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NF90 Exerts Antiviral Activity through Regulation of PKR Phosphorylation and Stress Granules in Infected Cells

Xi Wen,*† Xiaofeng Huang,*† Bobo Wing-Yee Mok,*† Yixin Chen,‡ Min Zheng,*† Siu-Ying Lau,*† Pui Wang,*† Wenjun Song,*† Dong-Yan Jin,§ Kwok-Yung Yuen,*† and Honglin Chen*†

NF90 was shown to exhibit broad antiviral activity against several viruses, but detailed mechanisms remain unclear. In this study, we examined the molecular basis for the inhibitory effect of NF90 on virus replication mediated through protein kinase (PKR)-associated translational regulation. We first verified the interaction between NF90 and PKR in mammalian cells and showed that NF90 interacts with PKR through its C-terminal and that the interaction is independent of NF90 RNA-binding properties. We further showed that knockdown of NF90 resulted in significantly lower levels of PKR phosphorylation in response to dsRNA induction and influenza virus infection. We also showed that high concentrations of NF90 exhibit negative regulatory effects on PKR phosphorylation, presumably through competition for dsRNA via the C-terminal RNA-binding domain. PKR activation is essential for the formation of stress granules in response to dsRNA induction. Our results showed that NF90 is a component of stress granules. In NF90-knockdown cells, dsRNA treatment induced significantly lower levels of stress granules than in control cells. Further evidence for an NF90–PKR antiviral pathway was obtained using an NS1 mutated influenza A virus specifically attenuated in its ability to inhibit PKR activation. This mutant virus replicated indistinguishably from wild-type virus in NF90-knockdown cells, but not in scrambled control cells or Vero cells, indicating that NF90’s antiviral function occurs through interaction with PKR. Taken together, these results reveal a yet-to-be defined host antiviral mechanism in which NF90 upregulation of PKR phosphorylation restricts virus infection. *The Journal of Immunology, 2014, 192: 3753–3764.

NF90 (also known as NFAR1 or DRBP76) was first identified as an IL-2 promoter-binding protein in activated T cells and was found to regulate IL-2 gene expression via stabilization of IL-2 mRNA (1–5). A variety of independent studies subsequently found that NF90 has dsRNA-binding properties. NF90 is predominantly localized within the nucleus (6, 7), although it also has been found in the cytoplasm (8). It interacts with PKR and is a substrate for phosphorylation by PKR (7–11). Further studies (12, 13) revealed that phosphorylation of NF90 by PKR is necessary for association of the NF90/NF45 complex, shuttling of NF90 between the nucleus and cytoplasm, and NF90 function in translational regulation and host antiviral defense.

NF90 was found to be involved in host antiviral mechanisms targeting various viruses. It suppresses the function of Ebola virus polymerases through interaction with VP35 (14), inhibits HIV replication through interaction with HIV-1 TAR RNA (15, 16), represses internal ribosome entry sites in rhinoviruses (17, 18) and negatively regulates influenza virus replication through interaction with viral nucleoprotein (NP) (19). However, other studies (20–23) found that NF90 is required for the replication of some positive-stranded RNA viruses and is important for expression of E6 protein in human papillomavirus–infected cells. It is postulated that, although members of the NF90 family generally serve as components of host antiviral responses, some viruses may have adapted a mechanism to hijack NF90, retasking it for viral replication and weakening host defenses (20). A study in which NF90 (NFAR) was depleted in mouse embryonic fibroblast (MEF) cells suggested that NF90 exerts its antiviral activity through modulation of translation in host cells in a PKR-dependent manner (12, 13).

In addition to its involvement in the regulation of the IL-2 promoter in T cells, dsRNA-dependent protein kinase (PKR) is the most well-defined host factor interacting with NF90. PKR is an IFN-inducible gene that plays a critical role in host antiviral responses (24, 25). It is natural to hypothesize that the antiviral function exerted by NF90 may be signaled through PKR-related pathways. The primary role of PKR in the antiviral response is its inhibition of translation of viral mRNAs through phosphorylation of eukaryotic initiation factor 2 α (eIF2α) (26). PKR is also recognized for its role in regulating cellular inflammatory signals (27, 28). A previous study (29) found that NF90 is able to inhibit yeast growth when coexpressed with PKR, but not on its own, implying that NF90 may activate PKR to cause translational inhibition. However, this same study (29) found no evidence of NF90 activation of PKR in an in vitro assay. Conversely, it was found that NF90 inhibited PKR phosphorylation, presumably through competition for...
dsRNA binding, because the C-terminal of NF90 possesses RNA-binding ability (6).

Activation of PKR leads to the phosphorylation of eIF2α and results in the stalling of mRNA translation in cells (30). Activation of eIF2α triggers formation of stress granules that are composed of multiple RNA-binding proteins, including TIA-1 and Ras GAP

SH3-domain–binding protein (G3BP)-1 (31, 32). Interaction between influenza A virus and host stress granules was demonstrated recently (33, 34). Activation of PKR to induce the formation of stress granules is regarded as a hallmark of the cellular response to virus infection (34–36). This study explored the possibility of NF90 involvement in PKR activation and the formation of stress granules in response to dsRNA treatment or virus infection of cells. We found that NF90 interacts with PKR via its C-terminal domain and further demonstrated that NF90 is a previously uncharacterized component of cellular stress granules formed when cells are treated with stress-inducing agents, such as polyinosinic-polycytidylic acid [poly(I:C)] or arsenite. NF90 may inhibit virus replication by regulating levels of PKR phosphorylation. Compared with control cells, stable NF90-knockdown 293T cells exhibit lower levels of activated PKR and form significantly fewer stress granules following treatment with poly(I:C). Using an NS1 mutant influenza A virus, which is specifically attenuated in its ability to antagonize PKR activation but unaltered with respect to other functions, this study confirms that NF90 is required for PKR activation in response to virus infection. NF90 may serve as a regulator for PKR activation in response to dsRNA in cells.

**Materials and Methods**

**Cells and viruses**

HEK 293T cells, MEF cells, HeLa cells, and Vero cells were maintained in Dulbecco’s minimal essential medium, whereas MDCK cells were cultured in Eagle’s MEM. Culture media were supplemented with 10% FBS, 100 IU penicillin G/ml, and 100 μl streptomycin sulfate/ml, and cells were incubated at 37°C in a 5% CO2 atmosphere. NF90-knockdown or scrambled control 293T cells were kindly provided by Dr. Christopher Basler (Mount Sinai School of Medicine, New York, NY) (14). PKR-knockout (PKR<sup>−/−</sup>) and wild-type (WT) (PKR<sup>+/+</sup>) primary MEF cells were a generous gift from Drs. John C. Bell (Ottawa Health Research Institute, Ottawa, ON, Canada) and Craig McCormick (Dalhousie University, Halifax, NS, Canada) (34).

WT and NS1-mutated influenza viruses (A/WSN/1933 [H1N1]) were propagated in MDCK cells cultured in MEM containing antibiotics and 1 μg TPCK-trypsin/ml at 37°C for 48 h. Supernatants were harvested at designated time points, and viruses were titrated in MDCK cells by plaque assay. Briefly, virus culture supernatant samples were serially diluted in PBS and adsorbed onto confluent MDCK cells for 1 h at 37°C. The inoculum was removed, and the cells were washed with PBS and covered with 2 ml an agar medium (1% agarose, 1 μg TPCK-trypsin/ml in MEM). After 2 d of incubation, plaques were counted, and the virus titer (PFU/ml) was calculated.

**Plasmid construction**

The NP, PB1, PB2, and PA genes, derived from the H5N1 strain A/VM/1194/2004 and the H1N1 strain A/WSN/1933, were cloned into pCDNA3.1-Flag/V5 and pCMV-Flag vectors, respectively. The NS1 gene was cloned into a Flag-tagged vector (33). For construction of recombinant virus, the viral genomes were cloned into the pHW2000 vector (37). The QuikChange II Site-Directed Mutagenesis Kit (Stratagene) was used to introduce R38A and R55A (19, 33) and N123A/M124A/K126A/N127A (33, 38) point mutations into NS1, as well as to construct an NS1-deletion mutant (delNS1) with stop codons introduced into residues C13 and L15. Plasmids pCDNA3.1-NP90-V5 (NP90-V5) and pCDNA3.1-NP90-Flag were generated by cloning the NP90 gene (GenBank accession number NM_004516; https://www.ncbi.nlm.nih.gov/genbank/) into pCDNA3.1-V5 and pCDNA3-1-Flag vectors, respectively. NP90-R38-A/38A mutant was generated using the primer 5′-GAGAAGACCTGGACCGCCGAGATGCGACG-3′ and 5′-ACGATCGGACCAGGAGAAGTATGGGAGGACGACG-3′ as reverse complementary primer. NP90-R38A-S255A mutant was made using primers 5′-AGGAGAGGACCTGGACCGCCGAGATGCGACG-3′ and 5′-ACGATCGGACCAGGAGAAGTATGGGAGGACGAGC-3′ and its reverse complementary sequence

**Transfection and coimmunoprecipitation**

For transfection experiments, subconfluent (70%) monolayer cells were transiently transfected or cotransfected with plasmids using TransIT-LT1 transfection reagent (Mirus). A total of 10–500 ng plasmid was added to cells in accordance with the manufacturer’s protocol for dsRNA treatment, 100 ng poly(I:C) (Invitrogen) and 400 ng pUC19 carrier DNA were combined to make a mix containing 500 ng DNA, which was then transfected into cells (34).

Coimmunoprecipitation (co-IP) was performed using Dyna Beads (Invitrogen). Specific Abs were added to the beads and incubated at room temperature for ≥20 min. Cell lysates were obtained by adding lysis buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100 and protease inhibitor mixture [Roche]) to the cells, shaking for 15 min on ice, and then centrifuging. Cell lysate supernatants were added to the Ab-coupled beads, and the mixture was incubated at room temperature for 2 h or overnight at 4°C. Finally, beads were washed three times with lysis buffer, and Western blotting was performed.

Immunoprecipitation products or diluted cell lysate supernatants were mixed with SDS loading buffer and heated at 95°C for 10 min. Samples were fractionated by 10% SDS-PAGE and then blotted onto nitrocellulose membranes (Bio-Rad). After blocking with 3% skim milk, membranes were incubated with the primary Abs anti–Flag M2 (Sigma) at 1:2000 dilution, anti-V5 (Invitrogen) at 1:2000 dilution, anti-PKR (Santa Cruz) at 1:1000 dilution, anti-p–PKR (T446; Abcam) at 1:1000 dilution, anti-NS1 (33) at 1:2000 dilution, anti-tubulin (Sigma) at 1:5000 dilution, or anti-NS1 (33) at 1:2000 dilution. IRDye 680– or IRDye 780-labeled donkey-antimouse or -antirabbit secondary Abs (Li-Cor Biosciences) were used at a dilution of 1:5000. Membrane blots were scanned to visualize Ab–protein bands using the Odyssey imaging system (Li-Cor Biosciences). Relative levels of protein present in bands were analyzed by estimation of band density using ImageJ software.

Immunofluorescence assay and microscopy

Cells were grown on Millicell EZ slides (Millipore) and transiently transfected with plasmids or infected with virus. For indirect immunofluorescence assay, cells were fixed for 15 min using 4% paraformaldehyde in PBS, followed by permeabilization with 0.2% Triton X-100 in PBS for 20 min. The cells were washed with PBS and incubated with 2 ml an agar medium (1% agarose, 1 μg TPCK-trypsin/ml in MEM). After 2 d of incubation, plaques were counted, and the virus titer (PFU/ml) was calculated.

Reverse plasmids

Transcriptome analysis of PKR

was calculated.

After 2 d of incubation, plaques were counted, and the virus titer (PFU/ml) was calculated. NP90-R38A-S255A mutant was generated using the primer 5′-GAGAAGACCTGGACCGCCGAGATGCGACG-3′ and 5′-ACGATCGGACCAGGAGAAGTATGGGAGGACGAGC-3′ and its reverse complementary sequence, which were obtained from Life Technologies and Sigma, respectively. HEK 293T cells were transfected with 50 nM siRNA using Lipofectamine RNAiMAX transfection reagent (Invitrogen). After 24 h of incubation, NF90-silenced cells were transfected with 100 ng the different NF90 constructs and incubated for an additional 24 h. Cells were subsequently treated with 150 ng poly(I:C) for 6 h. The effect of the various NF90 constructs on stress granule formation was visualized using an LSM700 confocal microscope in the Core facility at Li Ka Shing Faculty of Medicine, University of Hong Kong.
Quantitative real-time PCR

NF90-knockdown or scrambled 293T cells were infected with WSN WT or mutant viruses at a multiplicity of infection (MOI) of 0.1. Total RNAs were extracted at 24 h postinfection using RNAiso (Takara). cDNAs were synthesized by reverse transcription using SuperScript II Reverse Transcriptase (Invitrogen) and random primers. A SYBR Green–based real-time PCR method (Roche) was used to detect target mRNAs with the LightCycler system (Roche). Primers for detecting IFN-β were 5’-GCCGCA-TTGACCATCT-3’ and 5’-CACAGTGACTGACTCCT-3’. Actin mRNA was quantified, and the information was used to normalize the total RNA concentration between different samples, as described previously (19). A reaction mix of 20 μl was composed of 10 pmol each gene-specific primer, 10 μl SYBR Green Master Mix, and 2 μl cDNA. The amplification program was as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 s, 60˚C for 10 s, and 72˚C for 15 s. The specificity of the assay was confirmed by melting-curve analysis at the end of the amplification program.

Results

NF90 interacts with PKR

NF90 was shown previously to interact with PKR using yeast two-hybrid and GST pull-down assays (6, 29). To verify their interaction in mammalian cells and map the interacting domains on both proteins, we first tested interactions between Flag-tagged full-length NF90 and V5-tagged full-length and C-terminal– and N-terminal–truncated versions of PKR (Fig. 1A). NF90 and PKR plasmids were coexpressed in 293T cells, and co-IP with anti-V5 Ab showed that both N- and C-terminals of PKR interact with NF90 (Fig. 1B). NF90 contains a DZF motif in the N-terminal and an RNA-binding domain (RBD) in the C-terminal (Fig. 1A). We then investigated the PKR-interacting domain on the NF90 molecule by constructing 1–369 N-terminal and 370–702 C-terminal truncates and RBD mutant (RBDm) of NF90. These Flag-tagged truncated or mutated forms of NF90 were coexpressed with V5-tagged full-length PKR in 293T cells and coprecipitated with anti-Flag. Our result showed that only the C-terminal of NF90 interacts with PKR and that the interaction is independent of NF90 RNA-binding properties (Fig. 1C).

NF90 enhances phosphorylation of PKR upon induction by dsRNA

Previous reports (6, 7, 9) demonstrated that NF90 interacts with PKR and is one of the substrates of PKR. The phosphorylated form of NF90, MPP4, also was identified as a phosphoprotein detected during the M phase of the cell cycle (9, 39). Other studies indicated that NF90 may activate PKR and increase eIF2α phosphorylation
in yeast, but these positive effects on PKR phosphorylation could not be seen when purified proteins were used in vitro (29, 40), suggesting that additional factors may be missing in the in vitro assay. However, in the in vitro study, it was demonstrated that high concentrations of NF90 inhibited PKR activation, possibly through competitive binding to dsRNA (40). These observations from yeast and in vitro studies prompted us to investigate the effect of NF90 on PKR phosphorylation in mammalian cells. In this study, we examined the levels of the phosphorylated form of PKR in both NF90-knockdown and scrambled control 293T cells following PKR activation induced by transfection of either dsRNA [poly(I:C)] or viral RNA. We found that, although expression of PKR protein is not changed, the level of the phosphorylated form of PKR is significantly lower (3–4-fold) in NF90-knockdown cells compared with that in scrambled siRNA-treated cells (Fig. 2A). The observation that NF90 is required for PKR phosphorylation in response to dsRNA treatment was confirmed in a pair of 293T cell lines: one in which NF90 was stably knocked down with specific short hairpin RNA and the other a control created using a scrambled short hairpin RNA (14) (Fig. 2B). To delineate the mechanism for the regulation of PKR activation by NF90, we transfected increasing amounts of NF90 into NF90-knockdown cells to reveal the effect of NF90 on PKR phosphorylation in response to dsRNA. We found that PKR phosphorylation was enhanced in response to low amounts of transfected NF90, but the positive effect disappeared when higher concentrations (500 ng) of NF90 were introduced into the NF90-knockdown cells (Fig. 2C). To test whether PKR and NF90 may be competing for dsRNA binding in poly(I:C)-treated cells, we used NF90 truncated at either the N-terminal or RBD (C-terminal) or containing mutations abolishing RNA-binding ability (RBDm) (Fig. 1A) (41). We first confirmed that the full-length and N-terminal forms of NF90 bind poly(I:C) and that NF90 RBDm does not bind dsRNA by performing poly(I:C) bead pull-down assays (data not shown). Interestingly, it was found that, at the NF90 concentration that exhibited inhibitory effects (500 ng), WT (full-length) NF90 and the mutant form containing only the C-terminal significantly inhibited PKR activation following poly(I:C) treatment of 293T cells, whereas N-terminal and RBDm NF90 exerted little or no inhibitory effect, respectively (Fig. 2D) (41). The different p-PKR expression levels associated with the NF90 mutants also may be due to the positive or negative effect of these clones on PKR activation. It appears that NF90 has dual and conflicting functions, in

![FIGURE 2](http://www.jimmunol.org/)
that it can support PKR phosphorylation (Fig. 2A) while inhibiting the level of PKR phosphorylation through sequestration of dsRNA and consequent prevention of PKR activation via the C-terminal RBD (Fig. 2C, 2D). A study (13) found that PKR phosphorylated T188 and T315 of NF90 and regulated NF90 cellular shuttling. The T188A/T315A (attenuated) and the T188D/T315D (constitutively active) form of NF90 did not exhibit any altered activity with effect on PKR phosphorylation in response to dsRNA treatment compared with WT NF90 (data not shown). These results establish a mechanism in which NF90 is involved in a complex process of host regulation of gene expression exerted through the PKR pathway.

NF90 is a component of stress granules

NF90 was shown to inhibit replication of a broad range of viruses and to form complexes with NF45 and shuttle between the nucleus and cytoplasm (13–19). However, a detailed mechanism describing how NF90 negatively regulates virus replication remains unclear. One of the well-defined pathways set in motion following PKR activation was found to associate with the regulation of mRNA translation through induction of stress granules in virus-infected cells (25, 34, 35). Taken together with the observed interaction between NF90 and PKR, a mechanism for regulation of protein synthesis involving these two proteins seems likely. This study tested the possibility that NF90 is an undefined component of PKR-induced stress granules. We examined stress granules induced by treatment with arsenite and poly(I:C) for the presence of NF90. NF90 was found to colocalize with the stress granule markers PABP1 and G3BP (Fig. 3A), as well as TIA-1 (data not shown), in both arsenite- and poly(I:C)-induced granules. In untreated control cells, NF90 predominantly localized in the nucleus and did not colocalize with PABP1 in the cytoplasm. To investigate whether NF90 is involved in the formation of stress granules, we used the pair of NF90 stable knockdown 293T cell lines described above (14) (Fig. 2B). It is notable that significantly more stress granules were observed in the scrambled control cells compared with the NF90-knockdown cells following induction with poly(I:C) (Fig. 3B, p < 0.05). Interestingly, there was no significant difference in granule formation between NF90-knockdown and scrambled cells

![FIGURE 3. NF90 colocalizes with markers of stress granules. (A) 293T cells were transfected with poly(I:C) for 6 h, treated with sodium arsenite for 30 min, or left untreated, and immunofluorescence assays were performed as described previously (33). Stress granules were examined using Abs specific for G3BP or PABP1 and NF90 (anti-DRBP76) and visualized using a confocal microscope. DAPI is shown in blue in merged images (original magnification ×63). (B) Scrambled or NF90 stable knockdown (kd) cells were treated with poly(I:C) or arsenite and examined for the formation of stress granules (SG) by staining with anti-G3BP and DAPI. Stress granules (G3BP) present in the cytoplasm are shown in green. (C) 293T cells were treated with poly(I:C) or mock treated for 4 h; treatment medium was removed and replaced with medium containing 2-AP (with final working concentration of 10 mM) in vehicle (acetic acid) or vehicle only. For statistical analysis of cells containing stress granules (SG), ~100–200 cells were examined for the presence of SG in each of three independent experiments, and the average percentage of SG+ cells was calculated. Error bars represent SD. (D) Enlarged images of stress granules in scrambled and NF90-knockdown (kd) cells after treatment with poly(I:C) (original magnification ×40). *p < 0.05, **p < 0.01.](http://www.jimmunol.org/DownloadedFrom)
treated with arsenite (Fig. 3B, lower panels). Because poly(I:C), but not arsenite, activates PKR (42), these results suggest that NF90’s role in the formation of stress granules requires PKR. To further verify that NF90’s facilitation of stress granule induction in cells in response to poly(I:C) treatment is mediated by PKR, we tested whether treatment with the PKR inhibitor, 2-AP (43), affected stress granule induction. Our results showed that 2-AP, but not the vehicle-only control, reduced stress granule formation in NF90 WT cells treated with poly(I:C) (Fig. 3C, p < 0.01). These results clearly demonstrate that NF90-regulated stress granule formation in response to dsRNA induction is mediated by PKR.

In contrast to the scrambled 293T cells, in which distinct stress granules were observed, PABP1 exhibited a diffuse pattern of expression in NF90-knockdown cells (Fig. 3D), suggesting that NF90 may be a core nucleating component for the formation of stress granules in response to dsRNA induction. Just as we established that the C-terminal of NF90 is required for interaction with PKR (Fig. 1C), it was important to identify which domain of NF90 is associated with stress granules. Because NF90 predominantly localizes to the nucleus in normal conditions, we created a mutant NF90 protein that does not have the ability to localize to the nucleus to better understand NF90’s association with stress granules. When four substitutions were introduced into the putative nuclear-localization signal of NF90 (residues 385–388) (Fig. 1A), this mutated NF90 (NF90-NLSm) was found exclusively in the cytoplasm of poly(I:C)-treated cells, where it localized to stress granules (Fig. 4A). This seems to suggest that the nuclear-localization ability of NF90 is not associated with its interaction with stress granules. In contrast, an RBDm (NF90-RBDm) that lacked RNA-binding ability was not able to localize to stress granules (Fig. 4A). PKR was reported to phosphorylate T188 and T315 of NF90, thereby regulating NF90 cellular shuttling (13). We found that the phosphorylation-deficient T188A/T315A mutant (NF90-A) (Fig. 1A) exhibited a deficiency in localization to stress granules in response to dsRNA treatment (Fig. 4A). The control, 293T cells transfected with vector, showed no stress granules (data not shown). By using siRNA targeting the 3′-untranslated region of NF90, we were able to knock down NF90 expression to a minimal level in 293T cells (Fig. 4B). We then examined different NF90 mutants for the ability to restore formation of stress granules in NF90-knockdown cells. Although both RBDm (NF90-RBDm) and phosphorylation mutant (NF90-A) possess low-level stress granule formation activity, the ability of these mutants to restore stress granule formation in poly(I:C)-treated NF90 knockdown cells was considerably weaker than that of WT NF90 (Fig. 4C, 4D). Taken together, these results suggest that RNA binding and phosphorylation of NF90 are required for induction of stress granules via dsRNA activation of PKR.

**Mutant of influenza A virus associated with PKR activation**

NF90 was shown to be involved in the host antiviral response and to limit influenza virus replication (12, 19). We attempted to determine whether there is an NF90–PKR antiviral pathway that enhances activation of PKR, using influenza virus as a model. The nonstructural protein 1 (NS1) of influenza A virus is able to inhibit PKR activation and stress granule formation induced by PKR activation, thereby supporting virus replication (33, 34). We previously described a panel of NS1 mutant viruses that is not able to inhibit formation of stress granules (33) (Fig. 5A). We confirm in...
this study that the NS1 mutants that cannot inhibit stress granule formation also have lost the ability to inhibit PKR activation induced by poly(I:C) or virus infection (Fig. 5B, 5C). Consistent with previous observations, the R38A mutant form of NS1, which is attenuated in its ability to suppress host IFN expression, retains the ability to inhibit PKR phosphorylation similarly to WT NS1 (33, 44). The R35A mutant, which has lost both RNA-binding and dimerization ability (45), was used as a nonfunctional NS1 control and showed no activity in inhibiting PKR activation. Although RNA-binding activity is not affected, as described in a previous study (33), NS1 mutants that combine I123A/M124A/K126A/N127A (123-127A) quadruple mutations exhibited a reduced ability to inhibit PKR activation induced by poly(I:C) or virus infection (Fig. 5B, 5C). Using this PKR-specific NS1 mutant, we further demonstrated that knockdown of NF90 produced a negative effect on PKR phosphorylation upon virus infection (Fig. 5D).

We then examined whether the 123-127A quadruple mutant had lost the ability to suppress formation of stress granules, in addition to being unable to inhibit PKR activation. We first examined whether any influenza A virus components were targeted to stress granules. We showed previously that NP interacts with RAP55 and colocalizes to P-bodies and stress granules (33). Moreover, NP also interacts with NF90 (19). Because NF90 is associated with stress granules, as shown above, it seems possible that it negatively regulates influenza A virus replication by targeting viral NP to stress granules. To test this idea, individual PB1, PB2, PA, and NP influenza virus proteins were expressed in cells, and their colocalization with stress granules was examined following treatment with poly(I:C). Only NP colocalized with the stress granule marker G3BP following poly(I:C) induction (Fig. 6A, upper panel). A colocalization assay with another stress granule marker, PABP1, showed a similar result (data not shown). When RNP (composed of PB1, PB2, PA, and NP) were coexpressed with NF90, only NP was targeted to stress granules (Fig. 6A, lower panel). Using NP as a marker for influenza A virus–induced stress granules, we found that, although WT virus suppresses formation of stress granules, NP was clearly detectable in stress granules in 293T, MEF, and HeLa cells infected with 123-127A mutant influenza virus at 24 h postinfection. In addition, the nonfunctional R35A NS1 and del-NS1 mutants also failed to inhibit formation of NP-associated stress granules in infected cells (Fig. 6B–D). Quantitation of stress granule–forming 293T cells infected with WT or mutant viruses further confirmed that inhibition of PKR blocks induction of stress granules upon virus infection (Fig. 6E). The characteristics of these NS1 mutants of influenza A virus indicated their suitability for further use in studying the PKR-related antiviral pathway.

NF90 antiviral activity is not associated with expression of IFN-β

We next performed a series of infections, using WT and mutant influenza A viruses, to demonstrate the role of NF90 in the PKR-induced antiviral pathway and to confirm that it is independent of IFN expression. We previously showed that there is a significant increase in the replication of PR8 (H1N1) and 1194 (H5N1) influenza viruses in cells in which NF90 has been transiently knocked down using siRNA targeting NF90 compared with cells treated with control siRNA (19). Using stable NF90-knockdown cells, we observed a similar significant negative effect (p = 0.0001) of NF90 on virus replication in cells infected with another H1N1 virus strain, A/WSN/1933 (Fig. 7A). To verify that the altered virus-replication efficiency is associated with the NF90–PKR pathway, we compared the growth kinetics of WT WSN virus with those of mutant WSN viruses containing different NS1 mutations, which were attenuated in inhibition of PKR activation (123-127A) or

![FIGURE 5](https://example.com/figure5.png)

**FIGURE 5.** Characterization of an NS1 mutant of influenza A virus that is attenuated in the ability to inhibit PKR phosphorylation in response to dsRNA activation. (A) Illustration of the NS1 protein of influenza A virus (A/WSN1933), showing locations of mutations used in this study. (B) 293T cells were transfected with pCMV expression vectors containing WT or R35A, R38A, or 123-127A quadruple NS1 mutants or with vector expressing GFP, or they were left untransfected prior to treatment with poly(I:C). Cells were harvested and lysed for Western blot analysis of NS1 and PKR expression and PKR phosphorylation. Expression of β-tubulin was analyzed for normalization of protein loading. (C) 293T cells were infected with WT or R38A or 123-127A NS1 mutant viruses for 24 h, and PKR activation levels were examined. Virus infection is shown by viral NP expression. (D) 293T cells were treated with siRNA targeting NF90 or scrambled siRNA for 56 h and then infected with WT or 123-127A NS1 mutant virus or were mock infected. Cell lysates were examined for levels of PKR phosphorylation at 24 h postinfection. Levels of total PKR also were estimated as a control. Relative levels of protein present in bands were analyzed by estimation of band density using ImageJ software in Western blots, as described above.
IFN expression (R38A) (as described in Fig. 5A), in both NF90 stable-knockdown and scrambled 293T cells. In the scrambled 293T cells, antiviral mechanisms involving expression of IFN-β and activation of PKR are intact, with both 123-127 and R38A mutant viruses replicating to a significantly lower level ($p = 0.0001$) compared with the WT virus (Fig. 7B). In the NF90-knockdown cells, it is notable that 123-127A quadruple mutant virus showed no sign of attenuation and replicated to a level similar to the WT virus, whereas replication of R38A mutant virus was severely attenuated, presumably due to the antiviral effects of IFN (Fig. 7C).

The R38A mutant is well recognized for its reduced ability to inhibit the host IFN antiviral response (46). To differentiate the effects caused by the IFN antiviral response from those associated with the NF90–PKR pathway, we compared virus-replication efficiency in Vero cells, which are deficient in IFN expression. In contrast to the result obtained in NF90-knockdown cells, R38A mutant virus is able to replicate to a level close to that of WT virus, whereas the 123-127A quadruple mutant is significantly attenuated ($p = 0.0001$) (Fig. 7D).

PKR-knockout (PKR$^{-/-}$) and WT (PKR$^{+/+}$) primary MEF cells were used to further verify the linkage of NF90 and PKR. Western blot showed NF90 expression in both PKR-knockout (PKR$^{-/-}$) and WT (PKR$^{+/+}$) primary MEF cells. Using this influenza A virus–infection model, we further demonstrated that PKR is a downstream effector for NF90 antiviral activity, because infection with mutant viruses that are unable to inhibit PKR activation (NS1 R35A or 123-127A) induces stress granules in PKR WT (PKR$^{+/+}$) cells, but not in PKR knockout (PKR$^{-/-}$) cells with normal NF90 (Fig. 7F, 7G), and expression of PKR by transfection restores stress granule–forming ability in PKR$^{-/-}$ cells (Fig. 7G, lower panel). PKR is an IFN-inducible gene, and it is not known whether the differences in stress granule induction may be due to variations in inhibition of IFN expression by WT and mutant NS1 proteins. However, examination of inhibition of IFN-β expression in WT and mutant virus-infected cells revealed that both the WT and 123-127A quadruple mutant viruses are able to inhibit IFN-β expression, but R38A virus cannot (Fig. 7E). Taken together, these results support the idea of an underlying mechanism whereby NF90 is associated with PKR activation in the host antiviral response, and the related antiviral signaling is independent of induction of IFN-β expression (Fig. 8).

Discussion

NF90 was found to play an important role in host innate immunity against various virus infections (12–15). We (19) previously found that NF90 negatively regulates influenza A virus replication through interaction with viral NP. Although the significance of NF90 in restricting virus replication is evident from the magnitude of virus replication increase in NF90-knockdown cells (12, 19), the molecular
details of the NF90-associated antiviral mechanism remain largely unknown. NF90 is one of the evolutionarily conserved members of the dsRNA-binding protein family and is expressed abundantly in various human cells. Previous studies (6, 7, 9, 10, 13, 40) identified and confirmed PKR as the key interacting partner for NF90 in cells, in addition to its function in regulating IL-2 gene expression in T cells (4). PKR plays an important role in mediating innate immunity to viral infection and is required for NF90 antiviral activity, as demonstrated in a vesicular stomatitis virus infection experiment using PKR-knockout (PKR−/−) and control (PKR+/+) primary MEFs (13, 25). Thus, it is natural to reason that the antiviral activity exerted by NF90 may lie within the PKR-signaling pathway.

Phosphorylation of PKR is essential for activation of its function, and it can be regulated by host RNA-binding proteins other than dsRNA (47–49). Two models currently describe PKR activation: the autoinhibition and dimerization models (50). It was suggested that binding of either dsRNA or PACT to PKR may interrupt intramolecular interaction between the kinase and dsRNA-binding domains, leading to an open conformation of the PKR molecule and allowing autophosphorylation to occur (49). NF90 is phosphorylated by PKR in its RBD (6). Previous studies (7, 9, 29) showed that NF90 interacts with PKR in the yeast two-hybrid and GST-pull down assays. We confirmed interaction between NF90 and PKR in 293T cells using a co-IP assay and showed that it is independent of RNA binding of NF90, because the RBDm could interact with PKR (Fig. 1C). On testing the effect of NF90 on PKR activation, we found that low concentrations of NF90 restore some PKR activity in response to poly(I:C) induction in NF90 stably knocked-down cells. However, when high concentrations of NF90 were introduced by transfection, PKR phosphorylation was inhibited in these cells. Furthermore, the results showed that the inhibitory effect is exerted by the dsRNA-binding domain, presumably

**FIGURE 7.** NF90 inhibition of influenza A virus is mediated via the PKR pathway. (A) Growth kinetics in NF90 stable-knockdown and scrambled 293T cells infected with the influenza A virus A/WSN/1933 at an MOI of 0.005. Growth kinetics of influenza viruses containing specific mutations that attenuate their ability to inhibit PKR activation were determined in different cell lines. Scrambled (B) and NF90-knockdown (C) 293T cells and Vero cells (D) were infected with WT A/WSN/1933 (H1N1) or mutant viruses containing NS1 R38A or 123–127A quadruple mutations at an MOI of 0.05. Supernatants were collected at 8, 24, and 48 h postinfection (h.p.i.) for titration by plaque assay. (E) NF90 inhibition of influenza A virus is not caused by expression of IFN-β. NF90-knockdown (kd) cells and scrambled control cells were infected with WT or NS1 R38A or 123-127A mutant viruses at an MOI of 0.1 or were mock infected (PBS). At 24 h postinfection, cellular RNAs were extracted, and quantitative real-time PCR was performed. IFN-β mRNA levels were determined after normalization. (F) Western blot showing NF90 and PKR expression in both PKR-knockout (PKR−/−) and WT (PKR+/+) primary MEF cells. Expression of β-tubulin was assessed as a loading control. (G) PKR-knockout (PKR−/−) and WT (PKR+/+) primary MEF cells were infected with R35A or 123–127A virus for 24 h before being subjected to immunofluorescence assay for visualization of stress granules (upper panel, original magnification ×63). G3BP, NP, and DAPI are shown in green, red, and blue, respectively. PKR expression in PKR-knockout MEF cells was restored by transfection of a V5-tagged PKR construct (lower panel, original magnification ×63). MEF cells with restored PKR expression were infected with 123–127A mutated influenza A virus. Formation of antiviral stress granules was examined by immunostaining using anti-V5 (PKR), anti-NP, and anti-G3BP Abs. Error bars represent SD. *p < 0.05, **p < 0.01, ***p < 0.001, t test.
through competition with PKR for dsRNA ligand in an RNA-binding–dependent manner, as was suggested previously (6). This hypothesis was confirmed in this study using an NF90 mutant containing mutations in the RBD, which shows no inhibition of PKR phosphorylation when transfected into 293T cells at high concentrations (Fig. 2D). NF90 predominantly localizes to the nucleus but may be exported to the cytoplasm to perform its functions (5,8). NF90 is also phosphorylated by PKR, and activated NF90 does not inhibit PKR activation (6). It was proposed that shuttling of NF90 between nucleus and cytoplasm is regulated through phosphorylation of NF90 by PKR (13). However, these studies did not investigate whether NF90 exerted any effect on PKR. Our results suggest that a physiological concentration of NF90 is required for optimal activation of PKR in response to stimuli, such as cellular dsRNA or intermediate dsRNA products generated during virus replication. Because PKR phosphorylation can activate downstream antiviral cascades, including NF-κB and inflammasomes (28, 51), it is postulated that NF90 may serve as a regulator for PKR activation in response to cellular dsRNA stimuli or virus infection. Our findings suggest a mechanism in which NF90 may act to regulate PKR activity in cells through balancing PKR phosphorylation in response to dsRNA via its N- and C-terminal functions. The PKR-activation function of NF90 lies in the N-terminal region. In contrast, interaction between NF90 and PKR also may modulate the activation of PKR through competition with dsRNA via its C-terminal RBD; this also was reported in an in vitro study (6).

Hosts use multiple antiviral mechanisms to restrict virus infection; formation of stress granules is an important antiviral response that limits virus replication by stalling viral protein synthesis (52, 53). Activation of PKR is required for the formation of stress granules in response to virus infection (34–36, 54). This study attempts to delineate the mechanism of NF90 antiviral activity through examination of NF90 involvement in PKR activation and formation of antiviral stress granules using the influenza A virus–infection model.

We found that NF90 is regularly associated with stress granules formed in response to treatments with oxidative substances (arsenite) or dsRNA mimics [poly(I:C)]. It was further demonstrated that NF90 may be required for the formation of stress granules in the dsRNA-induced host response, because there is a significant reduction in the levels of stress granules formed in response to poly(I:C) treatment in NF90 stably knocked-down cells. However, there was no significant difference in granule formation between NF90 knockdown and scrambled controls treated with arsenite, which does not involve PKR activation (42) (Fig. 3B), whereas treatment with a PKR phosphorylation inhibitor, 2-AP (43), inhibited stress granule induction (Fig. 3C). It seems likely that NF90’s association with stress granules is coupled with its ability to interact with PKR and regulate its activation. Stable knockdown of NF90 renders 293T cells ineffective in induction of phosphorylation of PKR in response to poly(I:C) treatment.

To further verify the linkage between NF90 and PKR signaling in the host antiviral response, we generated an influenza A virus specifically mutated in the NS1 gene that is attenuated in its ability to inhibit PKR activation but otherwise functions normally. We found that NP of this mutant is targeted to stress granules, presumably through interaction with NF90 and/or other NP-interacting proteins. Using this PKR-specific virus model, this study clearly demonstrated that this mutant virus is able to replicate to a similar level as WT virus in NF90-knockdown cells but not in scrambled control cells or in Vero cells, which are deficient in expression of IFN-β. Our experiments also showed that this specific mutant virus is able to replicate to a similar level as WT virus in NF90-knockdown cells but not in scrambled control cells or in Vero cells, which are deficient in expression of IFN-β. Our experiments also showed that this specific mutant virus is able to inhibit IFN-β expression just as WT virus does but that the R38A mutant, which has lost RNA-binding ability, is attenuated in terms of suppression of IFN. These lines of evidence strongly support the hypothesis that NF90 antiviral function is signaled through the PKR pathway.

Although further studies are needed to reveal the details of how NF90 enhances PKR phosphorylation and how NF90 is recycled back to the nucleus after engaging in PKR activation in the

![Diagram](http://www.jimmunol.org/Downloadedfrom/fig8.gif)

**FIGURE 8.** Working model of PKR-mediated NF90 antiviral pathway. In normal conditions, NF90 migrates to the cytoplasm to facilitate PKR phosphorylation in response to virus infection or the presence of dsRNA during virus replication. Activation of PKR leads to the phosphorylation of eIF2α and subsequently triggers formation of stress granules that involve NF90, PKR, and other stress granule components. However, viruses adapt different mechanisms to antagonize NF90–PKR antiviral activity; for instance, influenza A viruses express NS1 to inhibit phosphorylation of PKR, which also may interact with NF90, to enhance virus replication. PKR also may be activated by IFN to exert antiviral activity in an NF90-independent manner.
cytoplasm, it is tempting to postulate that, in normal conditions, NF90 migrates to the cytoplasm to facilitate PKR phosphorylation in response to virus infection or the presence of dsRNA during virus replication. Activation of PKR leads to the phosphorylation of eIF2α and subsequently triggers formation of stress granules that involve both NF90 and PKR (Fig. 8). We also found that phosphorylation of NF90 by PKR is required for NF90–PKR signaling to induce antiviral stress granules (Fig. 4). Based on a previous study (13) that reported that NF90 is phosphorylated by PKR and retained in the cytoplasm during the interaction, it seems reasonable to suggest that NF90 and PKR are mutually regulated. It may be speculated that an accumulation of NF90 in the cytoplasm may subsequently compete with PKR for dsRNA binding and, thereby, form a feedback loop to restore PKR back to its latent form; similarly, NF90 would be recycled back to the nucleus after the stimulii dissipates. Further studies are needed to unveil the regulatory loop of the NF90–PKR pathway. Although we showed that IFN-β is not involved in NF90–PKR activation, it would be interesting to study how NF90 and IFN coordinately activate PKR in the antiviral state induced during virus infection. Host innate immunity relies on pattern recognition receptors, which reside primarily in the cytosol or cellular membranes, to recognize pathogen-associated molecule patterns that are usually intermediate products generated from the viral replication process. Because NF90 predominantly resides in the nucleus, it would be important to examine whether it is a yet-to-be-defined nuclear pattern recognition receptor, like IFI16 (55).

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Disclosures

The authors have no financial conflicts of interest.

References


