Biochemical and Biophysical Research Communications 457 (2015) 227–233

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Development of a functional cell-based assay that probes the specific interaction between influenza A virus NP and its packaging signal sequence RNA

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ARTICLE INFO

Article history:
Received 5 December 2014
Available online 2 January 2015

Keywords:
Influenza
Nucleoprotein
Packaging signal sequence
Cell-based protein–RNA interaction

ABSTRACT

Although *cis-acting* packaging signal RNA sequences for the influenza virus NP encoding vRNA have been identified recently though genetic studies, little is known about the interaction between NP and the vRNA packaging signals either *in vivo* or *in vitro*.
Here, we provide evidence that NP is able to interact specifically with the vRNA packaging sequence RNA within living cells and that the specific RNA binding activity of NP in vivo requires both the N-terminal and central region of the protein. This assay established would be a valuable tool for further detailed studies of the NP–packaging signal RNA interaction in living cells.

1. Introduction

The nucleoprotein (NP) of influenza A virus, a member of the *Orthomyxoviridae* family, is a single-strand RNA-binding protein comprised of 498 amino acids that serves multiple functions necessary for transcription and replication of viral RNA (vRNA) [1,2]. NP is the major protein in the viral ribonucleoprotein complexes (vRNPs) assembled on the viral RNA genomes in conjunction with the heterotrimeric viral polymerase subunit proteins PA, PB1, and PB2 within the virion [3,4].

The NP is encoded by the influenza vRNA segment number 5 that must be packaged specifically along with seven other influenza virus RNA genome segments into the virion [5,6]. Through a number of elaborate reverse genetic assays, it has been proposed recently that the vRNA encoding NP includes two specific regions that harbor so-called a packaging signal sequence required for efficient packaging of the RNA segment into the virion. These regions have been mapped to 60 nucleotides and 120 nucleotides from the 3′ and 5′ end of the open reading frame of the NP-encoding viral RNA, respectively [7–9]. However, the exact molecular mechanism(s) of specific vRNA packaging and whether the packaging signal RNA of NP vRNA could be recognized and interact with its cognate NP protein remain to be determined.

With regard to the RNA binding characteristics of NP, it has been shown *in vitro* that purified recombinant NP proteins from various sources (insect cells or *Escherichia coli*) could bind to single-stranded RNA and synthetic homopolymeric RNAs [10–13]. The RNA binding region of NP was reported initially to map to the region between amino acid residues 91 and 188 [11], which contains a stretch of residues highly conserved among NPs from A-, B-, and C-type influenza viruses, but it was extended further subsequently to include the entire one-third N-terminal portion (180 amino acids) of the protein as an RNA-binding motif [12]. However, an *in vitro* UV cross-linking study later reported that the contact points between NP and the vRNA could occur throughout the length of the polypeptide rather than at a particular discrete domain [13].

Recent characterization of the 3.2-Å resolution X-ray crystallographic structure of the NP from H1N1 influenza virus A has revealed that the protein is composed of a head domain, a body domain, and a flexible tail loop responsible for the protein oligomerization [14]. It has been also proposed that a possible RNA-binding groove could exist between the head and body domains at the exterior of the nucleoprotein oligomer, which is lined with a large number of highly conserved basic residues distributed widely along the primary sequence [14], supporting the notion that the NP–RNA interaction could be mediated by more than a discrete N-terminal domain of the NP. However, a similar 3.3-Å resolution crystallographic characterization and SPR analysis with an H5N1 NP suggested that a flexible and basic loop structure

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http://dx.doi.org/10.1016/j.bbrc.2014.12.092
0006-291X/

(residues 73–91) located in an arginine-rich groove surrounding Arg150, but mostly a protruding element containing Arg174 and Arg175, is important for RNA binding by NP [15]. Therefore, it appears that the RNA binding characteristics of the NP and the regions involved in RNA binding still remain to be understood further.

Thus far, studies of characterizing NP–RNA binding have been relied on systems using either unnatural synthetic RNA or non-NP vRNA substrates *in vitro* [10–13,15]. Moreover, Determination of the specific ability of NP to interact with the packaging signal in living cells is particularly important as it would address whether the NP protein alone could recognize and interact specifically with the packaging RNA element amongst a pool of diverse cellular RNAs *in vivo*. Thus, the development of a functional cell-based assay that can probe the specific interaction between NP and the packaging signal sequence RNA *in vivo* is urgently needed.

2. Materials and methods

2.1. Plasmid construction

2.1.1. Construction of reporter plasmids

The LacZ reporter plasmids used in this study and the primers used to generate them are listed in Fig. 1 and Supplementary Table 1, respectively. The packaging signal encoding DNA sequences of both the 3′ and 5′ end of the NP gene segment of influenza A/WSN/33 virus (Genbank accession number CY034135) were chemically synthesized with *SnaB* I and *Avr* II restriction enzyme recognition sequences at the 5′ and 3′ ends, respectively (Bioneer, Daejeon, Republic of Korea). After digestion with the respective restriction enzymes, the fragments were inserted into the corresponding sites of pMV1 plasmid described previously [16], resulting in pMV1-F and pMV1-R. Reporter plasmids, pMV1-F1, pMV1-F2, pMV1-F3, pMV1-F4 and pMV1-F5 were constructed by inserting each of two complementary oligonucleotides synthesized and annealed into pMV1 using the same *SnaB* I and *Avr* II recognition sites. pMV1-R1, pMV1-R2, pMV1-R3, pMV1-R4 and pMV1-R5 were constructed by cloning each insert DNA amplified by PCR using the respective primer sets listed in Supplementary Table 1 and inserted into pMV1-R.

2.1.2. Construction of NP expression plasmids

The gene encoding the NP of influenza virus strain A/WSN/33 with codon-optimization for *E. coli* was synthesized chemically (GenScript, Piscataway, NJ, USA) and inserted into the *Bgl* II and the *Pst* I site of pJC1 as described previously [16], resulting in pJC1-NP plasmid. The NP deletion constructs, pJC1-D1, pJC1-D2, pJC1-D3, pJC1-D4 and pJC1-D5, were generated by inserting PCR amplified DNAs using the primers shown in Supplementary Table 1 and pJC1-NP as a template. All plasmids used in this study are available upon request.

2.2. Surface Plasmon Resonance (SPR) assay

The RNAs used in this analysis were chemically synthesized (ST Pharm Co., Ltd., Seoul, Republic of Korea) as follows: NP-F1 (30-nucleotide) 5′-GCG ACC AAA GGC ACC AAA CGA UCU UAC GAA-3′, and NP-F3 (30-nucleotide) 5′-GAA CAG AUG GAG ACU GAU GGA GAA CGC CAG-3′, NP-R3 (60-nucleotide) 5′-GAA AAG GCA ACG AGC CCG AUC GUG CCC UCC UUU GAC AUG AGU AAU GAA GGA UCU UAU UUC-3′ and Poly U (30-nucleotide) RNA was purchased from Bioneer Inc., (Daejeon, Republic of Korea).

The CM5 sensor chip for Biacore T100 (GE Healthcare, Buckinghamshire, UK) was first immobilized with the recombinant influenza A virus H1N1 NP (Sino Biological Inc., Beijing, China) by EDC/NHS amine coupling in 10 mM sodium acetate buffer (pH 4.5). The subject single strand RNAs (F1, F3, R3) for SPR assay were diluted with HBS-EP buffer in RNase free water (10 mM HEPES, pH 7.4, 3 mM EDTA, 150 mM NaCl, 0.05% surfactant p20) serially and injected at 10 μl/min for 90 s. HBS-EP buffer was used as the running buffer and 10 mM NaOH was used to regenerate the NP immobilized CM5 chip. Binding kinetics between NP and each of

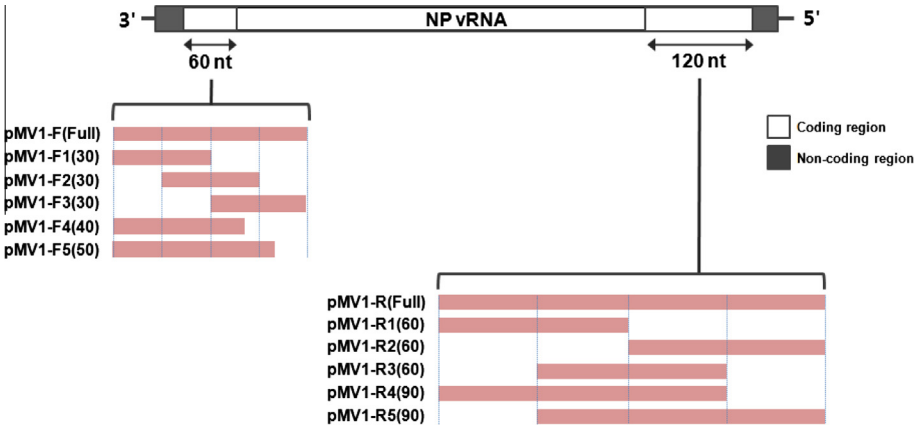

Fig. 1. Schematic representation of the location of the packaging signal sequence regions present in 5′ and 3′ end of influenza A virus NP vRNA genome segment. Shown also are both full length 60 nucleotides of 3′ end (designated as F) and 120 nucleotides of 5′ end (designated as R) of the packaging signal sequences, as well as their respective deletion sequences used to construct various *LacZ* reporter gene plasmids. Numbers in parenthesis indicate nucleotide lengths of the sequences designated.

Table 1
Binding kinetic and affinity constants of NP–RNA interactions.

RNA	Mean K_a (1/ms) ± SE	Mean K_d (1/s) ± SE	Mean k_D (nM) ± SE	R_{max} (RU) ± SE
F1	$1.31 \times 10^6 \pm 0.01 \times 10^6$	$1.97 \times 10^{-3} \pm 0.01 \times 10^{-3}$	1.59 ± 0.11	49.70 ± 0.05
F3	$4.17 \times 10^5 \pm 0.02 \times 10^5$	$3.33 \times 10^{-3} \pm 0.02 \times 10^{-3}$	8.49 ± 0.59	26.27 ± 0.06
R3	$4.82 \times 10^5 \pm 0.05 \times 10^5$	$1.95 \times 10^{-3} \pm 0.03 \times 10^{-3}$	4.04 ± 0.10	46.53 ± 0.18
Poly (U)	$1.35 \times 10^5 \pm 0.09 \times 10^5$	$4.01 \times 10^{-3} \pm 0.03 \times 10^{-3}$	29.72 ± 2.01	9.20 ± 0.03

the RNAs were analyzed using the Method Builder tool and Control & Evaluation software (BIA evaluation software) for the Biacore T100 system (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions.

2.3. Others

Details of other assays used are also provided in the Supplementary Methods.

3. Results

3.1. In vivo interaction between the NP vRNA packaging signals and NP protein

In order to examine the specific ability of the NP protein to interact with NP vRNA packaging signal sequence RNA *in vivo*, we exploited the cell-based translation inhibition assay described previously that could probe a specific interaction of an RNA-binding protein to its interacting sequence [16–18]. In this assay, if an RNA binding protein expressed in the cell recognizes and binds to its specific target RNA sequence(s) placed upstream of an engineered *LacZ* reporter gene, it inhibits translation of the reporter that harbors the target RNA. Thus, the degree of translation inhibition of the *LacZ* reporter vector is dependent on and directly proportional to the strength of a specific interaction *in vivo*.

To characterize the NP specific interaction, we first prepared *LacZ* reporter vectors, pMV1-F and -R as schematically shown in Fig. 1, in which a *lacZ* ORF is fused immediately to either a full length 3′ or 5′ end packaging signal sequence from NP vRNA and placed downstream of the *trc* promoter and operator. In addition, a control pMV1 vector lacking the packaging sequences but instead harboring a nonspecific vector-origin sequence (about 100 nucleotides of multi-cloning site) upstream of *lacZ* ORF was also constructed. For analysis, four different sets of double-transformants were prepared as follows: transformants having both a *LacZ* reporter plasmid harboring the full length packaging sequence of 3′ end NP vRNA (denoted as F) placed immediately upstream of the *LacZ* gene (pMV1-F) as shown in Fig. 1, and either the NP protein expression vector (pJC1-NP) or a control vector without the NP expression (pJC1(−)). As for specificity controls, transformants having both a *LacZ* gene reporter plasmid without the packaging RNA sequence (pMV1) and either the NP protein expression vector (pJC1-NP) or a control vector (pJC1(−)) were also prepared. Likewise, the same set of transformants was constructed for reporter vectors with and without the 5′ end full length packaging sequence of NP vRNA (denoted as R) and tested for *LacZ* (β-galactosidase) activity.

As shown in Fig. 2A, the transformants not expressing both NP protein and packaging RNA sequence (pJC1(−) + pMV1: circle) or only expressing NP but no packaging RNA sequence (pJC1-NP + pMV1: diamond) showed a nearly equivalent level of *LacZ* production (or lack of *LacZ* translation inhibition), indicating that NP over-expression itself does not affect either translation of β-gal from pMV1 control vector or any cell growth (by causing cellular toxicity) under the conditions examined. However, when the cells express both NP and the 3′ end RNA packaging sequence (pJC1-NP + pMV1-F: square) together, β-galactosidase activity was reduced by 68% compared to that of the transformant without the NP protein (pJC1(−) + pMV1-F: triangle). A little reduction in the level of β-galactosidase activity obtained with the transformant that expressed only the packaging RNA sequence without NP (pJC1(−) + pMV1-F) indicates the degree of translation efficiency of the reporter mRNA having the packaging RNA sequence. The western blot result shown on a right-hand side confirmed the nice correlation between β-galactosidase activity measured by the ONPG assay and the amount of β-galactosidase protein expressed in the transformants of the cell-based assay (compare lanes 2 and 3). Thus, the result that the inhibition *of* β-galactosidase activity is observed only in the presence of both NP and its 3′ packaging sequence RNA indicates nicely the specificity of the NP interaction with the packaging signal *in vivo*, as demonstrated with other types of specific RNA binding proteins previously [16–18].

To examine further the specific interaction between the NP and 3′ end packaging RNA sequence, we investigated the specific and major region(s) in the 3′ end packaging RNA sequence (F) that is critical for the NP interaction. Thus, we generated a number of reporter constructs harboring various deletions of the 3′ end packaging RNA sequence that were named pMV1-F1(30), -F2(30), -F3(30), -F4(40), and -F5(50) as shown in Fig. 1. Each reporter construct showed varying degrees of β-galactosidase activity with the NP protein. While pMV1-F1(30), the first half only of the 3′ end full length RNA, showed the greatest inhibition (62%) of β-gal activity with NP as nearly as observed with the 3′ end full length RNA (Fig. 2B), pMV1-F2(30) and pMV1-F3(30) that harbors the middle and the distal half of the 3′ end packaging RNA sequence showed approximately 26% and 31% inhibition of β-gal activity, respectively (Fig. 3C and D), suggesting that the F1 region (the proximal half) is more important than the other regions. This finding was confirmed when we examined further pMV1-F4(40) and pMV1-F5(50) reporters, which all have an extension of 10 or 20 more nucleotides including the F1(30) sequence, showed up to 54% and 55% translation inhibition, respectively (Fig. 2E and F). Therefore, these results indicate that the F1 region is the major determinant sequence region for the specific interaction between NP and the 3′ end packaging signal RNA sequence of NP vRNA.

Next, we also examined the effect of NP on the 5′ end full length RNA packaging sequence (R) and found a result similar but the degree of inhibition was much greater than the 3′ full packaging sequence. As shown in Fig. 3A, inhibition of *LacZ* expression was 89% in the presence of NP and R sequence RNA, suggesting thus that NP could interact more strongly with the 5′ full length packaging RNA sequence. This result was also confirmed by western blot analysis (Fig. 3A). To investigate the specific region(s) important for the interaction that reside in the 5′ end packaging RNA sequence of the NP vRNA, we also constructed and tested pMV1-R1(60), -R2(60), -R3(60), -R4(90), and -R5(90) reporter plasmids that harbor various regions of the R packaging RNA sequence element as shown in Fig. 1. Among the constructs, pMV1-R3(60), which expresses only the middle half of the 5′ end packaging RNA sequence showed the strongest inhibition (61%) and pMV1-R2(60) yielded only 46% inhibition, respectively (Fig. 3C and D). In the case of pMV1-R1(60), the first half of the 5′ end packaging RNA sequence, we were unable to positively identify the interaction to NP because the expression of *LacZ* by the reporter vector itself was too low (Fig. 3B). However, further examination of the reporter vectors R4 (which has the sequence of R1 + R3 region) and R5 (which has the sequence of R2 + R3 region) which are longer than R3 showed 45% and 50% inhibition of β-gal activity, respectively (Fig. 3E and F). No further increase in the inhibition of β-gal activity resulted with these constructs suggests thus that the R3 region is likely the major site for the NP interaction in the 5′ end packaging sequence of NP vRNA and other regions would contribute in minor.

To further verify the specificity of NP interaction to its packaging signal sequences, we also tested the effect of NP on other unrelated viral packaging sequence having a similar length, the HIV-1 Psi packaging signal sequence (130 bases long), placed upstream of *lacZ* as described previously [16]. The result showed that, unlike observed with R(120) RNA sequence, NP affected hardly inhibition of *LacZ* production with the HIV-1 Psi signal sequence as found similarly with pMV1 control vector (Fig. 3G).

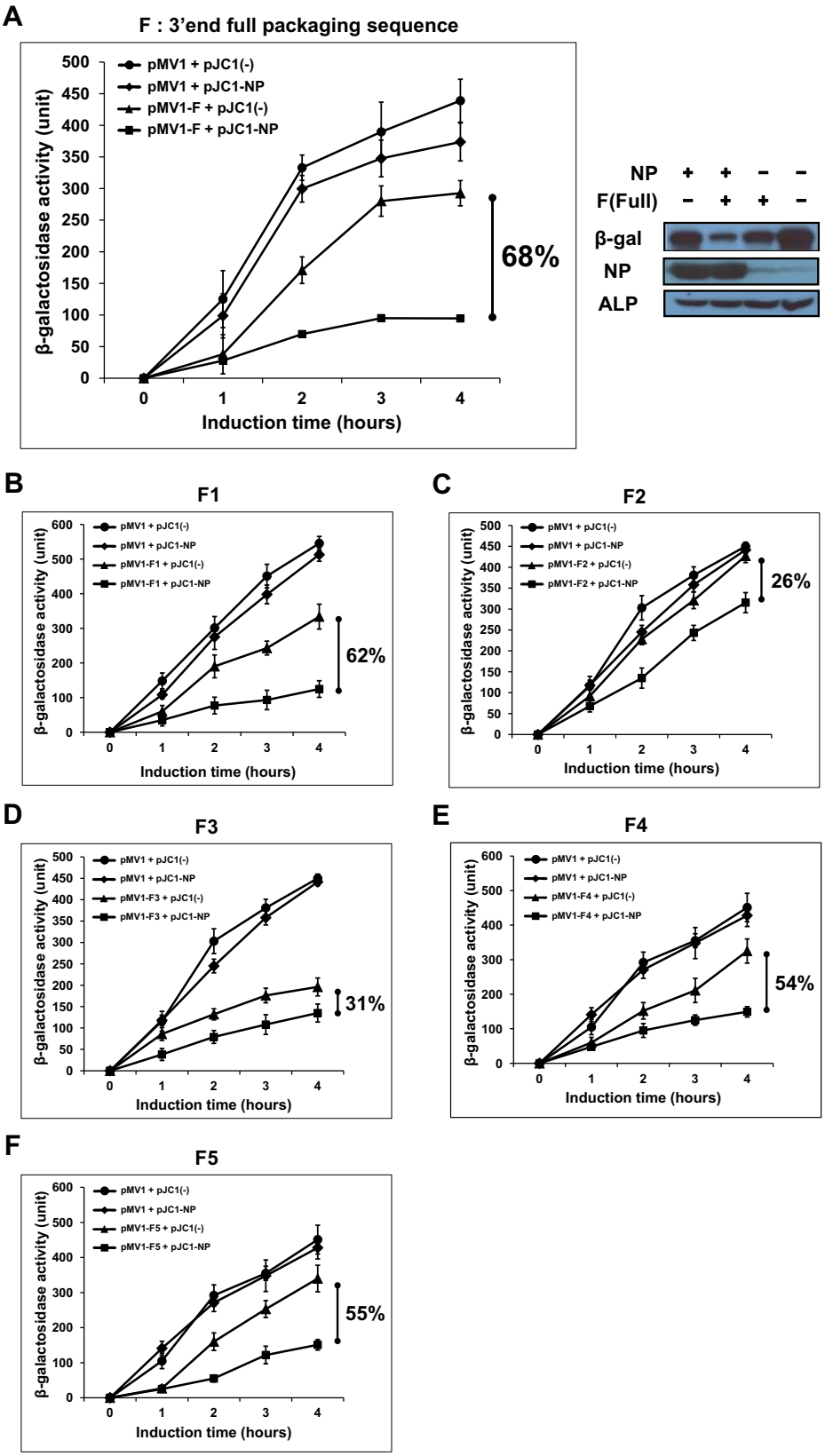

Fig. 2. Determination of *LacZ* inhibition by specific interaction between the NP protein and full length or different regions of F packaging sequences of influenza NP vRNA. (A) Effect of NP on full length F packaging sequence-*lacZ* reporter gene expression, and (B–F) various regions of the F packaging RNA sequence. The regions of F packaging RNA in the reported vector pMV1-F1 (B), pMV1-F2(C), pMV1-F3 (D), pMV1-F4 (E), and pMV1-F5 (F) are as described in Fig. 1. Shown are β-galactosidase activities of individual reporter as well as control vectors examined in the presence (square) and absence (triangle) of NP, respectively. pMV1 and pJC1(−) are control plasmids that lack expression of either the packaging signal sequences or NP protein. Shown on the right in (A) is the western blot result of the expression of *LacZ* (β-galactosidase) at 4 h after IPTG induction. Alkaline phosphatase (ALP) indicated was probed as an internal control for the amount of cell lysates loaded in the analysis. All experiments were performed at least three times. Data are presented as the mean ± SD (standard deviation) of three separate experiments.

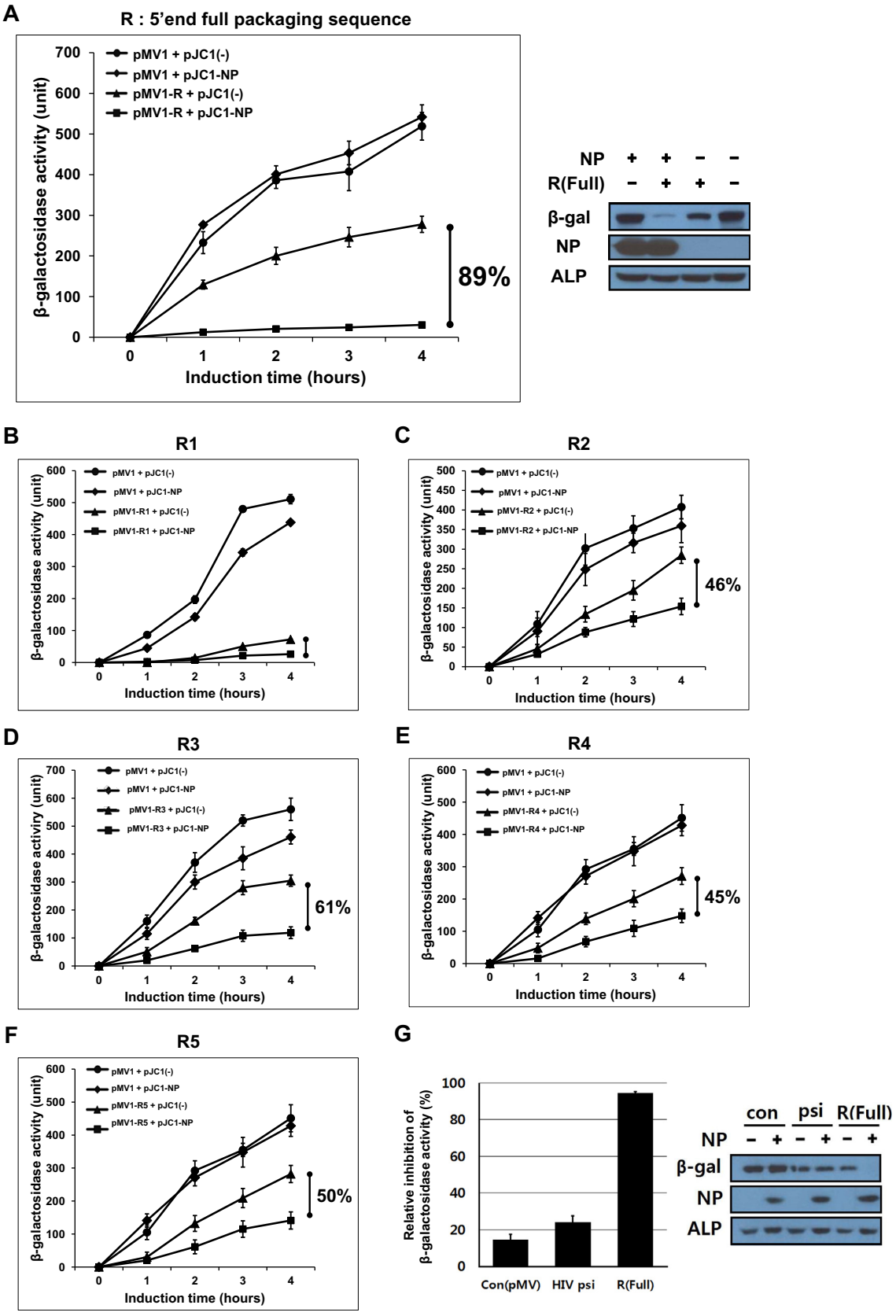

Fig. 3. Determination of the specific interaction between the NP protein and either full length or different regions of R packaging sequences of NP vRNA. (A) Effect of NP on full length R packaging sequence-*lacZ* reporter activity, and (B–F) the interaction of NP with various regions of the R sequence. The regions of R packaging RNA sequence in the reported vectors pMV1-R1 (B), pMV1-R2 (C), pMV1-R3 (D), pMV1-R4 (E), and pMV1-R5 (F) are as described in Fig. 1. Translation inhibition assay was performed as described in the legend of Fig. 2 and in the Materials and Methods. (G) Comparison of interaction specificity of NP to NP vRNA full length R and HIV Psi packaging sequence. Percent inhibition, an indicative of interaction specificity of NP protein to each packaging sequence, was calculated by reduction in *β*-galactosidase activity over that of the same reporter vector without NP. The results are the averages of three independent assays.

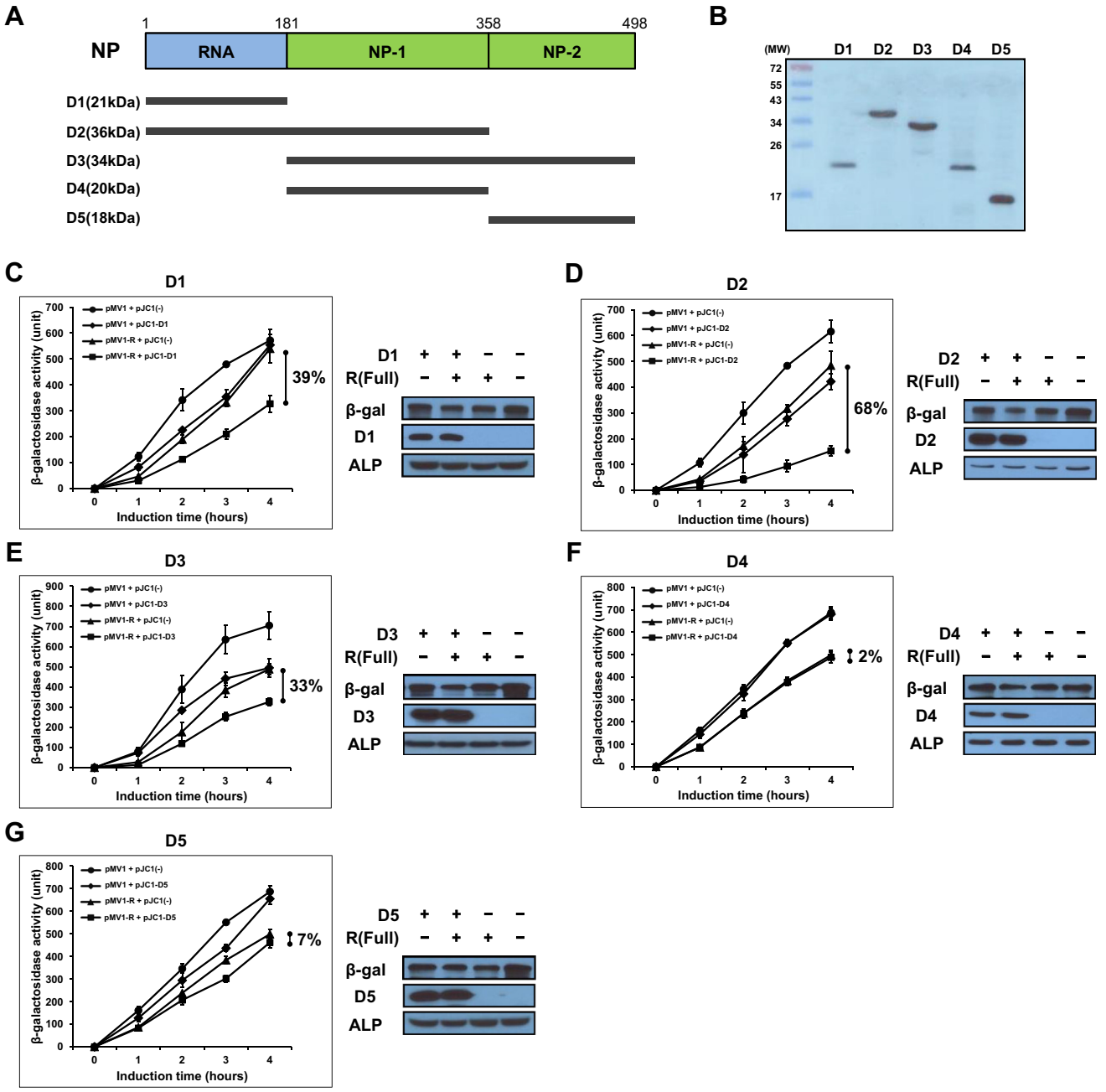

Fig. 4. The effect of various NP deletion mutants on the specific interaction with the R packaging RNA. (A) Schematic diagram of various NP deletion constructs made and (B) western blot analysis of the expression of mutant constructs in *E. coli*. The molecular weights of each deletion construct are indicated in parentheses. Lane 1: protein standard marker and lanes 2–5: D1, D2, D3, D4, and D5 protein bands detected. (C–G) Translation inhibition caused by interaction of the various truncated forms of NP with the R packaging signal sequence. Western blot results for β-galactosidase activity are shown on the right. Data are presented as the mean ± SD (standard deviation) of three independent experiments.

Taken together, these results indicate that (1) NP protein by itself is able to recognize specifically each of the packaging signal RNA sequences of NP vRNA within living cells; and (2) the ability to measure this interaction strength quantitatively provides a nice and powerful cell-based assay to probe further for the specific interaction *in vivo*.

3.2. Determination of NP binding efficiency to the packaging signal RNAs in vitro

To evaluate and verify further the observed *in vivo* specificity and binding efficiency of NP protein to the packaging signal RNA sequence, we examined *in vitro* NP binding to packaging sequence RNAs (F1, F3, and R3) using a SPR assay. The results demonstrated that NP binds to all of the packaging signal RNA sequences in a linear NP dose-dependent binding pattern with a much stronger binding than a nonspecific control RNA, poly (U) (Supplementary Fig. 1). When the result of the binding kinetics were analyzed using a 1:1 Langmuir binding model as summarized in Table 1, the K_D (K_d/K_a) values of all of the packaging RNAs were calculated to be in the range of 10^{-8} M and NP binding to F1 or R3 being a little higher than F3 RNA, but overall exhibited stronger affinity of NP binding to these RNAs over a non-specific control RNA. Thus, these *in vitro* results confirm further that NP interacts with the packaging signal sequence RNA with a very high affinity as observed in the cell-based assay.

3.3. Characterization of the specific RNA-binding determinant region(s) of NP protein

Next, we examined domain(s) of NP for the specific RNA binding, if any. To this end, a series of NP deletion constructs was prepared as shown in Fig. 4A: **D1**-a previously designated RNA binding domain only; **D2**-the RNA binding domain plus the first NP interaction domain; **D3**-only the first and second NP interaction domains; **D4**-the first NP protein interaction domain only; and **D5**-the second NP protein interaction domain only. Protein expression of each of the individual constructs was confirmed by SDS–PAGE and western blot analysis (Fig. 4B). The NP deletion constructs were then co-transformed into JM109 cells with the pMV1-R reporter plasmid (which harbors the packaging signal RNA sequence that showed the strongest interaction with NP) and examined for translation inhibition. As shown in Fig. 4, we observed the following results: D2 showed the highest level of inhibition (68%) followed by D1 (39%) and D3 (33%), both with a moderate level of inhibition. The D4 and D5 domains showed minimal or a lack of binding activity, as scoring 2% and 7% inhibition, respectively (Fig. 4C–G). A similar result was also obtained with these constructs for interaction to 3′ end packaging signal RNA (data not shown). Thus, the result indicates that while individual regions of D4 and D5 had no activity, D3 region, composed of only the first and second NP protein oligomerization interaction domains without the N-terminal region, had an activity level equivalent to D1, suggesting that the D1 region alone is not sufficient for the NP interaction with the packaging signal RNA. Rather D2, which harbors the N-terminal but also a central region of NP, constitutes together the most important region for the specific ability of NP to bind to the packaging signal RNA sequence in the cells.

4. Discussion

In this study, we found that NP interacts specifically with the packaging signal sequences located at the 5′ end of the vRNA with a greater affinity than the 3′ end, demonstrating for the first time the specific interaction between NP and the NP packaging signal sequences *in vivo*. Deletion analysis of individual NP packaging signal sequences at both ends revealed that a 30-base F1 region in the N-terminus or a 60-base R3 region in the C-terminus of the NP vRNA are the major determinants for the NP interaction.

We also investigated whether the NP has any specific RNA binding region(s) within the protein *in vivo*. Employing various NP deletion mutants as shown in Fig. 4, we found that each individual N-terminal (residues of 1-181), middle (central, residues of 182–358) and C-terminal portion (region, residues of 359–498) of NP had low or no significant binding activity to the 5′-end NP vRNA packaging signal sequence RNA. However, when the N-terminal and middle regions were combined as in the case of the D2 (residues 1–358) construct, the result showed nearly the same level of activity as the full-length wild type NP. The result suggests that RNA binding by NP is not limited to only the N-terminal region of the protein and requires at least the central portion of the protein for the interaction to the packaging RNA sequence *in vivo*.

Previously, mutational analysis of the influenza A virus NP showed that mutations in the N-terminal as well as amino acids in the central region such as residues R195, R199, S314, A332 K325, and R361 resulted in impaired vRNA packaging and RNA-binding activity [2,19], which are proposed to cluster around a possible RNA-binding groove that lies between the head and body domains at the exterior of the NP oligomer [14]. Moreover, it also has been determined recently that a loop spanning residues 200–214 of the H1N1 NP forms an important groove for RNA binding *in vitro* [20]. Thus, our results that RNA binding activity is beyond the N-terminal region of the protein appear to agree with these genetic and *in vitro* RNA binding assay results and reveal the importance of the central region of NP (known as NP interaction domain) for the recognition and interaction with NP vRNA packaging signal RNA *in vivo*.

Acknowledgments

This work was supported by National Research Foundation grants (No. NRF-2013068353 and 20084522004183) funded by the Korean Ministry of Education, Science and Technology – South Korea.

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.2014.12.092.

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