博士論文

Identification and Characterization of GPS1 as a Host Factor Involved in Influenza Virus Replication.

（インフルエンザウイルス感染環における宿主因子 GPS1 の同定と機能解析）

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PREFACE

The first appearance of influenza in human history can be traced back to 412 B.C [1]. Yet, despite rapid advances in science and medicine, influenza has not been conquered. One of the most catastrophic events in the 20th century, the “Spanish flu”, resulted in an estimated 20-40 million deaths [2-5]. In this century, we have also experienced an influenza pandemic. In March 2009, an influenza outbreak began in Mexico. After only 3 months, the infection had spread throughout the world, and the World Health Organization (WHO) declared this worldwide influenza outbreak as the first pandemic of this century. Because of the overwhelming numbers of cases being reported to the WHO each day, they stopped counting them. However, a group of researchers has estimated that over 20,000 people died as a result of their influenza virus infection during this pandemic [6].

One of the major reasons why it is so difficult to control influenza is that the influenza virus in humans frequently undergoes mutations. Influenza virus possesses eight segmented negative-sense, single-stranded RNAs as its genome. Many RNA viruses lack proofreading-repair mechanisms during RNA synthesis [7], and, therefore, have higher mutation rates than do DNA viruses [8,9]. Influenza viruses in humans

mutate rapidly, which leads to two major issues when attempting to counter influenza: the need for yearly vaccine renewals and the acquisition of resistance to antiviral drugs. In recent years, the emergence of antiviral drug-resistant viruses has become a serious public health concern. During the avian influenza A H7N9 epidemic, which started in March 2013, viruses that were resistant to an NA inhibitor (oseltamivir) were found in April 2013 [10]. This is a notable example of how quickly influenza viruses can acquire resistance to antiviral drugs and highlights the need for new influenza virus treatment options.

One of the ideal attributes of an anti-influenza virus drug is that it not be affected by mutations in the virus genome. For this reason, host proteins are now being considered as potential candidates for new antiviral drugs. Along with other viruses, influenza viruses are obligate intracellular parasites. From entry to budding, viruses rely heavily on their host's cellular mechanisms to replicate. In recent years, several large-scale screenings have been performed to identify host factors that could serve as targets for the development of new anti-influenza drugs [11-17]. As the result of these screenings, 1,449 candidate host proteins have been identified. However, how these host proteins function in the virus replication cycle remains largely unknown.

In our laboratory, 207 host cellular proteins that were immunoprecipitated with

influenza virus protein M2, but not other viral proteins, were identified by using mass spectrometry analysis. For my doctoral studies, I focused on the host protein G protein pathway suppressor 1 (GPS1) as an interacting partner of M2 and an important factor for influenza virus replication. I examined which virus replication step involves GPS1 and found that GPS1 is essential for efficient influenza virus genome transcription or replication.

Further studies of the functional mechanisms involving GPS1 in the virus replication cycle could lead to the development of an anti-influenza virus drug that targets GPS1.

Abstract

Influenza virus relies heavily on cellular machinery to replicate in host cells. Therefore, to better understand the influenza virus life cycle, it is important to identify which host proteins are involved and how they function in virus replication. In this study, I focused on the multi-functional viral protein M2, and attempted to identify the host proteins with which it interacts. To identify M2-interacting host proteins, I used co-immunoprecipitation and mass spectrometry; siRNA interference was also used to analyze the functions of the host proteins. I identified 207 M2-interacting host proteins; of these, I focused on G protein pathway suppressor 1 (GPS1). GPS1 is a component of the COP9 signalosome, which regulates the NF-κB signaling pathway. Down-regulation of GPS1 expression reduced influenza virus replication by more than two log units. Although GPS1 was not involved in the early and late stages of virus replication such as viral entry, uncoating, assembly or budding, I found that the viral polymerase activity was impaired in GPS1 down-regulated cells. Moreover, expression levels of viral RNAs and proteins were significantly decreased in GPS1 down-regulated virus-infected cells. These results suggest that GPS1 is involved in the transcription and replication of influenza virus genomic RNA.

Introduction

Influenza A viruses belong to the *Orthomyxoviridae* family, whose viral genomes consist of single-stranded, negative-sense, segmented RNAs. From the eight-segmented viral RNAs (vRNAs) of influenza A virus, 10 major viral proteins, which are essential for efficient viral replication *in vitro* and *in vivo*, are produced. Polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1) and polymerase acidic protein (PA) form a hetero-trimeric RNA-dependent RNA polymerase complex. Nucleoprotein (NP) is a RNA-binding protein, which forms a viral ribonucleoprotein complex (vRNP) along with the vRNA and RNA-dependent RNA polymerase complex. The vRNP is the minimal unit responsible for vRNA transcription and replication. Hemagglutinin (HA), Neuraminidase (NA), and matrix protein 2 (M2) are integral transmembrane proteins that are present on the lipid envelope of the virion (Fig. 1A). Matrix protein 1 (M1) is a peripheral membrane protein that lines the inside of the virion envelope, forming and maintaining the spherical or filamentous shell of the virion.

M2 is a tetrameric transmembrane protein, which possesses ion channel activity (Fig. 1B) [23,24]. The most studied function of M2 is its ion channel activity,

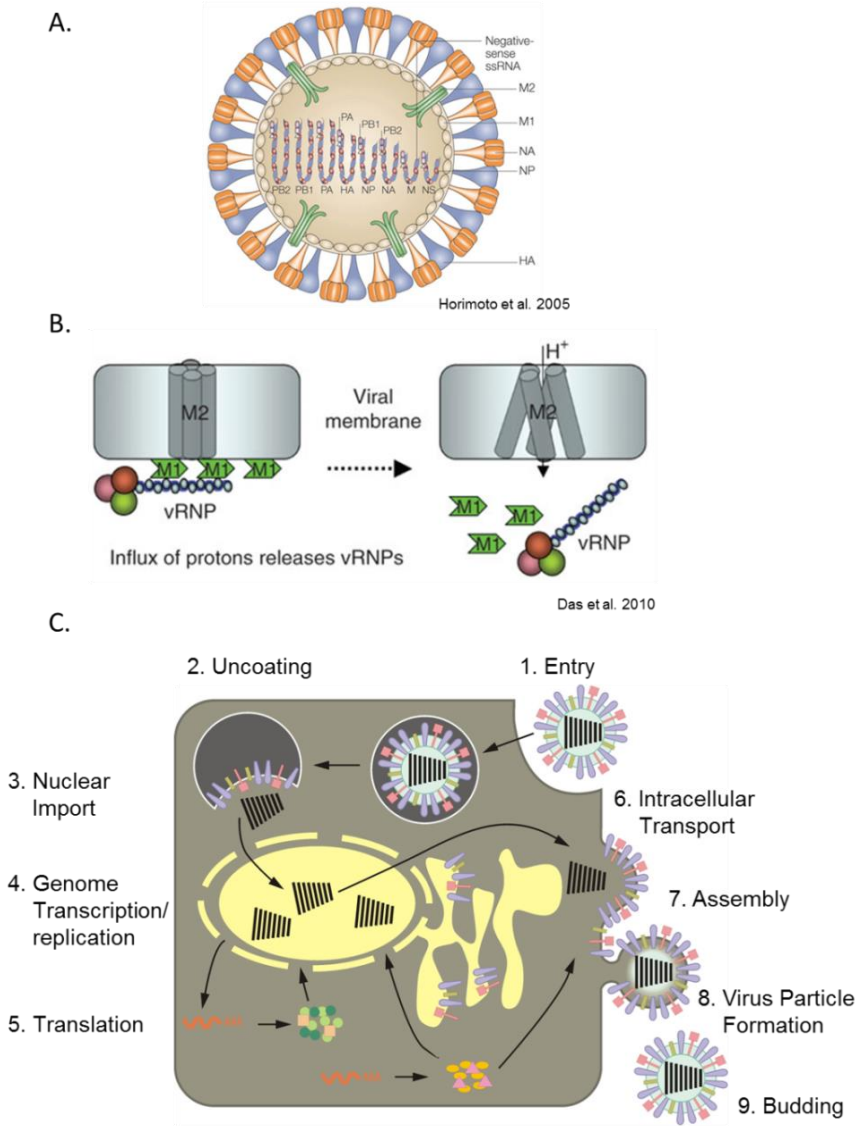

Figure 1. Schematic diagram of influenza virus and its replication steps. (A) Influenza virus and its genomic structure. Influenza virus consists of 8 segmented negative-sense single-strand RNAs and an outer shell, which is lined with M1 on the inside and with HA, NA, and M2 on the outside. (B) Structure and ion channel activity of M2. The influx of protons through the M2 ion channel allows the detachment of M1 and vRNPs from the endosome. Thus, vRNPs are released into the cytoplasm and imported to the nucleus. (C) Influenza virus replication steps. 1) Virus enters the host cell by endocytosis. 2) Viral membrane fusion with the endosomal membrane results in the release of vRNPs into the cytoplasm. 3) The vRNPs are imported to the nucleus by active transport. 4) Replication of the viral genome take place in the nucleus. 5) Viral protein translation takes place in the cytoplasm. 6) Newly synthesized RNPs are transported to the plasma membrane. 7) vRNPs and viral proteins synthesized in the cytoplasm are assembled at the plasma membrane. 8) vRNPs are selectively packaged into budding virions. 9) Progeny virus buds from the budding site.

which is highly selective for hydrogen ions, in the viral uncoating process. The acidic environment of the late endosome induces viral membrane fusion with the endosomal membrane, and the conformational change in the transmembrane region of M2 allows the influx of protons into the interior of the virions, which then leads to the dissociation of vRNPs from M1 molecules, resulting in the release of vRNPs into the cytoplasm (Fig.1B) [25]. It is also involved in other steps of virus replication including packaging of RNPs into progeny virions, and budding of progeny virions (Fig. 1C). When a mutant virus lacking the cytoplasmic tail of M2 is generated by reverse genetics, vRNPs are not incorporated into the progeny virions, resulting in the release of empty virus particles [26,27]. During progeny virion formation, M2 localizes to the neck of budding virions and alters the membrane curvature, which enables the scission and release of budding virions from the surface of virus-infected cells. Thus, M2 works multi-functionally at different subcellular locations and at different virus replication steps.

Several host proteins have been reported to interact with M2. Hsp40 is involved in Protein kinase-R (PKR) signaling and is thought to affect the life cycle of virus infected-cells [20]. Annexin A6 impairs the budding efficiency of progeny virions from virus-infected cells, thus acting as a negative regulator that targets the release of progeny virions [22]. Caveolin-1 seems necessary for efficient virus replication,

although its molecular functions in this process remain unknown [21]. Because M2 has multiple functions in the virus life cycle, there must be many more host proteins that interact with M2. Accordingly, my colleague and I sought M2-interacting host proteins by using co-immunoprecipitation and mass spectrometry and identified 207 host proteins that interacted specifically with influenza virus M2 but not with the other influenza virus proteins [28]. I then categorized these host proteins according to their known functions (Fig. 2) and found that a large proportion of them were related to the ubiquitin proteasome system (Table 1). In particular, I noted that all 8 subunits of the COP9 signalosome were M2-interacting partners.

The COP9 signalosome, which is a multi-functional protein complex, was first discovered in Arabidopsis [29] and then found to be highly conserved among most eukaryotes [30]. Perhaps the best-known function of the COP9 signalosome is in the regulation of protein degradation [31-35]. It has been reported that most of the proteins that interact with the COP9 signalosome are transcriptional regulators, indicating the importance of the COP9 signalosome in transcriptional regulation [36]. Importantly, activation of the transcription factor NF-κB, which is a critical regulator of multiple signaling pathways, is regulated by the COP9 signalosome (Fig. 3) [37]. The COP9 signalosome induces ubiquitination and degradation of the inhibitor of κB (IκB), which

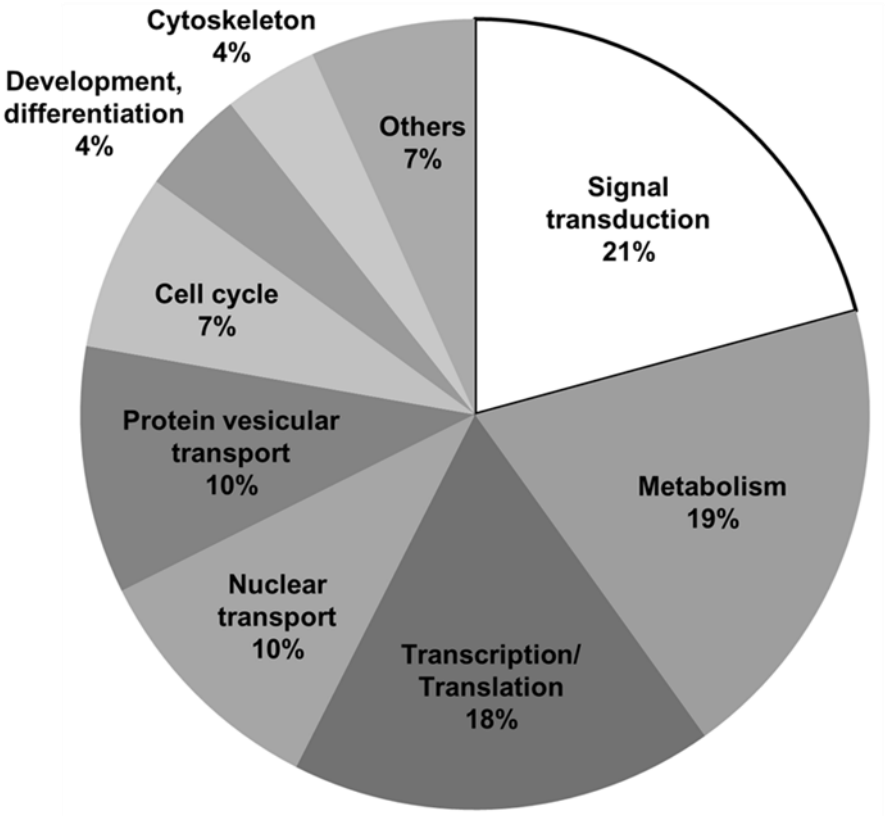

Figure 2. Categorization of host proteins with M2-specific interactions. A total of 207 candidate host proteins were categorized according to their known functions.

Table 1. Signal transduction-related host proteins that specifically interact with M2.

ID	Name	Function
GPS1	G protein pathway suppressor 1 isoform 1/ COP9 signalosome subunit 1	ubiquitin, signaling pathways
COPS2	COP9 signalosome subunit 2 isoform 1	ubiquitin, signaling pathways
COPS3	COP9 signalosome subunit 3	ubiquitin, signaling pathways
COPS4	COP9 signalosome subunit 4	ubiquitin, signaling pathways
COPS5	COP9 signalosome subunit 5	ubiquitin, signaling pathways
COPS6	COP9 signalosome subunit 6	ubiquitin, signaling pathways
COPS7B	COP9 signalosome subunit 7B	ubiquitin, signaling pathways
COPS8	COP9 signalosome subunit 8 isoform 1	ubiquitin, signaling pathways
USP9X	ubiquitin specific protease 9, X-linked isoform 3	Deubiquitinase
RNF160	zinc finger protein 294	E3 ubiquitin-protein ligase
COPS7B	COP9 constitutive photomorphogenic homolog subunit 7B	ubiquitin, signaling pathways
HECTD1	HECT domain containing 1	HECT domain containing E3 ubiquitin protein ligase 1
CUL4A	cullin 4A isoform 2	ubiquitin ligase component
DDB1	damage-specific DNA binding protein 1	an adaptor molecule for the cullin 4 (CUL4) ubiquitin E3 ligase complex
USP22	ubiquitin specific protease 22	nuclear receptor-mediated transactivation and cell cycle progression
DCUN1D4	DCN1, defective in cullin neddylation 1, domain containing 4 isoform 1	DCN1, Defective In Cullin Neddylation 1, Domain Containing 4
UBR5	retinoblastoma-associated factor 601	E3 ubiquitin-protein ligase
TPP2	tripeptidyl peptidase II	Component of the proteolytic cascade acting downstream of the 26S proteasome
PPP2R1B	beta isoform of regulatory subunit A, protein phosphatase 2 isoform b	signal transduction
RAP1GDS1	RAP1, GTP-GDP dissociation stimulator 1 isoform 1	Stimulates GDP/GTP exchange reaction
GPN3	GPN-loop GTPase 3	GTPase
RRAS2	related RAS viral (r-ras) oncogene homolog 2 isoform a	plasma membrane-associated GTP-binding protein with GTPase activity
IMPA2	inositol(myo)-1(or 4)-monophosphatase 2	inositol monophosphatase. important role in phosphatidylinositol signaling.
UCK2	uridine-cytidine kinase 2	pyrimidine ribonucleoside kinase/catalyzes phosphorylation of uridine and cytidine
PELO	pelota homolog	conserved nuclear localization signal
ATAD1	ATPase family, AAA domain containing 1	ATPase that plays a critical role in regulating the surface expression of AMPA receptors
HM13	minor histocompatibility antigen 13 isoform 1	catalyzes intramembrane proteolysis of some signal peptides
RAC1	ras-related C3 botulinum toxin substrate 1 isoform Rac1b	GTPase /regulate a diverse array of cellular events
NCDN	neurochondrin isoform 1	regulates Ca/calmodulin-dependent protein kinase II phosphorylation
FASTKD5	FAST kinase domains 5	poly (A) RNA binding/protein kinase activity
CTNNAL1	catenin, alpha-like 1	Rho pathway signaling
AIMP1	small inducible cytokine subfamily E, member 1 isoform a precursor	multisynthase complex/Possesses inflammatory cytokine activity
FASTKD2	FAST kinase domains 2	mitochondrial apoptosis
ABHD12	abhydrolase domain containing 12 isoform b	May be a regulator of endocannabinoid signaling pathways
ABR	active breakpoint cluster region-related protein isoform b	GTPase-activating protein
MFN1	mitofusin 1	transmembrane GTPase, which mediates mitochondrial fusion
OR4A15	olfactory receptor, family 4, subfamily A, member 15	G-protein-coupled receptors (GPCR)
PLCD3	phospholipase C delta 3	catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate
GNB3	guanine nucleotide-binding protein, beta-3 subunit	G protein
PDCD6	programmed cell death 6	accelerate apoptotic cell death
GJB2	gap junction protein beta 2	provide direct intracellular communication
TRAPPC9	trafficking protein particle complex 9	activator of NF-kappa-B
PRC1	protein regulator of cytokinesis 1 isoform 1	Key regulator of cytokinesis

Ubiquitin-proteasome system-related host proteins among the signal transduction-related host proteins. Host proteins known to be part of the ubiquitin-proteasome system represent approximately 44% of the signal transduction-related host proteins (gray). These include all 8 subunits of the COP9 signalosome.

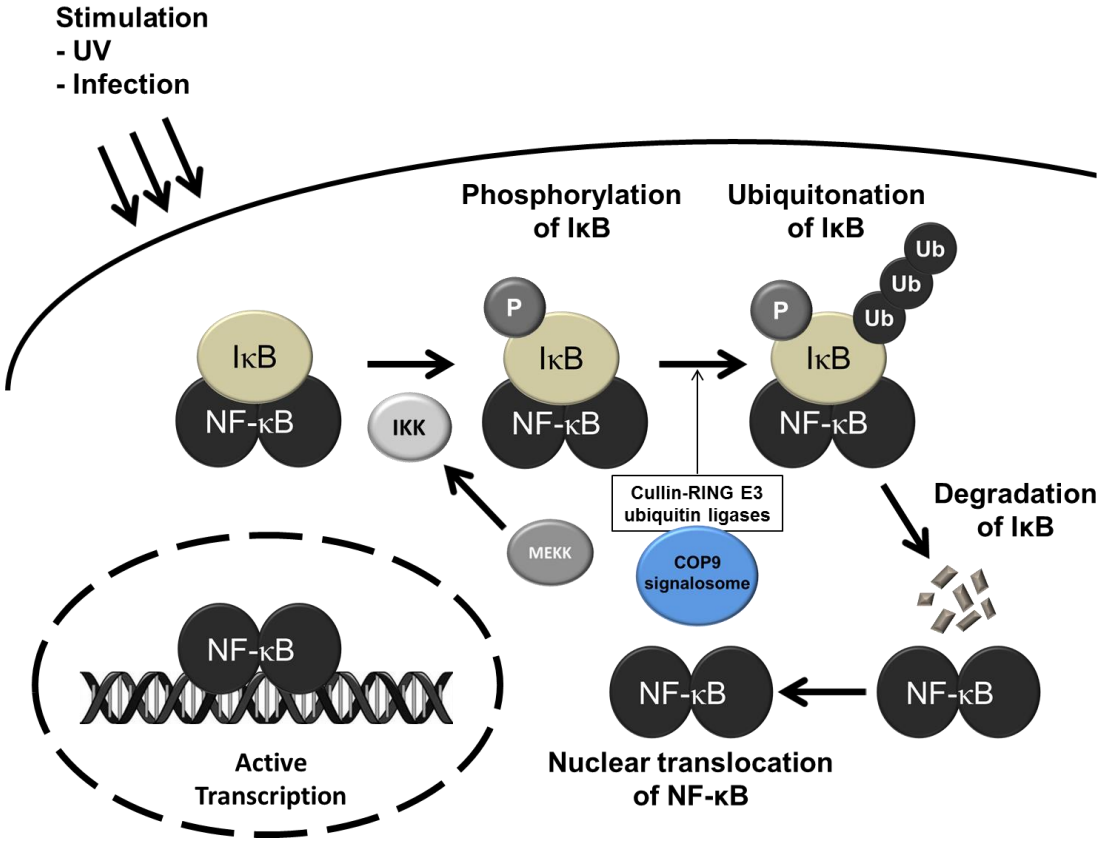

Figure 3. NF-κB signaling pathway. Upon extracellular stimulation, IκB is phosphorylated by IκB kinase (IKK), which leads to recognition by SCF-type Cullin-RING E3 ubiquitin ligases (CRL). Once IκB is recognized and poly-ubiquitinated by SCF-type CRL, it is degraded by the proteasome. This degradation of IκB exposes the nuclear localization signal of NF-κB and leads to the translocation and activation of NF-κB. The COP9 signalosome is involved in NF-κB signaling via its regulation of CRL.

forms a complex with NF-κB. Degradation of IκB causes translocation of NF-κB from the cytoplasm to the nucleus, where NF-κB activates numerous transcriptional activities of various host genes including genes involved in inflammation and the immune response.

G protein pathway suppressor 1 (GPS1) is the largest subunit of the COP9 signalosome. It localizes to both the nucleus and the cytoplasm and is essential for the maintenance of the COP9 signalosome as a complex. The N-terminal region is important for the regulation of several transcription factors such as activating protein-1 (AP-1) and serum response element (SRE), which responds to cellular stresses caused by various extracellular stimuli such as infection [38]. The C-terminal region of GPS1 is responsible for its integration into the COP9 signalosome complex [38-40]. A recent structural study of GPS1 revealed that its C-terminal tail interacts with IκB, suggesting the possible involvement of GPS1 in the regulation of the NF-κB signaling pathway [41].

Because previous studies have suggested that influenza viruses utilize NF-κB for their efficient replication [42-46], I selected GPS1, which interacts with M2 and may regulate NF-κB, for further functional analysis. To better understand the mechanisms of influenza virus replication with respect to virus-host interactions, I attempted to

characterize the function of GPS1 in influenza virus replication by down-regulating GPS1 with siRNA.

Results

Identification of candidate host proteins that interact with M2

In our laboratory, a total of 1,292 host proteins that interacted with 11 viral proteins PB2, PB1, PA, HA, NP, NA, M1, M2, NS1, NS2, and PB1-F2, were identified by use of co-immunoprecipitation and mass spectrometry analyses (Fig. 4). Among these proteins, 207 were identified as interacting only with M2. I categorized these M2-binding host proteins by their known cellular functions and found that signal transduction-related proteins were the most abundant group (21%) among the 207 host proteins (Fig. 2). I then, further sub-classified these signal transduction-related proteins and found that ubiquitin proteasome system-related proteins occupied a large proportion of the signal transduction-related proteins (Table 1) and that all 8 subunits of the COP9 signalosome were part of this group. Based on these results, I hypothesized that the COP9 signalosome plays important roles in influenza virus replication by interacting with M2 and examined the role of GPS1, an essential subunit of the COP9 signalosome, in the context of influenza virus replication in cultured cells.

GPS1 interacts and co-localizes with M2

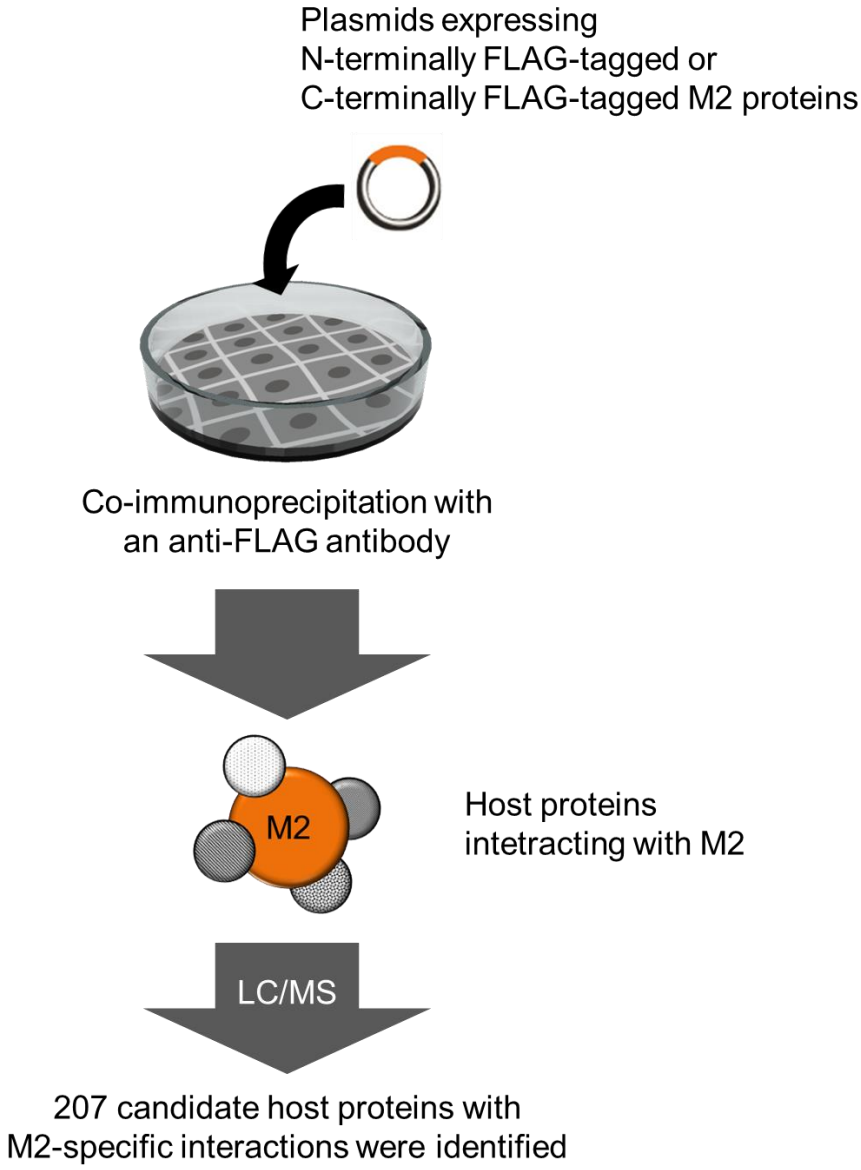

Figure 4. Schematic diagram of the method used to identify host proteins that specifically interact with M2. M2-expressing plasmids with a FLAG-tag at either the N-terminal or C-terminal end were individually expressed in HEK293 cells and immunoprecipitated with an anti-FLAG antibody. Then, mass spectrometry analysis was performed, and 207 candidate host proteins that interacted specifically with M2 were identified.

To confirm the interaction between M2 and GPS1, I performed an immunoprecipitation assay. Plasmids expressing FLAG-tagged M2 or wild-type M2 were transfected into 293T cells, and the immunoprecipitation assay was conducted with an anti-FLAG antibody at 48 h postinfection (hpi). The results showed that endogenous GPS1 was co-immunoprecipitated with FLAG-tagged M2, but not with wild-type M2, confirming the interaction of GPS1 with M2 (Fig. 5A). Next, to examine the intracellular localization of GPS1, I infected HEK293 cells with the virus at a multiplicity of infection (MOI) of 10, and performed an immunofluorescence assay with anti-M2 mouse and anti-GPS1 rabbit antibodies at 12 hpi. GPS1 localized to the nucleus, juxtanuclear region, and plasma membrane in mock-infected cells (Fig. 5B), and although I did not observe any changes in its subcellular localization in virus-infected cells, GPS1 co-localized with M2 mainly in the juxtanuclear region (Fig. 5B), suggesting that GPS1 interacts with M2 at the juxtanuclear region; however, the exact location of the co-localized M2-GPS1 remains to be determined.

GPS1 is essential for efficient virus replication in cultured cells

Next, I evaluated the effect of GPS1 down-regulation on influenza virus replication. GPS1 expression was down-regulated by GPS1-targeting siRNA, but not by

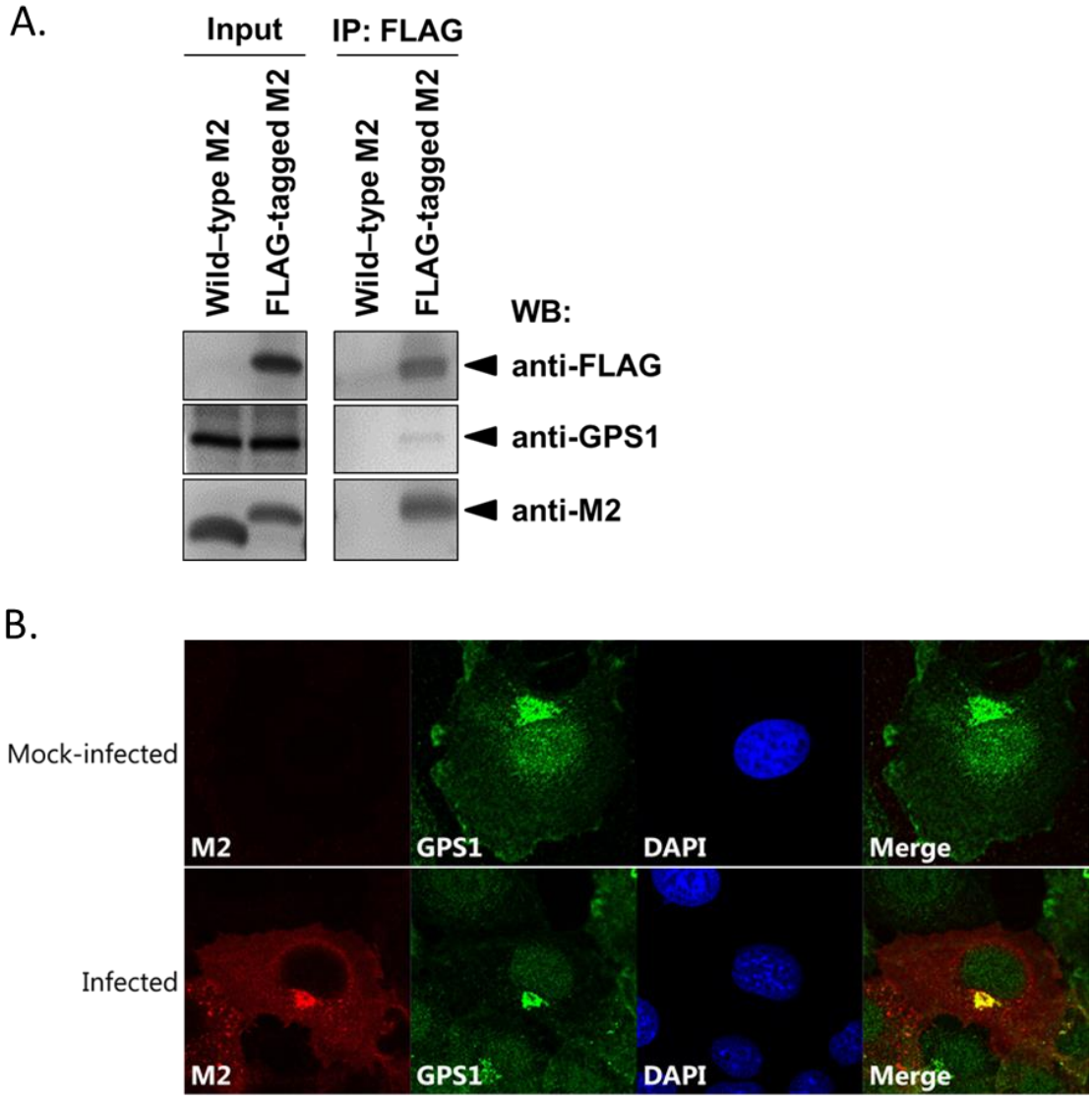

Figure 5. The interaction of GPS1 with M2. (A) The interaction between GPS1 and M2 was assessed by use of a co-immunoprecipitation assay. Either a wild-type M2- or a FLAG-tagged M2-expressing plasmid was transfected into HEK 293 cells. The cells were lysed 24 h after the plasmid transfection and immunoprecipitated with an anti-FLAG monoclonal antibody conjugated with magnetic beads. FLAG M2, GPS1, and M2 were detected by western blotting (WB). IP indicates immunoprecipitation. (B) The cellular localizations of GPS1 and M2 in mock-infected and virus-infected cells were analyzed by using an indirect immunofluorescence assay. M2 was detected by an anti-M2 antibody (red), endogenous GPS1 was detected by an anti-GPS1 antibody (green) and nuclei were stained with Hoechst 33342 (blue).

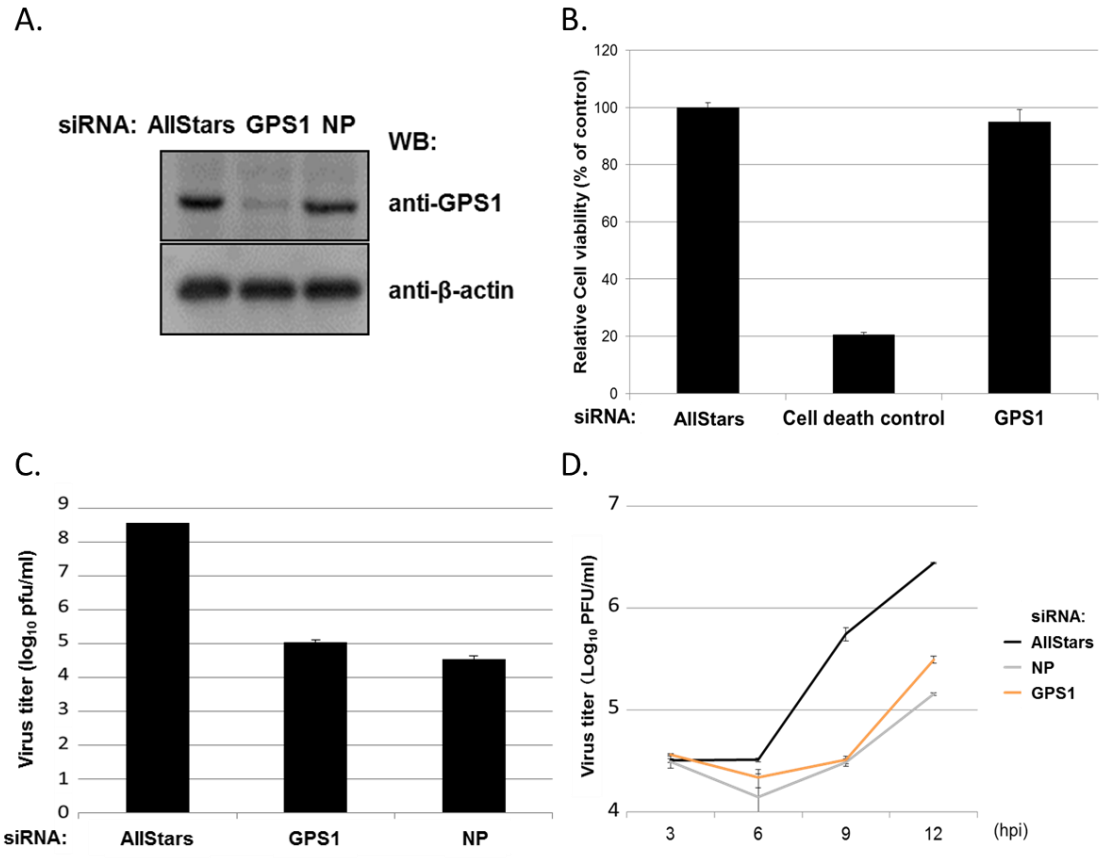

Figure 6. Effect of GPS1 down-regulation on virus replication. (A) Down-regulation of GPS1 expression by siRNA for GPS1 was examined by western blotting (WB). (B) A cell viability assay was performed on the siRNA-treated cells. Cell viability was assessed by measuring the amount of ATP in each siRNA-transfected cell. The experiments were performed in triplicate, and the error bars represent the standard deviation of triplicate samples. (C) Virus titers in the supernatants of respective siRNA-transfected cells. The experiments were performed in triplicate, and the error bars represent the standard deviation of triplicate samples. (D) The effect of GPS1 down-regulation on one replication cycle. The siRNA-transfected cells were infected with viruses at an MOI of 10, and virus titers were determined by use of plaque assays.

other siRNAs (Fig. 6A); AllStars siRNA has a sequence that is unrelated to any mammalian gene and was used as a negative control, while siRNA for the influenza virus NP gene was used as a positive control for the reduction of virus growth.

To eliminate the possibility that the effect of GPS1 down-regulation on the virus titer was caused by cellular toxicity, I examined the viability of the siRNA-transfected cells. The cells were transfected with AllStars siRNA, the siRNA for the GPS1 gene, or the siRNA Cell Death control, which targets cell survival genes, and the amounts of ATP in each sample were measured at 48 h after siRNA transfection. The amount of ATP in the GPS1 down-regulated cells was almost the same as that in the AllStars siRNA-transfected cells, whereas the amount of ATP in the siRNA Cell Death control-transfected cells was around 20% of that of the AllStars siRNA-transfected cells (Fig. 6B). These results indicate that GPS1 down-regulation does not affect cell viability.

Then, to evaluate the effect of GPS1 down-regulation on virus replication, siRNA-treated cells grown in 24-well plates were infected at an MOI of 0.001. At 48 hpi, the supernatants were collected, and virus titers were measured by means of plaque assays. The mean virus titer in the supernatant of the AllStars siRNA-transfected cells was 3.6×10^{8} PFU/ml, and that of the NP down-regulated cells was 3.5×10^{4} PFU/ml (Fig.

6C). The virus titer in the supernatant of the GPS1 down-regulated cells decreased by more than three log units compared with that of the AllStars siRNA-transfected cells (Fig. 6C). This decrease in virus titer was also observed in GPS1 down-regulated cells, when the cells were infected at an MOI of 10. (Fig. 6D).

Then, to confirm that the decrease in the virus titer was caused by the down-regulation of GPS1, and not by some other factors, such as off-target effects, I assessed the correlation between the degree of GPS1 protein down-regulation and the decrease in virus titer. Four kinds of siRNAs that had different target sequences to the GPS1 gene, were each transfected into HEK293 cells, and then virus infection and titration assessments were performed as in the previous experiments. The results showed that siRNA GPS1_2 suppressed neither GPS1 protein expression (Fig. 7A) nor the virus titer (Fig. 7B). The siRNA GPS1_3 and siRNA GPS1_5-transfected cells showed modest degrees of suppression in terms of both GPS1 protein expression and virus titer. The siRNA GPS1_6 showed the greatest degree of suppression of GPS1 protein expression and the greatest decrease in virus titer (Fig. 7A and 7B). Thus, there was a clear correlation between the degrees of GPS1 down-regulation and the suppression of virus titer, suggesting that the decrease in virus titer in GPS1 down-regulated cells was most likely caused by the down-regulation of GPS1, and not

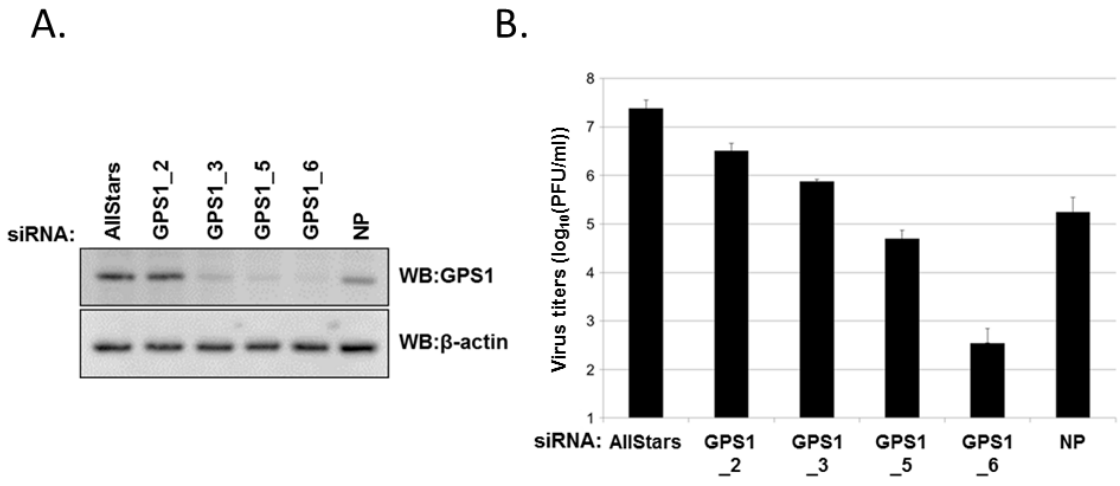

Figure 7. Correlation between the degree of GPS1 down-regulation and virus titers. (A) The degree of GPS1 down-regulation was assessed by using 4 different siRNAs for GPS1. GPS1 was detected by western blotting using an anti-GPS1 antibody. (B) Virus titers in the supernatants of the siRNA-treated cells were examined by using plaque assays at 48 hpi. The experiments were performed in triplicate; the error bars represent the standard deviation of triplicate samples.

by off-target effects.

GPS1 does not affect the early or late steps of the virus replication cycle

To determine whether GPS1 plays a role in the early steps of influenza virus replication, such as virus entry, uncoating, or the nuclear import of the viral genome (Fig. 1C), an assay using a replication-incompetent PB2 knockout virus (PB2-KO/Rluc virus) was performed. The coding region of the PB2 gene of this virus is replaced with the *Renilla* luciferase reporter gene [47]. Since this mutant virus lacks a functional PB2 gene and does not express the PB2 protein, it can go through the steps of host cell entry, endosomal internalization, uncoating of the viral genome, nuclear import of the viral RNA, and initial transcription of vRNA, but it cannot perform *de novo* transcription and replication of the viral genome (Fig. 1C). At 8 hpi, the virus-infected cells were subjected to the luciferase assay and the levels of luminescence in the virus-infected cells were quantitated. Amantadine, an inhibitor of ion channel activity that inhibits viral uncoating, was used as a control. The relative luciferase activity in the amantadine-treated cells was hardly detected (Fig. 8). In contrast, relative luciferase activities were only slightly reduced in the GPS1 down-regulated cells compared with the AllStars siRNA-transfected cells. These findings suggest that GPS1 is unlikely to be

involved in the early steps of influenza virus replication such as virus entry, uncoating, nuclear import of the viral genome, or the initial transcription of vRNA.

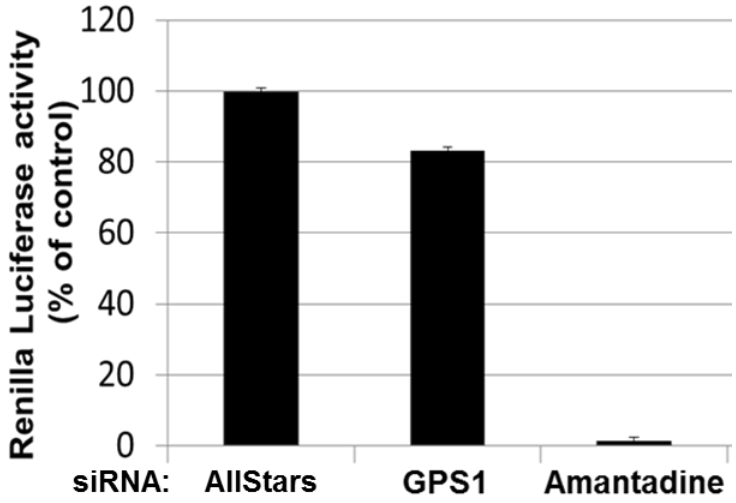

Figure 8. Effect of GPS1 down-regulation on the early steps of influenza virus infection. siRNA-treated cells were infected with PB2-KO/Rluc virus, and luciferase activities in the virus-infected cells were measured at 8 hpi. Amantadine was used as a positive control for the inhibition of viral uncoating.

Next, the involvement of GPS1 in the late stages of infection, such as intracellular transport, viral assembly, viral particle formation, and budding, was assessed by examining the efficacy of the formation of virus-like particles (VLPs), which are induced by the co-expression of the viral proteins HA, NA, and M1 [48]. Following the siRNA treatment, viral protein-expressing plasmids for HA, NA, and M1 were transfected into 293 cells, and the supernatant and cell lysates were collected at 24 h after the plasmid transfection. The supernatants were concentrated by ultracentrifugation through a 20% sucrose cushion, and the viral proteins in the supernatants and the cell lysates were detected by western blotting. When the expression of HA and M1 in the plasmid-transfected cells was examined, similar levels of HA and M1 proteins were detected in the GPS1 down-regulated cells and in the AllStars siRNA-transfected cells. Similar levels of HA and M1 proteins were also detected in the supernatants of GPS1 down-regulated cells and the AllStars siRNA-transfected cells (Fig. 9), suggesting that there were no significant differences in the efficiency of VLP formation and release between the negative control cells and the GPS1 down-regulated cells. Therefore, GPS1 is not involved in intracellular transport, viral assembly, viral particle formation, or virus particle budding.

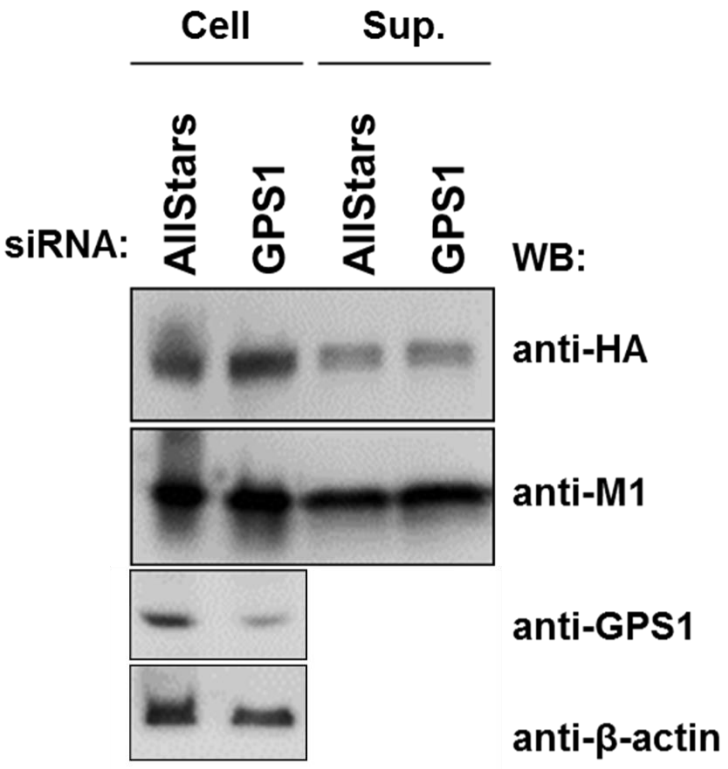

Figure 9. VLP formation in GPS1 down-regulated cells. Viral protein expression plasmids for HA, NA, and M1 were transfected into siRNA-treated cells. The supernatants were harvested at 24 hpi, and HA and M1 were detected by western blotting using anti-HA and anti-M1 antibodies.

GPS1 plays a role in the transcription and replication of the virus genome

To determine whether GPS1 plays a role in the transcription and replication of the virus genome, I used mini-replicon assay to compare the luciferase activities in GPS1 down-regulated cells and AllStars siRNA-transfected cells. In the mini-replicon assay, plasmids for the expression of influenza virus polymerase PB2, PB1, PA, and NP, which are necessary for the transcription and replication of vRNA, and a vRNA-expressing plasmid, pPolI NP(0)luc2(0), which encodes the firefly luciferase gene between the noncoding regions of the influenza virus NP gene, were co-transfected into siRNA-transfected cells. After 48 h of the plasmid transfection, the cells were subjected to the luciferase assay and the levels of luminescence were quantitated. The luciferase activity in the NP-gene down-regulated cells was almost 30% of that of the AllStars siRNA-transfected cells. Similarly, the luciferase activity in the GPS1 down-regulated cells was almost 20% of that in the AllStars siRNA-transfected cells (Fig. 10A).

Because GPS1 is involved in protein degradation via the ubiquitin proteasome system, I wanted to eliminate the possibility that the observed decrease in luciferase activity in GPS1 down-regulated cells was caused by protein degradation. I, therefore,

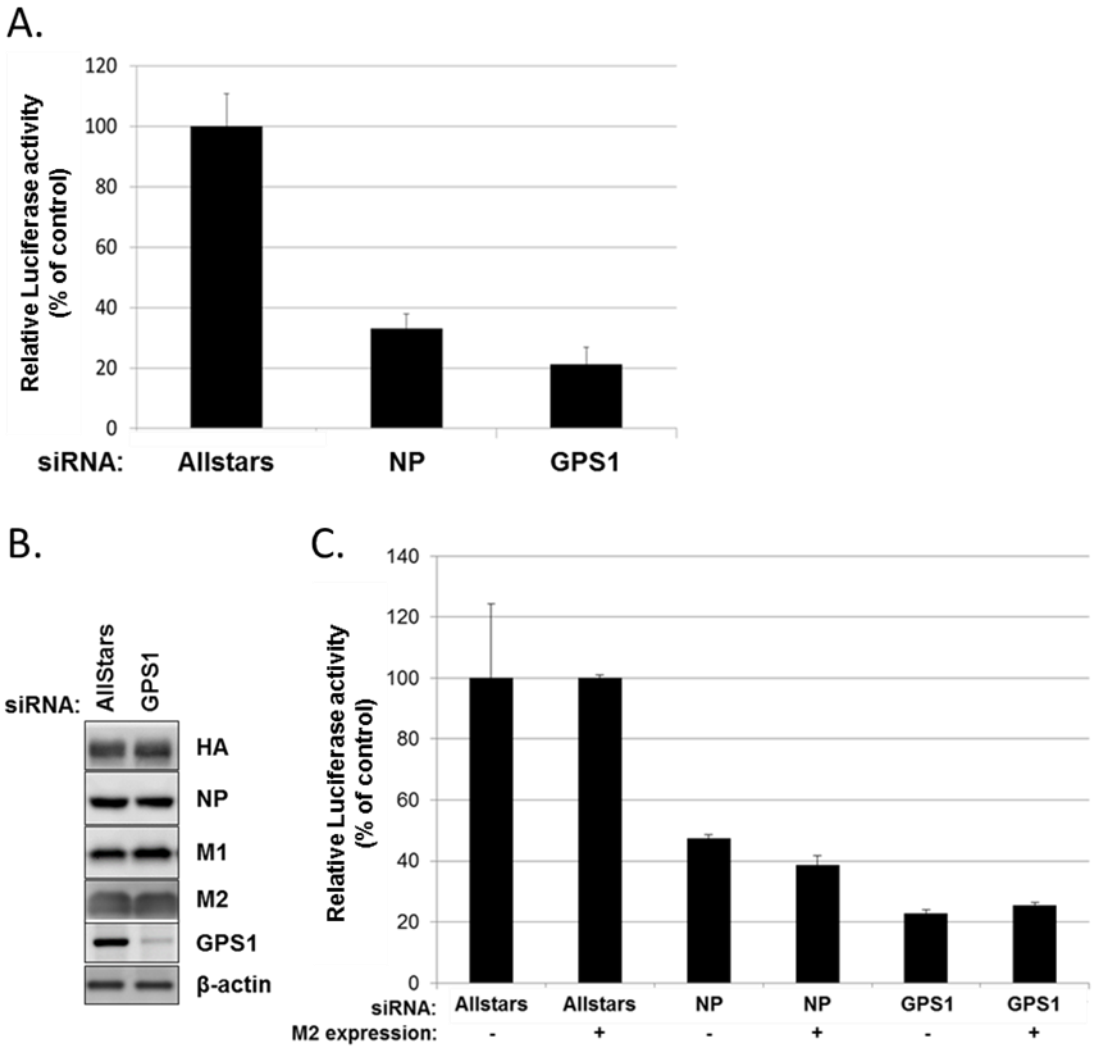

Figure 10. Effect of GPS1 down-regulation on influenza virus polymerase activity. (A) Virus polymerase activities in GPS1 down-regulated cells were assessed by using a mini-replicon assay. The luciferase activity of the AllStars siRNA-transfected cells was set as 100%. The tests were performed in triplicate, and the error bars represent the standard deviation of triplicate samples. (B) The stability of the viral proteins was also examined. siRNA-treated cells were transfected with HA, NP, M1, or M2 protein expressing plasmids. The cell lysates were harvested 24 h after the plasmid transfection, and viral proteins were detected by western blotting. (C) The role of M2 in virus polymerase activities. A mini-replicon assay was performed as described except that an influenza virus M2 protein-expressing plasmid was added. The experiments were performed in triplicate, and the error bars represent the standard deviation of triplicate samples.

examined the expression levels of viral proteins in siRNA-treated cells by transfecting them with pol II-dependent protein expression plasmids for the expression of HA, NP, M1, and M2. The cell lysates were harvested 24 h after the plasmid transfection, and the viral proteins were detected by western blotting. There were no appreciable differences between the expression levels of HA, NP, M1, or M2 in the AllStars siRNA-transfected cells compared with those in the GPS1 down-regulated cells (Fig. 10B). These data suggest that the decrease in viral protein expression levels in the GPS1 down-regulated cells was not caused by protein degradation. These results also indicate that protein expression under the control of the pol II promoter is not affected by GPS1 down-regulation.

The lack of involvement of M2 in the transcription and replication of vRNA begs the question: how is the GPS1-M2 interaction involved in the polymerase activity of influenza virus? To examine whether the addition of M2 has any effect on the viral polymerase activity, I performed the mini-replicon assay in the presence and absence of M2. I found no appreciable differences between the luciferase activities in the AllStars siRNA-transfected cells and the influenza virus NP down-regulated cells in the presence or absence of M2 (Fig. 10C). Moreover, there was no appreciable difference between the luciferase activity in the GPS1 down-regulated cells in the presence of M2

compared with that in these cells in the absence of M2 (Fig. 10C).

Therefore, these data suggest that GPS1 is involved in influenza virus polymerase activity but that this involvement does not directly involve M2 or protein expression.

Effect of GPS1 down-regulation on viral protein and RNA expression.

Because the mini-replicon assay showed a significant decrease in viral polymerase activity in GPS1 down-regulated cells, I next examined the effects of GPS1 down-regulation on the expression levels of virus proteins and RNAs in virus-infected cells. First, to confirm whether GPS1 plays a role in virus transcription and translation, viral protein expression levels were assessed in the context of the virus infection. Cells transfected with each siRNA were infected with virus at an MOI of 10, and cell lysates were collected at 0, 3, 6, 9, and 12 hpi. The viral proteins PA, HA, NP, and M2 were then detected by western blotting. The results showed that viral proteins started to be detected from 9 hpi in both AllStars siRNA-transfected cells and in GPS1 down-regulated cells, but that viral protein expression levels were significantly lower in GPS1 down-regulated cells compared with in AllStars siRNA-transfected cells (Fig. 11).

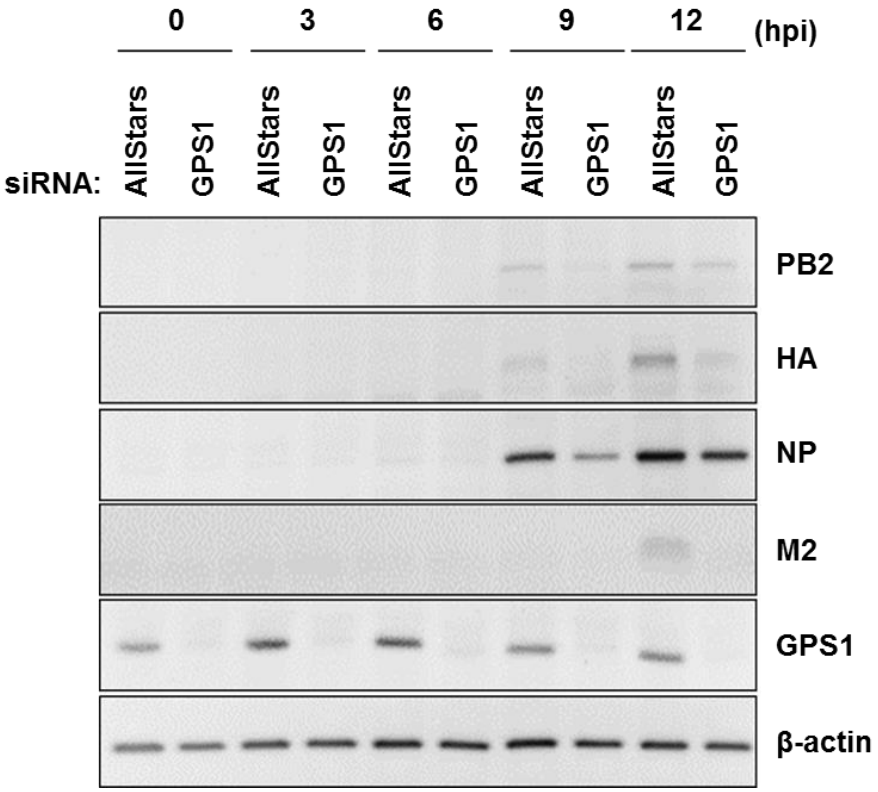

Figure 11. Effect of GPS1 down-regulation on viral protein expression. Expression levels of viral proteins were examined in virus-infected cells. siRNA-treated HEK 293 cells were infected with virus at an MOI of 10. The cells were lysed at 0, 3, 6, 9, and 12 hpi, and viral proteins were subjected to western blotting.

To examine whether viral RNA expression levels were also affected by GPS1 down-regulation, I next quantitated the expression levels of the vRNA, complementary RNA (cRNA), and messenger RNA (mRNA) of the NP segment by using real-time PCR. siRNA-treated cells were infected with virus in the same manner as in the previous experiments, and total RNA was extracted at 0, 3, 6, 9, and 12 hpi. The numbers of RNA copies of all vRNA, cRNA, and mRNA in the NP-gene down-regulated cells were 10%–20% of that those in the AllStars siRNA-transfected cells at 12 hpi. The number of vRNA copies in GPS1 down-regulated cells was almost the same as that in AllStars siRNA-transfected cells until 3 hpi, but decreased to 59% of that in AllStars siRNA-transfected cells at 12 hpi (Fig. 12A). The number of cRNA copies in GPS1 down-regulated cells started to decrease at 6 hpi, and the relative number of cRNA copies in GPS1 down-regulated cells decreased to 65% of that in AllStars siRNA-transfected cells at 12 hpi (Fig. 12B). The number of mRNA copies in GPS1 down-regulated cells showed a similar decreasing tendency to that seen for the cRNA copies. The number of mRNA copies in GPS1 down-regulated cells decreased to 39% of that in AllStars siRNA-transfected cells at 12 hpi (Fig. 12C). Therefore, all vRNA, cRNA, and mRNA expression levels were impaired in GPS1 down-regulated cells, demonstrating that GPS1 is essential for efficient viral transcription and replication.

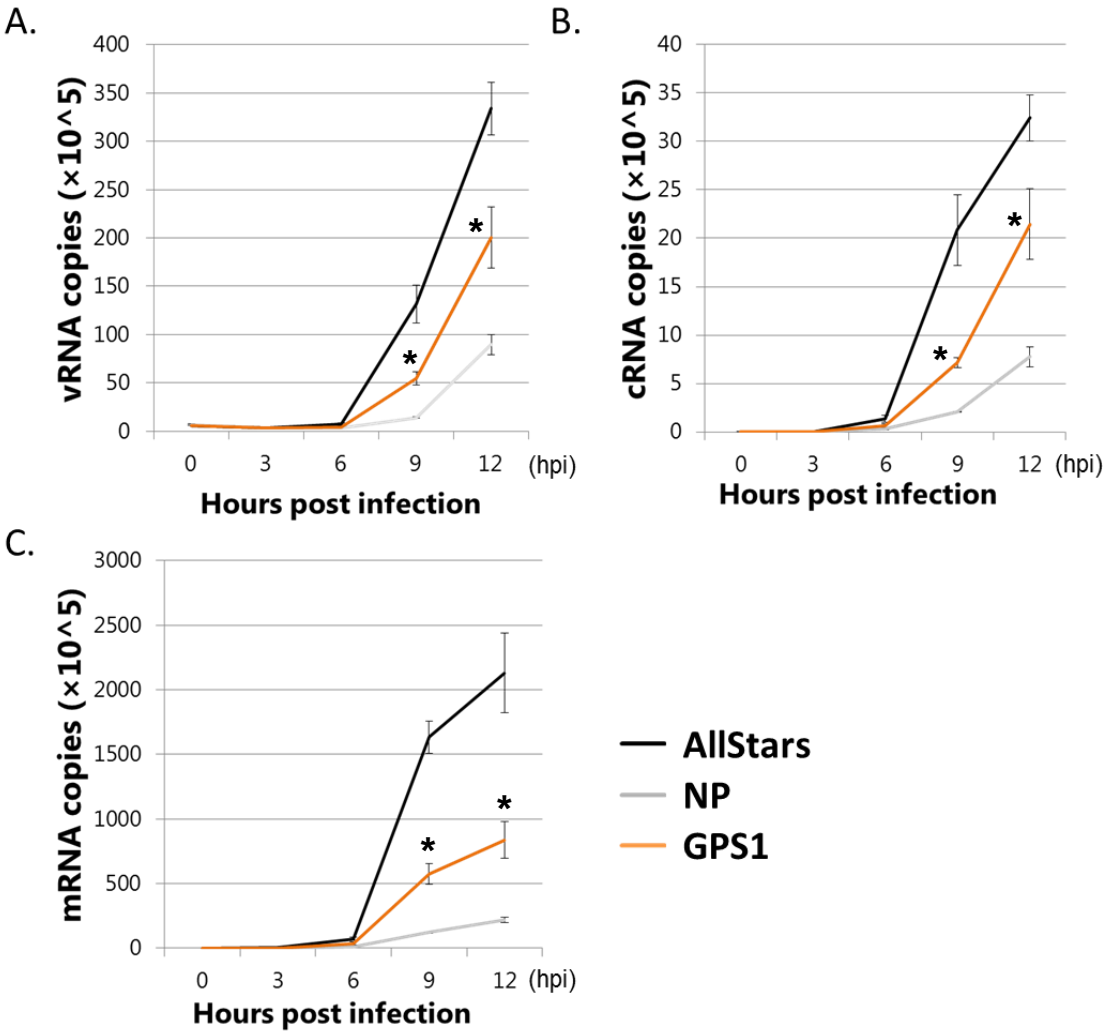

Figure 12. Effect of GPS1 down-regulation on viral RNA expression. Kinetics of synthesis of (A) vRNA, (B) cRNA, and (C) mRNA of the NP segment were examined by quantitative real-time PCR. The experiments were performed in triplicate; error bars represent the standard deviation of triplicate samples. Asterisks (*) indicate a p value < 0.05, for three independent experiments. The statistical analysis was carried out by using ANOVA followed by Dunnet's test.

GPS1 and viral polymerase proteins.

Because my findings suggested the involvement of GPS1 in virus polymerase activity, I used an immunofluorescence assay to examine the intracellular localization of GPS1 and the influenza virus polymerase proteins PB2, PB1, PA, and NP, which are essential for viral genome transcription and replication. At 12 hpi, GPS1 as well as PB2, PB1, and NP were mainly localized in the nucleus of virus-infected cells, but PA localized only in the cytoplasm (Fig.13A). Then, to determine whether these viral proteins co-localize with GPS1 in the nucleus of virus-infected cells, I performed a three-dimensional analysis by using the z-stack function of the confocal microscope. This anaylsis showed that the polymerase subunits and NP proteins did not co-localize with GPS1 in the nucleus (Fig. 13B), suggesting that GPS1 does not interact directly with the influenza virus polymerases or NP.

GPS1 is also important for the replication of VSV

To determine whether the down-regulation of GPS1 reduces the growth of other viruses, I examined the effect of GPS1 down-regulation on the growth of vesicular stomatitis virus (VSV). As with influenza virus, VSV is an enveloped virus possessing a single-stranded, negative-sense RNA as its genome; however, VSV replicates in the

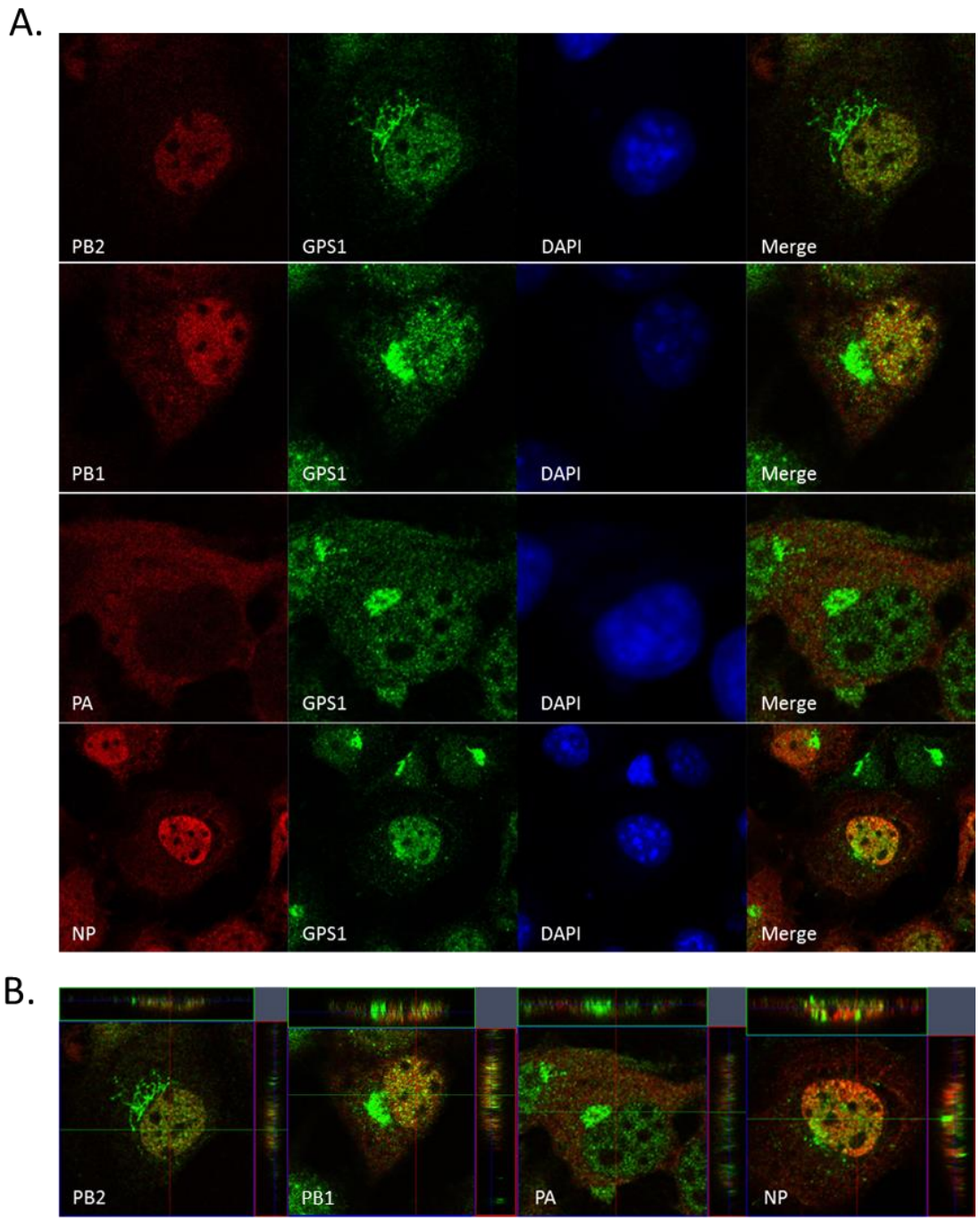

Figure 13. Intracellular localization of GPS1, NP, and viral polymerase proteins. (A) The intracellular localization of GPS1 and PB2, PB1, PA, and NP were analyzed by use of an indirect immunofluorescence assay. HEK 293 cells were infected with influenza virus at an MOI of 10, and PB2, PB1, PA, and NP were detected by antibodies for each protein (red) at 12 hpi. Endogenous GPS1 was detected by an anti-GPS1 antibody (green), and the nuclei were stained with Hoechst 33342 (blue). (B) z-stack analysis of the intracellular localization of GPS1, NP, and the viral polymerase proteins.

cytoplasm. HEK293 cells were treated with AllStars siRNA, siRNA for the GPS1 gene, or siRNA for the VSV polymerase L gene. siRNA for the VSV polymerase L gene was used as a positive control for the reduction of virus growth. siRNA-treated cells were infected with VSV at an MOI of 0.001, and the cell supernatants were harvested at 24 hpi. A plaque assay was then performed to determine the virus titer. The virus titer was 1.2×10^{9} PFU/ml in the AllStars siRNA-transfected cells (Fig. 14). The virus titers of the supernatant of the VSV L gene down-regulated cells and of the GPS1 down-regulated cells were reduced by about one to three log units compared with that of the AllStars siRNA-transfected cells (Fig. 14). These data demonstrate that GPS1 also plays an important role in VSV replication.

GPS1 plays a role in the activation of the NF-κB signaling pathway

The COP9 signalosome regulates the NF-κB signaling pathway (Fig. 3), in part because GPS1 interacts with IκB, which negatively regulates NF-κB transcription. To elucidate whether GPS1 down-regulation influences the activation of the NF-κB signaling pathway, I examined the activation of the NF-κB signaling pathway by performing a reporter assay. Cells were treated with either AllStars siRNA or siRNA for GPS1. Twenty-four hours later, pNF-κB-luc plasmid, which encodes the luciferase gene controlled by an NF-κB enhancer element, and a plasmid for the expression of MAP

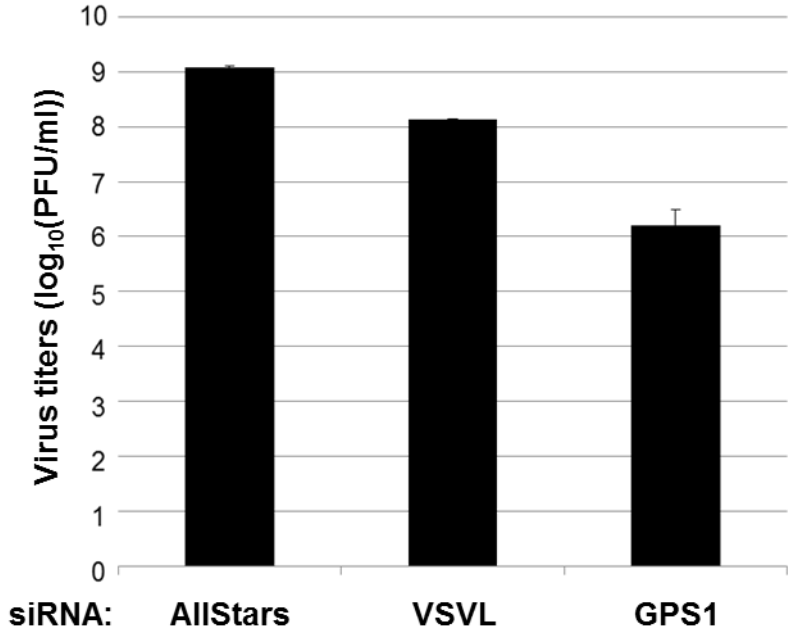

Figure 14. Effect of GPS1 down-regulation in VSV. The virus titer of VSV in GPS1 down-regulated cells was assessed by means of a plaque assay. VSVL represents the polymerase L protein of VSV, which is responsible for virus genome transcription and replication.

kinase kinase kinase (MEKK), which activates the NF-κB signaling pathway, were co-transfected into the siRNA-treated cells. The cells were lysed after another 24 h, and luciferase activities were measured. The luciferase activities were not detected in either the AllStars siRNA-transfected cells or the GPS1 down-regulated cells without MEKK stimulation (Fig.15). On the other hand, when the AllStars siRNA-transfected cells and the GPS1 down-regulated cells were stimulated with MEKK, there was a significant increase in luciferase activities in both cells. Importantly, the luciferase activity in the GPS1 down-regulated cells was 47% of that in the AllStars siRNA-transfected cells. These results indicate that GPS1 is required for the effective activation of the NF-κB signaling pathway.

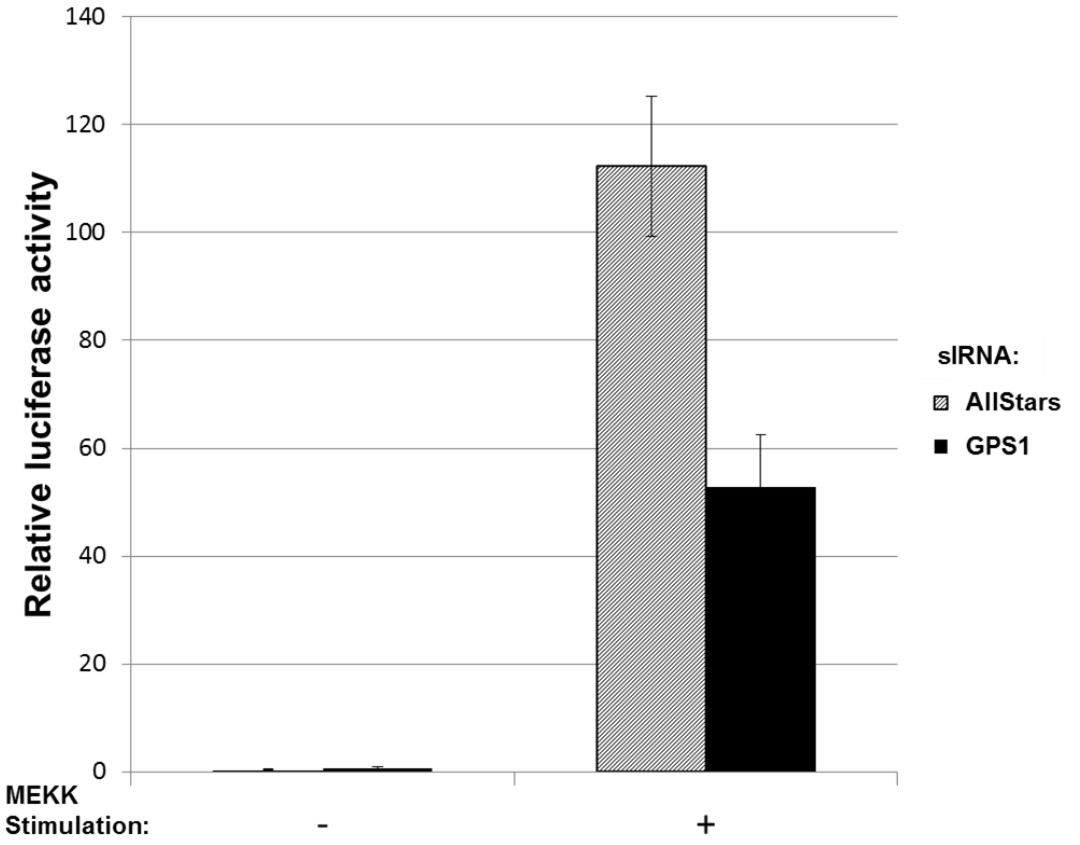

Figure 15. Effect of GPS1 down-regulation on NF-κB signaling. The activity of the NF-κB signaling pathway was assessed by using a lucifearse assay. HEK293 cells were treated with siRNA AllStars, or siRNA for GPS1. Twenty-four hours later, an NF-κB reporter plasmid encoding luciferase and an NF-κB signaling pathway activating protein MEKK expressing plasmid were transfected; luciferase activities were measured 24 h after the plasmid transfection.

Discussion

In recent years, several genome-wide screens have been conducted to identify host proteins that are important for influenza virus replication [11-17,28]. However, the host-mediated mechanisms involved in influenza virus replication remain largely unknown. Here, I demonstrated that GPS1 interacts with the influenza virus protein M2 (Fig. 5A) and is essential for efficient virus replication (Fig. 6). Further analysis showed that influenza virus polymerase activity (Fig. 9A) and the expression levels of viral proteins (Fig. 11) and viral RNAs (Fig. 12) were significantly decreased in GPS1 down-regulated cells. These data demonstrate that GPS1 is required for the efficient transcription and replication of the influenza virus genome.

How does GPS1 contribute to the transcription and replication of the influenza virus genome? We know that NF-κB signaling is activated upon influenza virus infection [49,50] and that the major inducer of NF-κB signaling upon infection is reported to be the accumulation of single-stranded vRNAs [51]. Although NF-κB signaling is an important inducer of the host innate immune response, previous studies have shown that active NF-κB signaling is required for the efficient replication of influenza virus [42-46]. Kumar *et al*. showed that inhibition of NF-κB signaling by the

NF-κB inhibitor PDTC, which inhibits IκB-ubiquitin ligase activity, or by the down regulation of the NF-κB subunit p65 via siRNA, results in the suppression of vRNA synthesis as well as virus replication [44]. Measurement of vRNA, cRNA, and mRNA expression levels in virus-infected cells at 5 hpi showed that vRNA synthesis, but not that of cRNA or mRNA, is impaired by the NF-κB inhibitor – a finding that was confirmed in a plasmid-based RNA transcription assay. Suppression of vRNA synthesis eventually should lead to the suppression of cRNA and mRNA synthesis, which is consistent with my result that all vRNA, cRNA, and mRNA expression was suppressed upon virus infection (Fig. 12). Therefore, the suppression of the viral polymerase activity, as shown by the mini-replicon assay (Fig. 9A), would be related to the role of GPS1 or the COP9 signalosome in NF-κB signaling via interactions with IκB.

Another question that needs to be answered is how does the M2-GPS1 interaction affect viral replication? I showed that GPS1 co-precipitates with FLAG-tagged M2 in plasmid-transfected cells by using an anti-FLAG antibody, although co-precipitation of GPS1 with M2 in virus-infected cells has not yet been observed (data not shown). This disparity may be due to the anti-M2 and anti-GPS1 antibodies used here that were not available for the immunoprecipitation assay. Although I attempted to determine the role of M2 in viral transcription and replication

by comparing viral polymerase activity with or without M2 protein expression, I found no appreciable difference (Fig. 10C). This result suggested that the M2-GPS1 interaction may play a role in the transcription and replication of vRNA in a somewhat indirect way, such as the regulation of NF-κB signaling. Given that GPS1 interacts with IκB [41] and is required for NF-κB signaling activity (Fig.15), I propose the following hypothesis: Newly synthesized M2 binds to GPS1, which is present as a complex with IκB-NF-κB. The binding of M2 with the complex via GPS causes the dissociation of IκB from NF-κB. Dissociation of IκB exposes the NF-κB nuclear localization signal (NLS), resulting in the nuclear translocation of NF-κB and the enhancement of viral transcription and replication. Further investigations are required to test the validity of this hypothesis especially the mechanism on the role of M2 in GPS1 mediated viral transcription and replication. Because M2 is highly conserved among influenza A viruses, it is likely that influenza A viruses commonly utilize GPS1 in their replication cycle. In future studies, the effect of GPS1 overexpression in a mini-replicon assay and an NF-κB reporter assay should also be examined.

Many viruses, such as human immunodeficiency virus type 1 (HIV-1) cytomegalovirus, herpes virus, human papillomavirus type 16, hepatitis B virus, and Epstein–Barr virus (EBV) utilize the NF-κB signaling pathway in their replication

cycles [52]. For example, HIV-1, which possesses two NF-κB-binding sites in its long terminal repeat [53], manipulates the NF-κB signaling pathway during the transcription of its viral genome. When NF-κB binds to the binding site on the long terminal repeat region, viral transcription is promoted. It seems reasonable that DNA viruses and RNA viruses with reverse transcriptase activity would also have NF-κB-binding sites in their genomes that could be utilized for viral transcription. Although it remains unknown whether NF-κB activates the transcription and replication of RNA viruses such as influenza virus and VSV by directly interacting with the viral RNAs, it could be the case, as has been shown with DNA viruses. Further investigation of the mechanisms involving NF-κB signaling and the transcription and replication of RNA viruses is warranted.

In conclusion, I demonstrated that the host protein GPS1 contributes to influenza virus replication by supporting the transcription and replication of the influenza virus genome, possibly through NF-κB signaling. Since GPS1 also contributes to VSV replication, this host protein may be used by many negative-strand RNA viruses. Further unveiling of the functions of GPS1 may lead to the discovery of an anti-viral drug that is effective in a variety of viral infections.

Materials and Methods

Cells and Viruses

Human embryonic kidney cells (HEK293 cells) were cultured in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS) and a penicillin-streptomycin solution (Sigma-Aldrich, St. Louis, MO). Madin-Darby canine kidney (MDCK) cells were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum (FCS). Baby hamster kidney cells (BHK cells) were cultured in Dulbecco's modified Eagle medium containing 5% FCS. Cells were incubated at 37 °C in 5% CO_2. Influenza A/WSN/33 (WSN; H1N1) was generated by use of reverse genetics asdescribed previously [54] and propagated in MDCK cells. VSV was propagated in BHK cells.

Plasmids and transfection reagents

Plasmids for the expression of GPS1 were constructed by using RNA extracted from HEK293 cell as a template for RT-PCR. The PCR product was inserted into pCAGGS/MCS [55]. Viral RNA expressing plasmids or viral protein expressing plasmids were generated as described elsewhere [54]. pNF-κB-luc and pMEKK were

purchased from Stratagene. Plasmids were transfected to HEK293 cells with Trans IT 293 reagent according to the manufacturer's protocol (Mirus, Wisconsin, WI).

Antibodies

The rabbit anti-GPS1 (16-30), mouse anti-β actin (AC-74), mouse anti-FLAG epitope (F1804), and anti-FLAG M2 magnetic beads were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). The mouse anti-M2 antibody (SS23R15-1) was kindly provided by Ayato Takada, Hokkaido University. The mouse anti-M1 antibody (WS-27/52), mouse anti-HA antibody (WS3-54), mouse anti-PB2 antibody (21/3), mouse anti-PB1 antibody (45/10), mouse anti-PA antibody (65/4), and mouse anti-Aichi NP antibody (2S-347/3) were available in our laboratory.

Western blotting

Protein samples were lysed with 1×Tris-Glyscine SDS sample buffer (Invitrogen, Carlsbad, CA) with 10 mM dithiothreitol. Then, the lysates were treated at 95 °C for 10 min and were immediately placed on ice. When the samples were prepared, each sample was applied to an Any KD Mini-PROTEAN TGX gradient gel (BioRad, Hercules, CA) and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was

performed. Following SDS-PAGE, the proteins in the gel were transferred electrophoretically to PVDF membranes (Millipore, Bedford, MA) with transfer buffer (100 mM Tris, 190 M Glycine, 10% methanol). The membranes were blocked by using Blocking One (Nacalai Tesque, Kyoto, Japan), and the proteins were detected by using Chemi-Lumi One Super (Nacalai Tesque, Kyoto, Japan) and visualized with the VersaDoc Imaging System (BioRad, Hercules, CA).

Indirect-Immunofluorescence Assay

HEK 293 cells were infected with influenza virus at MOI of 10. Cells were fixed with 4% paraformaldehyde (PFA) and permeabilized with 0.2% Triton X-100. A confocal microscope with an LSM510 system (Carl Zeiss, Oberkochen, Germany) was used for microscopic examinations. Nuclei were stained with Hoechst 33342 (Invitrogen, Carlsbad, CA).

Immunoprecipitation assay

FLAG M2 and M2 protein expressing plasmids were each transfected into HEK 293 cells by using the Trans IT 293 transfection reagent. Twenty-four hours later, the cells were washed with PBS and lysed with 1 ml of lysis buffer [50 mM Tri HCl

(pH7.5), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and protease inhibitor mixture Complete Mini (Roche)] for 1 h at 4 °C. Then, the cellular debris in each sample was removed by centrifugation. Supernatants were then incubated with 10 μl of anti-FLAG M2 magnetic beads (Sigma-Aldorich, St. Louis, MO) overnight at 4 °C. After the affinity gels were washed three times with lysis buffer, 50 μl of 1×sample buffer was added to the samples. Finally, the samples were subjected to SDS-PAGE followed by western blotting.

siRNA treatment

siRNAs for GPS1 were purchased from QIAGEN as part of a predesigned genome-wide human siRNA library (FlexiTube siRNA; QIAGEN). The following siRNA target sequences for GPS1 were used: GPS1_2 (AAG AGC AGA CTC AGC GTT AAA), GPS1_3 (CAA GTG GGC GGT GTC CAT TAA), GPS1_5 (AAC CTT TAA CGT GGA CAT GTA), and GPS1_6 (CAG CCT GGA TCT GGA ACA GTA). For the negative control, AllStars Negative Control siRNA (QIAGEN, Tokyo, Japan) was used. siRNA targeting influenza virus WSN strain NP gene (GGA UCU UAU UUC UUC GGA GUU) was purchased from Sigma-Aldrich. HEK 293 cells, cultivated in 24-well plates, were transfected with 25 nM of the indicated siRNA by using the

Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA). The down-regulations of the targeted genes were evaluated by western blotting.

Virus Titration

To assess influenza viral replication, two parallel sets of siRNA-transfected cells were infected with WSN virus at an MOI of 0.001 per well of a 24-well tissue culture plate at 24 h after the second siRNA transfection. Forty-eight hours post-infection, supernatants were harvested and virus titers were determined by means of plaque assays in MDCK cells.

Minireplicon and Cell-Viability Assays

Following siRNA treatment, influenza virus RNA polymerase activity was assessed by using a minipoplicaon assay as described previously [57].

To evaluate the cell viability of siRNA-transfected cells, cell lysates were collected after 48 h of siRNA transfection, and cell viability was measured by using the CellTiter-Glo assay system (Promega, Madison, WI) according to the manufacturer's instructions.

Quantitative reverse transcription-PCR

siRNAs for the negative control, the influenza WSN NP gene, and GPS1 were transfected into HEK 293 cells as described previously. siRNA-transfected cells were then infected with WSN virus at an MOI of 10 at 4°C for 1 h, and cell lysates were collected at 0, 3, 6, 9, and 12 h post-infection. Total RNA was extracted by using the Maxwell 16 LEV simplyRNA Tissue Kit (Promega, Madison, WI). Reverse transcription and quantitation of vRNA, cRNA, and mRNA by realtime PCR were performed as previously described [58].

PB2-KO/Rluc virus assay

AllStars siRNA or siRNA for GPS1 was transfected into HEK 293 cells as described previously. A replication-incompetent PB2-knockout virus (PB2-KO/Rluc virus) [47], which possesses the *Renilla* luciferase reporter gene in the coding region of the PB2 gene, was used to infect siRNA-transfected cells at an MOI of 1. At 8 h post-infection, cells were lysed and the relative luciferase activities were measured by using the *Renilla* Luciferase Assay System (Promega, Madison, WI). Amantadine ion channel inhibitor (100 μg/ml) was used as a control.

Virus-like Particle (VLP) formation assay

HEK 293 cells were treated with the siRNA AllStars negative control and siGPS1 as described previously. siRNA-treated HEK 293 cells were then transfected with viral protein HA, NA, M1, and M2 expressing plasmids by using TransIT293 (Mirus, Madison, WI) according to the manufacturer's instructions. Forty-eight hours after the plasmid transfections, the cell lysates and supernatants were collected. The supernatants were centrifuged at 3000×*g* for 5 min at 4°C to remove cell debris. Then, the supernatant was layered over a 20% sucrose cushion, concentrated by ultracentrifugation at 50,000 rpm for 2 h at 4°C, and pelleted. The cell lysates and the supernatant were then analyzed by SDS-PAGE and western blotting.

NF-κB signaling pathway reporter assay

HEK 293 cells were treated with the siRNA AllStars negative control and siRNA targeting the GPS1 gene. The siRNA-treated cells were then transfected with 250 ng of pNF-κB-Luc and 25 ng of pMEKK. Twenty-four hours later, the cells were lysed and the levels of luciferase activities were determined by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

CONCLUDING REMARKS

Even though influenza viruses have existed in human history for a long time, annual epidemics and occasional pandemics are still serious public health concerns. In recent years, the emergence of anti-viral resistant viruses is an especially grave challenge that needs to be addressed. Currently available antiviral drugs target viral protein functions such as M2 ion channel activity (amantadine and remantadine), NA neuraminidase activity (oseltamivir, zanamivir, peramivir, and laninamivir), and polymerase activity (favipiravir). Given that most influenza viruses have already acquired resistance to amantadine and remantadine, these drugs are now rarely prescribed for the treatment of influenza. Currently, NA inhibitors are the primary treatment option for influenza virus infection; however, viruses that are resistant to these antiviral drugs have already been reported. This year, a polymerase inhibitor was approved for production, but it can be produced only when the highly pathogenic avian influenza viruses that are resistant to other antiviral drugs emerge. In other words, it is only a matter of time before viruses acquire resistance to the antiviral drugs that target viral protein functions. In the virus life cycle, influenza virus needs host protein functions to replicate. Therefore, the development of antiviral drugs that target host

proteins has potential as a countermeasure to the emergence of resistant viruses. Therefore, it is important to study host-mediated virus replication mechanisms.

In this thesis, I identified GPS1 as a host protein that interacts with M2 and supports the transcription and replication of influenza virus. Furthermore, I demonstrated that GPS1 is also necessary for proper NF-kB signaling, which is important for the efficient replication of influenza viruses. Although further analysis is required, the data obtained in this study provide a better understanding of host-mediated virus replication mechanisms.

Although the influenza virus polymerase inhibitor favipiravir has been approved with limitations, it is now attracting international attention as a potential treatment for Ebola virus infections. Moreover, it has been reported that favipiravir is also effective against norovirus and Lassa virus infections [59,60]. These reports suggest that antiviral drugs that target virus polymerase activity have the potential to be effective against more than one type of RNA viruses. Therefore, further studies on host-mediated influenza virus replication mechanisms may lead to the development of an antiviral drug that is effective for a broad range of viruses and has a low propensity to produce resistant strains.

ACKNOWLEDGEMENTS

I would like to acknowledge Prof. Yoshihiro Kawaoka, Dr. Takeshi Noda, and Seiya Yamayoshi, Department of Microbiology and Immunology, Institute of medical Science, The University of Tokyo, for their guidance and generous support during the course of this work. I also wish to thank, Dr. Hiroko Kozuka-Hata and Dr. Masaaki Oyama for conducting the mass spectrometry analysis. I thank Atsushi Nagata for performing the initial screening for the host factors that interacts with M2. I also thank Susan Watson for scientific editing. I am grateful for all the members of Dr. Kawaoka's labotarory for supporting me in every way during the years of my PhD course.

I am grateful for my parents, Masatoshi Kuwahara and Setsuko Kuwahara, and my brother Hidemi Kuwahara for their every support and encouragement. I would also like to express my sincere gratitude for my grandmother Toshi Maruyama for her mental and physical support. I am thankful for my special friends for helping me to get through tough times.

Lastly and most importantly, I am grateful for God for providing me with this opportunity and every needful thing during my graduate work and throughout my life. I will try to go forth and serve others using all what I have been blessed with.

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