ウイルス増殖に影響を与える宿主タンパク質の解析

The analysis of host proteins affecting the growth of viruses

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CONTENTS

INTRODUCTION

Influenza viruses were thought to be responsible for the disease described by Hippocrates in 412 BC (Hoehling, 1961) and thus they have been with us for a long time. Influenza remains a major cause of morbidity and mortality worldwide, and large segments of the human population are affected every year.

Influenza A viruses belong to the *Orthomyxoviridae* family. Influenza A viruses are further classified into subtypes based on the antigenicity of their HA and NA molecules; currently, 16 HA subtypes (H1–H16) and 9 NA subtypes (N1–N9) are known. Type A Influenza viruses have been isolated from various animals, including humans, pigs, horses, sea mammals and birds. Phylogenetic studies of type A isolates have revealed that the viral genes form species-specific lineages, and aquatic birds are thought to be the source of all influenza A viruses in other animal species (Webster *et al*, 1992).

Pandemics are outbreaks that impact large geographic area and large portions of the population in a short period of time. Three influenza pandemics emerged during the 20th century, the most devastating of which was the Spanish influenza, which was caused by an H1N1 virus and was responsible for the deaths of at least 40 million people in 1918–1919 (Johnson *et al*, 2002). Other, less serious pandemics occurred in 1957 (Asian influenza, H2N2), 1968 (Hong Kong influenza, H3N2), of which mortality were estimated to be approximately 1 million deaths or 750 thousand death, respectively.

Influenza pandemics are caused by viruses that possess an HA molecule to which most of the human population lacks immunity. Recently, purely avian influenza viruses, including the H5N1 subtypes, have been directly transmitted to humans, raising concern

over the possibility of a new influenza pandemic among the world's immunologically naïve populations. To control both a possible new pandemic influenza and seasonal influenza, we need to develop new anti-influenza drug as well as vaccine preparation.

After binding of cell surface receptors, influenza virus is introduced into cells via receptor-mediated endocytosis. The viral ribonucleoproteins (vRNP), which is composed of viral RNA, three polymerase subunit proteins (PA, PB1 and PB2) and nucleoprotein (NP), are then released from virions and transported to the nucleus, where viral RNA (vRNA) transcription and replication occur (Palese, 2007). : Once in the nucleus, the incoming negative-sense vRNA is transcribed into messenger RNA (mRNA) by a primer-dependent mechanism. These mRNA products are incomplete copies of the vRNA template and are capped and polyadenylated, unlike vRNA. Replication occurs via a two-step process. A full-length, positive-sense copy of the vRNA is first made that is referred to as complementary RNA (cRNA) and is in turn used as a template to produce more vRNA. Since the vRNA transcription and replication are essential steps in viral propagation, these steps are attractive targets for antiviral drug development. Therefore, it is important to identify these host proteins, which support or inhibit the function of vRNP, for accumulation of knowledge about the mechanism of viral replication.

To this end, I have developed an expression cloning method to identify host proteins that affect viral RNA and proteins synthesis. Conventional methods of the screening for such host proteins had been mainly by proteomics approach. Limitation of this approach is that one would not know whether such interaction is essential for viral replication. On the other hand, the expression cloning strategy I developed is based on evaluating reporter gene expression of influenza virus minigenome. This method is

useful for identification of host proteins involving in viral polymerase reaction regardless of their direct interaction.

In this thesis, I described two host proteins I identified by the expression cloning as regulatory factors to influenza virus, RuvB-like 2 and RSK2. RuvB-like 2, which is a members of the AAA+ family of DNA helicases, have roles in diverse cellular processes, including the response to DNA double-strand breaks and the control of gene expression (Gallant, 2007). I demonstrated that the protein directly interacts with vRNP, interfering vRNP assembly and leading to inhibition of virus replication and transcription. RSK2, a downstream mediator of mitogen-activated protein kinase (MAPKs) pathways, functions in the regulation of cell growth and differentiation (Carriere *et al*, 2008). The kinase was found to activate NF-κB- and IFN-β-dependent promoters and antiviral protein PKR in the RSK2 knockdown experiments, indicating that the kinase has important roles in innate immunity.

These studies provide new insight into not only the mechanism of viral replication but also the targets for the development of anti-virus drug.

RESULTS

To identify host proteins that regulate viral replication, I developed a screening system in which human embryonic kidney 293T cells are transfected with a plasmid for the synthesis of a virus-like RNA encoding EGFP (polI-EGFP) and with plasmids for the expression of the influenza A virus polymerase and NP proteins (Fig. 1A). The PB2 protein possesses glutamic acid at position 627, which attenuates replication in mammalian systems at 33°C, but allows efficient replication in avian systems (Shinya K *et al*, 2004). To identify avian host factors that 'rescue' efficient replication, I cotransfected cells with a cDNA expression library derived from quail QT6 cells. Cells were incubated for two days at 33˚C, then GFP-expressing cells were sorted by FACSCaliber (BD). After three rounds of selection, I extracted plasmid DNAs and sequenced them (Fig. 1B). To confirm that these proteins affect influenza virus RNA replication, I expressed them in human embryonic kidney 293 cells that also expressed the polymerase and NP proteins and a virus-like RNA encoding luciferase. A truncated RuvB-like 2 protein (qRBL2) that lacked 217 N-terminal amino acids (ΔN-qRBL2 ; Fig. 2A) was identified as one of the quail proteins that most upregulated viral replication.

RuvB-like proteins (RuvB-like 1 and RuvB-like 2; RBL1 and RBL2) are members
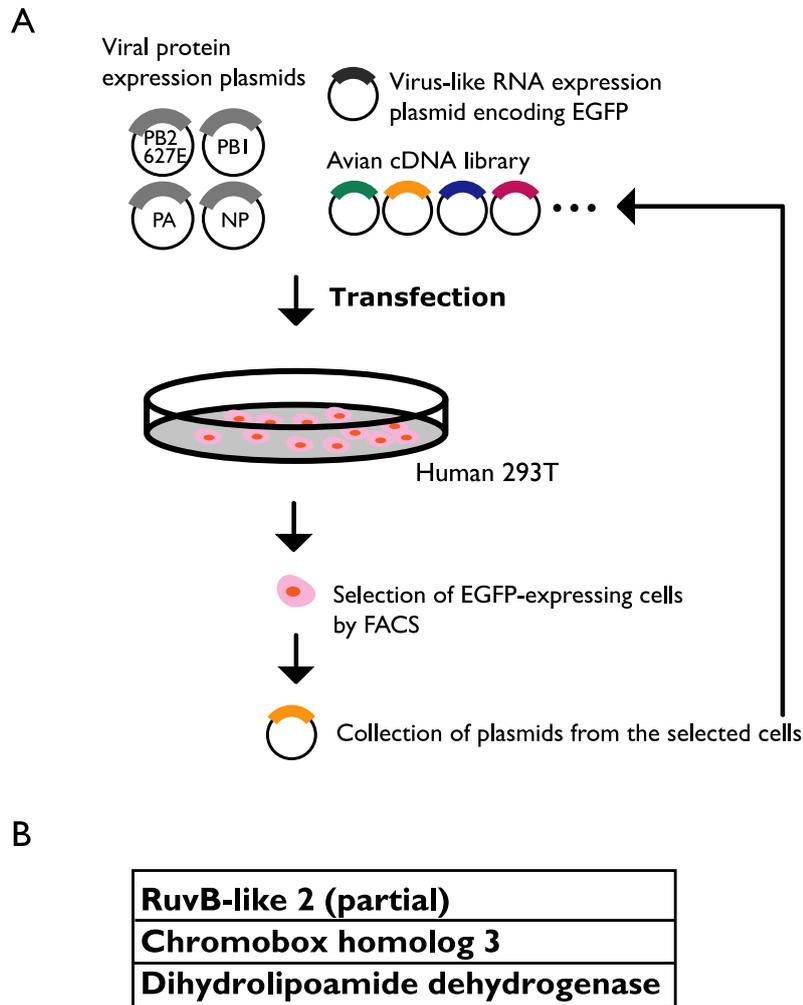
Figure 1. Identification of cellular proteins that enhance influenza virus replication.

(A) Cells were transfected with plasmids encoding the A/Hong Kong/483/97 (H5N1) PB2, PB1, PA, and NP proteins (the PB2 protein possesses glutamic acid at position 627; PB2-627E). Cells were cotransfected with polI-EGFP for the synthesis of a virus-like RNA, and with an avian (quail cell) cDNA expression library. Cells expressing avian proteins that support efficient replication by PB2-627E in mammalian cells at 33°C produce increased amounts of EGFP. GFP-expressing cells were selected by a FACS cell sorter. After three rounds of selection, plasmid DNA was extracted from the cells and sequenced.

(B) Quail proteins were identified that upregulated reporter gene expression from the influenza virus minigenome by use of the screening approach shown in (A).

A

B

C

hpi	4	6	8	4	6	8
M1/actin	1.0	7.1	91	1.0	6.2	54
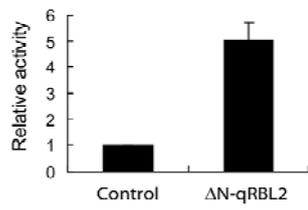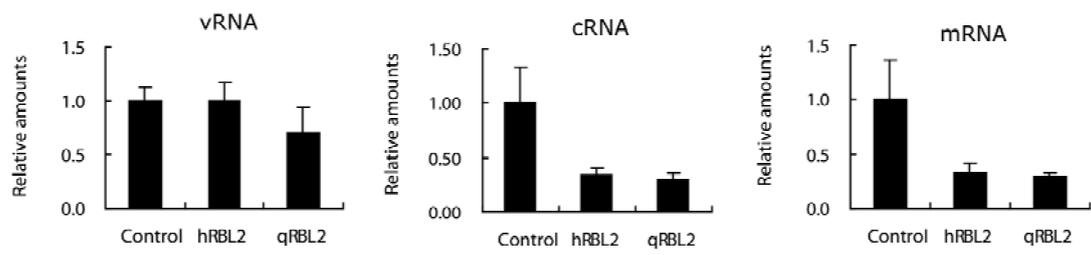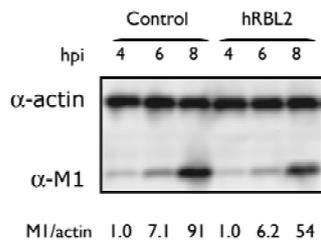
D
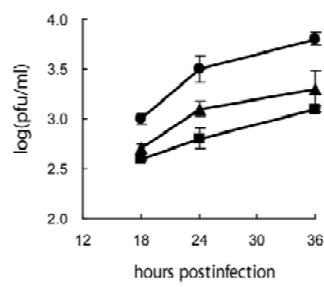
Figure 2. Effect of RBL2 expression on influenza virus replication and growth.

(A) N-terminally deleted qRBL2 increases influenza virus replication in a minireplicon assay. 293T cells were transfected with plasmids expressing HK483 PB1, -PB2, -PA, and -NP proteins, pPolI-Luc, and N-terminally deleted quail RBL2. Twenty-four hours after incubation at 33˚C, luciferase expression was detected.

(B) Viral polymerase activity in cells expressing hRBL2 or qRBL2. 293 cells were transfected with plasmids expressing qRBL2 or hRBL2, or a control vector. Twenty-four hours later, cells were infected with WSN (moi = 1). Three hours later, RNA was extracted and quantified by real-time RT-PCR with primer sets specific for NP vRNA, cRNA, or mRNA. These values were normalized to beta-actin. The error bars represent standard deviations (n=3).

(C) Viral M1 protein production in cells overexpressing hRBL2. 293 cells expressing hRBL2 or a control vector were infected with WSN (moi = 3). At the indicated hours post-infections (hpi), cell lysates were subjected to western blot analysis with antibodies against M1 and beta-actin. The values show the ratio of M1 to actin normalized to control cells 4 hours after infection.

(D) Influenza virus titers in 293 cells overexpressing hRBL2 or qRBL2. 293 cells overexpressing hRBL2 (closed square), qRBL2 (closed triangle), or a control vector (closed circle) were infected with WSN virus (moi = 0.05). The cells were incubated at 37˚C for the indicated time periods. Virus titers in the supernatant were determined by plaque assays in Madin Darby canine kidney (MDCK) cells. The error bars represent standard deviations (n=3).

of the AAA+ (ATPases associated with diverse cellular activities) family of helicases. They share moderate homology with bacterial RuvB, the ATP-dependent motor of the RuvAB complex that drives branch migration of the holiday junction (Gallant, 2007). RBL1 and RBL2 are essential for viability in yeast and *Drosophila melanogaster*, and may have similarly important roles in humans (Kanemaki *et al*, 1999; Bauer *et al*, 2000).

In mammalian cells, the RuvB-like proteins modulate cellular transformation, signaling, apoptosis, and the response to DNA damage by interacting with proteins such as β-catenin, c-Myc, and ATF2 (Bauer *et al*, 2000; Wood *et al*, 2000; Cho *et al*, 2001). Moreover, they modulate ribosomal RNA processing and small nucleolar RNA maturation (King *et al*, 2001; Watkins *et al*, 2004), and function in complexes such as the chromatin remodeling complex INO80 (Shen *et al*, 2000) and the histone acetylase Tip60 complex (Ikura *et al*, 2000). A role for RuvB-like proteins in the regulation of viral infections has not been described to date. However, using a genome-wide RNA interference (RNAi) screen in *Drosophila* to identify host genes important for influenza virus replication, I found that knockdown of the *Drosophila* homolog of human RBL2 (hRBL2) enhanced reporter gene expression of the influenza virus replicon (Hao L *et al*, 2008). In addition, Mayer *et al.* (2007) identified hRBL2 as a cellular interaction partner of influenza vRNPs. However, neither Hao *et al.* nor Mayer *et al.* assessed the biological

significance of the RBL2 interaction with influenza vRNPs.

I overexpressed the full-length hRBL2 and qRBL2 in 293 cells that were subsequently infected with influenza A/WSN/33 virus (H1N1; WSN) at a multiplicity of infection (moi) of 1. Assessment of viral RNA (vRNA), complementary RNA (cRNA), and mRNA levels by Real-Time RT-PCR three hours postinfection revealed downregulation of polymerase activity upon overexpression of hRBL2 or qRBL2 (Fig. 2B). As a consequence, viral protein synthesis (Fig. 2C) and replication (Fig. 2D) were restricted. These findings indicate that ΔN-qRBL2 (identified in our screen) likely enhances influenza viral replication by acting as a dominant negative protein that competes with the endogenous RBL2, and that RBL2 is a general, rather than host-species specific, host factor that suppresses influenza virus replication.

To further demonstrate that RBL2 interferes with influenza viral replication, I knocked it down in 293 cells by use of a specific siRNA (Fig. 3A, left and middle lanes). In these cells, the amounts of viral transcripts were increased relative to control cells (Fig. 3B, compare white and black bars), an effect that was partially reversed upon transfection of siRBL2-treated cells with a plasmid expressing hRBL2 (pChRBL2; Fig. 3A, right lane; and Fig. 3B, light gray bar). Further, viral protein production and replication increased in siRBL2-treated cells (Fig. 3C and 3D), demonstrating that hRBL2 has an antiviral effect

A

siRBL2   -   +   +
pChRBL2   -   -   +

α-RBL2

α-actin

B

vRNA

Relative amounts

15

10

5

0
          3h   5h

cRNA

Relative amounts

150

120

90

60

30

0
          3h   5h

mRNA

Relative amounts

160

120

80

40

0
          3h   5h

☐ si Control + Empty vector   ■ si RBL2 + Empty vector   ☐ si RBL2 + pChRBL2

C

                si Control   siRBL2
hpi   4   6   8   4   6   8

α-actin

α-M1

M1/actin   1.0 47 131 1.7 73 132

D

log(pfu/ml)

4.0

3.5

3.0

2.5
          15h   20h
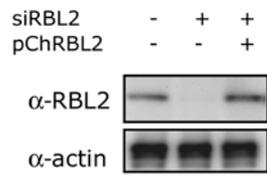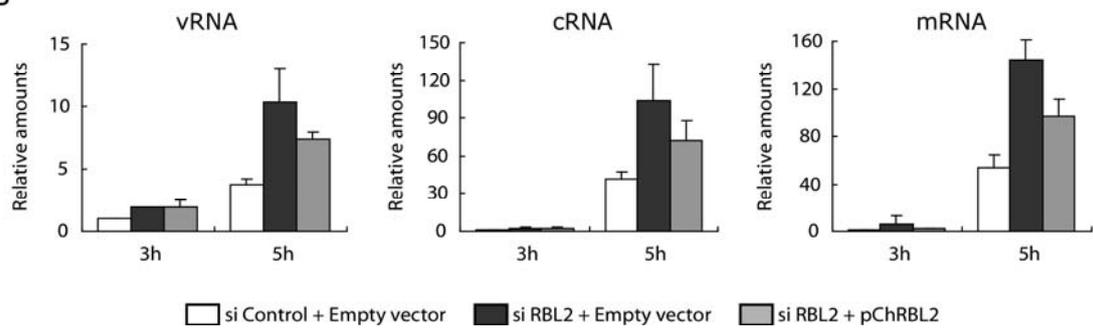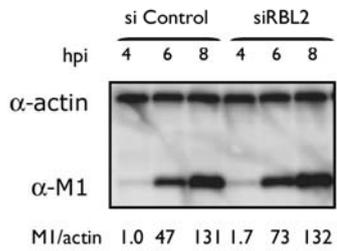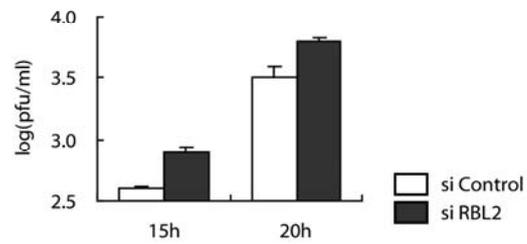
☐ si Control   ■ si RBL2

Figure 3. Effect of hRBL2 knockdown on influenza viral replication.

(A) Knockdown of hRBL2 in 293 cells. 293 cells were transfected with an siRNA specific to hRBL2, or a non-specific control siRNA. Cells were also transfected with a plasmid expressing hRBL2 (pChRBL2) or a control vector. Two days later, hRBL2 expression levels were assessed by western blot analysis. Beta-actin expression levels served as an internal control.

(B) Viral polymerase activity in cells treated with an siRNA to hRBL2. 293 cells were transfected with hRBL2-specific or control siRNAs and incubated at 37˚C for 48 hours. The transfectant cells were then infected with WSN (moi = 1). The amounts of vRNA, cRNA, and mRNA were determined as described for Fig. 2b.

(C) Viral M1 protein production in hRBL2 knockdown cells and control cells. Cells were transfected with siRNAs as described above and infected with WSN virus at an moi of 3. M1 protein levels were assessed as described for Fig. 2c.

(D) Influenza virus titers in hRBL2 knockdown cells and control cells. Cells were treated with siRNAs as described above, infected with WSN virus at an moi of 0.05, and incubated at 37˚C for the indicated time periods. Virus titers were determined in MDCK cells. The error bars represent standard deviations (n=3).

on influenza virus replication.

RBL2 possesses ATPase activity, which is critical for its biological function (Gallant, 2007; Cho *et al*, 2001). I, therefore, assessed a dominant negative form of RBL2 that lacks ATPase activity (hRBL2-DN; Cho *et al*, 2001) for its effects on influenza viral growth. I found that hRBL2-DN interfered with polymerase activity and viral growth at almost the same level as the wild-type protein (data not shown). Hence, the ATPase activity of hRBL2 is not critical for its antiviral effect.

To investigate the interaction of hRBL2 with influenza vRNPs, I expressed hRBL2, the three polymerase subunits, and cMyc-tagged NP protein in the absence or presence of a virus-like RNA. hRBL2 interacted with the viral replication complex regardless of the presence of vRNA (Fig. 4A).

To assess which viral protein interacts with hRBL2, cMyc-tagged polymerase and NP proteins were separately expressed with hRBL2 and immunoprecipitated with beads coated with an anti-cMyc antibody. Interestingly, western blot analysis showed hRBL2 interaction with three different vRNP components – NP, PB2, and PB1 (Fig. 4B).

To further study the interaction of hRBL2 with vRNP components, I used a bimolecular fluorescence complementation (BiFC) assay (Hu *et al*, 2003; Kerppola, 2006) in which the proteins of interest were fused to N-terminal and C-terminal portions
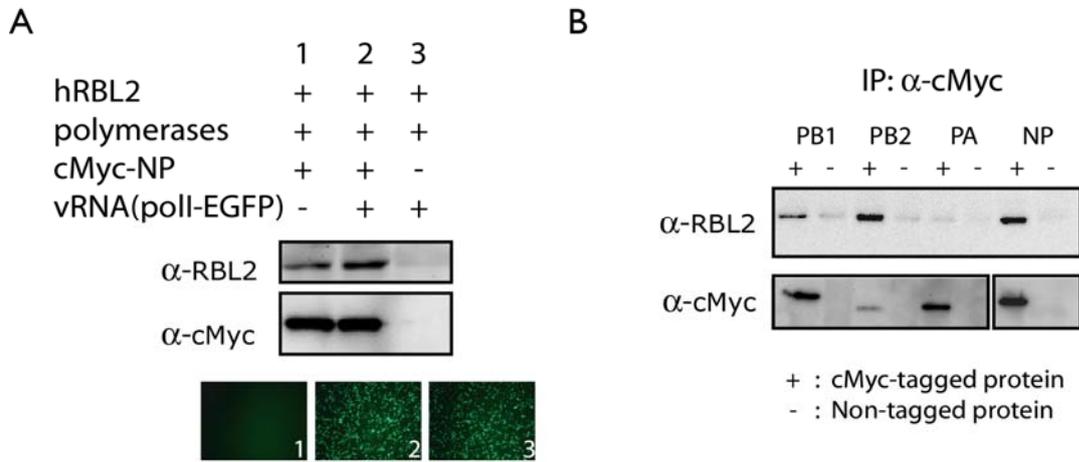
Figure 4. Interaction of hRBL2 with viral proteins.

(A) hRBL2 interacts with viral RNP complexes. The three viral polymerase proteins and cMyc-tagged NP were expressed in 293T cells in the absence or presence of virus-like RNA (lanes 1 and 2). Non-tagged NP was expressed as a negative control (lane 3). One day later, cell lysates were immunoprecipitated with anti-cMyc beads. Western blot analysis was carried out with anti-hRBL2 and anti-cMyc antibodies. EGFP expression in transfected cells indicates the functionality of cMyc-tagged NP protein.

(B) hRBL2 interacts with NP, PB2 and PB1. The three polymerase and NP proteins were individually tested for their interaction with hRBL2 as described above.

of Kusabira-Green (resulting in 'GN' and 'GC' fusion proteins, respectively); interaction of the proteins of interest thus resulted in Kusabira-Green fluorescence (Fig. 5A, upper panel). Strong fluorescence was detected for the hRBL2 interaction with NP (Fig. 5A); whereas, the level of fluorescence suggested no or moderate interaction of hRBL2 and the polymerase proteins in this assay. NP was found to interact with hRBL2 in both the immunoprecipitation and BiFC assays. To assess if hRBL2 interferes with NP oligomerization, which is important for its biological activities (Elton *et al*, 1999; Ye *et al*, 2006), I tested the NP-NP interaction in cells overexpressing hRBL2. As expected, I detected NP-NP interaction in the BiFC assay (Fig. 5B, upper panel); however, hRBL2 overexpression decreased the levels of fluorescence (indicative of NP-NP interaction) in a dose-dependent manner (Fig. 5B, lower left panel). Similarly, reporter gene expression from a virus-like RNA was also reduced by hRBL2 expression in a dose-dependent manner (Fig. 5B, lower right panel), demonstrating the functionality of NP fusion products and the biological significance of the hRBL2 interference with NP oligomerization. Collectively, these data suggest that hRBL2 disrupts the proper assembly of NP oligomers and, thereby, interrupts its biological activities. In addition to identifying this new host antiviral mechanism, our study also suggests RBL2 as a promising target for antiviral drug development.
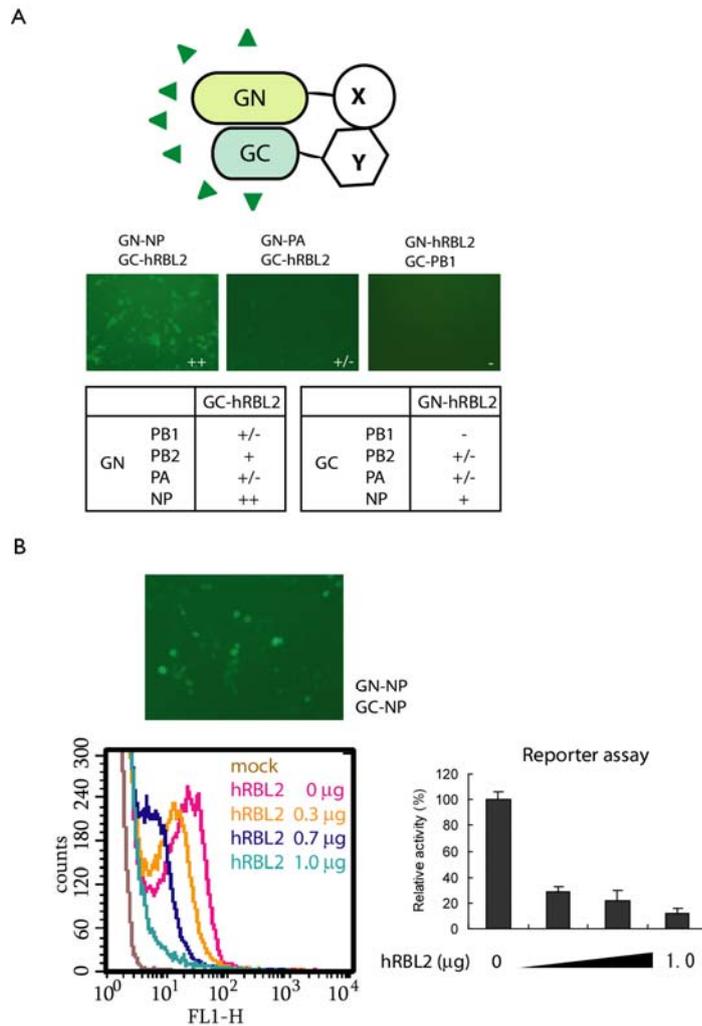
Figure 5. hRBL2 interferes with NP oligomerization.

(A) BiFC assay to assess interactions between hRBL2 and vRNP components. The proteins of interest were fused to the N- or C-terminal portions of Kusabira Green (GN or GC). Interaction between the proteins of interest thus results in fluorescence. hRBL2 interacts with NP, but not with the polymerase subunits.

(B) hRBL2 inhibits NP oligomerization in a dose-dependent manner. Cells were transfected with plasmids for the expression of PB2, PB1, PA (each 0.5μg), pPolI-GFP (0.05 μg), pGN-NP (0.25μg) and pGC-NP (0.25μg) in 12-well plates. NP-NP interaction was detected by microscopy and FACS analysis (red curve). This interaction decreased with increasing amounts of hRBL2 (see FACS analysis). Reporter gene expression in transfected cells indicates the functionality of the GN-NP and GC-NP fusion proteins and the biological significance of hRBL2 interference with NP oligomerization.

To identify the other host factor(s) that regulate influenza virus RNA synthesis, I modified the original expression cloning method (see Fig. 1) for regulatory factors to influenza virus as shown in Fig. 6. In this screen, I used feline CD2 (fCD2) as a reporter protein (Shimojima *et al*, 2003) to assess viral replication efficiencies. I cotransfected cells with plasmids for expression of the A/Hong Kong/483/97 (H5N1) virus NP, PB1, and PA proteins; and a plasmid for expression of a mutant PB2 possessing PB2-627E (Fig. 6A). The PB2-627E mutation restricts amplification of the virus-like RNA in human cells at 33°C. To identify the avian host factor(s) that 'rescues' efficient replication, even with PB2-627E, I cotransfected human 293T cells with a cDNA expression library derived from quail QT6 cells. Avian host factors that mediate efficient replication of PB2-627E virus should support high levels of fCD2 expression from the virus-like RNA. The fCD2-positive cells were selected with an antibody against fCD2. After four rounds of selection, I extracted plasmids from the fCD2-positive transfectants and subjected them to sequencing (Fig 6A, lower panel). Since the cDNAs for Protein-O-mannosyltransferase 1 and Reticulon 4 were inserted in the anti-sense orientation, I focused on the quail homolog of the ribosomal protein S6 kinase alpha 3 (Gallus gallus RSK2). However, this protein was missing 310 N-terminal amino acids (avian ΔN-RSK2; Fig. 6B). To confirm that avian ΔN-RSK2 enhances influenza virus

A

Viral protein
expression plasmids

PB2E PB1

PA NP

Virus-like RNA expression
plasmid encoding fCD2

Avian cDNA library

• • •

Transfection

Human 293T

Selection of fCD2 expressing cells

Collection of plasmids from the selected cells
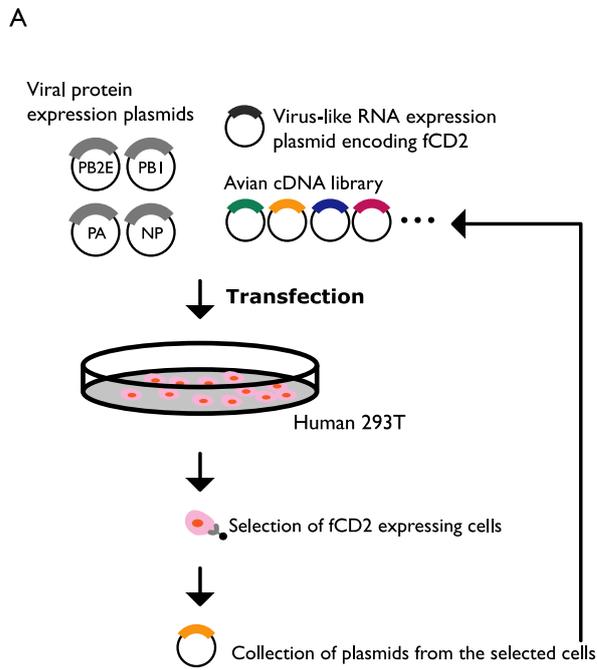
| Protein-O-mannosyltransferase 1 (antisense) |
| Reticulon 4 (antisense) |
| Ribosomal protein S6 kinase alpha 3 (partial) |

B

S227 T365 S369 S386 T577

NKD CKD

CKD

C

Relative activity (%)

120
100
80
60
40
20

pCAGGS   pCAGGSΔN-RSK2

D

Relative activity (%)

100

50

0

pCAGGS   pCAGGS pCAGGS
          -hRSK2   -gRSK2

1 2 3

RSK2

β-actin

1 : pCAGGS
2 : pCAGGS-hRSK2
3 : pCAGGS-gRSK2
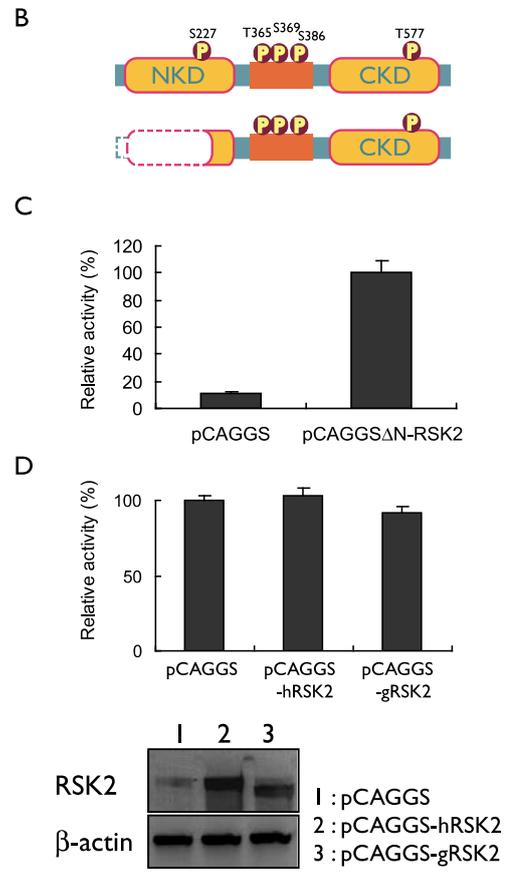
Figure 6. Identification of cellular proteins that enhance influenza virus replication and RSK2 down-regulates influenza virus replication.

(A) Human embryonic kidney 293T cells were transfected with plasmids encoding the components of the influenza viral replication complex (PB2, PB1, PA, and NP). The PB2 protein possesses glutamic acid at position 627 (PB2-627E), which supports efficient replication in avian cells, but not in mammalian cells at 33°C. Cells were cotransfected with a plasmid for the synthesis of a virus-like RNA encoding feline CD2 (polI-fCD2), and with an avian (quail cell) cDNA library. Cells expressing avian proteins that support efficient replication by PB2-627E in mammalian cells at 33°C will produce increased amounts of fCD2. Cells with high levels of fCD2 were selected by immunoaffinity using anti-fCD2 antibody. After a total of four rounds of selection, plasmid DNA was extracted from the cells and sequenced (lower panel). cDNAs for Protein-O-mannosyltransferase 1 and Reticulon 4 were inserted in the anti-sense direction.

(B) Schematic diagram of human RSK2 (upper portion) and the N-terminally deleted avian RSK2 protein selected in our screening approach (lower portion). The C-terminal kinase domain (CDK) is activated by ERK1/2, resulting in the activation of the N-terminal kinase domain (NKD). The NKD domain subsequently phosphorylates target proteins.

(C) Influenza viral polymerase activity in 293T cells expressing avian ΔN-RSK2. Cells were transfected with plasmids expressing PB2E, PB1, PA, and NP, a virus-like RNA encoding luciferase (pPolI-luc), and the N-terminally deleted avian RSK2 protein (pCAGGS-ΔN-RSK2) or the 'empty' control vector (pCAGGS). Cells were maintained at 33°C for 24 hours, and were then subjected to luciferase assays. The error bars represent standard deviations from three independent experiments.

(D) Influenza viral polymerase activity in 293T cells expressing human RSK2 (pCAGGS-hRSK2) and Gallus gallus RSK2 (pCAGGS-gRSK2). Luciferase assays were performed as described above. The error bars represent standard deviations from three independent experiments. In parallel, RSK2 and Beta-actin (as a internal control) expression levels were assessed by western blotting (lower panel).

replication in mammalian cells at 33°C, I overexpressed this protein in human cells that expressed the viral replication complex components (i.e., PB2-627E, PB1, PA, and NP) and a virus-like RNA that encodes the luciferase reporter protein. Our results indicate that luciferase expression was elevated in cells expressing avian ΔN-RSK2 protein relative to control cells expressing the 'empty' expression vector (Fig. 6C).

Next, I cloned the full-length avian and human RSK2 proteins and tested their ability to enhance PB2-627E-mediated replication in human cells at 33°C; however, I did not detect a significant effect for either protein (Fig. 6D). RSK family members are unusual among serine/threonine kinases in that they contain two distinct kinase domains that are sequentially activated (Fig. 6B). The C-terminal kinase domain (CKD) is activated by ERK1/2, which then triggers subsequent activation of the N-terminal kinase domain (NKD) (reviewed in Carriere *et al*, 2008). The NKD of RSK2 then phosphorylates a broad range of substrates, including cAMP response element-binding protein, c-Fos, glycogen synthase kinase-3, and many others (reviewed in Carriere *et al*, 2008). ΔN-RSK2 encodes the entire C-terminal kinase domain of this protein (Fig. 6B) and may sequester free ERK1/2, thereby preventing activation of functional RSK2. Thus, I speculated that full-length RSK2 has antiviral activity, and that ΔN-RSK2 acts as a dominant-negative factor that suppresses RSK2 and its antiviral activity, resulting in

increased viral replication.

To test our hypothesis that RSK2 has antiviral activity against influenza virus, I knocked down RSK2 in 293 (human embryonic kidney) cells using a retroviral vector expressing shRNA specific to human RSK2; these cells are termed shRSK2 cells. RSK2 expression was reduced to approximately 15% in shRSK2 cells relative to the expression level in control shGFP cells, which express shRNA against green fluorescent protein (Fig. 7A). As speculated, RSK2 knockdown resulted in increased viral polymerase activity for PB2-627E at 33°C (Fig. 7B) and, as a consequence, resulted in increased production of the viral M1 protein (Fig. 7C).

The data obtained thus far indicate that RSK2 suppresses influenza virus replication. My finding that full-length avian or human RSK2 expression did not have a significant effect on viral replication (Fig.6D) suggested that just because the kinases are overexpressed does not mean that they are sufficiently activated (i.e., phosphorylated) to suppress viral replication. Although RSK2 is thought to be a suppressor of influenza virus replication, whether it interferes with influenza virus replication in general, or in a strain- or host-specific manner, remains unclear. To address this question, I generated influenza viruses that possessed either a lysine at position 627 of PB2 (PB2-627K), which confers efficient replication in mammalian cells at 33°C and 37°C,
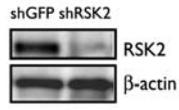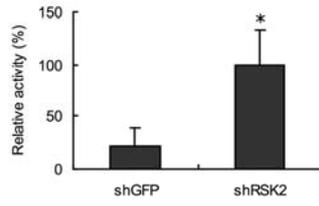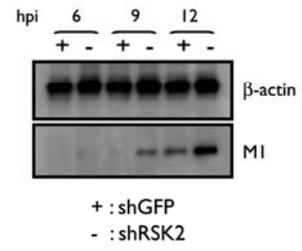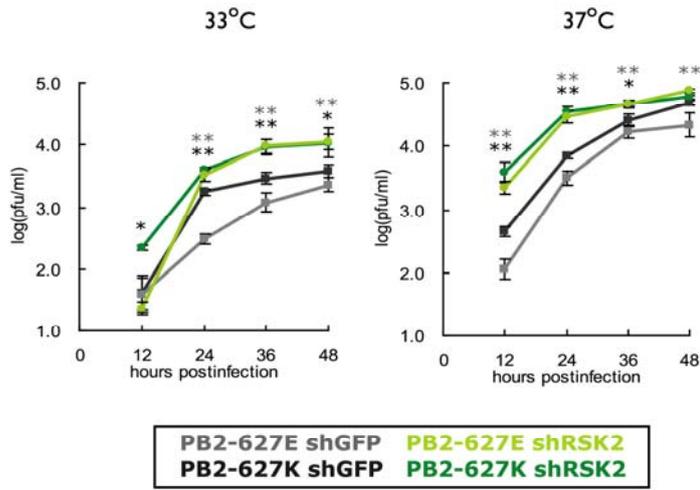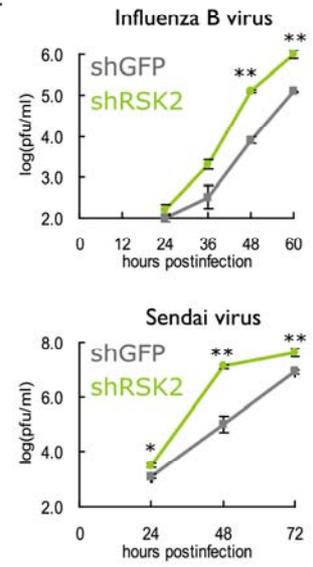
Figure 7. Effect of RSK2 knockdown on influenza viral replication.

(A) Knockdown of human RSK2. 293 cells were transduced with retroviral vectors for shRNAs to human RSK2 or GFP (as a control), resulting in shRSK2 and shGFP cells. RSK2 expression levels were assessed by western blotting with an antibody to this protein. Beta-actin expression levels served as an internal control.

(B) Viral polymerase activity in shRSK2 and shGFP cells. Cells were transfected with plasmids that directed synthesis of PB2-627E, PB1, PA, NP, and pPolI-luc. After incubation at 33°C for 24 hours, cell lysates were prepared and submitted to luciferase assays. Knockdown of RSK2 resulted in more efficient replication of the virus-like RNA, suggesting that RSK2 suppresses influenza virus replication. The error bars represent standard deviations from three independent experiments. The statisitcal significance of the difference between the control and test samples is shown by the *P* value, which was determined by the Student's *t*-test (*; *P*< 0.05).

(C) Viral protein production in shRSK2 cells and shGFP cells. shRSK2 and shGFP cells were infected with virus possessing PB2-627E and incubated at 33°C. At the indicated time points post-infection, western blot analysis was carried out with antibodies against M1 and beta-actin.

(D) Influenza A virus growth in shRSK2 cells. shRSK2 (green) or control shGFP cells (gray) were infected at an m.o.i. of 0.05 with PB2-627E or PB2-627K virus, which possess glutamic acid or lysine at position 627 in the PB2 protein, respectively. Cells were incubated at 33°C or 37°C for the indicated time periods. Virus titers were determined in MDCK cells. The error bars represent standard deviations from three independent experiments. The statisitcal significance of the difference between the control and test samples is shown by *P* values, which were determined by the Student's *t*-test (**; *P*<0.01, *; *P*< 0.05; black asterisks indicate the PB2-627K virus and gray asterisks indicate the PB2-627E virus).

(E) Influenza B virus and Sendai virus growth in shRSK2 cells. shRSK2 (green) and control shGFP cells (gray) were infected at an m.o.i. of 0.05 with influenza B virus (upper panel) or Sendai virus (lower panel). At the indicated time points after infection, cells were harvested and the virus titers were determined in MDCK cells. The error bars represent standard deviations from three independent experiments. The statisitcal significance of the difference between the control and test samples is shown by the *P* value, which was determined by the Student's *t*-test (**; *P*<0.01, *; *P*< 0.05).

or a glutamic acid at this position (PB2-627E), which results in attenuation of replication

in mammalian cells at 33°C and, to a lesser extent, at 37°C. Growth of both viruses was

tested at 33°C and 37°C in shRSK2 cells and control shGFP cells. Briefly, cells were

infected at a multiplicity of infection (m.o.i.) of 0.05 with PB2-627E or PB2-627K virus,

and virus titers in the culture supernatant were determined by plaque formation assays in

MDCK cells at various times post-infection. As expected, the PB2-627K virus grew

more efficiently than the PB2-627E virus in control shGFP cells at 33°C (Fig. 7D, left

panel), while only minor differences in growth were observed for PB2-627K and

PB2-627E viruses in control shGFP cells at 37°C (Fig. 7D, right panel); these findings

are consistent with the earlier data (Hatta *et al*, 2007). When comparing viral growth

properties in control shGFP and shRSK2 cells (i.e., gray versus green growth curves), I

found more efficient growth in shRSK2 cells for both viruses and at both temperatures,

demonstrating that the nature of the amino acid at PB2-627 does not affect the antiviral

effect mediated by RSK2; all subsequent infection experiments were therefore carried

out with PB2-627K virus. Together, these findings indicate that RSK2 is a general

antiviral host factor that suppresses influenza A virus replication.

To further examine the antiviral function of RSK2, I assessed the growth kinetics

of an influenza B virus (Fig. 7E, upper panel) and Sendai virus (a negative-sense RNA

virus belonging to the family *Paramyxoviridae*; Fig. 7E, lower panel) in shRSK2 and

shGFP control cells. These viruses grew more efficiently in shRSK2 cells as compared

to control shGFP cells. Hence, RSK2 may have broad antiviral effects; for example, it

may trigger an innate immune responses.

In quiescent cells, RSK2 is maintained in an inactivate form in a complex with

ERK1/2 (reviewed in Roux *et al*, 2003). Interaction with phosphorylated ERK1/2 results

in RSK2 phosphorylation, dissociation from ERK1/2, and (partial) translocation to the

nucleus (Chen *et al*, 1992). To assess whether RSK2 is activated upon influenza virus

infection, I immunoprecipitated RSK2 from cell lysates obtained from influenza virus-

or mock-infected cells and subsequently evaluated the phosphorylation status of RSK2

using antibodies specific to distinct phosphorylated forms of RSK2. Influenza virus

infection resulted in increased levels of phosphorylated RSK2 as compared to

mock-infected cells (Fig. 8A, B). The most efficient induction of phosphorylation was

observed for Thr577. This residue is phosphorylated by ERK1/2, resulting in the

activation of RSK2 and the subsequent autophosphorylation of other phosphorylation

sites, such as Ser386 (reviewed in Carriere *et al*, 2008). The observed phosphorylation of

RSK2 upon influenza infection may result from direct interaction with a viral component,

or from ERK1/2 signaling, as the 'classical' MAPK (Raf/MEK/ERK) pathway is known
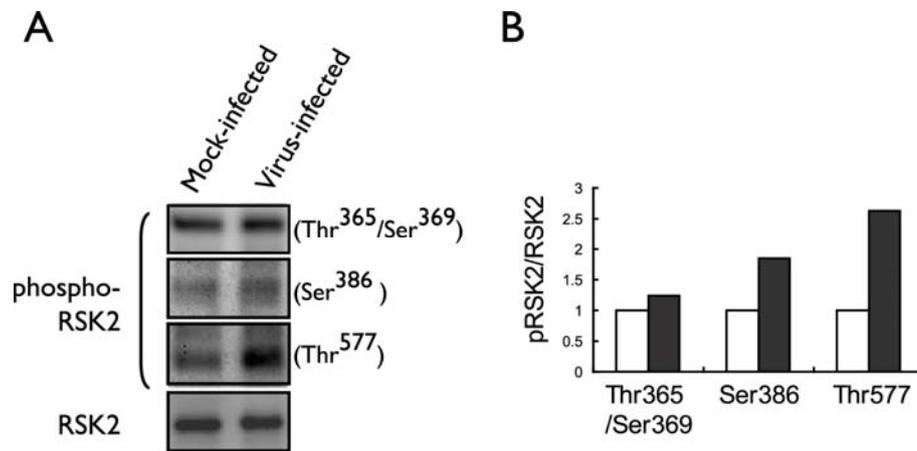
Figure 8. Influenza virus–induced phosphorylation of RSK2.

(A) 293 cells were infected with the PB2-627K virus (m.o.i. of 3.0) or remained mock-infected. Three or five hours later, cells lysates were immunoprecipitated with anti-RSK2 antibody, followed by western blot analysis with the indicated antibodies to phosphorylated forms of RSK2.

(B) Graphical representation of data shown in (A). The relative ratios of phosphorylated to non-phosphorylated RSK2 are depicted. Influenza virus infection results in phosphorylation of RSK2.

to be activated by influenza virus infection (Pleschka, 2008).

Next, I assessed the mechanism(s) by which RSK2 affects influenza virus replication. RSK2 activates NF-κB (Ghoda *et al*, 1997; Schouten *et al*, 1997), a major player in innate immune responses to viral infections (reviewed in Hiscott *et al*, 2001). Moreover, NF-κB is known to be activated upon influenza virus infection (Pahl *et al*, 1995). Therefore, I speculated that influenza virus-induced activation of RSK2 may lead to the stimulation of NF-κB and subsequent induction of an antiviral response. To test this assumption, I expressed the luciferase reporter protein under the control of an NF-κB-dependent promoter in virus- or mock-infected shRSK2 and control shGFP cells (Fig. 7A). As expected, influenza A virus infection induced NF-κB promoter activity in shGFP cells (Fig. 9A, white bar); the level of promoter stimulation was similar to that published elsewhere (Wurzer *et al*, 2004). In contrast, no significant increase in NF-κB-dependent promoter activity was observed in virus-infected shRSK2 cells (Fig. 9A, black bar), suggesting that virus-induced NF-κB stimulation relies on RSK2 activation. This lack of activation was partially restored following transfection of shRSK2 cells with pmRSK2, which encodes a human RSK2 cDNA containing a silent mutation within the siRNA target sequence (Fig. 9A, gray bar). In conclusion, these findings suggest that RSK2 may affect influenza virus replication, at least in part,
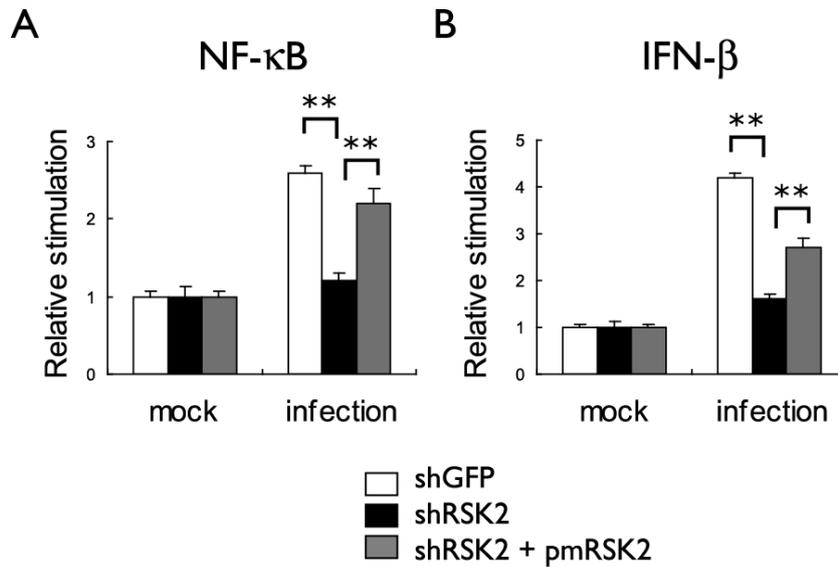
Figure 9. Effect of RSK2 knockdown on influenza virus-induced activation of NF-κB- and IFN-β-dependent promoters.

Plasmids that express luciferase under the control of NF-κB- (A) or IFN-β-dependent (B) promoters were transfected into shRSK2 (black bars) or control shGFP cells (white bars). Nine hours post-transfection, cells were infected with PB2-627K virus at an m.o.i. of 1; control cells remained mock-infected. After twelve hours of incubation at 33°C, cell lysates were prepared and submitted to luciferase assays. In a parallel experiment, cells were also transfected with pmRSK2 (gray bars), which encodes a silently mutagenized human RSK2 that is not recognized by the RSK2 shRNA. Influenza virus induced stimulation of NF-κB- and IFN-β-dependent promoters in shGFP cells (white bars); this stimulation is blocked by RSK2 knockdown (black bars), but can be partially restored upon expression of RSK2 that is insensitive to the RSK2 shRNA (gray bars). The error bars represent standard deviations from three independent experiments. P values were determined by the Student's t test (**; $P<0.01$).

through NF-κB.

NF-κB stimulation leads to the expression of multiple cellular factors, including IFN-β, a central player in the innate immune response that is activated upon virus infection. I asked whether RSK2 activates IFN-β-stimulated promoters. I carried out experiments essentially as described in the previous section. That is, I cloned the luciferase gene under the control of an IFN-β-stimulated promoter and assayed luciferase expression in virus- and mock-infected shRSK2 and control shGFP cells (Fig. 9B). Influenza virus infection activated IFN-β-stimulated promoter activity in control shGFP cells (Fig. 9B, white bar), but not in shRSK2 cells (Fig. 9B, black bar). This impediment was partially overcome by transfection of shRSK2 cells with pmRSK2 (Fig. 9B, gray bar). These results demonstrate that RSK2 activates both NF-κB and IFN-β signaling pathways upon influenza infection in 293 cells.

PKR is an important component of IFN-mediated antiviral responses (reviewed in Haller *et al*, 2007). This kinase phosphorylates the α subunit of initiation factor eIF2 (eIF2α), resulting in rapid inhibition of translation and restriction of the spread of the virus (Balachandran *et al*, 2000; Barber *et al*, 2005; Gale Jr *et al*, 1998; Goodman *et al*, 2007). Recently, PKR was also identified as an RSK2 substrate (Zykova *et al*, 2007). I tested whether RSK2 affects influenza virus replication through PKR phosphorylation

and activation. As expected, phosphorylation of PKR upon influenza virus infection was apparent in control shGFP cells (Fig. 10A; see also Fig. 10B, white bars, for an increasing ratio of phosphorylated vs. non-phosphorylated PKR upon virus infection). In contrast, PKR phosphorylation was suppressed in shRSK2 cells (Fig. 10A and black bars in Fig. 10B), indicating that influenza virus-induced activation of RSK2 results in PKR phosphorylation in 293 cells.
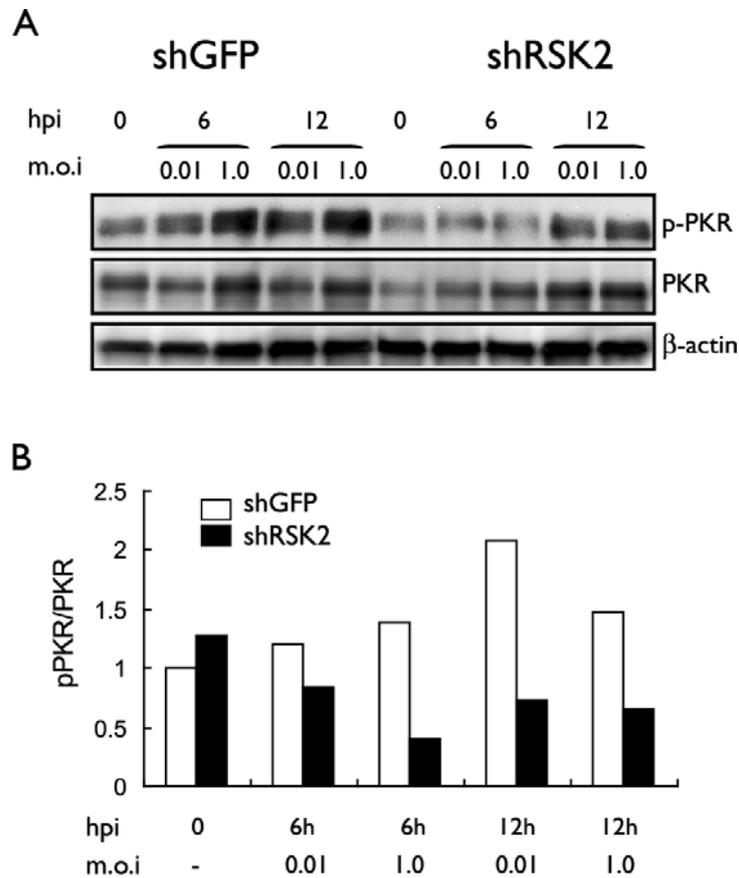
Figure 10. Effect of RSK2 knockdown on influenza virus-induced phosphorylation of PKR.

(A) shRSK2 cells or control shGFP cells were infected with PB2-627K virus at an m.o.i. of 0.01 or 1 and incubated at 33°C. At the indicated time points post-infection, cell lysates were prepared and subjected to western blot analysis with antibodies against PKR, phosphorylated PKR, or β-actin, which served as an internal control.

(B) Graphical representation of data shown in (A). The relative ratios of phosphorylated to non-phosphorylated PKR are depicted. Influenza virus infection results in increased levels of phosphorylated PKR in control shGFP cells, but not in shRSK2 cells.

DISCUSSION

To identify host proteins that affect viral RNA and proteins synthesis, I developed an expression cloning method. Of the candidate proteins identified with this system, I analyzed two host proteins, RuvB-like 2 and RSK2. I demonstrated that RBL2 inhibits influenza virus polymerase activity by disrupting NP formation. During viral replication and transcription, newly-synthesized polymerase subunits and NP protein enhance viral RNA replication and transcription. RBL2 likely acts by "catching " each new NP, resulting in disruption of the NP oligomer and the inability of the oligomer to execute its proper function.

Mammalian RuvB-like proteins are implicated in a wide range of cellular activities, including DNA replication and repair, cell cycle progression, and chromatin remodeling (Gallant, 2007). Structural analysis revealed that RBL2 and RBL1 can assemble into a double hexameric ring complex *in vitro* and that the dodecamer is likely one of the functional forms of the proteins (Puri, 2006; Gribun, 2008). Currently, it is not clear whether one of the hexamers contains exclusively RBL2 and the other RBL1, or whether both contain equimolar amounts of RBL2 and RBL1 (Gallant, 2007). However, each protein is thought to have specific roles, and some data suggest subunit-specific

functions that may not require RBL1/RBL2 complex formation (Gallant, 2007). This is in line with findings by Mayer *et al.* who identified hRBL2, but not hRBL1, as a cellular interaction partner of vRNPs (Mayer *et al.*, 2007). Moreover, I did not detect a role for hRBL1 in influenza viral infection (data not shown). Hence, the inhibitory effect of hRBL2 on influenza virus seems to be a specific function of RBL2, rather than of the RBL1/RBL2 hetero-oligomer.

I demonstrated that the MAPK-activated protein kinase RSK2 plays a role in the innate immune response to influenza virus infection as shown in Fig. 11. RSK2 is known to phosphorylate cellular factors involved in cell proliferation, regulation of transcription, regulation of translation, cell survival and apoptosis (reviewed in Carriere *et al*, 2008), and may affect viral growth through these processes. The HIV Tat protein is known to interact with RSK2 (Hetzer *et al*, 2007), resulting in RSK2 activation. Activated RSK2 is, in turn, critical for the transcriptional activity of Tat (Hetzer *et al*, 2007). Another study demonstrated that ORF45 of the Kaposi's sarcoma-associated herpesvirus (KSHV) interacts with RSK1 and RSK2, resulting in their stimulation (Kuang *et al*, 2008). Further studies suggest that RSK1/2 upregulation plays a critical role in the lytic replication of KSHV (Kuang *et al*, 2008). Another study suggested that RSK2 signaling is important for efficient vaccinia virus amplification (Andrade *et al*, 2004). It is
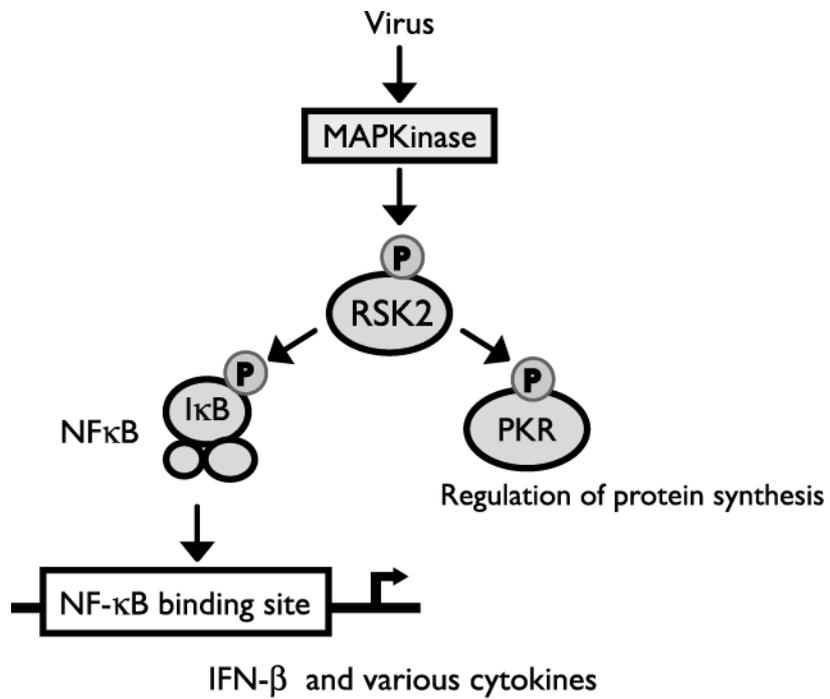
Figure 11. Putative role of RSK2 in antiviral signaling.

Influenza virus infection may activate RSK2 through MAPK kinase signaling pathways, or through direct interaction with a viral component. Our data suggest that RSK2 activation is needed for efficient stimulation of NF-κB, IFN-β, and PKR, all of which play major roles in the cellular antiviral response.

interesting to note that these studies ascribe a virus-supportive role to RSK2 activation.

In contrast to the above-mentioned studies, which found a virus-supportive role for RSK2, our findings suggest an antiviral activity for this kinase. In particular, I found that the C-terminal domain of RSK2, which seems to act in a dominant-negative manner, and an siRNA to RSK2 increased influenza virus polymerase activity in *in vitro* assays and/or enhanced influenza virus replication. RSK2 is known to be activated by the Raf/MEK/ERK signaling cascade (reviewed in (Lu *et al*, 2008)). Studies by Pleschka et al. (Pleschka *et al*, 2001) suggest that this pathway has a virus-supportive function. This finding seems to contradict our finding that RSK2 has antiviral activity. However, the virus-supportive function of the Raf/MEK/ERK signaling cascade was established by use of an inhibitor to MEK (Pleschka *et al*, 2001), which may affect several pathways that are controlled by MEK/ERK. In addition, activation of RSK1/2 is controlled not only by ERK1/2, but also by other MAPKs, such as p38 (reviewed in Gaestel *et al*, 2008; Raman *et al*, 2007), which is activated upon influenza virus infection and triggers an antiviral response (Conze *et al*, 2000). Collectively, the current data suggest that RSK2 is activated by both virus-supportive and -antagonistic signaling pathways, and that the complex interplay of these factors and pathways determines its downstream effects.

Our data suggest that RSK2 may execute its antiviral function through NF-κB. Some studies have found a supportive role for NF-κB in influenza virus infection (Nimmerjahn *et al*, 2004; Wei *et al*, 2006), while another study suggested that NF-κB activation is not required for efficient influenza virus replication (Bernasconi *et al*, 2005). In general, the role of NF-κB in the regulation of IFN-β responses is still not well understood. Recent studies found that NF-κB stimulates the expression of certain interferon-stimulated genes, while it suppresses others (Pfeffer *et al*, 2004). As discussed earlier, viral infections trigger multiple cellular pathways that stimulate both agonistic and antagonistic functions; the outcome of a viral infection may ultimately be determined by the complex and the as-yet poorly understood interplay between these virus-supportive and -antagonistic factors.

I found that RSK2 affects influenza virus replication through innate immune response pathways (Fig. 11). Although RSK2 is known to activate NF-κB (Ghoda *et al*, 1997; Schouten *et al*, 1997) and PKR (Zykova *et al*, 2007), it had not been recognized as a critical signaling component in innate immune responses to viral infections. I found, however, that RSK2 knockdown in 293 cells affected the influenza virus-induced activation of NF-κB and IFN-β, and the influenza virus-induced phosphorylation of PKR, suggesting a critical role for RSK2 in innate immune responses to viral infections. In

fact, I found that RSK2 knockdown not only increased influenza A virus titers, but also stimulated influenza B virus and Sendai virus replication. In conclusion, I have identified a role for RSK2 in innate immune responses to influenza A virus infection, which may be executed through the regulation of IFN-β, NF-κB, and PKR responses. Other MAPK-activated protein kinases may have similar, as of yet unidentified, functions in the innate immune response and antiviral defense mechanisms.

METHODS

Cell culture

Human embryonic kidney cells (293T cells and 293 cells) were cultured in Dulbecco's

modified Eagle's medium (DMEM; Sigma) supplemented with 10% heat-inactivated

fetal calf serum (FCS) and antibiotics. Plat-GP (murine leukemia virus [MLV]-based

packaging) cells were kindly provided by T. Kitamura (University of Tokyo, Tokyo,

Japan) and cultured in DMEM with 10% FCS and 10 μg/ml blasticidin (Invitrogen).

QT6 quail fibrosarcoma cells were maintained in Ham's F12K medium (MP

Biomedicals) supplemented with 10% FCS and 10% tryptose phosphate broth (Sigma).

Madin Darby canine kidney (MDCK) cells were cultured in minimal essential medium

(MEM) containing 5% newborn calf serum and antibiotics.

Viruses

Influenza viruses A/WSN/33 (H1N1), PB2-627K virus and PB2-627E virus were

generated by reverse genetics (Neumann *et al*, 1999) and propagated in MDCK cells.

PB2-627K virus and PB2-627E possess the HA and NA genes of A/WSN/33 (H1N1)

virus, while the remaining genes were derived from A/Hong Kong/483/97 (H5N1) virus;

the PB2 protein of this virus possesses a lysine at position 627 in the PB2 protein, resulting in PB2K virus. PB2E virus is a derivative that possesses glutamic acid at position 627 in the PB2 protein. Influenza virus B/Hong Kong/73 and Sendai virus (Enders strain; kindly provided by Allan Portner, St. Jude Children's Research Hospital, Memphis, TN, USA) were also propagated in MDCK cells. Viruses were titrated by plaque assay in MDCK cells.

Plasmids

The PB1, PA, and NP proteins of A/Hong Kong/483/97 (H5N1; HK483) virus were expressed using the pMX vector (Onishi *et al*, 1996), which was kindly provided by T. Kitamura, University of Tokyo, Tokyo, Japan or using the pCAGGS vector (Hatta *et al*, 2001). For the PB2 protein, I used a variant that possesses glutamic acid at position 627 (PB2E). pPolI-fCD2 and pPolI-Luc drive the synthesis of negative-sense viral RNAs comprising the 3' noncoding region of the NA (A/Hong Kong/483/97) vRNA, the complementary coding sequence of feline CD2 (Shimojima *et al*, 2003) or luciferase, respectively, and the 5' noncoding region of the NA vRNA.

The three polymerase cDNAs from the WSN strain fused toa cMyc-tag sequence at the 3' termini, and the NP cDNA from the WSN strain fused to a cMyc-tag sequence at the

5' terminus were also inserted into pCAGGS. pPolI-GFP drives the synthesis of negative-sense viral RNAs comprising the 3' noncoding region of the NA (A/Hong Kong/483/97) vRNA, the complementary coding sequence of EGFP and the 5' noncoding region of the NA vRNA. Similarly, polI-Luc drives the transcription of a virus-like RNA expressing luciferase.

Quail RBL2 was cloned from QT6 cells by using the 5' RACE System (Invitrogen) according to the manufacturer's instructions. Human RBL2 and RBL1 were cloned from human 293T cells. Briefly, RNA was extracted from these cells by use of the RNeasy mini kit (Qiagen). RT-PCR was performed using an oligo-dT primer followed by PCR with gene-specific primers. hRBL2-DN (D299N) (Cho *et al*., 2001), which is designed to inactivate helicase activity, was constructed with site-directed mutagenesis. These PCR products were cloned into the pCAGGS vector and then sequenced.

To assess the interaction between hRBL2 and the viral polymerase in living cells, I used a *CoralHue*®Fluo-Chase kit (Amalgaam). Using the vectors in this kit, I constructed plasmids for the expression of the three polymerases, NP, or hRBL2 fused with the N- or C-terminal portion of Kusabira-Green, resulting in 'GN' and 'GC' fusion proteins (for example, pPB1-GN or pPB1-GC).

Gallus gallus RSK2 and human RSK2 were cloned from chicken embryo fibroblast or

human 293T cells, as appropriate. Briefly, RNA was extracted from these cells by use of the RNeasy mini kit (Qiagen). RT-PCR was performed using an oligo-dT primer followed by PCR with gene-specific primers. The PCR products were cloned into the pCAGGS vector and then sequenced. To escape the knockdown effect of the shRNA, a silent mutation was introduced into the human RSK2 protein expression plasmid, yielding pmRSK2.

pNF-κB-Luc, which expresses luciferase upon promoter activation by NF-κB, was purchased from Stratagene. pIFN-luc, which express luciferase under the control of an IFN-β-dependent promoter, was derived from the following components: the bacteria artificial chromosome (BAC) clone RP11-113D19 was used as the source of the promoter and terminator regions of the human interferon-beta gene (Invitrogen) and pGEM-luc (Promega) as the source of the luciferase gene. These components were joined using PCR, and the resulting construct was cloned into the pUC19 vector.

cDNA library

A cDNA library was prepared from quail QT6 cells by isolating mRNA (using the FastTrack 2.0 mRNA Isolation Kit; Invitrogen). cDNA was synthesized by use of the SuperScript Choice System for cDNA Synthesis (Invitrogen), according to the

manufacturer's instructions. The resulting cDNAs were size-fractionated by agarose gel electrophoresis, and cDNA fragments longer than 1 kbp were extracted from the gel using the QIAEX II Gel Extraction kit (Qiagen). The respective cDNA fragments were then inserted into the *Bst*XI sites of pCAGGS-Kan (a pCAGGS variant that carries the kanamycin resistance gene) by using *Bst*XI adapters (Invitrogen). The ligated DNA was ethanol-precipitated and then electroporated into DH10B-competent cells.

Library screening

-Screeing 1(as shown in Fig.1)-

Human embryonic kidney 293T cells were transfected with A/Hong Kong/483/97 (H5N1) polymerase and NP protein expression plasmids (note that PB2 possesses glutamic acid at position 627), with a plasmid for the synthesis of a virus-like RNA encoding EGFP (polI-EGFP), and with the quail QT6 cDNA library. Cells were incubated for two days at 33°C, collected, and GFP-expressing cells were sorted by FACSCaliber (BD). Plasmid DNA was then extracted from the cells and amplified in *E. coli*. Cells that expressed high levels of GFP underwent selection three times, at which point their plasmid DNA was extracted and sequenced.

-Screening 2(as shown in Fig. 6)-

293T cells were transfected with plasmids for the expression of the polymerase and NP proteins, i.e., pMX-PB2E (possessing glutamic acid at position 627), -PB1, -PA, -NP; with the plasmid for the synthesis of a virus-like RNA encoding feline CD2 (polI-fCD2); and with the quail QT6 cDNA library. Cells were incubated for two days at 33°C, collected, and treated with an antibody against fCD2 (Shimojima *et al*, 2003 and 2004). After incubation at 4°C for 30 min, fCD2-positive cells were selected immunoaffinity using Bio-Adembeads goat anti-mouse IgM antibody (Ademtech) according to the manufacturer's instructions. Plasmid DNA was then extracted from the cells and amplified in *E. coli* in LB medium supplemented with kanamycin. Selection of cells that expressed high levels of fCD2 was repeated four times, at which point plasmid DNA was extracted from the cells and sequenced.

Luciferase assay

Luciferase assays were performed by use of a dual-luciferase reporter assay system (Promega) on a microplate luminometer (Veritas; Turner Biosystems, Sunnyvale, CA), according to the manufacturer's instructions. As an internal control for the dual-luciferase assay, pGL4.74[hRluc/TK] (Promega) was used.

Knockdown of human RBL2 by use of siRNA

siRNA specific to RBL2 (HSS116737) was purchased from Invitrogen. At 48 hours post-transfection with the siRNA, cells were tested for RBL2 expression levels by western blot analysis.

Construction of RSK2 knockdown cells by use of a retroviral vector

Short hairpin RNA (shRNA) with a human RSK2 target sequence (5'- gatgctgcttgtgatatatgg - 3') was flanked by the mU6 promoter and terminator. The resulting cDNA was inserted into the pSSSP vector, which was kindly provided by H. Iba (University of Tokyo, Tokyo, Japan). A similar plasmid, pSSSP-shGFP, with a target sequence for GFP (5'-gccacaacgtctatatcatgg- 3') was also kindly provided by H. Iba (University of Tokyo, Tokyo, Japan). Using these plasmids, MLV-based viruses were produced in Plat-GP cells, as described (Kitamura *et al*, 2000 ; Shimojima *et al*, 2003), and then used to transduce 293 cells.

Quantification of viral RNA products

To assess viral polymerase activity, cells transfected with protein expression plasmids or siRNAs were infected with WSN virus at an moi of 2. At various time points, cells were

washed three times with PBS, after which the RNA was extracted and subjected to RT-PCR with NP gene specific primers or oligo-dT primers. The resultant cDNAs were quantified with LightCycler® 2.0 (Roche) by NP gene specific primers; beta-actin specific primers served as an internal control.

Analysis of virus propagation

To establish virus growth rates, three wells of cells were infected in parallel with the indicated virus at a multiplicity of infection (m.o.i.) of 0.05 and incubated at 33˚C or 37˚C. At various time points, supernatants were assayed to determine the titer of the infectious virus by plaque assay in MDCK cells.

Bimolecular fluorescence complementation (BiFC) assays

To examine the interaction of hRBL2 with viral proteins in living cells, I performed BiFC assays using the *CoralHue*®Fluo-Chase kit (Amalgaam). Briefly, the respective proteins were fused to N- or C-terminal portions of Kusabira-Green, resulting in GN- or GC-fusion proteins. Pairs of GN- and GC-fusion protein were transfected into 293T cells and subjected twelve or twenty-four hours later to luciferase assays or FACS analysis, respectively.

NF-κB and IFN-β promoter activity

pNF-κB-Luc and pIFN-luc were transfected into shRSK2 cells and shGFP cells, respectively. Nine hours later, cells were infected with the indicated virus at an m.o.i. of 1.0 and incubated at 33°C. Twelve hours later, the levels of luciferase expression were determined.

Western blot analysis

To assess hRBL2 expression levels, cells transfected with the respective hRBL2 protein expression plasmid were suspended in Tris-Glycine SDS Sample Buffer (Invitrogen), and subjected to western blot analysis with anti-hRBL2 (BD Transduction Laboratories™) and anti-beta-actin (as an internal control; Sigma) antibodies, according to the manufacturer's instructions. Biotinylated anti-mouse IgG antibody (Vector) was used as a secondary antibody. Bands were detected using the VECTASTAIN ABC kit (Vector) and ECL Plus™ Western Blotting Detection Reagents (GE Healthcare); the VersaDoc Imaging System (BioRad) was used to quantify band intensities.

To analyze the expression of the viral M1 protein, cells transfected with protein expression plasmids or siRNA were infected with WSN virus at a multiplicity of 3 and

incubated at 37˚C. At the indicated time points, the cells were washed three times with PBS and resuspended in Tris-Glycine SDS sample buffer. Western blot analysis was then performed with monoclonal antibodies specific to the M1 protein; beta-actin served as a control. Biotinylated anti-mouse IgG antibody (Vector) was used as a secondary antibody. Bands were detected as described above.

To assess the interaction of hRBL2 with viral polymerase complexes, 293T cells were transfected with plasmids for the expression of hRBL2 (pChRBL2), the viral polymerase proteins (pCAGGS- PB1, PB2, PA), myc-tagged NP protein, and, in a subset of experiments, a plasmid for the synthesis of virus-like RNA (polI-GFP). Twenty-four hours later, myc-tagged proteins were immunoprecipitated with anti-cMyc agarose (Sigma). Non-tagged NP was used as a negative control. The beads were resuspended in Tris-Glycine SDS sample buffer and western blot analysis was performed with an antibody specific for hRBL2. Biotinylated anti-mouse IgG antibody (Vector) was used as a secondary antibody. Bands were detected as described above.

To assess the interaction of hRBL2 with individual components of the replication machinery, pChRBL2 was co-transfected with plasmids for the synthesis of myc-tagged polymerase and NP proteins (pCPB1-Myc, -PB2-Myc, -PA-Myc or -Myc-NP). Immunoprecipitations were performed as described above.

To assess RSK2 expression levels, shRSK2 cells and shGFP cells were suspended in Tris-Glycine SDS Sample Buffer (Invitrogen), and western blot analysis was performed with anti-RSK2 (E1; Santa Cruz) and anti-β-actin (as an internal control; Sigma) antibodies, according to the manufacturer's instructions. Biotinylated anti-mouse IgG antibody (Vector) was used as a secondary antibody. Bands were detected as described above.

To analyze expression of the viral M1 protein, shRSK2 cells and control shGFP cells were infected with a PB2-627E-expressing virus at an m.o.i. of 1.0 and incubated at 33°C. At the indicated times, the cells were washed three times with PBS and resuspended in Tris-Glycine SDS sample buffer. Western blot analysis was performed with monoclonal antibodies specific to M1 protein, using beta-actin as a control. Biotinylated anti-mouse IgG antibody (Vector) was used as a secondary antibody. Bands were detected as described above.

To assess RSK2 phosphorylation levels, 293 cells (7.5×10^5 cells) were plated in 60 mm plates and cultured overnight at 37°C. The growth medium was replaced with DMEM containing 4% BSA and cells were infected with influenza virus (m.o.i. of 3.0) twelve hours later; control cells remained uninfected. Three hours post-infection, RSK2 was immunoprecipitated with anti-RSK2 antibody (E1; Santa Cruz) coupled to protein G

beads. The beads were resuspended in Tris-Glycine SDS sample buffer and western blot analysis was performed with antibodies specific for RSK phosphorylated at Thr365/Ser369, Ser386 or Thr577 (antibodies obtained from Cell Signaling). Biotinylated anti-rabbit IgG antibody (Vector) was used as a secondary antibody. Bands were detected as described above.

To assess the phosphorylation levels of PKR, shRSK2 and control shGFP cells were infected at an m.o.i. of 0.01 or 1.0 with influenza virus and incubated at 33˚C. Infected cells were collected at 0, 6, and 12 hours post-infection. Western blot analysis was performed with anti-PKR (Santa Cruz), anti-phospho PKR (Biosource), and anti-β-actin (as an internal control; Sigma) antibodies according to the manufacturer's instructions. Biotinylated anti-rabbit IgG antibody or anti-mouse IgG antibody was used as a secondary antibody. Bands were detected as described above.

Statistical analysis.

The Student's *t* test was used to compare the means of two groups.

CONCLUDING REMARKS

To control influenza, it is important to collect basic information of the mechanism of influenza virus propagation. In particular, the replication and transcription, which polymerase subunits and nucleoprotein execute, are fundamental stages in viral replication cycle. Therefore, these steps are attractive targets for antiviral drug development. I focused on host proteins affecting viral replication and transcription, developing an expression cloning strategy to identify them. The expression cloning I have established can identify not only host proteins which have direct interaction with viral proteins, but also proteins which have no direct interaction but affect viral growth. Therefore, this method is applicable for screening host factors in a wide range of viruses.

One of the proteins I identified as a suppressor of influenza virus polymerase activity is RuvB-like 2. It directly interacted with vRNP, disordering NP self-association. RuvB-like 2 is reported to be a cellular interaction partner of the vRNP by another group (Mayer *et al*, 2007) and also that the knockdown of a *Drosophila* homologue of hRBL2 enhanced reporter gene expression of influenza virus replicon by our group (Hao *et al*, 2008). All together, it is strongly demonstrated that RBL2 is an inhibitor to influenza virus polymerase.

I also analyzed the role of MAPK-activated protein kinase, RSK2, in viral infection. In RSK2-knockdown experiment, I found that RSK2 was one of activators to NF-κB, and IFN-β promoter. In addition, RSK2 was found to phosphorylate PKR as a substrate in viral infection. PKR phosphorylates the α subunit of initiation factor eIF2 (eIF2α), resulting in rapid inhibition of translation and restriction of the spread of the virus. As a result, RSK2-knockdown enhanced influenza virus replication. Thus, I

revealed that RSK2 mediates its antiviral activity through innate immune response pathways.

Further studies of host factors supporting or inhibiting influenza virus replication in cells would provide us with a better understanding on the properties of the virus and lead to effective control measures for the pathogen.

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