The role of Ran-binding protein 3 during influenza A virus replication

Rey Predicala and Yan Zhou

Vaccine and Infectious Disease Organization, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 5E3, Canada

Influenza A virus vRNP nuclear export is CRM1-dependent. Ran-binding protein 3 (RanBP3) is a Ran-interacting protein that is best known for its role as a cofactor of CRM1-mediated cargo nuclear export. In this study, we investigated the role of RanBP3 during the influenza A virus life cycle. We found that RanBP3 was phosphorylated at Ser58 in the early and late phases of infection. Knockdown of RanBP3 expression led to vRNP nuclear retention, suggesting that RanBP3 is involved in vRNP nuclear export. Moreover, we demonstrated that the function of RanBP3 during vRNP nuclear export is regulated by phosphorylation at Ser58, and that RanBP3 phosphorylation is modulated by both PI3K/Akt and Ras/ERK/RSK pathways in the late phase of viral infection.

INTRODUCTION

Influenza A viruses are important pathogens that are contagious and cause acute respiratory disease in humans and different animal species. Influenza A viruses are enveloped particles belonging to the RNA virus family Orthomyxoviridae (Palese & Shaw, 2007). The genome consists of eight segmented RNA molecules of negative polarity. The majority of influenza A virus strains encode 12–14 viral proteins (Muramoto et al., 2012). Of those, NP and RNA polymerase subunits PB2, PB1 and PA are associated with the RNA genome, forming a viral nucleoprotein (vRNP) complex. A unique feature of the influenza virus life cycle is that virus replication and transcription take place in the nucleus of infected cells. Thus, newly synthesized vRNP complex must be exported from the nucleus to the cytoplasm before assembling into viral particles. Although the detailed mechanism of vRNP nuclear export is not yet fully elucidated, several studies on this process have begun to emerge. At the late-stage of infection, influenza vRNPs are transported across the nuclear envelope through the nuclear pore complex (NPC) in an active, energy-dependent process. Studies have shown that leptomycin B, which specifically inactivates the chromosome region maintenance 1 protein (CRM1), resulted in the nuclear retention of vRNP in virus infected cells, indicating a role for the CRM1 nuclear export pathway in influenza vRNP nuclear export (Elton et al., 2001; Watanabe et al., 2001). The viral M1 protein and nuclear export protein (NEP) are also involved in this process. Even though no quadruple complex containing vRNP, CRM1, M1 and NEP has been isolated from the infected cells, a study by Akarsu et al. (2003) supported a ‘daisy-chain’ model where CRM1 binds to the nuclear export signal located in NEP, and thus bridges the complex between M1 and vRNP (Boulo et al., 2007).

CRM1 is a member of the importin β family of nuclear transport factors, which facilitate the nuclear export of many proteins and RNPs that bear a leucine-rich nuclear export signal (Petosa et al., 2004). In the nucleus, CRM1 associates in a cooperative manner with RanGTP and with a nuclear export signal (NES)-bearing cargo to form a ternary CRM1/Ran/cargo complex that translocates through the NPC and is disassembled in the cytosol after GTP hydrolysis by Ran (Bischoff & Görlich, 1997; Kutay et al., 1997). In the absence of Ran, CRM1 has low binding affinity for most NES-bearing cargos. It was later found that the binding of CRM1 to this diversity of export substrates requires cofactors.

Ran-binding protein 3 (RanBP3) is a Ran-interacting protein that is best known for its role as a cofactor of CRM1-mediated export. It enhances the rate of nuclear export by increasing the affinity of CRM1 for RanGTP, thereby stabilizing the ternary CRM1/Ran/cargo complex in the nucleus (Englmeier et al., 2001; Lindsay et al., 2001). Recently, Yoon et al. (2008) showed that the function of RanBP3 is regulated by phosphorylation via the Ras/ERK and PI3K pathways. In human cells, RanBP3 is phosphorylated at Ser58 when it interacts with either RSK or Akt, which are activated downstream of Ras/ERK and PI3K, respectively. The Raf/MEK/ERK and PI3K/Akt signalling pathways were previously shown to be activated upon influenza A virus infection (Ehrhardt et al., 2006; Hale et al., 2006; Pleschka et al., 2001; Shin et al., 2007c), and their inhibition resulted in nuclear retention of vRNPs. These findings prompted us to investigate the role of RanBP3 in regulating influenza vRNP nuclear export.
RESULTS

RanBP3 is activated during the early and late phases of influenza A virus infection

Given that RanBP3 is phosphorylated by RSK and Akt (Yoon et al., 2008), and influenza virus infection activates the Ras/ERK and PI3K/Akt pathways, we sought to determine whether RanBP3 is activated during virus infection.

Serum-starved A549 cells were mock-infected or infected with wild-type (wt) PR8 virus at an m.o.i. of 1, and cell lysates prepared at the predetermined times were subjected to immunoblotting using antibodies against phospho-RanBP3 (Ser58), total RanBP3 and viral M1 protein. As shown in Fig. 1(a), infection of cells leads to a biphasic activation of RanBP3, as manifested by a brief period of RanBP3-S58 phosphorylation during the early phase \([15–30 \text{ min post-infection (p.i.)}]\) of infection and again at the late phase, which started at 6 h. p.i. and was sustained for the remainder of infection. Immunoblotting with total RanBP3 showed equal amounts of RanBP3 throughout the course of infection, indicating that the changes in phosphorylation were not the result of altered cellular protein levels. When the amount of phospho-RanBP3 was normalized against total RanBP3, and the fold-change of phosphorylation relative to initial value was calculated, the biphasic mode of RanBP3 activation was more apparent (Fig. 1a lower panel). A similar pattern of RanBP3 phosphorylation was observed during the course of Halifax-210 infection (Fig. 1b), demonstrating that this phenomenon was not limited to a specific virus strain.

As we observed biphasic RanBP3 phosphorylation, we postulated that the early phase activation might be associated with the initial virus–host cell interactions, whereas the late phase activation might be virus replication dependent. To test this hypothesis, we inactivated the virus by UV irradiation, since UV irradiation blocks viral RNA transcription, and mRNA and protein syntheses, but has no effect on virus receptor binding and subsequent entry into host cells (Shin et al., 2007b). A549 cells were infected with UV-inactivated PR8 at an m.o.i. of 1. Cells were

![Graphs showing phosphorylation of RanBP3](image)

**Fig. 1.** Influenza virus infection activates RanBP3 phosphorylation. Serum-starved A549 cells were infected by wt PR8 virus (a), Halifax-210 virus (b) or UV-inactivated PR8 virus (c) at an m.o.i. of 1 for the time indicated. Phosphorylation of RanBP3 at Ser58, total RanBP3 and ongoing viral replication demonstrated by viral M1 protein synthesis were detected by their respective antibodies. Quantification of band density was performed by Odyssey software. The level of phospho-RanBP3 was normalized against total RanBP3 and the fold-change of phosphorylation relative to the mock infected cells was plotted.
harvested at early (15 min p.i.) and late (9 h p.i.) phases of infection, and the phosphorylation of RanBP3 and total RanBP3 was determined by Western blotting. As seen in Fig. 1(c), while infection by UV-inactivated viruses did not alter total RanBP3, replication-deficient virus could activate the early phase of RanBP3 phosphorylation, but failed to trigger late phase phosphorylation of RanBP3. No M1 protein could be detected in UV-inactivated virus-infected cells (data not shown).

Knockdown of endogenous RanBP3 results in reduction of progeny virus titre

To examine the biological function of RanBP3 during influenza A virus replication, we assessed the gene knockdown effect on virus yield and NP expression. A549 cells were transfected with an siRNA mixture that targeted four different regions of RanBP3. Forty-eight hours post-transfection, A549 cells were harvested, and the knockdown effect was determined by immunoblotting with RanBP3 antibody. As shown in Fig. 2(a), transfection with off-target siRNA (siOT) did not alter the expression level of RanBP3 as compared to that in the non-transfected cells [si(−)]. However, transfection with RanBP3 siRNA led to a significant suppression in RanBP3 expression. siRNA treatment did not result in concomitant reduction in the level of non-targeted cellular proteins, as indicated by consistent β-actin levels.

After confirming that siRanBP3 suppressed endogenous RanBP3 expression in A549 cells, the siRNA-treated cells were then infected with PR8 virus at an m.o.i. of 1. At 8 and 16 h p.i., supernatant was harvested and virus titres were determined by plaque assay. The cells were harvested at the end-point and viral protein expression was determined by immunoblotting with NP antibody. As shown in Fig. 2(b), at 8 h p.i., virus titre did not decrease significantly in RanBP3 siRNA-transfected cells compared with off-target siRNA-transfected or non-siRNA-transfected cells. However, at 16 h p.i., siRanBP3-treated cells exhibited 1 log reduction in progeny virus titre relative to off-target siRNA-treated control cells. Concomitantly, NP protein levels in siRanBP3-treated cells were lower than that in control cells.

Knockdown of endogenous RanBP3 impairs influenza virus RNP nuclear export

Having observed that RanBP3 knockdown reduced progeny virus titre, we next assessed its effect on virus replication at the single-cell level. Given that RanBP3 is a cofactor of CRM1 and influenza vRNP nuclear export is CRM1 dependent, we thus examined whether RanBP3 is involved in regulating vRNP nuclear export. Using the same transfection conditions, A549 cells were treated with siRNA and then infected with PR8 virus. At 11 h p.i., the cells were fixed and stained with antibodies specific for NP and RanBP3. As seen in Fig. 3(a), endogenous RanBP3 was predominantly localized to the nucleus, which is consistent with the previous report of Yoon et al. (2008). In virus-infected cells, NP was found predominantly in the cytoplasm of non-siRNA, or off-target siRNA-transfected cells, indicating that the majority of NP had been exported from the nucleus. In contrast, the majority of NP accumulated in the nucleus of RanBP3-knockdown cells, indicating that NP nuclear export is disturbed (Fig. 3b). Cells were counterstained with DAPI. Of about 50 cells examined, 83 % of siRNA non-transfected cells and 90.3 % of off-target siRNA-transfected cells exhibited cytoplasmic NP staining, whereas 71.2 % of siRanBP3-transfected cells showed NP nuclear staining.

Phosphorylation of RanBP3 at Ser58 is essential for influenza vRNP transport

As we observed that RanBP3 is phosphorylated at Ser58 during virus infection and downregulation of RanBP3 expression impairs vRNP export, we next assessed whether RanBP3 phosphorylation at Ser58 could be responsible for vRNP nuclear export.

Using electroporation, Madin–Darby canine kidney cells (MDCK) cells were transfected with plasmids expressing
Flag-tagged wt RanBP3, dominant-negative RanBP3-S58A, or constitutively active RanBP3-S58D (Yoon et al., 2008), and allowed to express the exogenous proteins for 8 h. The transfected cells were then superinfected with PR8 virus, and at 10 h p.i. vRNP trafficking was assessed by immunofluorescence microscopy. For each type of plasmid transfection, about 100 transfected and superinfected cells were examined, where Flag-specific antibody was used to distinguish the transfected cells from non-transfected cells; and NP antibody was used to identify the superinfected cells.

As seen in Fig. 4, in non-transfected and infected cells, almost all vRNP was transported into the cytoplasm; 38.2% of the cells that expressed the dominant-negative RanBP3-S58A exhibited nearly complete retention of vRNPs in the nucleus. This was in stark contrast to vRNP distribution and trafficking in cells expressing wt RanBP3, in 75.5% of which the majority of vRNPs were exported to the cytoplasm. The vRNP distribution exhibited in cells expressing constitutively active RanBP3-S58D was similar to that in cells expressing wt RanBP3 (58.2% of RanBP3-S58D expressing cells showed vRNP cytoplasmic localization). It was interesting to note that the vRNP distribution in dominant-negative RanBP3-expressing cells closely resembled the vRNP distribution in RanBP3-knockdown cells (Fig. 3b), where vRNPs were retained in the nucleus.

Fig. 3. Knockdown of RanBP3 impaired influenza virus vRNP nuclear export. A549 cells were left untransfected, or transfected with off-target siRNA or siRNA targeting RanBP3. Forty-eight hours later, cells were mock infected (a), or infected with PR8 at an m.o.i. of 1 (b). At 11 h p.i., the intracellular localizations of RanBP3 and NP were stained with specific antibodies. Cells were counterstained with DAPI.
Phosphorylation of RanBP3 at Ser58 is regulated by both PI3K/Akt and Ras/ERK pathways in the late phase of infection

Because we observed that RanBP3 is activated during influenza virus infection, we sought to examine whether the upstream signals, specifically PI3K/Akt and Ras/ERK pathways, could regulate RanBP3 phosphorylation. We focused on the late phase of virus life cycle, since we could not detect PI3K/Akt pathway activation until 6 h p.i. (Shin et al., 2007b).

Serum-starved A549 cells were infected with PR8 virus, followed by treatment with Ras/ERK/RSK inhibitor U0126, and the PI3K inhibitor LY294002, individually or in combination. At 9 h p.i., cell lysates were prepared and subjected to Western blotting to assess the phosphorylation of RanBP3 on Ser58. As seen in Fig. 5, neither U0126 alone nor LY294002 alone could completely inhibit phosphorylation of RanBP3 (lanes 3 and 4). However, combination treatment of the cells with both inhibitors resulted in an inhibition of RanBP3 phosphorylation (lane 5). The levels of total RanBP3 and β-actin remained constant in all samples, indicating that the changes in RanBP3 phosphorylation were not due to changes in cellular protein levels. Phospho-Akt and phospho-ERK activations were monitored by their specific antibodies.

DISCUSSION

Influenza A virus transcription and replication occurs in the host nucleus, because the virus is dependent on the RNA processing machinery of the host cell. During the processes of importing viral genomic segments into the nucleus, exporting back to the cytoplasm, and then preventing them from re-entering the nucleus, the virus utilizes the cellular transport machinery. Active transport through the host cell nucleus via the NPC requires several host factors including RanBP3. In this study, we investigated the role of RanBP3 in influenza virus replication.

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**Fig. 4.** Phosphorylation of RanBP3 at Ser58 is essential for influenza vRNP nuclear export. MDCK cells were non-transfected or transfected with plasmids expressing Flag tagged wt RanBP3, dominant-negative construct RanBP3-Ser58A, or constitutive active construct RanBP3-S58D. Cells were then infected with PR8 virus at an m.o.i. of 1 and were fixed at 10 h p.i. The expression of exogenous RanBP3 and the subcellular localization of NP were stained with Flag antibody and NP antibody. Cells were counterstained with DAPI.
We initially examined whether influenza virus infection activates RanBP3. We found that infection of cells with influenza A led to a biphasic activation of RanBP3 during the early phase of infection and again at the late phase of infection starting at 6 h p.i. (Fig. 1). The early phase RanBP3 phosphorylation could not be eliminated in UV-inactivated cells, suggesting that it corresponds to initial virus–host cell interactions such as virus attachment and adsorption. UV-inactivated virus could not activate RanBP3 phosphorylation in the late phase of infection, suggesting that the late-phase RanBP3 phosphorylation is viral replication dependent. This late phase activation suggests that RanBP3 might be utilized by the virus.

We next examined whether RanBP3 has a biological function in virus replication by assessing the virus yield in RanBP3-knockdown cells. Knockdown of RanBP3 expression did not significantly alter expression levels of non-targeted proteins, but it reduced progeny virus yield by approximately 1 log and viral protein synthesis at 16 h p.i. (Fig. 2b). Upon examining the effect of RanBP3 knockdown at the individual cell level by immunofluorescence assay (Fig. 3), we found that vRNPs were retained in the nucleus of RanBP3-knockdown cells, while vRNPs were predominately in the cytoplasm of off-target siRNA-treated cells. These results provided more evidence and clearly demonstrated that RanBP3 has a role in vRNP export. Recently, Chase et al. (2011) reported a unique chromatin-targeting strategy to facilitate vRNP export, where the chromatin association of vRNPs leads to retention of CRM1 and Ran, which confers the advantage of the nuclear export of vRNPs over that of other CRM1 substrates. This study further confirmed the role of CRM1 and Ran in facilitating vRNP nuclear export. Since RanBP3 could increase the affinity and association of CRM1 for RanGTP, therefore stabilizing the ternary CRM1/Ran/cargo complex in the nucleus (Langer et al., 2011; Nemergut et al., 2002), it is possible that in the RanBP3-knockdown cells, CRM1/Ran/vRNP complex was unstable, leading to vRNP nuclear retention.

Upon observing that RanBP3-Ser58 is phosphorylated during virus infection and that RanBP3 knockdown abrogates vRNP export, we further investigated whether Ser58 is a regulatory site for the function of RanBP3 in vRNP export. We found that overexpression of dominant-negative RanBP3-S58A in MDCK cells resulted in a nearly complete retention of vRNPs in the nucleus, which is similar to the result shown for vRNP distribution in RanBP3-knockdown cells (Figs 4 and 3b, respectively). In contrast, the cells overexpressing wt RanBP3, constitutively active RanBP3-S58D, and endogenous RanBP3 all had vRNPs exported to the cytoplasm (Fig. 4). These results suggested that the function of RanBP3 in vRNP export is regulated by phosphorylation at Ser58. Yoon et al. (2008) reported that Ser58 is the only site in RanBP3 phosphorylated by the kinases RSK and Akt; phosphorylation at this site regulates nuclear export by modulating the Ran gradient across the cytoplasm and nucleus, in part by controlling RCC1 activity. More detailed study is required in the future to determine whether this is the mechanism by which RanBP3 phosphorylation regulates influenza vRNP nuclear export.

To identify the upstream signals that regulate RanBP3 phosphorylation upon influenza virus infection, we were particularly interested in the PI3K/Akt and Ras/ERK pathways, since previous studies showed that blockage of these pathways by their specific inhibitors led to vRNP nuclear retention (Pleschka et al., 2001; Shin et al., 2007b).

We found that at the late phase of infection, a single treatment of either PI3K/Akt or Ras/ERK inhibitor only partially decreased RanBP3 activation. In contrast, combination treatment of the cells resulted in a complete inhibition of RanBP3 activation (Fig. 5). RSK and Akt can recognize and phosphorylate serine or threonine residues in the consensus sequence RXRXXS/T, and RanBP3 has such a consensus sequence: $^{53}$RERTS$^{58}$. Our data suggested that for full phosphorylation of RanBP3, both PI3K/Akt and Ras/ERK pathways are required. Pleschka et al. (2001) reported that inhibition of the Raf/MEK/ERK signalling pathway by U0126 impairs vRNP nuclear export, and proposed it was through some cellular factors. Shin et al. (2007b) proposed that inhibition of the PI3K/Akt pathway by LY294002 led to vRNP retention in the nucleus and might be due to reduced M1 protein synthesis. In the present study, our results may support a novel mechanism whereby inhibition of vRNP export when the aforementioned pathways are blocked results from RanBP3 inactivation. We also detected RanBP3 phosphorylation at the early phase of infection. This early
activation might be triggered by virus attachment, since UV-inactivated virus was still able to activate RanBP3 phosphorylation (Fig. 1). However, the early activation of RanBP3 seems to be independent of both Ras/ERK and PI3K/Akt pathways, as U0126 and LY294002 did not inhibit RanBP3 phosphorylation either individually or synergistically (data not shown). While the late phase activation of RanBP3 is regulated by both PI3K/Akt and Ras/ERK/RSK pathways and contributes to regulation of vRNP nuclear export, the mechanism and function of early phase RanBP3 phosphorylation remain to be elucidated.

In conclusion, we have identified RanBP3 as a host factor that has a vital role during influenza A virus replication. Phosphorylation of RanBP3 is regulated by both Ras/ERK/RSK and PI3K/Akt pathways, and is involved in CRM1-mediated shuttling of viral RNPs from the nucleus to the cytoplasm. Although RanBP5 was reported to have a key role in the influenza life cycle by interacting with viral PB1 protein (Hutchinson et al., 2011), this is the first report that RanBP3 is involved in regulation of viral RNP nuclear export.

METHODS

Cell, viruses and infections. A549 (human lung carcinoma cells) were maintained in Kaighn’s modification of Ham’s F-12 medium (ATCC) containing 10% FBS (Invitrogen). MDCK were cultivated in minimum essential medium (MEM; Sigma) supplemented with 10% FBS. All media were supplemented with 50 µg gentamicin (Invitrogen) ml⁻¹ and maintained at 37 °C in an atmosphere of 5% CO₂.

Influenza A/PR/8/34 (H1N1) (referred to as PR8) was propagated at 37 °C in 11 day-old-embryonated chicken eggs. A/Halifax/210/2009 (H1N1) (referred to as Halifax210) was cultivated in MDCK cells. Virus titres were determined in MDCK cells by plaque assay as described previously (Shin et al., 2007a). UV irradiation-inactivated virus was prepared by exposing virus solution (0.5 ml in a 35 mm tissue-culture dish) to a 30 W UV light at a distance of 20 cm for 20 min.

Antibodies and inhibitors. Rabbit polyclonal NP and M1 antibodies were generated in our laboratory as previously described (Shin et al., 2007b). Monoclonal NP antibody (Serotec) and monoclonal anti-Flag antibody (Sigma) were used in immunofluorescence staining analysis. Rabbit polyclonal phospho-RanBP3 (Ser58) and rabbit monoclonal anti-RanBP3 antibodies were purchased from Invitrogen. Mouse monoclonal anti-β-actin, rabbit monoclonal anti-phospho-Akt (Ser473) antibody and rabbit polyclonal anti-phospho-ERK1/2 (Thr202/Tyr204) antibody were purchased from Cell Signaling Technology. IRDye 680-conjugated polyclonal donkey anti-rabbit IgG and IRDye 800-conjugated donkey polyclonal anti-mouse IgG were purchased from LI-COR Biosciences. Secondary antibodies used for immunofluorescence staining were Alexa-Fluor (AF) 594-conjugated goat anti-rabbit IgG, AF488-conjugated goat anti-mouse IgG and AF594-conjugated goat anti-mouse IgG, which were purchased from Invitrogen. Cy2-conjugated goat anti-rabbit antibodies were purchased from Jackson Immunoresearch Laboratories.

PI3K-specific inhibitor LY294002 was purchased from Sigma. Ras/ERK/RSK pathway inhibitor U0126 was purchased from Calbiochem. Treatment of cells with LY294002 or DMSO was performed at concentrations of 20 µM and 0.4% (v/v), respectively, while treatment with U0126 was performed at a concentration of 10 µM. Chemicals were added to the cells 1 h prior to infection, removed during virus adsorption and added again 1 h p.i.

siRNAs and transfections. An siRNA set targeting human RanBP3 (catalogue numbers SI04149418, SI04223443, SI04241398, SI04261236) was purchased from Qiagen. An off-targeting siRNA (Negative Control Stealth RNAi duplex, catalogue number 12935400) was obtained from Invitrogen. A549 cells seeded in 24-well plates or four-well chamber slides (4 × 10⁵ cells per well) were transfected with siRNAs at a concentration of 40 nM (10 nM each siRNA) using X-tremeGene siRNA transfection reagent (Roche) according to the manufacturer’s protocol with minor modifications. Briefly, 40 nM of siRNA and 10 µl of X-tremeGene siRNA transfection reagent were diluted in OptiMEM in separate vials. The diluents were mixed immediately, and the mixture was further incubated at room temperature for 20 min before addition to cells at 30–40% confluence. At 48 h post-transfection, cells were superinfected with influenza A virus.

Plasmids and transfections. Plasmids encoding HA-tagged wild-type RanBP3 (pKH3-HA-RanBP3), dominant-negative RanBP3 (pKH3-HA-S58A-RanBP3) and constitutively active RanBP3 (pKH3-HA-S58D-RanBP3) proteins were a gift from Dr John Blenis (Yoon et al., 2008). Flag-tagged versions of these RanBP3 proteins were created by PCR amplifying the RanBP3 genes with primers (forward 5'-ATAGCGAATTCATGCGACCTGGCAATACTGGGAAAC-3' and backward 5'-AACTAGTACCTATGTGTCGCCGCGTCTG-3') carrying EcoRI and BamHI sites. The respective genes were then cloned into the pCMV-3 × Flag (Li et al., 2008) at the same sites.

MDCK cells were transfected with the Flag-tagged plasmids by electroporation with Amaxa Nucleofector kit I (Lonza). Five micrograms of each plasmid was used per 5 × 10⁵ cells, and electroporations were performed according to the appropriate Nucleofector program. Pre-warmed antibiotic-free medium was immediately added to electroporated cells, and about 1 × 10⁵ cells per well were transferred to a four-well chamber slide and incubated for 10 h. Transfected cells were then superinfected by PR8 virus at an m.o.i. of 3. At 10 h p.i. cells were fixed and subjected to immunofluorescence staining as described below.

Immunofluorescence and microscopy. Influenza virus-infected A549 or MDCK cells grown on a four-well chamber slide were fixed in a mixture of aceton : methanol (1 : 1) for 15 min at −20 °C. Cells were rehydrated with PBS, and non-specific protein binding was blocked by incubation with PBS containing 10% normal horse serum (blocking solution) for 1 h at room temperature. Primary and secondary antibody dilutions were prepared in blocking solution and incubated with cells for 1 h at room temperature. Between staining steps, cells were rinsed three times with PBS. For A549 cells, the primary antibodies used were mouse monoclonal anti-NP antibody (1 : 250) and rabbit anti-RanBP3 antibody (1 : 100). For MDCK cells, the primary antibodies used were rabbit polyclonal anti-NP antibody (1 : 400) and mouse anti-Flag antibody (20 µg ml⁻¹). Secondary antibodies included AF594 goat anti-rabbit (1 : 400), AF488 goat anti-mouse (1 : 400), AF594 goat anti-mouse (1 : 400) and Cy2 goat anti-rabbit (1 : 400). Stained slides were mounted with glass coverslips using Invitrogen Prolong Gold antifade reagent with DAPI, which stained cell nuclei. All fluorescence images were captured with a Leica TCS SP5 laser confocal microscope.

Immunoblotting. About 5 × 10⁵ A549 cells were grown on 35 mm dishes overnight and then incubated in serum-free media for 20 h. During the last 1 h, cells were left untreated or treated with inhibitors, then mock-infected or infected with influenza virus at an m.o.i. of 1. At the indicated times, cell monolayers were washed with cold PBS and lysed with cell lysis buffer [20 mM Tris/HCl (pH 7.5), 150 mM...
NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 μg leupeptin ml⁻¹. Cell Signaling Technology] supplemented with 1 mM PMSF (Sigma). The lysates were collected, incubated on ice for 10 min, sonicated, and further homogenized by passage several times through a 0.5 ml syringe with 28 gauge needle. The lysates were analysed for total protein content using the Bradford assay (Bio-Rad). Western blotting was performed as previously described (Shin et al., 2007b) with minor modifications. Briefly, 20 μg of total protein was resolved by SDS–12% PAGE and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked for non-specific binding with Tris-buffered saline (TBS) [0.1 M Tris (pH 7.6), 0.9% NaCl] containing 5% BSA for 1 h at room temperature, and then incubated with primary antibody diluted in TBS overnight at 4 °C. Infrared dye-linked secondary antibody (1:5000) was then added, and membranes were incubated at room temperature for 1 h. The immunoblots were visualized using an Odyssey infrared imaging system (LI-COR Biosciences).

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REFERENCES


