Andes Virus Nucleocapsid Protein Interrupts Protein Kinase R Dimerization To Counteract Host Interference in Viral Protein Synthesis

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ABSTRACT

Pathogenic hantaviruses delay the type I interferon response during early stages of viral infection. However, the robust interferon response and induction of interferon-stimulated genes observed during later stages of hantavirus infection fail to combat the virus replication in infected cells. Protein kinase R (PKR), a classical interferon-stimulated gene product, phosphorylates the eukaryotic translation initiation factor eIF2 α and causes translational shutdown to create roadblocks for the synthesis of viral proteins. The PKR-induced translational shutdown helps host cells to establish an antiviral state to interrupt virus replication. However, hantavirus-infected cells do not undergo translational shutdown and fail to establish an antiviral state during the course of viral infection. In this study, we showed for the first time that Andes virus infection induced PKR overexpression. However, the overexpressed PKR was not active due to a significant inhibition of autophosphorylation. Further studies revealed that Andes virus nucleocapsid protein inhibited PKR dimerization, a critical step required for PKR autophosphorylation to attain activity. The studies reported here establish a hantavirus nucleocapsid protein as a new PKR inhibitor. These studies provide mechanistic insights into hantavirus resistance to the host interferon response and solve the puzzle of the lack of translational shutdown observed in hantavirus-infected cells. The sensitivity of hantavirus replication to PKR has likely imposed a selective evolutionary pressure on hantaviruses to evade the PKR antiviral response for survival. We envision that evasion of the PKR antiviral response by NP has likely helped hantaviruses to exist during evolution and to survive in infected hosts with a multifaceted antiviral defense.

IMPORTANCE

Protein kinase R (PKR), a versatile antiviral host factor, shuts down the translation machinery upon activation in virus-infected cells to create hurdles for the manufacture of viral proteins. The studies reported here reveal that the hantavirus nucleocapsid protein counteracts the PKR antiviral response by inhibiting PKR dimerization, which is required for its activation. We report the discovery of a new PKR inhibitor whose expression in hantavirus-infected cells prevents the PKR-induced host translational shutdown to ensure the continuous synthesis of viral proteins required for efficient virus replication.

Hantaviruses are segmented negative-strand RNA viruses of the *Bunyaviridae* family. Their genomes are composed of three RNA segments, S, L, and M, encoding the viral nucleocapsid protein (NP), the viral RNA-dependent RNA polymerase (RdRp), and the glycoprotein precursor (GPC), respectively (1). The GPC is posttranslationally cleaved at a conserved WAASA motif into two glycoproteins: Gn and Gc (2). Hantaviruses are carried by rodents. Humans are infected by the inhalation of aerosolized excreta of infected rodent hosts. Hantavirus infections cause hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS), with mortality rates of up to 12% and 50%, respectively, in certain outbreaks (3). Annually, 150,000 to 200,000 cases of hantavirus infection are reported worldwide (4). There is no FDA-approved vaccine or antiviral therapeutic against hantavirus infections. Hantaviruses usually are not transmitted from human to human. However, Andes virus (ANDV), a New World hantavirus species, has been reported to undergo human-to-human transmission (5). Hantaviruses primarily target endothelial cells (ECs) with the receptor (β 3 integrin) for virus attachment and entry. Their replication occurs exclusively in the host cell cytoplasm. Hantaviral RdRp initiates transcription by a unique cap-snatching mechanism to generate 5'-capped viral mRNAs (6–8). Despite their 5' caps, viral mRNAs

must actively compete with host cell transcripts for the same translation machinery. Our recently published findings suggest that hantaviruses use a novel NP-mediated translation initiation mechanism that lures the host translation apparatus for the preferential translation of viral mRNA (9).

ECs respond differently to pathogenic and nonpathogenic hantavirus infections. Previous studies have shown that the nonpathogenic virus Prospect Hill virus (PHV) strongly stimulates the expression of interferon (IFN) and interferon-stimulated genes (ISGs) during the early stage of viral infection, limiting PHV replication in ECs (10, 11). In contrast, the pathogenic viruses Hantaan virus (HTNV), Sin Nombre virus (SNV), New York-1

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virus (NY-1 virus), and ANDV induce very weak innate immune responses during the early stages of infection. As a result, pathogenic hantaviruses successfully replicate in ECs (10, 11). Moreover, both pathogenic and nonpathogenic hantaviruses replicate to the same titers in IFN-deficient Vero E6 cells (10). These observations suggest that pathogenic hantaviruses have evolved a strategy to delay early interferon induction for efficient replication in ECs. Further studies revealed that the Gn cytoplasmic tail domain inhibits IFN induction (12). Interestingly, both pathogenic and nonpathogenic hantaviruses strongly induce the expression of both IFN and ISGs at later stages of viral infection, but this fails to combat pathogenic hantavirus replication (11). Moreover, pathogenic hantaviruses are sensitive to IFN pretreatment or posttreatment within 12 h of virus infection. IFN treatment at 15 to 24 h post-virus infection induces an ISG response which fails to combat virus replication (10, 13). These observations suggest that pathogenic hantaviruses have evolved strategies to counteract antiviral effects of ISGs by an unknown mechanism. One of the crucial ISGs implementing the antiviral actions of interferon is protein kinase R (PKR), a double-stranded RNA (dsRNA)-activated protein kinase that phosphorylates and inactivates the α subunit of eukaryotic translation initiation factor 2 α (eIF2 α) (14). The phosphorylation of eIF2 α inhibits translation initiation, which imposes restrictions on the synthesis of viral proteins in the host cell. The PKR antiviral response promotes the establishment of an antiviral state in the host cell, aimed to limit virus replication and dissemination in the host (15). The hantavirus NP-mediated translation strategy is also sensitive to PKR activation due to its dependence upon eIF2 α . However, viruses have evolved numerous strategies to counteract the PKR antiviral response. These include the expression of virus-encoded decoy dsRNA, PKR degradation in virus-infected cells, sequestration of viral dsRNA, inhibition of PKR dimerization, dephosphorylation of downstream target eIF2 α , and production of PKR pseudosubstrates (16–18). It is well understood that hantavirus-infected cells do not undergo translational shutdown even though PKR is overexpressed due to the virus-induced IFN response during later stages of infection. This led to the hypothesis that hantaviruses have evolved a strategy to counteract the PKR-induced translational shutdown in virus-infected cells that ensures continuous manufacture of viral proteins during the course of viral infection. In this study, we show that ANDV NP interferes with PKR dimerization, a critical step for its activation. The resulting PKR inactivation prevents translational shutdown in virus-infected cells and facilitates viral protein synthesis during the course of infection.

MATERIALS AND METHODS

Cell culture and virus propagation. Human embryonic kidney 293T (HEK293T), African green monkey kidney (Vero E6), and human hepatocarcinoma Huh7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (HyClone) supplemented with 10% fetal bovine serum (FBS) (HyClone), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and cultured in EGM BulletKit medium (Lonza). Lentiviruses were packaged in HEK293T cells. Briefly, the gene of interest was cloned into the pLenti-CMV vector. The resulting expression plasmid was cotransfected into HEK293T cells along with the packaging plasmid psPAX2 (Addgene) and the envelope plasmid pMD2.G (Addgene). Supernatants from transfected cells were harvested at 48 and 72 h posttransfection. Lentivirus particles were concentrated by ultracentrifugation and quantified by a quantitative PCR (qPCR)-based assay (19). ANDV (strain

Chile-9717869) was propagated in Vero E6 cells. Briefly, ANDV was inoculated into Vero E6 cells at a multiplicity of infection (MOI) of 0.03. The cells were cultured in DMEM containing 2.5% FBS. Supernatant from cultured cells was collected at 13 days postinfection. The viral titers in the supernatant were determined by plaque assay (20). All work with infectious ANDV was performed in a biosafety level 3 laboratory at the University of Kansas Medical Center.

Antibodies. The primary antibodies for PKR (12297), eIF2 α (5324), and phosphorylated eIF2 α (9721) were from Cell Signaling Technologies. The primary antibody for phosphorylated PKR (ab81303) was from Abcam. The primary antibodies for the FLAG tag (clone M2; F1804), β -actin (A5441), and enhanced green fluorescent protein (EGFP) (G6795) were from Sigma. The primary antibodies for the c-Myc tag (clone 9E10; sc-40) and normal mouse IgG (sc-2025) were from Santa Cruz. The primary antibody for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (A01622) was from GenScript. The rat antiserum used for the detection of NP was from our lab.

Plasmids. Total RNA purified from ANDV-infected Vero E6 cells was reverse transcribed using random primers. The resulting cDNA was used to PCR amplify the ANDV NP open reading frame (ORF). The ORF encoding SNV NP was similarly PCR amplified from the pTSNV N vector (21). The ORF encoding the nucleocapsid protein of HTNV strain M14626 was synthesized by Integrated DNA Technologies (IDT). Each ORF was inserted into a pcDNA3.1(+) backbone that was previously modified to incorporate either a Myc or FLAG tag at the N terminus of the ORF. The pHis-NP plasmid, expressing C-terminally His-tagged ANDV NP, was constructed by insertion of the NP ORF into the pTriEx1.1 backbone. The pMyc-NP (Δ 175-230) and pHis-NP (Δ 175-230) plasmids, expressing ANDV NP mutants lacking the RNA binding domain, were subcloned by overlapping PCR as previously reported (22). Lentiviral vectors expressing N-terminally Myc-tagged wild-type or mutant NP were subcloned from the pMyc-NP or pMyc-NP (Δ 175-230) plasmid into the pLenti-CMV backbone (Addgene). The pMyc-PKR and pFLAG-PKR plasmids, expressing Myc- and FLAG-tagged PKR, respectively, were constructed by inserting the PKR ORF into the modified pcDNA3.1(+) backbone as mentioned above. The PKR ORF was amplified from cDNA generated by reverse transcription of an RNA sample obtained from HUVECs pretreated with IFN- α . All plasmids were sequenced to verify sequence integrity.

Transfection and virus infection. Plasmid and poly(I:C) transfections were carried out using Turbofect transfection reagent (Thermo) and Lipofectamine 2000 (Invitrogen), respectively, following the manufacturers' instructions. Lentiviral infection was carried out in a biosafety level 2 environment. Briefly, Polybrene (8.0 μ g/ml) was added to endothelial cell growth medium (EGM, cc-3124; Lonza) followed by the addition of lentivirus at the required MOI, using a high-concentration stock. The resulting mixture was added to HUVECs, incubated for 12 h, and then replaced with fresh medium. Cells were harvested at 48 h postinfection. For lentivirus-based stable cell line generation, HEK293T cells were infected with lentivirus by the same method as that mentioned above, except that the cells were selected with puromycin (3 μ g/ml). ANDV infection was carried out in a biosafety level 3 environment. Briefly, ANDV from a high-concentration stock was diluted in DMEM containing 2.5% FBS to achieve the required MOI. Cells were incubated with the diluted virus preparation for 1 h with periodic rocking. Cells were rinsed twice with phosphate-buffered saline (PBS) to remove the unabsorbed virus. Cells harvested at this time point are referred to as cells at 1 h postinfection and were used as the baseline for virus replication. The remaining cells were allowed to grow in fresh medium and harvested at different time points postinfection.

Coimmunoprecipitation. HEK293T cells seeded in 6-cm dishes were cotransfected with the required plasmids. Cells were carefully washed once with PBS at 48 h posttransfection, followed by lysis in 600 μ l of NP-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM EDTA) supplemented with protease and phosphatase

inhibitor cocktails (Roche). Cell lysates were clarified by centrifugation at $16,000 \times g$ for 15 min at 4°C , and supernatants were collected. Fifty microliters of the supernatant was mixed with an equal amount of $2\times$ SDS loading buffer and saved as input. The remaining supernatant was pre-cleared with protein G agarose beads (Invitrogen) for 30 min and incubated for 4 h with $1\ \mu\text{g}$ of required antibody. The mixture was further incubated with $40\ \mu\text{l}$ of protein G agarose beads by continuous rotation for 1 h at 4°C . The beads were pelleted down by a brief centrifugation and washed four times with lysis buffer. The material bound to washed beads was eluted by boiling with $45\ \mu\text{l}$ of $1\times$ SDS loading buffer. The boiled samples were briefly centrifuged, and $15\ \mu\text{l}$ of the supernatant was examined by Western blot analysis.

Western blot analysis. Cells were washed once with PBS and lysed with RIPA buffer supplemented with protease inhibitor cocktail (Roche). Cell lysates were mixed with an equal volume of $2\times$ SDS loading buffer and boiled at 95°C for 5 min. Samples were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked with 5% nonfat milk in PBST buffer ($1\times$ PBS, 0.05% Tween 20). The membrane was incubated with primary antibody, followed by washing and further incubation with the secondary antibody conjugated to horseradish peroxidase (HRP). The antibody concentrations used were those suggested by the manufacturer. The protein signal was detected by chemiluminescence.

^{35}S metabolic labeling. Cells grown in 6-well plates were washed twice with starvation medium (DMEM containing 10% FBS and deficient in methionine and cysteine) and then cultured in starvation medium for 30 min to deplete the intracellular pools of methionine and cysteine. Cells were then incubated for 40 min with 1 ml of starvation medium containing $300\ \mu\text{Ci}$ ^{35}S -labeled methionine and cysteine (PerkinElmer). Cells were washed once with PBS and lysed with $100\ \mu\text{l}$ RIPA buffer. The cell lysates were mixed with an equal volume of $2\times$ SDS loading buffer and boiled at 95°C for 5 min. Protein samples were separated by 10% SDS-PAGE. The gel was stained with Coomassie brilliant blue dye, dried on filter paper, and exposed to X-ray film overnight at -80°C .

Real-time PCR. Total RNA was extracted from cells by use of an RNeasy kit (Qiagen) following the manufacturer's instructions. One microgram of purified RNA was reverse transcribed in a total volume of $20\ \mu\text{l}$, using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) according to the manufacturer's protocol. The cDNA was diluted 10-fold, and $5\ \mu\text{l}$ of the diluted cDNA sample was used in a $20\text{-}\mu\text{l}$ real-time PCR mixture for quantitative estimation of the mRNA of interest, using an appropriate primer set. Each reaction was performed in triplicate. Similarly, the mRNA levels of a housekeeping gene (β -actin) were quantified as an internal control. Real-time PCRs were performed on an ABI 7500 real-time PCR system (Applied Biosystems), using SYBR green PCR master mix (Roche). We used the relative quantification method for data analysis, as previously mentioned (23). Fold changes in mRNA levels and standard deviations (shown as error bars) were calculated as previously reported (6). The primer pairs used for the quantitative estimation of mRNA levels for PKR (5'-GCC GCT AAA CTT GCA TAT CTT CA-3' and 5'-TCA CAC GTA GTA GCA AAA GAA CC-3'), IFN- β (5'-GCT TGG ATT CCT ACA AAG AAG CA-3' and 5'-ATA GAT GGT CAA TGC GGC GTC-3'), and β -actin (5'-GAG CAC AGA GCC TCG CCT TT-3' and 5'-TCA TCA TCC ATG GTG AGC TGG-3') were selected from PrimerBank and have previously been verified for use in real-time PCR analysis (24). The sequences of the primers used for the quantitative estimation of ANDV S-segment RNA levels by real-time PCR analysis were 5'-CAG CTC GTG ACT GCT CGG C-3' and 5'-GTA GAC ACA GCT GCC CGT CTA C-3'. These primers have been verified for use in real-time PCR.

RESULTS

ANDV induces PKR overexpression but fails to shut down the host translation machinery. Pathogenic hantaviruses, such as ANDV and SNV, are well known to inhibit the early IFN response

after infection (11, 25, 26). However, strong expression of both IFN and ISGs is observed in the later stages of infection. Interestingly, the delayed but strong interferon response does not inhibit virus replication (11). This observation is consistent with the fact that pathogenic hantaviruses are sensitive to early IFN treatment in cell culture. The IFN treatment remains ineffective beyond 15 h post-viral infection (10). These observations led to the hypothesis that pathogenic hantaviruses may have evolved strategies to antagonize the antiviral effects of some ISGs. To test this hypothesis, we infected HUVECs with ANDV and harvested the cells at different time points postinfection. The cells were examined for PKR expression at both the mRNA and protein levels. We observed that ANDV infection induced PKR expression at the transcriptional level (Fig. 1B). The PKR protein levels in virus-infected cells started to rise from 24 h postinfection and remained steadily high onwards (Fig. 1A). PKR is a classical ISG that promotes the establishment of an antiviral state in virus-infected cells by shutting down the host cell translation machinery to create barriers for viral protein synthesis. An examination of virus-infected cell lysates by Western blot analysis revealed that PKR overexpression had no impact upon the endogenous steady-state levels of ANDV NP (Fig. 1A). This observation suggested that PKR overexpression may not affect the host translation machinery. To test this hypothesis, we monitored *de novo* protein synthesis in virus-infected HUVECs at different time points postinfection by using [^{35}S]methionine-cysteine labeling as described in Materials and Methods. We did not observe any change in the rate of host protein synthesis due to virus infection, suggesting that ANDV has evolved strategies to counteract the PKR antiviral response (Fig. 1C and D).

ANDV regulates PKR activation. We next asked how ANDV counteracts the overexpressed PKR antiviral response to maintain the cellular translation machinery in the functional state. PKR undergoes dimerization and autophosphorylation for activation. Activated PKR then phosphorylates the downstream target eIF2 α to induce a transient shutdown of the host translation machinery. To determine whether ANDV interferes in the activation of the PKR-eIF2 α pathway, we infected HUVECs with ANDV or treated them with either IFN- α or IFN- α along with poly(I-C), as positive controls. Cells were harvested at different time points postinfection. The expression of phosphorylated PKR and eIF2 α was examined by Western blotting. As expected, both positive controls induced PKR overexpression and phosphorylation of both PKR and eIF2 α (Fig. 2A). However, while ANDV infection induced PKR overexpression similar to that in the positive control, the overexpressed PKR was poorly phosphorylated, leading to the poor phosphorylation of eIF2 α (Fig. 2A). This observation clearly demonstrates that ANDV has evolved strategies to inhibit PKR activation in virus-infected cells.

It was previously reported that lentivirus infection triggers an IFN response that activates the PKR-eIF2 α pathway (27–30). We next compared the activation levels of the PKR-eIF2 α pathway in lentivirus- and ANDV-infected cells at two different time points postinfection. It is evident from Fig. 2B that lentivirus and ANDV infections induced PKR overexpression to similar levels. However, the PKR-eIF2 α pathway was poorly activated in ANDV-infected cells compared to cells infected with lentivirus, as evident from poor phosphorylation of both PKR and eIF2 α (Fig. 2B). These results further support the hypothesis that ANDV has evolved strategies to inhibit PKR activation.

To determine whether ANDV replication was sensitive to PKR

FIG 1 ANDV stimulated PKR expression but did not affect host protein synthesis. (A) HUVECs were infected with ANDV at an MOI of 0.5, and cells were harvested at different time points postinfection. PKR and NP expression was analyzed by Western blotting. GAPDH was used as a loading control. (B) HUVECs were infected with ANDV and harvested as described for panel A. PKR mRNA levels were examined by real-time PCR, normalized to β -actin mRNA levels, and shown as fold changes relative to the level in mock cells. Results from three independent experiments were used to calculate standard deviations, shown as error bars. The significance of differences (***) was calculated by the *t* test. (C) HUVECs were infected with ANDV as described above. Cellular *de novo* protein synthesis was monitored by [35 S]methionine-cysteine incorporation at different time points postinfection. Protein samples were separated by SDS-PAGE and visualized by Coomassie blue staining (right) and autoradiography (left). (D) The band intensities from the autoradiogram were quantified by Image-Pro Plus software, averaged, and normalized to that for the mock sample. Results from three independent experiments were used to calculate the standard deviations, shown as error bars. NS, not significant.

activation, we asked whether overexpression of PKR from a transfected plasmid would interfere with ANDV replication. The expression and activation of PKR were tested in Huh7 cells either lacking or harboring replicating ANDV. Huh7 cells were used in this experiment because of their better transfection efficiency and poor expression of Toll-like receptor 3 (TLR3) (31), which is important for hantavirus recognition (32). Unlike the case in HUVECs, ANDV infection of Huh7 cells did not induce high PKR expression (Fig. 2C, compare lanes 1 and 2), likely due to the mild innate immune response in Huh7 cells. It is evident from Fig. 2C (lane 3) that PKR was overexpressed and phosphorylated in cells lacking ANDV infection. However, the overexpressed PKR was poorly phosphorylated in cells containing replicating ANDV (Fig. 2C, lane 4), consistent with similar observations shown in Fig. 2A and B.

To test the sensitivity of ANDV to PKR's antiviral effects, we transfected Huh7 cells with a PKR expression plasmid 24 h before or after ANDV infection. Virus replication was monitored in cells at 48 and 72 h postinfection by quantitative estimation of viral S-segment RNA, using real-time PCR. As shown in Fig. 2D, the prior expression of PKR inhibited ANDV replication by ~75% (Fig. 2D). In comparison, the later ex-

pression of PKR did not affect ANDV replication (Fig. 2E). These results suggest that ANDV likely does not inactivate PKR that has been activated previously.

ANDV NP inhibits PKR autophosphorylation to prevent PKR-induced translational shutdown in cells. We next asked whether ANDV NP inhibits PKR autophosphorylation, especially because of its high expression level throughout the ANDV replication cycle. Since lentivirus infections are known to induce the innate immune response and promote the expression and activation of PKR in cells, we asked whether NP expression through the lentivirus delivery system would inhibit the PKR-eIF2 α pathway in lentivirus-infected cells. To test this hypothesis, HUVECs were infected with lentivirus at increasing MOIs, with expression of either NP or EGFP (as a negative control). Cells were harvested at 48 h postinfection, and activation of the PKR-eIF2 α pathway was examined by Western blot analysis. We observed that lentiviral vectors expressing either EGFP or NP induced PKR expression in a dose-dependent manner (Fig. 3A). The infection with EGFP-expressing lentivirus activated the PKR-eIF2 α pathway, as evident from the remarkable phosphorylation of both PKR and eIF2 α (Fig. 3A, lanes 2 to 4). In comparison, the activation of the PKR-eIF2 α pathway was significantly inhibited in cells infected with

FIG 2 ANDV infection interfered with PKR phosphorylation. (A) HUVECs in six-well plates were infected with ANDV, and cells were harvested as described in the legend to Fig. 1A. In addition, HUVECs were treated with IFN- α (1,000 U/ml) for 16 h. Following IFN- α treatment, cells were either mock transfected or transfected with poly(I:C) (200 ng/ml) for an additional 2 h before harvesting. Cell lysates were examined for the expression of PKR, eIF2 α , and their phosphorylated forms by Western blot analysis using appropriate antibodies. GAPDH was used as a loading control. (B) HUVECs were infected with ANDV at an MOI of 0.5 or with lentivirus at an MOI of 30 to stimulate comparable expression levels of PKR. Cells were harvested at 48 and 72 h postinfection (p.i.). The levels of PKR, p-PKR, eIF2 α , p-eIF2 α , ANDV NP, EGFP, and GAPDH in cell lysates were detected by Western blotting using appropriate antibodies. (C) Huh7 cells were infected with Andes virus at an MOI of 1, followed by transfection with either empty vector or a plasmid expressing N-terminally Myc-tagged PKR at 24 h postinfection. Cells were harvested at 48 h postinfection. The levels of PKR, p-PKR, NP, and GAPDH were detected using appropriate antibodies. (D) Huh7 cells in six-well plates were transfected with the PKR expression plasmid, followed by ANDV infection at an MOI of 0.2 at 24 h posttransfection. Cells were harvested at 1, 48, and 72 h postinfection, and viral S-segment RNA was quantified by real-time PCR. The RNA levels were normalized to that at 1 h postinfection. Data from three independent experiments were averaged and used to calculate the standard deviations, shown as error bars. (E) Huh7 cells were infected with ANDV at an MOI of 0.2. Cells were transfected with a PKR-expressing plasmid at 24 h postinfection and harvested at 72 h postinfection. Viral S-segment RNA was quantified using the same method as that mentioned for panel D.

lentivirus expressing NP, as evident from significant reductions in the phosphorylation of both PKR and eIF2 α (Fig. 3A, compare lane 7 with lane 4). It was previously reported that the cytoplasmic tail domain of hantavirus glycoprotein Gn regulates the early interferon response during virus infection (12). To determine whether the Gn tail domain also regulates PKR activation similarly to that with NP, HUVECs were infected with a lentivirus expressing either the Gn tail domain or NP. An examination of cell lysates by Western blot analysis revealed that expression of the Gn tail domain induced PKR expression and activation of the PKR-eIF2 α pathway (Fig. 3B, lane 2). Consistent with the observations from Fig. 3A, infection with the lentivirus expressing NP induced PKR expression in a dose-dependent manner but failed to activate the PKR-eIF2 α pathway, as evident from negligible phosphorylation of both PKR and eIF2 α (Fig. 3B, compare lane 6 with lane 2). Based on the results from Fig. 3A and B, we envisioned that infection with a lentivirus expressing EGFP in HUVECs would inhibit the host translation machinery, whereas NP expression would rescue such translational shutdown. To test this hypothesis, HUVECs were infected with increasing amounts of lentivirus expressing either EGFP or NP. At 48 h postinfection, cells were

chased for *de novo* protein synthesis by metabolic labeling using [35 S]methionine-cysteine (Fig. 3C). As expected, the inhibition of *de novo* protein synthesis in a dose-dependent manner was observed in cells infected with lentivirus expressing EGFP (Fig. 3C). In comparison, infection with the lentivirus expressing NP did not affect the rate of host protein synthesis. These observations demonstrate that activation of the PKR-eIF2 α pathway by lentivirus infection induces host translational shutoff, whereas inhibition of PKR activation by NP expression rescues cells from such translational shutdown. Taken together, these studies demonstrate that NP is a PKR inhibitor.

ANDV NP selectively inhibits PKR without affecting the activity of other eIF2 α kinases. There are four eIF2 α kinases that phosphorylate eIF2 α under different stress conditions or in response to different stimuli and cause translational shutdown. These kinases include PKR, PKR-like endoplasmic reticulum kinase (PERK), heme-regulated inhibitor (HRI), and general control nonderepressible 2 (GCN2) (33). PKR is activated by dsRNA, mostly during viral infection. HRI is activated by heme deficiency to prevent the synthesis of globin peptides in response to elevated heme levels. HRI is also activated by oxidative stress. PERK is

FIG 3 ANDV NP inhibits PKR phosphorylation. (A) HUVECs were infected with a lentivirus expressing either EGFP (Lenti-EGFP) or NP (Lenti-NP) at increasing MOIs, ranging from 10 to 30. Cells were harvested at 48 h postinfection, and the expression of PKR, p-PKR, eIF2 α , p-eIF2 α , EGFP, and NP was detected using corresponding antibodies. Lysate from mock-infected cells was used as a negative control. The level of β -actin was used as a loading control. (B) HUVECs were infected with a lentivirus expressing the cytoplasmic tail domain of glycoprotein Gn (Lenti-Gntail) at an MOI of 40 or with Lenti-NP at increasing MOIs, ranging from 10 to 40. Cells were harvested at 48 h postinfection, and cell lysates were examined by Western blot analysis as mentioned for panel A. (C) HUVECs were infected with lentivirus (Lenti-EGFP or Lenti-NP) at increasing MOIs as mentioned for panel A, followed by metabolic labeling with [35 S]methionine-cysteine at 48 h postinfection. (Right) Cells were lysed, and protein samples were separated by SDS-PAGE and visualized by Coomassie blue staining. (Left) The same gel was dried and exposed to X-ray film. (D) The band intensities from the autoradiogram were quantified by Image-Pro Plus software and normalized to that of the mock control. Results from three independent experiments were used to calculate standard deviations, shown as error bars. The significance of differences (**) was calculated by the *t* test.

activated by the unfolded protein response, which is triggered by the accumulation of misfolded proteins in the endoplasmic reticulum (ER). GCN2 is activated by amino acid starvation. Viral infections can induce various stress responses in the host cell by the production of dsRNA intermediates, by competition with the cellular translation machinery and other resources for protein synthesis, or by the alteration of cellular metabolism (34). Since these diverse virus-induced stress conditions may activate multiple eIF2 α kinases, we asked whether ANDV NP can also antagonize other eIF2 α kinases and rescue cells from the induction of translational shutdown. We generated HEK293T stable cell lines constitutively expressing either EGFP or NP from ANDV, SNV, or HTNV. These cells were transfected with poly(I:C), stimulated with either dithiothreitol (DTT) or sodium arsenite, or subjected to amino acid starvation to activate PKR, PERK, HRI, or GCN2, respectively (33), as mentioned in the legend to Fig. 4. Cell lysates were examined for eIF2 α phosphorylation by Western blot analysis. It is evident from Fig. 4A that poly(I:C) transfection activated PKR in control cells expressing EGFP, as observed by significant phosphorylation of both PKR and eIF2 α (Fig. 4A, compare lane 4

with lane 1). However, cells expressing ANDV NP, SNV NP, or HTNV NP comparatively resisted the poly(I:C)-induced phosphorylation of both PKR and eIF2 α (Fig. 4A). This is consistent with similar observations from Fig. 3. Interestingly, the stimuli activating PERK (Fig. 4B), HRI (Fig. 4C), and GCN2 (Fig. 4D) equally induced the phosphorylation of eIF2 α in both EGFP- and NP-expressing cells, suggesting that NP likely does not inhibit the activation of these kinases. Based on these observations, it is likely that NP does not rescue cells from translational shutdown induced by the stimuli activating PERK, HRI, and GCN2. We used a luciferase reporter assay to test this hypothesis. Firefly luciferase has a short half-life (about 2 h), making it suitable for monitoring the rate of *de novo* protein synthesis in cells (35). The HEK293T stable cell lines constitutively expressing either EGFP or NP were transfected with pGL3-Fluc vector and subjected to different treatments at 10 h posttransfection for the activation of PKR, PERK, HRI, and GCN2, as described above. Cell lysates were examined for luciferase activity by use of a luciferase assay kit (Promega) following the manufacturer's instructions. Luciferase activity in each group was normalized to that of the untreated control

FIG 4 ANDV NP selectively inhibited PKR. (A) HEK293T cells stably expressing either EGFP, ANDV NP, SNV NP, or HTNV NP were transfected with poly(I:C) (400 ng/ml) for 2.5 h prior to lysis. Cell lysates were examined for the expression of PKR, p-PKR, eIF2 α , p-eIF2 α , EGFP, NP, and β -actin by Western blot analysis using appropriate antibodies. (B to D) The HEK293T stable cell lines mentioned above were treated with 2 mM DTT (B) or 0.1 μ M arsenite (C) for 2 h before lysis or cultured in starvation medium for 24 h before lysis (D). The expression of eIF2 α , p-eIF2 α , EGFP, NP, and β -actin was detected by Western blotting. (E to H) The HEK293T stable cell lines mentioned above were transfected with 100 ng pGL3-Fluc plasmid. At 10 h posttransfection, cells were transfected with 400 ng/ml poly(I:C) (E), treated with 2 mM DTT (F), or treated with 0.1 μ M arsenite (G) for 8 h before lysis or subjected to starvation medium for 24 h before lysis (H). The luciferase activities were measured and normalized to that of the no-treatment control in the same group. Results from three independent experiments were used to calculate standard deviations, shown as error bars. **, significant difference calculated by *t* test; NS, not significant.

(Fig. 4E to H). As shown in Fig. 4E, the poly(I:C) treatment reduced luciferase activity by 50% compared to that in EGFP-expressing control cells, indicating translational shutdown. In comparison, a marginal decrease in luciferase activity was observed in cells stably expressing ANDV NP, suggesting that NP counteracts the PKR-induced translational shutdown. The results from Fig. 4A and E suggest that NP expression inhibits PKR activation and rescues cells from PKR-induced host translational shutdown. However, the stimuli activating PERK (Fig. 4F), HRI (Fig. 4G), and GCN2 (Fig. 4H) equally reduced the luciferase activities in both EGFP- and NP-expressing HEK293T stable cell lines, suggesting that NP expression does not rescue cells from translational shutdown induced by PERK, HRI, or GCN2.

Inhibition of PKR and eIF2 α phosphorylation by NP does not require RNA sequestration or recruitment of cellular phosphatases. Viruses have evolved multiple strategies to antagonize PKR-mediated antiviral responses. Previous studies revealed that viruses either express a decoy dsRNA, promote PKR degradation, hide viral dsRNA, express pseudosubstrates, inhibit PKR dimerization, activate the PKR inhibitor P58^{IPK}, or directly dephosphorylate PKR or eIF2 α to overcome PKR-mediated antiviral responses (16–18). High-level PKR expression during ANDV infection and similar PKR induction in cells infected with lentivirus expressing either EGFP or NP suggest that NP likely does not target PKR for degradation (Fig. 2 and 3). The requirement of dsRNA for PKR autophosphorylation and the fact that NP is an

RNA binding protein (22) led to the hypothesis that sequestration of dsRNA by NP might result in the inhibition of PKR autophosphorylation. To test this possibility, we deleted the RNA binding domain in NP and examined the potential of the resulting deletion mutant to inhibit the phosphorylation of PKR in cell culture. The RNA binding domain of NP was previously mapped to the region of amino acids 175 to 230 (Fig. 5A) (22). HUVECs were infected with lentivirus expressing either EGFP, wild-type NP, or an NP mutant lacking the RNA binding domain. At 48 h postinfection, cells were harvested and PKR expression and activation were examined by Western blot analysis. As shown in Fig. 5B, similar levels of PKR induction were observed in HUVECs for infections with lentiviruses expressing either EGFP or wild-type or mutant NP. However, unlike EGFP, both wild-type and mutant NP equally inhibited the phosphorylation of both PKR and eIF2 α , suggesting that the RNA binding activity of NP likely does not play a role in the inhibition of PKR phosphorylation. To further verify this observation, HEK293T cells stably expressing either EGFP or wild-type or mutant NP were transfected with poly(I:C), and cells were harvested at different time points posttransfection. An examination by Western blot analysis revealed that poly(I:C) treatment triggered PKR phosphorylation as early as 1 h posttransfection in EGFP-expressing control cells, which was inhibited by the expression of both wild-type and mutant NP (Fig. 5C). The effect of poly(I:C) on PKR activation was time dependent: it stimulated more PKR phosphorylation at 2.5 h posttransfection (Fig. 5C),

FIG 5 The RNA binding activity of NP and cellular phosphatases are not involved in NP-mediated PKR inhibition. (A) Schematic representation of wild-type ANDV NP and the NP (Δ175-230) mutant. (B) HUVECs were infected with a lentivirus expressing either EGFP, NP, or the NP (Δ175-230) mutant at an MOI of 30. Cells were harvested at 48 h postinfection, and expression of PKR, p-PKR, eIF2α, p-eIF2α, EGFP, wild-type NP, and the NP (Δ175-230) mutant was examined by Western blot analysis. Cell lysate from uninfected cells was used as a control (mock). (C) HEK293T stable cell lines expressing either EGFP, wild-type NP, or the NP (Δ175-230) mutant were transfected with 400 ng/ml poly(I:C) for 1 or 2.5 h before harvesting. Cell lysates were examined for the expression of PKR, p-PKR, EGFP, NP, NP (Δ175-230), and β-actin by Western blot analysis using appropriate antibodies. (D) Poly(I:C) pulldown assays were carried out using a standard protocol (68). Briefly, HEK293T cells stably expressing either EGFP, NP, or NP (Δ175-230) were lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA). The resulting cell lysates were incubated with poly(I:C)-coated beads (Sigma) for 1 h at 4°C with gentle agitation. The beads were extensively washed with lysis buffer, and bound proteins were eluted by boiling with 1× SDS loading buffer. The eluted proteins were examined by Western blotting using appropriate antibodies. (E) HEK293T cells were transfected with poly(I:C) at increasing concentrations, ranging from 100 to 400 ng/ml, for 3 h, followed by transfection with plasmids expressing either EGFP, NP, or the NP (Δ175-230) mutant. Cells were lysed at 27 h post-poly(I:C) transfection, and protein samples were examined by Western blot analysis to monitor the expression levels of PKR, p-PKR, NP, the NP (Δ175-230) mutant, EGFP, and β-actin, using appropriate antibodies.

possibly due to a higher transfection efficiency of poly(I:C) with a longer incubation time. However, the activation of PKR was still equally inhibited by both wild-type and mutant NP, suggesting that RNA sequestration is likely not the mechanism by which NP inhibits PKR activation. To rule out the possibility that sequestration of poly(I:C) by NP inhibited PKR phosphorylation in this assay, HEK293T cell lysates expressing either wild-type or mutant NP or EGFP were incubated with poly(I:C) agarose beads. An examination of washed beads by Western blot analysis revealed that unlike PKR, wild-type or mutant NP or EGFP did not bind to poly(I:C), demonstrating that possible sequestration of poly(I:C)

by NP is not involved in the inhibition of PKR phosphorylation (Fig. 5D).

To delineate the mechanism for the inhibition of PKR signaling by NP, we asked whether NP recruits a phosphatase to dephosphorylate the activated forms of PKR and/or eIF2α (36–38). To test this hypothesis, HEK293T cells were first transfected with increasing amounts of poly(I:C) for 3 h to allow the phosphorylation of PKR and eIF2α, followed by transfection with plasmids expressing either EGFP, wild-type NP, or an NP mutant lacking the RNA binding domain. Cells were harvested at 27 h post-poly(I:C) treatment, and phosphorylation of both PKR and eIF2α

FIG 6 Andes virus NP inhibits PKR dimerization. (A) HEK293T cells were cotransfected with plasmids expressing either EGFP or wild-type or mutant His-tagged NP along with two additional plasmids: one expressing Myc-tagged PKR and another expressing FLAG-tagged PKR. Cells were lysed at 48 h posttransfection, and one-third of the cell lysate was treated with poly(I-C) (1 μ g/ml) for 30 min. Cell lysates were immunoprecipitated with either IgG or anti-FLAG antibody, and immunoprecipitated material was examined by Western blot analysis (IB) using anti-Myc antibody to detect Myc-tagged PKR, anti-FLAG antibody to detect FLAG-tagged PKR, anti-NP antibody to detect His-tagged NP, anti-GFP to detect GFP, or anti-GAPDH antibody to detect GAPDH. (B) HEK293T cells were cotransfected with plasmids expressing Myc-tagged PKR and FLAG-tagged NP. At 48 h posttransfection, cells were lysed with 0.5% NP-40 lysis buffer. Cell lysates were immunoprecipitated with either anti-FLAG antibody or IgG. The immunoprecipitated material was examined by Western blot analysis using either anti-Myc antibody (top) or anti-FLAG antibody (middle). The bottom panel shows the IgG light chain (IgG-L). (C) The experiment was performed in the same way as that for panel B, except that immunoprecipitation was carried out using anti-Myc antibody and Western blot analysis was carried out using anti-His antibody.

was monitored. As evident from Fig. 5E, transfection with poly(I-C) did not affect the intrinsic steady-state levels but induced the phosphorylation of both PKR and eIF2 α in a dose-dependent manner, although the effect was more pronounced with PKR. Interestingly, the expression of wild-type or mutant NP did not affect the phosphorylation status of prephosphorylated PKR or eIF2 α (Fig. 5E). This observation clearly demonstrates that inhibition of PKR and eIF2 α phosphorylation by the NP-mediated mechanism does not involve the recruitment of a possible phosphatase.

Nucleocapsid protein inhibits PKR dimerization. The dimerization-induced autophosphorylation of PKR is a critical step in its activation process. PKR has two dsRNA binding domains (RBDs) at the N terminus and one kinase domain (KD) at the C terminus (39). The intramolecular interaction between RBD and KD renders KD in an inactive state (40, 41). The binding of dsRNA or PKR-activating protein (PACT) to the RBDs induces a conformational change in PKR that relieves the intramolecular inhibition and promotes PKR dimerization by direct interaction between dimerization domains (42). The dimerization triggers PKR autophosphorylation and renders the enzyme in its fully active form (43–45). We used an immunoprecipitation approach to determine whether NP interferes in PKR dimerization. HEK293T cells were cotransfected with plasmids expressing either wild-type or mutant NP or EGFP along with two additional plasmids: one expressing Myc-tagged PKR and another expressing FLAG-tagged PKR. Cells were lysed at 48 h posttransfection. To promote PKR dimerization, one-third of the cell lysate was treated with poly(I-C) for 30 min at 30°C before the lysates were subjected to immunoprecipitation using anti-FLAG antibody. The immunoprecipitated material was examined by Western blot analysis using anti-Myc antibody. It is evident from Fig. 6A that Myc- and

FLAG-tagged PKR molecules in the EGFP control experiment underwent dimerization, which was significantly promoted by poly(I-C) treatment (compare lane 3 with lane 4). Interestingly, coexpression of His-tagged NP or mutant NP dramatically inhibited the interaction between Myc- and FLAG-tagged PKR molecules (compare lane 4 with lanes 8 and 12). This result demonstrates that NP inhibits PKR dimerization. Since both wild-type and mutant NP did not bind to poly(I-C) (Fig. 5D), the possible sequestration of poly(I-C) by NP is not likely the mechanism for inhibiting PKR dimerization. Moreover, it is evident from Fig. 6A (lanes 7, 8, 11, and 12) that FLAG-PKR does not bind to His-NP.

Since the hepatitis C virus (HCV) NS5A protein has been reported to inhibit PKR dimerization through binding to the PKR dimerization site (46, 47), we further confirmed that NP does not bind to PKR. We cotransfected HEK293T cells with Myc-tagged PKR and FLAG-tagged NP. Cell lysates were immunoprecipitated with either anti-FLAG antibody or IgG, as a negative control. The immunoprecipitated material was examined by Western blot analysis using anti-Myc antibody. It is evident from Fig. 6B that NP did not bind to PKR. To further confirm this observation, cell lysates were immunoprecipitated with either anti-Myc antibody or IgG. Immunoprecipitated material was again examined by Western blot analysis using anti-His antibody. This reverse immunoprecipitation experiment (Fig. 6C) further demonstrated that NP does not bind to PKR.

DISCUSSION

Modulation of the innate immune response is a common strategy employed by viruses to survive in infected hosts. Pathogenic hantaviruses, such as ANDV, SNV, and NY-1 virus, achieve this goal with the assistance of a virus-encoded glycoprotein to inhibit the type I interferon response during early stages of viral infection (10,

12, 25, 48). However, the inhibition of the early interferon response does not seem to be sufficient for pathogenic hantaviruses to be human pathogens. This is supported by the observation that Tula hantavirus (TULV) inhibits IFN- β production without causing a serious human disease (49–52). Thus, the virulence factors for hantaviruses remain incompletely defined. It is also well known that all hantaviruses strongly induce the expression of both interferon and ISGs during the later stages of virus infection, which does not have an effect on virus replication (11). Moreover, it has been known that establishment of an antiviral state initiated by the transient shutdown of the host translation machinery is not observed in hantavirus-infected cells (53). Consistent with these known findings, we observed that ANDV infection of HUVECs induced PKR overexpression, which failed to shut down the host translation machinery and remained ineffective in combating virus replication (Fig. 1). Further studies demonstrated that although PKR was overexpressed during hantavirus infection, the overexpressed PKR was not activated due to the lack of autophosphorylation (Fig. 2A). As a result, the activity of the downstream target eIF2 α was not impaired, as evident from the lack of translational shutdown in virus-infected cells (Fig. 1C). Not only did ANDV infection inhibit the activation of endogenous PKR, but the overexpressed PKR from a transfected plasmid was also inhibited in virus-infected cells (Fig. 2C). The studies reported here show for the first time that NP is a PKR inhibitor (Fig. 3). NP interferes with PKR dimerization, a prerequisite for PKR autophosphorylation and activation. A similar strategy is used by the influenza virus and HCV NS5A proteins to mitigate PKR antiviral responses (46, 54, 55). The mechanism by which NP inhibits PKR dimerization is unknown. We demonstrated that NP does not bind to PKR (Fig. 6B and C), ruling out a possible interference of NP-PKR interaction in PKR dimerization. Moreover, the possible recruitment of cellular phosphatases to dephosphorylate PKR or eIF2 α was also ruled out (Fig. 5E). In addition, the binding of PKR to dsRNA triggers PKR dimerization and autophosphorylation. However, an NP mutant lacking the RNA binding domain was able to inhibit PKR similarly to the case with wild-type NP, demonstrating that possible sequestration of dsRNA by NP is not the mechanism for inhibiting PKR dimerization. Viruses have evolved numerous strategies to inhibit the PKR antiviral response (16–18). However, PKR inhibition by members of the *Bunyaviridae* family has not been studied extensively. The only reported bunyavirus inhibiting PKR is Rift Valley fever virus (RVFV), whose NSs protein has been reported to promote PKR degradation (56, 57). However, the overexpression of PKR during ANDV infection suggests that unlike RVFV infection, hantavirus infection does lead to PKR degradation. Thus, the actual mechanism for the inhibition of PKR dimerization by NP remains a mystery. Nonetheless, the studies carried out here demonstrate that NP is a virulence factor of hantaviruses. These studies also shed light on the mechanism of hantavirus resistance to the host interferon response during later stages of infection and solve the mystery of the lack of translational shutdown in hantavirus-infected cells.

Hantavirus NP is a multifunctional protein that is primarily involved in the packaging of viral genomic RNA in viral nucleocapsids. However, our recent studies have shown that NP has a role in the cap-snatching mechanism of transcription initiation by viral RdRp (7). In addition, NP also facilitates mRNA translation without the requirement of the eIF4F cap binding complex (9, 58). Our previous studies showed that NP binds to both the mRNA 5'

cap and the viral mRNA 5'-untranslated region (5' UTR). In addition, NP also binds to the 40S ribosomal subunit by direct interaction with the ribosomal protein S19 (RPS19), located at the head region of the 40S ribosomal subunit. Our previous studies suggest that NP-associated ribosomes are preferentially loaded on the viral mRNA 5' UTR, thereby favoring their translation in the host cell cytoplasm, where cellular transcripts are competing for the same translation machinery (9, 59). However, both the canonical host translation mechanism and the NP-mediated translation strategy are dependent upon eIF2 α and thus sensitive to PKR-induced translational shutdown. This is indirectly supported by the fact that overexpression and activation of PKR in transfected cells prior to ANDV infection significantly impaired virus replication (Fig. 2D). The sensitivity of hantavirus replication to the PKR antiviral response likely imposed a selective evolutionary pressure on hantaviruses to evade the PKR antiviral response for survival. Our studies suggest that inhibition of the PKR antiviral response by NP has likely helped hantaviruses to exist and survive in infected hosts over the course of evolution.

PKR is a multifunctional protein which not only inhibits the host translation machinery but also plays critical roles in numerous signal transduction pathways. For example, PKR plays a positive-feedback role in the IFN signaling pathway, and its inhibition attenuates the induction of genes normally stimulated by IFN (60). PKR positively regulates the induction of IFN- β in response to viral infection via NF- κ B and IRF1 (61). PKR also functions as a cytosolic dsRNA sensor and activates NF- κ B by activating NIK and IKK kinases (62). Since NF- κ B broadly regulates the induction of antiviral genes and inflammatory cytokines, its activation favors the quick establishment of an antiviral state, which is not observed in hantavirus-infected cells (63). Moreover, PKR has been implicated to play a role in apoptotic pathways. Overexpression of PKR sensitizes cells to apoptosis induced by dsRNA, tumor necrosis factor alpha (TNF- α), and virus infection (64). Apoptosis is the ultimate cellular measure for controlling virus replication and spread (65). However, hantavirus-infected cells do not undergo apoptosis (66), likely due to the inhibition of PKR by NP. PKR also plays a key role in the formation of antiviral stress granules (avSGs), which serve as platforms for sensing of nonself RNAs by RIG-I-like receptors (67). These reported findings demonstrate that PKR is a multifunctional antiviral host factor. Since it is a PKR inhibitor, NP likely antagonizes the diverse PKR antiviral responses in virus-infected cells and creates a supportive environment for virus replication. Taken together, our results illustrate that the multifunctional nature of viral proteins help viruses to carry small genomes and still survive in hosts with a multifaceted antiviral defense.

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