon over-expression in various host cells, including *E. coli* [16], bacuolovirus [17] and yeast [18,19]. *E. coli*-expressed HPV16 L1 [20] was used for specific pull-down analysis. The HPV16 L1-specific mouse monoclonal antibodies (mAbs), denoted PB9 and PB11, were produced in-house using hybridoma technology. The purified IgG form of PB9 and PB11 was used. The rabbit anti-His and anti-GFP polyclonal antibodies (pAbs) and mouse anti-β-tubulin mAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-GAM-IgG and TRITC-GAR-IgG were purchased from Sigma–Aldrich Corporation (St. Louis, MO). IR Dye 800CW-conjugated goat (polyclonal) anti-rabbit and anti-mouse IgG were purchased from Invitrogen (Carlsbad, CA).

2.2. Plasmid construction

2.2.1. Reporter pPG plasmid and plasmid for hnRNP H expression

As shown in Fig. 1A, the polyadenylation site sequence (PAS) and GU-rich sequence (GRS) with six G-triplets, serving a similar function with early polyadenylation site (pAE) in HPV, were constructed upstream or at the 5′ to the GFP reporter gene. In the HPV genome, the GU-rich sequence was found in the part of the 5′ coding region for HPV16-L2 [3]. The PAS-GRS sequence was inserted into pcDNA3.1 at *BamHI* and *EcoRI* sites. The GFP sequence was amplified by PCR and inserted into pcDNA3.1-pAE-G at *EcoRI* and *XhoI* sites to generate pPG. The pHis-H-hnRNP H sequence was amplified by PCR using Pvl1392 H as a template with the primers H-F and H-R. All of the plasmids and primers used in the plasmid construction are listed in Supplementary Tables 1S and 2S.

2.2.2. Inhibitory plasmids for the investigation of hnRNP H activity

The oligo GGGCACAGGTATATTGAAA for RNAi of hnRNP H was designed using an algorithm (http://www.dharmacon.com/sidesign) and inserted into a pSUPER vector purchased from OligoEngine (Seattle, WA) to construct pSUPER-Hi by following established protocols. In brief, two complementary oligos, Hif and Hir, were designed and synthesized. The oligos were mixed and denatured at 90 °C for 10 min and then annealed at room temperature for 30 min to generate sticky ends at both the 3′ and 5′ ends. The annealed product was subsequently inserted into pSUPER at the *BglII* and *HindIII* sites to generate pSUPER Hi. The plasmid p16L1 h, containing the HPV16 L1 expression cassette [20], was transfected into mammalian cells to co-express HPV L1.

2.2.3. Control plasmid

The pHis-IR-human cytochrome P450 2A6 gene (CYP 2A6) expression plasmid pCW2A6-His was kindly provided by Prof. F.P. Guengerich (Vanderbilt University, Nashville, TN) [21]. It was tagged with a 6× His tag and then inserted into pcDNA3.1 to generate pHis-IR.

2.3. Flow cytometric analysis

HeLa cells (with or without pPG and/or p16L1 h transfection) were trypsinized, washed and re-suspended in PBS. The fluorescence intensity of the cells due to GFP expression was measured as previously described [22].

2.4. Northern blot analysis for RNA quantitation

Total cellular RNA was prepared from 5×10^5 transfected HeLa cells using a Trizol RNA extraction kit from Invitrogen (Grand Island, NY). Northern blot analysis to determine the GFP RNA levels was performed as previously described [23].

2.5. Immuno-capture of HPV 16 L1-interacting protein(s)

In the specific pull-down experiment, 8 μg of recombinant HPV16 L1, 6 μg of anti-L1 BP9, and 20 μL of protein A/G beads (Santa Cruz Biotechnology) were incubated for 1 h at 4 °C. The mixtures were then incubated with 2 mL of HeLa cell lysate for 1 h at 4 °C. The beads with anti-L1 mAb and HPV16 L1 protein attached were washed prior to the addition of cell lysate. The immunocomplexes were collected by centrifugation prior to denaturation for subsequent 2D gel electrophoresis [22] and MALDI-TOF-MS [22] to identify the L1-associating protein(s) pulled-down from the cell lysate.

2.6. Affinity co-immunoprecipitation

HeLa cells were co-transfected with either p16L1h (HPV16 L1) and pHis-H (hnRNP H) or pHis-CYP 2A6 (as a control). After re-culturing for 24 h, the cells were collected and lysed, prior to loading onto a 30-μl nickel column (Qiagen, Hilden, Germany), prepared according to the manufacturer's instruction. The mixtures were first washed 3 times with 20 mM imidazole (pH 8.0). The captured proteins were subsequently eluted with elution buffer (150 mM imidazole, pH 8.0) for further analysis.

2.7. Western blot analysis

The protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of protein for each sample were loaded onto an SDS–PAGE gel and subsequently electro-blotted onto a nitrocellulose membrane (Whatman, Dassel, Germany). The appropriate pAbs or mAbs were used to detect the blotted proteins as previously described [22].

2.8. Immunofluorescence analysis

HeLa cells were transfected with p16L1h and pHis-H, and the expressed proteins, HPV16 L1 and hnRNP H, were then specifically detected as previously described [22] with anti-L1 PB11 or anti-His pAbs from Santa Cruz Biotechnology.

3. Results and discussion

3.1. Plasmid construction and validation

A plasmid with multiple G3 elements was constructed to evaluate the impact of various factors on the functioning of hnRNP H. The reporter plasmid, pPG (Fig. 1A), linking the reporter gene expression to hnRNP H function, was constructed to elucidate the mechanism of late gene expression. The synergistic effect of hnRNP H and cleavage/polyadenylation specificity factor (CPSF) enhanced RNA splicing and adenylation, leading to the premature termination of GFP reporter transcription, as shown in Fig. 1B.

Because GFP is fluorescent, the gene expression level can be quantitatively analyzed with or without cell lysis. The synergistic effect of hnRNP H and CPSF should lead to a small amount of GFP transcription and translation, resulting in very low fluorescence signals. In the absence of hnRNP H or when hnRNP H forms complexes with other proteins, CPSF was not able to inhibit the gene transcription without a functioning hnRNP H [3,9]. Therefore, if the previously available hnRNP H were bound by another protein, the expression of the GFP reporter would be up-regulated, leading to enhanced fluorescence.

The validity of the reporter plasmid pPG was confirmed by the RNAi silencing of hnRNP H (Figs. 2 and 3, Table 1). The reporter gene demonstrated enhanced expression, with GFP fluorescence in approximately 79% of cells when hnRNP H was silenced by RNAi (Fig. 2). The up-regulation of the downstream GFP expression was due to the decreased suppression by hnRNP H.

3.2. Enhanced GFP expression in the presence of HPV 16 L1

A late viral protein (HPV16 L1) was co-expressed in HeLa cells with the plasmid pPG to investigate its impact on the GFP expression. The co-expression of HPV16 L1 induced an approximately 3-fold increase (21 vs 78%) in the percentage of cells positive for GFP fluorescence (Fig. 3A), similar to the result in the RNAi experiment. Not surprisingly, no additional enhancing effect on GFP expression

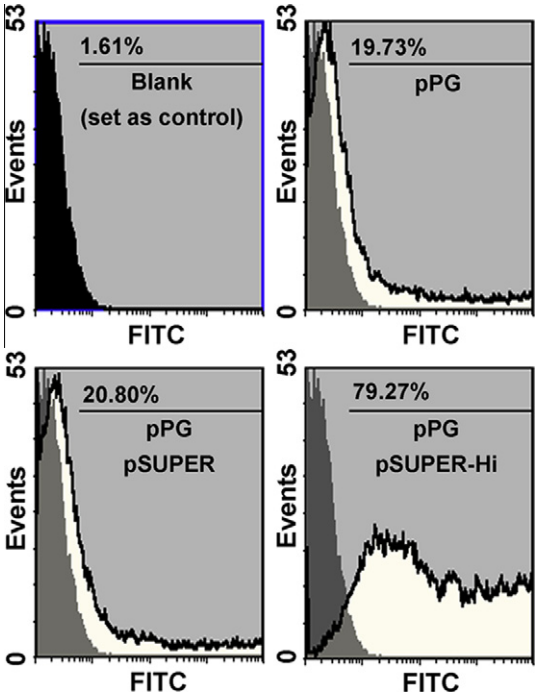

Fig. 2. Quantitative analysis of hnRNP H activity by the intracellular GFP fluorescence. Flow cytometric analysis of cells transfected with the reporter plasmid pPG with and without additional inhibitory plasmids. The control represents the background values detected for non-transfected cells.

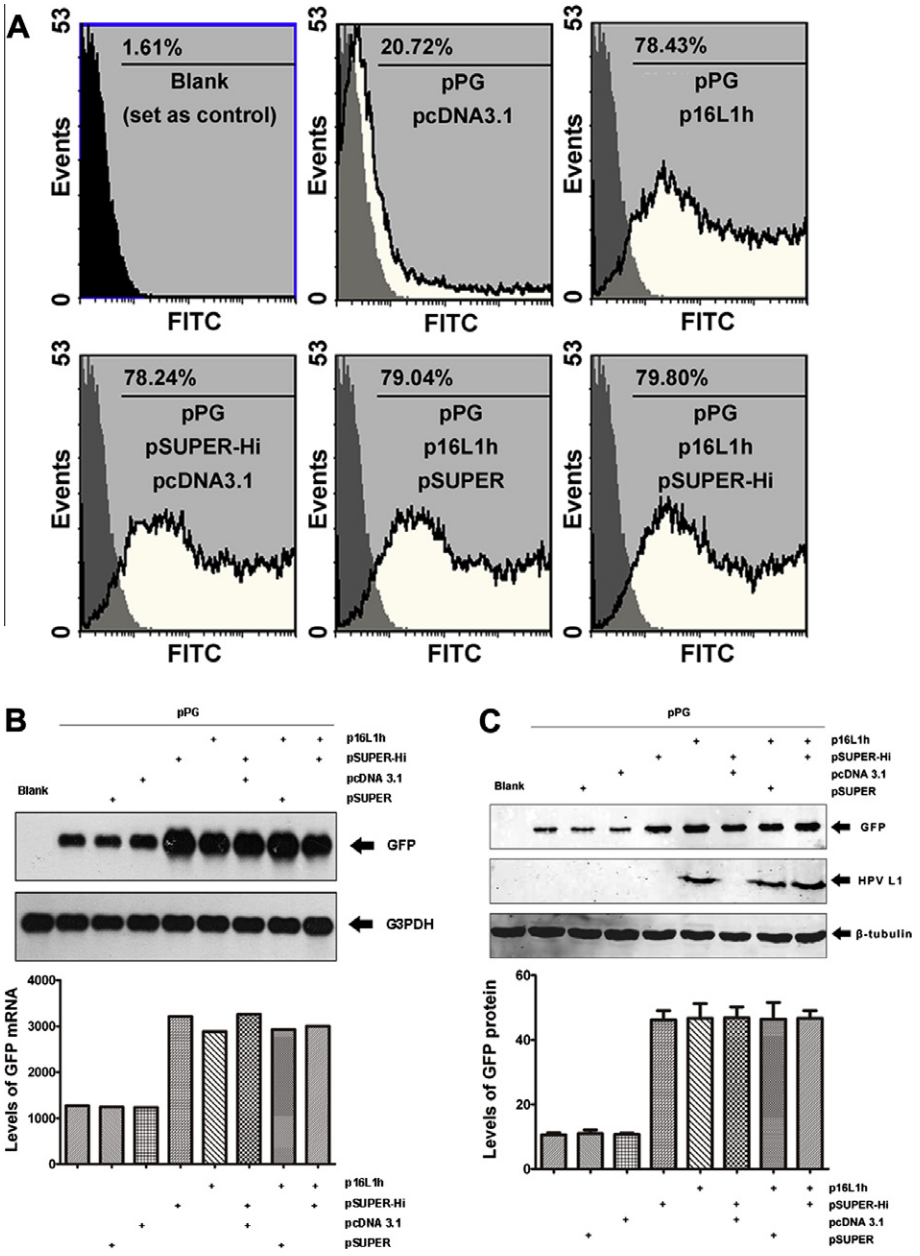

Fig. 3. (A) Flow cytometric analysis indicated enhanced GFP fluorescence when HPV16 L1 was co-transfected with pPG or when hnRNP H was specifically silenced by RNAi. (B) Enhanced GFP transcription when HPV16 L1 was co-transfected with pPG or by RNAi silencing. GFP in the reporter plasmid and baseline HeLa cell G3PDH transcripts (G3PDH functions as a baseline control) were examined by Northern blotting. GFP transcript levels were quantified with Quantity One® gel analysis software. (C) An enhanced level of GFP translation was noted when HPV16 L1 was co-transfected with pPG or with RNAi silencing. The expression of GFP, HPV16 L1 and β-tubulin in the cells was measured by Western blotting (upper panel). Data were analyzed with an Odyssey® Infrared Imaging System (mean of three independent experiments ± SE).

was observed when co-transfection was performed using pPG with 'p16L1h and pSUPER Hi' (Fig. 3A, marked panel). This result indicates that HPV16 L1 exerted its effect via the late gene suppressor hnRNP H.

The Northern blot showed levels of cellular GFP mRNA (Fig. 3B) that were consistent with the flow cytometry results, and the Western blot assay with cell lystates (Fig. 3C) showed concordant results at the protein level (Table 1).

3.3. Demonstration of the interaction between hnRNP H and HPV16 L1 at the protein level

To demonstrate the interaction between hnRNP H and HPV16 L1 at the protein level, we employed pull-down experiments using Protein A beads loaded with an anti-L1 mAb, followed by peptide mapping, to identify the HPV16 L1-associated protein(s) in the cell lysates. Purified recombinant HPV16 L1 produced in *E. coli* was added into the cell lysate. Then, the specifically enriched protein mixtures, following pull-down by functionalized anti-L1 mAb beads, were separated by 2D gel electrophoresis and visualized by silver staining. Compared with the control, an extra protein spot with a similar molecular weight and isoelectric point to hnRNP H (theoretical MW 49.2 kDa and pI 5.89) was identified in the L1-spiked mixture (Fig. 4A). The spot was excised from the gel, subjected to in-gel tryptic digestion and analyzed by MALDI-TOF-MS, as shown in Fig. 4A and Supplementary Tables 3S and 4S. hnRNP H was identified as a L1-associating protein with high confidence. In addition, co-immunoprecipitation and immunofluorescence analyses were performed to verify the indicated specific protein–protein interaction (see below and Table 1).

Table 1
Demonstration of the interaction between hnRNP H and HPV16 L1, a late viral protein, which is down regulated during the early stage of the virus life cycle, using a series of analytical, biochemical, and immunochemical methods intracellularly or with cell lysates.

Method (corresponding results)	Objective	Target of analysis	Method capability (qualitative/ quantitative)	Level of interaction
Flow cytometry (Figs. 2 and 3A)	Detection of the level of transcription/ translation with respect to the hnRNP H activity	GFP reporter in cells	Quantitative	Cellular level/ protein function
Northern blotting (Fig. 3B)		Cellular reporter mRNAs	Semi-quantitative	
Western blotting(Fig. 3C)		Cellular reporter protein	Semi-quantitative	
	Conclusion: A specific interaction exists between HPV16 L1 and hnRNP H via co-expression			
Specific pull-down with protein A beads and peptide mapping (Fig. 4A, Tables 3S and 4S)	Confirmation of the interaction between hnRNP H and HPV 16 L1	Identification of proteins associated with HPV16 L1	Qualitative	Protein level
Co-immunoprecipitation (captured by nickel resin) (Fig. 4B)[a]		Complex of HPV16 L1 and hnRNP H	Qualitative	
Immunofluorescence (Fig. 4B)[a]		Co-localization of HPV16 L1 and hnRNP H in HeLa cells	Qualitative	Cellular level
	Conclusion: An interaction exists between hnRNP H and HPV16 L1 in cell lysates, as demonstrated by co-immunoprecipitation, and intracellularly using confocal fluorescence microscopy.			

[a] The hnRNP H used in these experiments was over-expressed in HeLa cells transfected with the plasmid pHis-H, whereas in other experiments, endogenous hnRNP H in the mammalian cells was used.

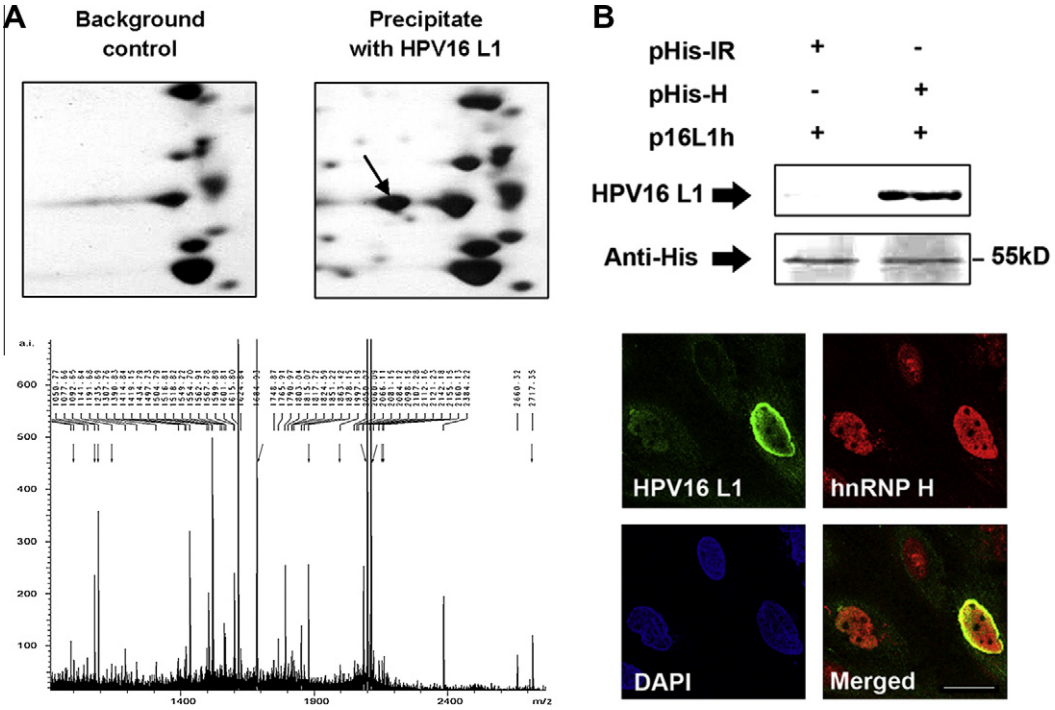

Fig. 4. (A) Confirmation of the intracellular interaction between hnRNP H and HPV16 L1. Upper panel: 2D gel electrophoresis map of proteins associated with HPV16 L1 isolated from HeLa cell lysates by co-immunoprecipitation. Proteins in HeLa cell sample lysates were co-immunoprecipitated with or without 'L1 and anti-L1 PB9' on protein A/G agarose beads. Lower panel: MALDI-TOF-MS tryptic peptide map of the extra protein spot was excised from the 2D gel (X: *m*/*z*, mass-to-charge ratio, Y: a.i., analysis intensity). (B) Upper panel: Co-immunoprecipitation of His-tagged hnRNP H and HPV16 L1. The upper panel displays HPV16 L1 captured by His-hnRNP H, but not by His-tagged CYP 2A6 (pHis-IR), when probed with an anti-HPV16 L1 mAb (PB11). His-tag fusion proteins (His-hnRNP H and His-tagged CYP 2A6) both presented MWs of approximately 55 kDa on SDS–PAGE after nickel column purification and probing with an anti-His pAb. Lower panel: immunofluorescence analysis indicated the co-localization of hnRNP H and HPV16 L1 in HeLa nuclei with specifically tagged hnRNP H and HPV16 L1. HeLa cells co-transfected with plasmids p16L1 h and pHis-H were stained with anti-HPV16 L1- and anti-His-specific pAbs and corresponding labeled secondary Abs and then examined by confocal microscopy. DAPI staining was used as a control. Scale bar: 10 μm.

3.4. Co-immunoprecipitation of co-expressed hnRNP H and HPV16 L1

To probe the specific intracellular interactions, HeLa cells were co-transfected with the plasmids p16L1h and pHis-H, with the putative binding partners HPV16 L1 and hnRNP H co-expressed in the same cells. The anti-His pAbs were used to detect His-hnRNP H or other His-containing proteins, such as His-CYP 2A6 (a His-tagged control with a similar MW), that were captured by the nickel column (Fig. 4B), and the L1-specific mAb PB11 (Fig. 4B, upper panel) was used to indicate whether HPV16 L1 had co-immunoprecipitated in the mixtures. Fig. 4B reveals equivalent anti-His signals among different lanes, confirming comparable protein loadings, but the L1-reacting band was observed only in lanes that contained His-hnRNP H. These data demonstrate that HPV16 L1 was specifically co-immunoprecipitated by His-tagged hnRNP H and further corroborate the notion that HPV16 L1 interacts specifically with hnRNP H intracellularly.

3.5. Intracellular co-localization of hnRNP H and HPV16 L1

The co-localization of His-tagged hnRNP H and HPV16 L1 was observed in the co-transfected HeLa cells. Confocal fluorescence

microscopy analysis indicated that the transfected His-tagged hnRNP H was diffused throughout the cell nucleus, whereas accumulated L1 protein appeared mainly on the nuclear membrane (Fig. 4B). The co-localization signal of the two proteins was observed in the cell nucleus, particularly near the nuclear membrane (Fig. 4B, merged image). These data demonstrate that these two proteins are co-localized intracellularly in proximity to the nuclear membrane. hnRNP H, an RNA-binding protein associated with gene regulation, needs a transport mechanism to cross the nuclear membrane and enter the nucleus. This specific association between HPV16 L1 and hnRNP H near the outer layer of the nuclear membrane may affect the regulatory capability of hnRNP H. This suppressive function was likely intended for the early stage of the viral life cycle, whereas in the late stage, late gene expression is essential for viral capsid assembly.

3.6. Implications of the positive feedback loop associated with capsid protein production

The multifaceted characterization of the direct association between hnRNP H and HPV16 L1 and the enhanced GFP expression was accomplished through the utilization of several methods for protein–protein and RNA–protein interaction analysis. The interaction between hnRNP H (an RNA-binding protein whose function is to suppress the read-through of late genes downstream of G3 elements) and HPV16 L1 (a late protein) implicates a positive feedback cycle that occurs once the L1 protein expression has been initiated during the late stage of the viral life cycle. This should promote late viral gene (L1 and L2) expression. This positive auto-regulation would favor rapid protein production, thus allowing efficient capsid assembly.

The RNA-binding proteins, including hnRNP H, were previously implicated in the binding to G3 and G6 motifs in the coding region of the L2 gene to suppress the gene expression down-stream from G-triplets [3]. The presence of the suppressors of late protein expression is crucial so that the virus can express the early proteins in the early phase upon cell attachment and initial un-coating, allowing the initiation of viral replication in the endoplasm. The HPV life cycle depends on epithelial differentiation and properly staged expression of certain viral genes. The expression of late viral capsid proteins, L1 and L2, is mainly observed in the most terminally differentiated keratinocytes in the upper layers of the epithelium [10]. Oberg et al. [3] demonstrated that hnRNP H plays a key role in the inhibition of transcription of the late viral genes of HPV.

A recent report by Somberg et al. [24] indicated that serine/arginine-rich protein 30c is the activator of the production of HPV16 L1 mRNA. Once translation of this mRNA is complete, with the L1 protein available in the cellular environment, the binding of L1 to hnRNP H renders hnRNP H incapable of binding to RNA or forming a complex with another factor, thereby impeding its function as the suppressor of late gene expression. Therefore, the efficient suppressive effect of hnRNP H and/or its associated complex, with hnRNP H being a crucial component, is weakened or depleted by the L1 protein. hnRNP H can enter the nucleus and bind to RNA, carrying out its function of gene regulation. The co-localization of hnRNP H and HPV16 L1, with the transcription/translation of HPV16 L1 previously suppressed by hnRNP H early in the viral life cycle, was demonstrated near the surface of the cell nucleus (as visualized in Fig. 4B). This work provides the first evidence of the potential self-up-regulation of a late viral protein via the weakening of the function of the late gene expression suppressor, hnRNP H, through a specific association.

4. Conclusion

Direct evidence of HPV16 L1: hnRNP H binding was obtained through a combination of biochemical, biophysical and immunochemical methods. The toolbox of methods that we used to probe interactions at different levels while studying a gene-regulating protein could be applied to other systems. Using HPV as a model system, the protein product of a gene that was regulated by a suppressor, hnRNP H, was shown to play a role in binding that suppressor and diminishing its function. This action, in turn, led to further enhanced expression of the late genes, which were suppressed in the early stage of the viral life cycle. From the evidence in current study is clear, we cannot extrapolate these findings to other viruses and draw a general conclusion regarding the autoregulation of a late viral protein by blocking the function of a suppressing factor, thus allowing more efficient read-through of the genes encoding late viral proteins. However, the definitive evidence obtained indicates direct association of a late protein (L1) with a suppressor that down regulated the expression of the same late protein (L1 in this case) earlier in the life cycle. Such an association enhances late capsid protein expression during the late stage of the HPV viral life cycle. Additional work is needed to ascertain whether this proposed positive feedback loop is present in biological systems, i.e., in HPV-infected human epithelial cells.

In summary, hnRNP H was shown to bind to a late capsid protein of HPV, and the data imply a positive feedback cycle for late protein expression. The tools presented here can be applied to study the function and modulation of RNA-binding proteins in gene regulation during the viral life cycle.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.042.

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