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Impairment of the Staufen1-NS1 interaction reduces influenza viral replication

Jun-Han Lee [a,1], Jun-Young Oh [a,1], Philippe Noriel Q. Pascua [a], Eung-Gook Kim [b], Young-Ki Choi [a], Hyong Kyu Kim [a,*]

[a] Department of Medicine and Microbiology, College of Medicine, Signaling Disorder Research Center, Chungbuk National University, Cheongju 361-763, Republic of Korea
[b] Department of Medicine and Biochemistry, College of Medicine, Signaling Disorder Research Center, Chungbuk National University, Cheongju 361-763, Republic of Korea

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ABSTRACT

Staufen1 (Stau1), a host cellular protein, along with non-structural protein 1 (NS1), an influenza viral protein, associate with each other during influenza viral infection and down-regulation of Stau1 by RNA interference reduces the yield of influenza A virus, suggesting a role for Stau1 in viral replication. In order to develop a new tool to control influenza A virus, we determined the specific regions of Staufen1 protein involved in the interaction with NS1. The linker between RBD3 and 4 was isolated as the binding regions. Expression of RBD3L, the linker including RBD3, inhibited the interaction between Stau1 and NS1, reducing the colocalization of the two proteins in the cytosol and nucleus regions. In addition, yield of influenza A virus in RBD3L-expressing cells was significantly reduced 36 h after infection. These results suggest that disruption of the Stau1-NS1 interaction can be used to control replication of influenza A virus, thereby providing a target for the development of antiviral drugs.

1. Introduction

Influenza A virus is a major pathogen that threatens human health through perennial epidemics or occasional pandemic outbreaks. Vaccination is the primary measure to control influenza virus infections in humans. However, in the event of pandemics, the development and production of vaccines that match the currently circulating strain require time (at least 3 months). Therefore, for individuals who have not been vaccinated, or when a vaccine is not available, antiviral agents can provide an important alternative as the first line of defense against newly emerging viruses.

The viral nucleoprotein (NP) and non-structural protein 1 (NS1) have recently been demonstrated as drug targets. Nucleozin, a small-molecule compound, was shown to trigger aggregation of NP by inhibiting nuclear accumulation [1]. On the other hand, the multifunctional role of NS1 from the early to late stages of viral infection in host cells has been determined for the design and discovery of antiviral compounds. Some of these compounds are designed to inhibit the interaction between NS1 protein and dsRNA [2], restore innate immune function [3], and block multi-cycle replication in an RNase L-dependent manner [4]. It has also been shown that blocking the CPSF30 binding site in NS1 is a potential antiviral target [5].

Although several drugs targeting viral proteins have been or are currently being developed, they are not effective for the frequent mutation of viral genomes or acquisition of resistance to the drugs [6–8]. In an effort to overcome these drawbacks, more conserved and critical mechanisms involved in the host response to influenza A virus infection should be elucidated. Several studies have isolated host factors required in viral replication by examining the interactions between viral proteins and host proteins [9], or genome wide RNA interference screenings [10,11].

Staufen, a double-stranded RNA-binding protein first isolated in *Drosophila*, is a critical protein regulating the anterior to posterior distribution of some mRNAs, such as *oskar* or *bicoid*, in oocyte development [12]. Two Staufen homologs, Staufen1 (Stau1) [13,14] and Staufen2 (Stau2) [15], have been isolated in mammals. In contrast to Stau1, which is expressed in almost all tissues [13], Stau2 is a brain-specific protein [15]. Stau1 has two isoforms, a 63 kDa ($Stau1^{63}$) variant that is 81 amino acid residues longer at its N-terminal region compared to the 55 kDa ($Stau1^{55}$) variant [13]. In initial studies, the function of both Staufens was shown to be transport of a subset of RNAs to specific regions, such as neuronal dendrites, by forming a ribonucleoprotein (RNP) complexes or RNA-transporting granules [15–17]. Besides RNA-transporting granules, Stau1 has also been found in a variety of RNA granules, stress granules, or processing bodies in cells [18]. In addition, Stau1 plays roles in non-sense mediated mRNA decay [19] and translational regulation [20,21]. Although Stau1 is mainly localized to the rough endoplasmic reticulum (RER) [13], a recent study

* Corresponding author. Address: Department of Medicine and Microbiology, College of Medicine, Signaling Disorder Research Center, Chungbuk National University, Naesoodong-ro 52, Heungduk-gu, Cheongju 361-763, Republic of Korea. Fax: +82 43 272 1603.
E-mail address: hkkim69@chungbuk.ac.kr (H.K. Kim).
[1] These authors equally contributed to this work.

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demonstrated its localization in the nucleus/nucleolus, suggesting nucleocytoplasmic shuttling or other functions [22].

In a series of studies, human Stau1 was identified as a binding partner of NS1 [23], and specific down-regulation of Stau1 via RNA interference was shown to reduce viral production, suggesting a relevant role for Stau1 in the influenza A virus replication [24]. Until now, little is known about the specific domain in Stau1 responsible for the interaction with NS1. Therefore, in this study, we tried to elucidate the specific region responsible for the interaction between Stau1 and NS1. We demonstrated that the 36-amino acid linker between RBD3 and RBD4 was required for binding. Expression of the linker containing RBD3 significantly reduced the colocalization of Stau1 with NS1 in the nucleus and cytosol of transfected cells, as well as the interaction between Staufen1 and NS1. In addition, introduction of the linker containing RBD3 significantly reduced viral replication. These results suggest that perturbation of the interaction could be a key tool in controlling replication of influenza A virus, thereby providing a target for development of antiviral drugs.

2. Material and methods

2.1. Cell cultures, viruses and transfection

Human embryonic kidney (HEK) 293T and human lung carcinoma (A549) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO®, Grand Island, NY) supplemented with 7–10% fetal bovine serum (GIBCO®) and supplied with 5% CO_2, at 37 °C. Madin-Darby canine kidney (MDCK) cells were grown in minimum essential medium (MEM) with Eagle salts containing 5% fetal bovine serum (FBS). The influenza A viruses A/Puerto Rico/8/1934 (H1N1) and A/WSN/1933 (H1N1) were propagated in 11-day-old embryonated chicken eggs at 37 °C. Cells were transfected with the respective plasmid constructs via calcium phosphate method or using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and incubated for 12–48 h to induce expression.

2.2. Plasmid construction

For the construction of plasmids, expressing the full-length and truncated forms of Stau1 (Fig. 1), full-length Stau1 cDNAs (Stau1^{55} and Stau1^{63}) were amplified by PCR, and then inserted into HindIII/XhoI (NEB, Ipswich, MA) sites of pCMV-Tag2B vector (Stratagene, La Jolla, CA), producing various Stau1 expression vectors for FLAG-tagged Staufen1 or mutants under regulation of the CMV promoter (see supplemental table 1 for primer sequences). For generation of Myc-tagged NS1 expression plasmid (Myc-PR8-NS1), the NS1 gene from A/Puerto Rico/8/1934 (H1N1) was amplified from cDNA templates that were synthesized from viral RNA extracts as described [25]. Amplicons were digested with EcoRI/KpnI (NEB), and then inserted into pcDNA3.1-Myc-His plasmid vector (Invitrogen).

The GFP-tagged Stau1^{63} expression plasmid was constructed by insertion of the amplified full-length human Stau1^{63} into the XhoI/KpnI (NEB) enzyme sites of pEGFP-C1 (Clontech Laboratories, Mountain View, CA) expression vector.

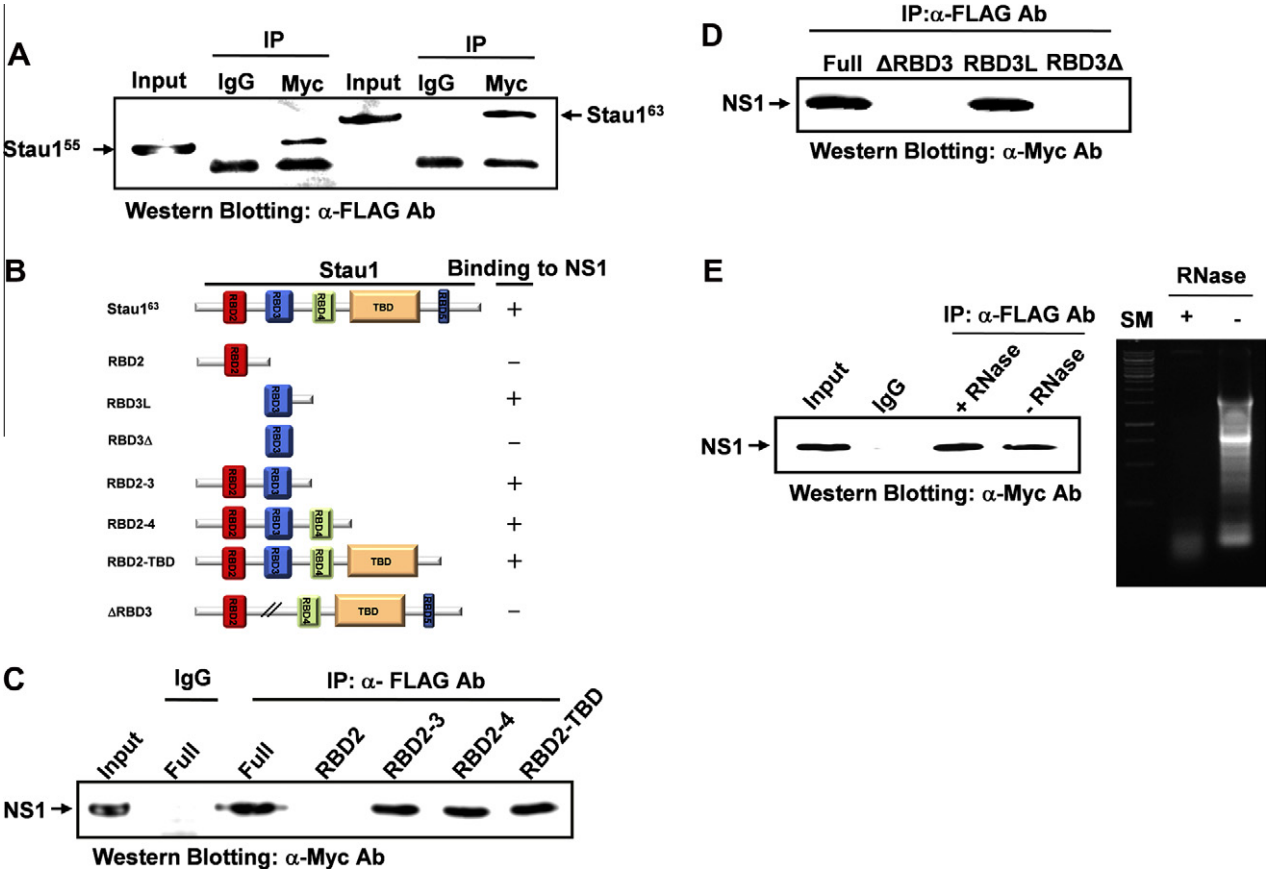

Fig. 1. Stau1 interacts with NS1 through the linker region between RBD3 and 4. (A) Two isoforms of Stau1 interact with NS1. Myc-tagged NS1 and FLAG-tagged Stau1^{63} or Stau1^{55} were co-expressed in cultured 293T cells, and cell lysates were used for immunoprecipitation using anti-Myc antibody. Coprecipitated protein was visualized by Western blotting using anti-FLAG antibody. (B) Schematic representation of Stau1 mutant proteins and association results with NS1 (+: interaction detected; −: interaction: not detected). RBD represents RNA-binding domain and TBD, tubulin-binding domain. (C) RBD2 and its subsequent linker region are not necessary for the Stau1-NS1 interaction. (D) Interaction of Stau1 with NS1 is mediated by the linker region between RBD3 and 4. (E) Interaction of Stau1 with NS1 is independent of the RNA binding of both proteins. Cell lysates were pretreated with 50 μg/ml of RNase A before immunoprecipitation. The right panel shows the efficiency of RNase A treatment to the lysates.

2.3. Immunoprecipitaion and RNase treatment

For coimmunoprecipitation (co-IP) analysis, 293T cell lysates were prepared by adding lysis buffer (150 mM NaCl, 1% IGEPAL® CA-630, 50 mM Tris–Cl [pH 8.0]), followed by immunoprecipitation with 2–3 μg of each antibody, as indicated in the figures, and incubation with 50 μl of protein A-Sepharose (Amersham Biosciences, Tokyo, Japan). The immunoprecipitates were washed three times with 1 ml of ice-cold lysis buffer and once with 1 ml of 50 mM Tris–Cl (pH 8.0), followed by 8% SDS–PAGE analysis. For Western blotting analysis, the blots were incubated with monoclonal anti-Myc antibody (1:2000) or anti-FLAG antibody (1:2000). As a control, the blot was washed with stripping buffer (Thermo Scientific, Rockford, IL) and re-probed. For RNase digestion, the cell lysates were incubated with 50 μg/ml of RNase A (TypeXII-A, Sigma) in lysis buffer for 15 min at 4 °C, as previously described [26]. Total RNA was purified using Trizol® reagent (Invitrogen) from 1/10 volume of the lysates and analyzed by 10% agarose gel electrophoresis. Unless stated otherwise, all reagents were purchased from Sigma–Aldrich.

2.4. Confocal microscopy and colocalization analysis

Cultured A549 cells were transfected with constructs of GFP-tagged full-length Stau1, Myc-tagged NS1, and pCMV-Tag2B vector as controls or constructs of RBD2-containing linker regions (RBD2) or RBD3-containing linker regions (RBD3L). After 24 h of transfection, the cultures were fixed, immunostained with monoclonal anti-Myc antibody (9E10, Sigma, St. Louis, MO), and subsequently stained with Cy3-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and 4′,6-Diamidino-2-phenyindole (DAPI, Sigma). In addition, cultures were immunostained with monoclonal anti-FLAG® antibody (M2, Sigma) to evaluate the expression of RBD2 or RBD3L.

Immunostained images were acquired by a confocal microscopy (TCS-SP2 AOBS, Leica Microsystems, Heidelberg, Germany), and colocalization was evaluated by colocalization threshold mode of NIH image analysis program (ImageJ ver. 1.42q).

2.5. Over-expression of target gene and viral infection

A549 cells were transfected with each plasmid (FLAG plasmid, FLAG-tagged full-length Staufen1, RBD3L, and RBD2) using Lipofectamine 2000 transfection reagent (Invitrogen) and were incubated for 24 h to induce expression of each protein. The transfected cells were washed with 1X PBS and subsequently infected with A/WSN/1933 (H1N1) virus at 0.01 of MOI. Unattached viruses were removed after incubation at 37 °C for 1.5 h, and the culture medium was replenished. Supernatants from virus-infected cells were collected at 12, 24, 48, and 72 h p.i., and virus was titrated in MDCK cells as previously described [27]. Individual sets of experiments were conducted at least three times under the same conditions.

2.6. Statistical analysis

All data are represented as the mean values ± SEM. Statistical significance of the two groups was analyzed by paired or unpaired Student's *t*-tests.

3. Results and discussion

A previous study reported that the influenza viral protein NS1 interacts with human Stau1 through its C-terminal region regardless of its RNA-binding ability [23]. However, since Stau1 has two splice variants, that study did not clearly discriminate which specific isoform was the binding partner of NS1. Therefore, we first co-expressed each Stau1 isoform with NS1 in HEK 293T cells and examined the protein–protein interaction via a series of co-IP assays. Visualization of the immunoprecipitates through Western blotting showed that both Stau1^{63} and Stau1^{55} could interact with NS1, suggesting that the interaction was not affected nor mediated by the residues at the N-terminal end (Fig. 1A).

In order to isolate the specific regions of Stau1 involved in the interaction with the viral protein NS1, we constructed a series of truncated mutants (Fig. 1B) and examined their interactions with NS1 through co-IP assay. As shown Fig. 1C, C-terminal truncated mutants containing RBD3 could bind with NS1. However, expression of the RBD3 region only (RBD3Δ) was not sufficient to co-precipitate with the viral protein. Additional expression of the linker region between RBD3 and RBD4 (RBD3L construct) was required to rescue the host-viral protein interaction (Fig. 1D), indicating a critical role for this linker region in binding to NS1. Interestingly, basic amino acids were found to be enriched in the linker region between RBD3 and RBD4. Ten out of the total 36 amino acids of the linker region are basic amino acids, and the region is overlapped by a bipartite nuclear/nucleolus localization signal (NLS) [22]. Deletion of the linker region between RBD3 and RBD4 in mutants also resulted in the deletion of NLS on the Stau1. Lack of this interaction could be attributed to different localization of each mutant with NS1. In order to rule out this possibility, we compared localization among all of the mutants. The mutant was found in the cytosol similar to that of full-length Stau1 (Full) or GFP-tagged Stau1 (GFP-Stau). However substantial amount of RBD2 or ΔRBD3, which is lack of RBD3 and the linker region, was found in the nucleus as well as in the cytosol. RBD3Δ, which contains RBD3 region only and is lack of the linker region, was dominantly localized to the cytosol (Supplementary Fig. 1). These recapitulate

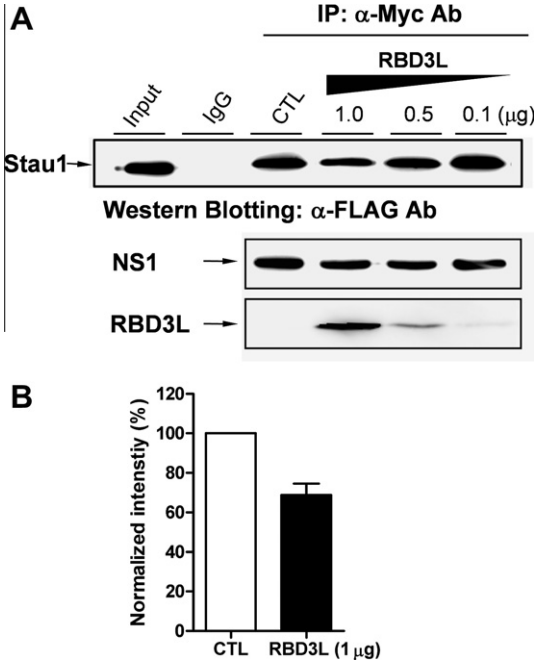

Fig. 2. Interaction of Stau1 with NS1 is inhibited by the presence of RBD3L in a dose-dependent manner. Cultured HEK 293T cells were co-transfected with NS1 and Stau1 expression vectors with or without RBD3L, as indicated. Cells from control groups were transfected with 1 μg of empty vectors. (A) Representative results of co-IP analysis. Lower panels show the expression level of each protein. (B) Bar graphic representation of data analysis. Band intensity of co-IP assay was quantified by densitometric analysis. The band intensity value upon RBD3L (1 μg) expression is represented as the percentage of that of each control group (***$p < 0.001$, ns: not significant compared with controls).

a previous result that RBD3 works as a main cytoplasmic retention signal [22]. Although NS1 mostly localizes to the nucleus at very early stage of viral infection or DNA transfection, cytoplasmic localization and nucleocytoplasmic shuttling of NS1 protein have been reported [28]. Indeed, we found almost the same amount of NS1 in the cytosol at 24 h after transfection (Supplementary Fig. 2). These results indicate that disruption of the Stau1-NS1 interaction was not due to the different localization of each protein.

As both proteins are known associate with other RNA molecules, we next examined their dependency on RNA for the interaction. However, RNase treatment did not significantly affect the Stau1-NS1 interaction. Consistent with a previous result [23], our data suggest that the interaction between Staufen1 and NS1 was independent of the RNA-bindings of Stau1 and NS1.

To determine whether or not expression of RBD3L can perturb the Stau1-NS1 interaction, we immunoprecipitated Myc-NS1 in 293T cells co-expressing FLAG-Stau1 with or without FLAG-RBD3L at various concentrations (1, 0.5, or 0.1 μg). Western blotting against FLAG antibody showed that additional expression of RBD3L decreased the coprecipitation of Stau1 with NS1 in a dose-dependent manner (Fig. 2). In particular, the amount of Stau1 coprecipitated with NS1 was reduced by approximately 30% in the presence of 1 μg of RBD3L compared to non-treated control lysates, indicating that RBD3L potentially inhibited the host-viral protein interaction by competitive binding (Fig. 2B).

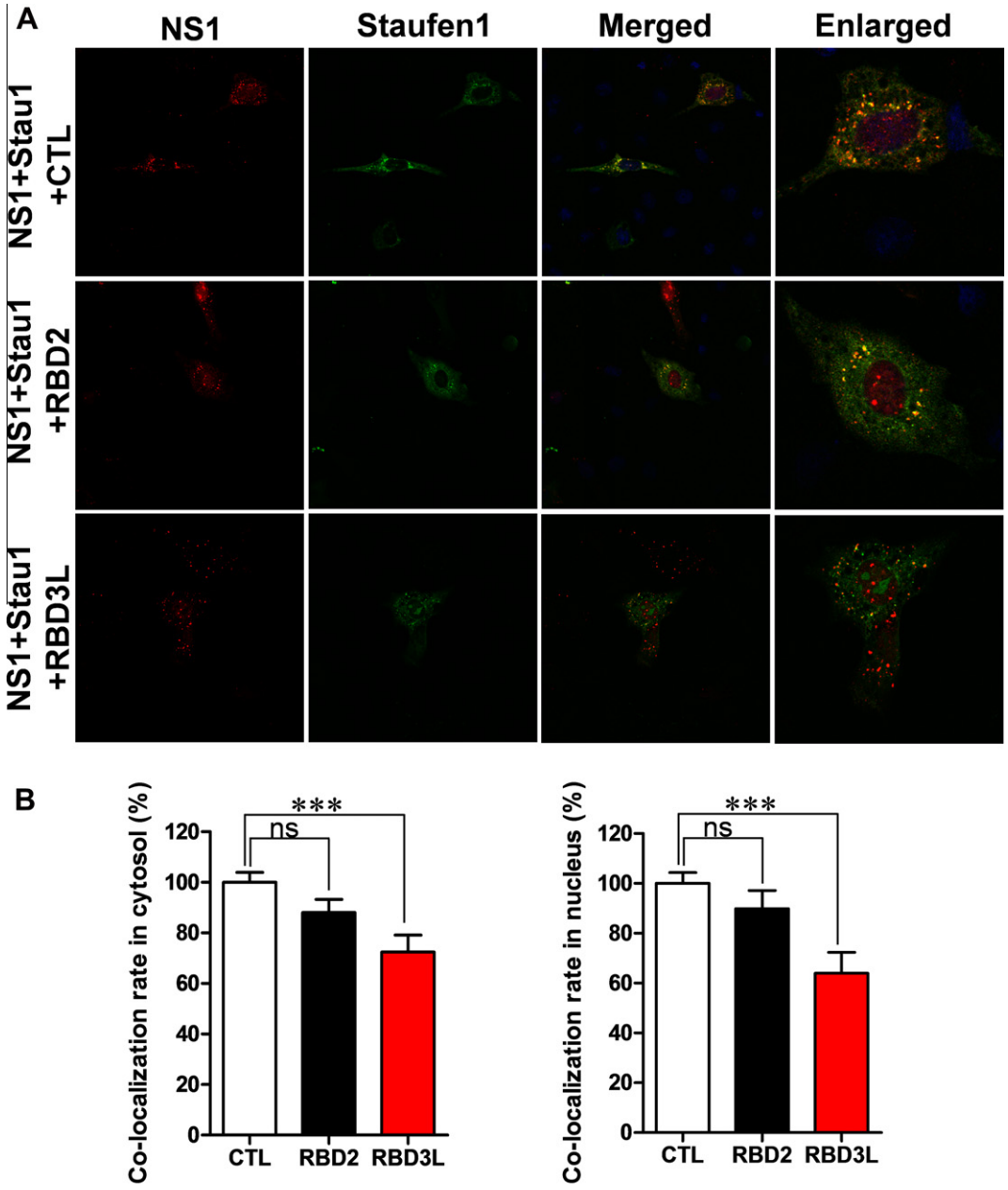

Fig. 3. RBD3L expression reduces colocalization of Stau1 with NS1. Cultured A549 cells were co-transfected with GFP-Stau1, Myc-NS1, and RBD3L or RBD2 as a control. After 24 h of incubation, cells were fixed, and then immunostained with anti-Myc antibody. DAPI was used to stain the nuclei. Colocalization of Stau1 with NS1 was measured in both the cytosol and nucleus. (A) Representative images of immunostained A549 cells. (B) Bar graphic representation of data analysis. Relative colocalization of Stau1 with NS1 compared with control is represented as the means ± SEM (%). Student's *t*-test was employed to determine the statistical difference between the groups (***$p < 0.001$, ns: not significant compared with controls).

Stau1 has been shown to be localized to the rough endoplasmic reticulum and ribosomes in the cytosol [13,14], but was noted to localized in the nuclear compartment in NS1-expressing cells [23]. It was also recently reported that Stau1 is imported into the nucleus and nucleolus by a bipartite nuclear localization signal [22]. In the following experiments, we explored and measured the colocalization of Stau1 with NS1 in both the cytosol and nucleus after expression of RBD3L. Cultured A549 cells were transfected with GFP- Stau1, Myc-NS1, and FLAG-RBD3L or FLAG-RBD2 as a negative control, followed by immunostaining with anti-Myc-antibody 24 h later. Confocal microscopy revealed co-localization of Stau1 and NS1 in both the cytoplasmic and nuclear compartments (Fig. 3A), in agreement with previous results [23,24]. Interestingly, co-expression of RBD3L but not RBD2 significantly decreased the colocalization of Stau1 with NS1 in both the cytosol (Fig. 3B, CTL: 100.0 ± 3.84%, $N = 22$, RBD2: 90.3 ± 5.06%, $N = 13$, RBD3L: 72.4 ± 6.71%, $N = 14$; $^{***}p < 0.001$, ns: not significant compared with controls) and nucleus (Fig. 3B, CTL: 100.0 ± 4.33%, $N = 26$, RBD2: 89.86 ± 7.31%, $N = 16$, RBD3L: 63.97 ± 8.40%, $N = 15$; $^{***}p < 0.001$, ns: not significant compared with controls). Taken together, these suggest that the presence of RBD3L perturbed the interaction of Stau1 with NS1 *in vivo*.

Finally, we examined whether or not the perturbation of the interaction between Stau1 and NS1 has biological significance for virus replication. Transfected A549 cells (FLAG-tagged Stau1, RBD3L, RBD2, or an empty vector control plasmid) were infected 24 h later with A/WSN/1933 virus at 0.01 MOI. Titration of virus-infected supernatants sampled at the indicated time points demonstrated that RBD3L-expressing cells significantly reduced virus titers by approximately 10-fold compared to control cells transfected with empty vector at almost all of the time points tested, except at 24 h (paired *t*-tests, 36 h: $^{*}p < 0.05$, 48 h: $^{**}p < 0.01$, 72 h: $^{***}p < 0.001$, $N = 3$) (Fig. 4). However, compared to full-length Stau1, viral titers in the RBD3L group significantly differed starting at 48 h p.i. In contrast, RBD2-transfected cells had comparable virus titers with those of control vector treatment during the entire course of the experiment. These results appear to be consistent with a previous report that found RNAi-mediated down-regulation of Stau1 significantly reduces influenza viral replication [24]. However, our data indicate that influenza viral production could potentially be reduced by specific perturbation of the interaction between Stau1 and NS1. Additionally, as with another possible mechanism of the reduction of viral production, the RBD3L expression might cause a steric effect on the interaction between Stau1 and NS1. However determining those are beyond the scope of this study.

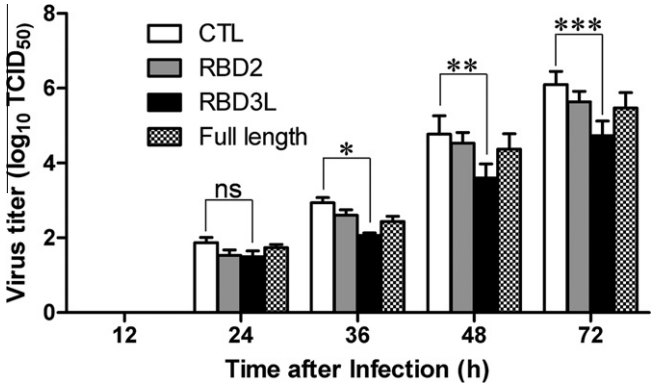

Fig. 4. RBD3L expression reduces viral replication. A549 cells were transfected with FLAG-tagged Stau1, RBD3L, RBD2, or an empty plasmid vector control, incubated for 24 h, and then infected with A/WSN/1933 (H1N1) virus at 0.01 of MOI. Virus titers in infected cell culture supernatants were measured at different time points (12, 24, 36, and 72 h p.i.) by titration in MDCK cells and expressed as $\log_{10}$ $TCID_{50}$/100 μl. Data are the means ± SEM from three independent titrations (paired *t*-test: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compare relative virus titers of compared with control transfections).

In this study, we identified the linker region between RBD3 and RBD4 in human Stau1 protein as the required site for binding to influenza A virus NS1 protein. It is noteworthy that this linker region is enriched in basic amino acids (10 out of 36 amino acid residues) and is overlapped by a bipartite nuclear/nucleolus localization signal responsible for the nuclear import of Stau1 [22]. Viral NS1 protein is capable of shuttling in and out of the nucleus, whereas the nuclear import of Stau1 may be modulated by the balance between its nuclear import domain and specific molecular determinants promoting cytoplasmic retention [22]. We therefore hypothesize that binding with NS1 at the linker region provides a stimuli for its nuclear import.

Aside from NS1, the influenza viral RNP and viral mRNA have also been shown to interact with Stau1, independent of NS1 [24]. At present, we could not confirm whether or not these molecules share similar interaction sites since it was beyond the scope of the current study. However, the RBD3 and/or RBD4 motifs, proposed to be involved in both RNA-binding and protein–protein interactions [22], were found to be potential alternative binding sites for viral RNP and mRNA but not NS1 (Fig. 1).

It was conceived that interaction of influenza virus components with Stau1 allows the virus to subvert the role of the host protein to improve RNP packaging into infectious viruses and at the same time properly manage mRNA localization and expression [24]. Suppression of Stau1 by RNA interference reduces virus particle production, suggesting its requirement for efficient virus replication [24]. Accordingly, our findings show that expression of the RBD3L region led to reduction of the Stau1-NS1 protein interaction and consequently attenuation of virus yield in A549 cells. Overall, these results provide a potential alternative approach for the development of antiviral drugs (i.e., RBD3L-like peptides or compounds) as an infection control measure. Further experiments are needed to elucidate the mechanism of how the linker region associated with the RBD3 motif of Stau1 modulates influenza A virus replication.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.09.042.

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