Andes and Prospect Hill Hantaviruses Differ in Early Induction of Interferon although Both Can Downregulate Interferon Signaling[▽]

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Hantavirus pulmonary syndrome (HPS) is a severe respiratory disease which is thought to result from a dysregulated immune response to infection with pathogenic hantaviruses, such as Sin Nombre virus or Andes virus (ANDV). Other New World hantaviruses, such as Prospect Hill virus (PHV), have not been associated with human disease. Activation of an antiviral state and cell signaling in response to hantavirus infection were examined using human primary lung endothelial cells, the main target cell infected in HPS patients. PHV, but not ANDV, was found to induce a robust beta interferon (IFN- β) response early after infection of primary lung endothelial cells. The level of IFN induction correlated with IFN regulatory factor 3 (IRF-3) activation, in that IRF-3 dimerization and nuclear translocation were detected in PHV but not ANDV infection. In addition, phosphorylated Stat-1/2 levels were significantly lower in the ANDV-infected cells relative to PHV. Presumably, this reflects the lower level of IRF-3 activation and initial IFN induced by ANDV relative to PHV. To determine whether, in addition, ANDV interference with IFN signaling also contributed to the low Stat-1/2 activation seen in ANDV infection, the levels of exogenous IFN- β -induced Stat-1/2 activation detectable in uninfected versus ANDV- or PHV-infected Vero-E6 cells were examined. Surprisingly, both viruses were found to downregulate IFN-induced Stat-1/2 activation. Analysis of cells transiently expressing only ANDV or PHV glycoproteins implicated these proteins in this downregulation. In conclusion, while both viruses can interfere with IFN signaling, there is a major difference in the initial interferon induction via IRF-3 activation between ANDV and PHV in infected primary endothelial cells, and this correlates with the reported differences in pathogenicity of these viruses.

Hantaviruses, family *Bunyaviridae*, are rodent-borne negative-stranded RNA viruses. Members of the genus *Hantavirus* have been identified as etiologic agents of two severe human diseases: hemorrhagic fever with renal syndrome (HFRS), which is caused by the Old World hantaviruses, and hantavirus pulmonary syndrome (HPS), which is caused by the New World hantaviruses (18, 24). Case fatality is considerably higher for HPS (up to 40%) than for HFRS (between 0.1 and 15%). The major target in human hantavirus infection is the microvascular endothelium, and severe hantavirus disease in humans has been attributed to microvascular leakage (20, 30). Several Old and New World hantaviruses have not been associated with any human disease. The basis for disease in humans, and differences between pathogenic and nonpathogenic hantaviruses, remains unclear; however, innate immune responses likely play an important role.

In general, type I interferons (IFNs) are considered the first line of host defense to RNA virus infections. The expression of type I interferons is regulated by the activation of preexisting transcription factors, such as interferon regulatory factor 3 (IRF-3), nuclear factor κ B, and mitogen-activated protein kinase (12). IRF-3 activation plays a major role in the induction of interferon by viruses. After viral infection, IRF-3 is phosphorylated, dimerizes, and translocates from the cytoplasm to

the nucleus (16). In the nucleus, IRF-3 recruits the transcriptional coactivator CREB-binding protein and initiates IFN- β mRNA synthesis (23, 29). Secreted IFN- β binds to its receptor and initiates the activation of the interferon signaling pathway and an antiviral state within infected and neighboring cells. Through the JAK-STAT pathway, phosphorylated Stat-1 and Stat-2 recruit IRF-9 to form the IFN-stimulated gene factor 3, which translocates to the nucleus to activate the expression of interferon-stimulated genes (15).

It is becoming evident that an increasing number of RNA viruses have evolved mechanisms to escape the interferon response. These mechanisms include the direct blocking of interferon induction and also inhibition of the interferon signaling pathway (e.g., degradation of Stat proteins or inhibition of Stat phosphorylation). For the RNA viruses studied so far, the viral nonstructural proteins have been the most common proteins implicated in downregulation of the host innate immune response (5, 8). As we begin to consider what interaction hantaviruses have with the host antiviral response, it's worth noting that the hantaviruses are known to encode only four structural proteins, the nucleocapsid (N), two glycoproteins (Gn and Gc), and the viral polymerase (L) (24).

While innate immune responses have been implicated as a major contributor to the outcome of hantavirus infections, results of earlier studies have failed to produce a clear unified view of precise responses or mechanisms involved. For instance, in studies of hantavirus-infected primary umbilical macrovascular endothelial cells, one study showed that the nonpathogenic Prospect Hill virus (PHV) induced higher interferon responses (detectable at 24 h postinfection) than the HPS-associated NY-1 virus or HFRS-associated Hantaan virus

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(HTNV) (6). In contrast, another study using human umbilical macrovascular endothelial cells showed that the HFRS-associated HTNV induced higher interferon responses (detectable at 24 h postinfection) than the nonpathogenic Tula virus (14). A third report showed that neither HPS-associated Sin Nombre virus nor nonpathogenic PHV induced any interferon response detectable at 12 h postinfection (13). Many of these reported discrepancies may simply reflect the experimental approach being carried out, the use of different hantaviruses or multiplicities of infection (MOIs), or the inherent variability in the methods employed (e.g., microarray assays). Based on such observations we decided to carefully examine the ability of the HPS-associated Andes virus (ANDV) and a nonpathogenic New World hantavirus, PHV, to induce interferon at early times postinfection of primary human lung microvascular endothelial cells (the major cell target in HPS). This provides a direct comparison of a relevant pair of pathogenic and non-pathogenic viruses in a relevant human cell type. It was found that while both viruses can interfere with IFN signaling, there is a major difference in the initial interferon induction via IRF-3 activation between ANDV and PHV in infected primary lung endothelial cells, and this correlates with the reported differences in pathogenicity of these viruses.

MATERIALS AND METHODS

Cells. Primary cultures of human pulmonary microvascular endothelial cells (HMVEC-L) isolated from individual donors were obtained at passage 4 from Clonetics Corporation (Walkersville, MD). The cells were seeded into tissue culture flasks (5,000 cells/cm²) precoated with 0.2% gelatin (Sigma, St. Louis, MO). Cells were grown in EGM-2MV growth medium (Clonetics) consisting of modified EGM basal medium supplemented with human recombinant epidermal growth factor (10 ng/ml), hydrocortisone (1 µg/ml), gentamicin (50 µg/ml), amphotericin B (50 µg/ml), bovine brain extract (3 mg/ml), and fetal calf serum (5% [vol/vol]). All experiments were performed at cell passages 5 to 7. African green monkey kidney (Vero-E6) cells were maintained in Dulbecco's modified Eagle medium (Gibco BRL, Grand Island, NY) containing 10% fetal calf serum (HyClone, Logan, Utah).

Viruses. Low-passage stocks of ANDV (strain Chile-9717869; Vero-E6+4) and PHV prototype (Vero-E6+3) were prepared in Vero-E6 cells. Virus titers were determined by limited dilution as described previously (9). Sendai virus strain Cantell was obtained from ATCC and propagated in 10-day-old embryonated chicken eggs. The work with infectious hantaviruses was done under biosafety level 3 conditions. Noninfectious virus controls were prepared by gamma inactivation of virus using a high-energy ⁶⁰Co source.

Antibodies. The following antibodies were used in this study: rabbit polyclonal antisera against ANDV or PHV, which recognized the viral glycoproteins as well as the nucleocapsid protein; a Puumala anti-N monoclonal antibody, GB04-BF07, which is cross-reactive with all hantaviruses; antihemagglutinin (HA) monoclonal antibody (Sigma); mouse anti-human IRF-3, rabbit anti-Stat-1, mouse anti-Stat-2, rabbit anti-IRF-9, and phospho-specific Stat-1 (pY701) (BD Biosciences, San Diego); polyclonal phospho-specific anti-Stat-2 (Upstate, New York); polyclonal anti-IRF-3 antibody (FL-425) and actin (I-19) (Santa Cruz Biotechnology, San Diego, CA); anti-Stat-1 (9H2) monoclonal antibody (Cell Signaling). Secondary antibodies used were Alexa Fluor 594 goat anti-mouse, Alexa Fluor 594 goat anti-rabbit, Alexa Fluor 488 goat anti-mouse, and Alexa Fluor 488 goat anti-rabbit (Molecular Probes, Invitrogen).

RPA and ELISA. Total HMVEC-L RNA was extracted by using Tri-Pure reagent (Boehringer Mannheim Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. Quantitative measurements of the IFN-β mRNA were determined using a RiboQuant RNase protection assay (RPA) kit and hCK-3 multiprobe (PharMingen, San Diego, CA). The amounts of IFN-β present in cell supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using a human IFN-β ELISA kit (Fujirebio, Tokyo, Japan).

IRF-3 dimerization. Cells were lysed in buffer containing 50 mM NaCl, 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 1% NP-40, protease inhibitors, and phosphatase inhibitors (Calbiochem, San Diego). The lysates were electrophoresed by native polyacrylamide gel electrophoresis (PAGE) with 1% deoxycholate in the cathode

FIG. 1. A. Induction of IFN-β mRNA by ANDV and PHV infection. Primary lung microvascular endothelial cells (HMVEC-L) were infected with ANDV or PHV at an MOI of 1 or were treated with inactivated ANDV. Total RNA was isolated at 24 h postinfection and analyzed for IFN-β mRNA induction in an RNase protection assay. The mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. B. Accumulation of virus nucleocapsid protein N. Equal amounts of cell lysates of ANDV- and PHV-infected HMVEC were prepared, and the amounts of nucleocapsid protein were detected by immunoblotting using the cross-reactive anti-N monoclonal antibody GB04-BF07. C. Secretion of IFN-β by HMVEC infected with ANDV or PHV. The amounts of IFN-β in the supernatants were determined by ELISA. The data represent the means and standard deviations of triplicate samples.

buffer in order to resolve IRF-3 monomers and dimers as described previously (11), followed by immunoblotting with IRF-3 antibody.

Western blotting. Cells were lysed in boiling buffer containing 10 mM sodium orthovanadate and 1% sodium dodecyl sulfate (SDS). Proteins were electrophoretically separated on 12% SDS-PAGE or 10% Nupage (Invitrogen) gels and transferred to nitrocellulose. To ensure that equal amounts of proteins were loaded for comparisons, cell lysates were split in half and separated gels were run and probed with different primary antibodies. In some experiments, membranes were stripped after the incubation with phospho-specific antibodies and were reprobed with antibodies for the detection of total protein. The membranes were blocked with buffer containing 1× Tris-buffered saline and 0.1% Tween 20 with 5% nonfat dry milk for 1 h and then probed overnight at 4°C with specific antibodies. Anti-mouse or anti-rabbit immunoglobulin G-horseradish peroxidase was used as secondary antibody. Lastly, membranes were incubated with an enhanced chemiluminescence detection kit (ECL-plus; Amersham, Biosciences, Piscataway, NJ) for 5 min and exposed on film.

Construction of plasmids encoding recombinant ANDV-GPC and PHV-GPC proteins. Total RNA was extracted from Vero-E6 cells infected with ANDV or PHV by using an RNeasy kit (QIAGEN). Ten µl of purified RNA was used as template in reverse transcription-PCRs (RT-PCRs) using the SuperScript III One-Step RT-PCR system with platinum *Taq* high fidelity (Invitrogen) following the manufacturer's protocols. Primers used in the one-step RT-PCRs were designed to amplify the entire glycoprotein precursor (GPC) open reading frames

FIG. 2. A. IRF-3 dimerization in ANDV- or PHV-infected primary cells. HMVEC-L cells were infected with virus at an MOI of 1 or treated with inactivated ANDV or PHV. Cell extracts were analyzed on a nondenaturing native gel followed by Western blotting with anti-IRF-3 antibody. Sendai virus infection of HMVEC-L cells was used as a positive control. IRF-3 dimer formation was evident in PHV-infected cells at 12 h postinfection, whereas in ANDV-infected cells it was barely detected even by 24 h postinfection. No IRF-3 dimerization was detected with either inactivated PHV or ANDV. B. IRF-3 nuclear translocation induced by PHV infection. PHV- or ANDV-infected HMVEC-L cells were examined at 24 h postinfection by double immunofluorescent staining using anti-IRF-3 antibody (left panels) and cross-reactive anti-N monoclonal antibody GB04-BF07 (middle panels). Right panels show the merged images. No nuclear staining of IRF-3 was observed in uninfected or cells treated with inactivated PHV or ANDV (data not shown).

from ANDV (position 52-3468) or PHV (position 50-3478). In addition, amplification primers contained different restriction sites chosen for cloning into a polymerase II-based expression plasmid, pCAGGS (19). Amplification products were analyzed by electrophoresis on agarose gels, and DNA bands of the correct size were cut and purified using a QIAquick gel extraction kit (QIAGEN). Amplified DNA products were digested with the corresponding restriction enzymes and further purified with a QIAquick PCR purification kit (QIAGEN). Digested DNA fragments were then ligated between convenient restriction sites of pCAGGS, and selected clones were sequenced using standard protocols (ABI).

Immunofluorescence. HMVEC-L or Vero-E6 cells grown on coverslips were infected with ANDV or PHV or were transfected with the recombinant plasmid DNA expressing ANDV-GPC, PHV-GPC, or HA-tagged IRF-3. At certain times postinfection or posttransfection, cells were washed twice with phosphate-buffered saline (PBS) and fixed with 2% formaldehyde at room temperature for 10 min. After formaldehyde fixation, the cells were washed three times with PBS and permeabilized with 0.1% Triton X-100 for intracellular staining. The primary antibodies were added at a 1:200 dilution in 1% bovine serum albumin in PBS for 30 min. The cells were then washed three times with PBS and incubated for 30 min with the secondary antibodies diluted 1:1,000 in 1% bovine serum albumin in PBS. Multiple final washes were done, and the cells were mounted on microscope slides and viewed using a Zeiss microscope.

RESULTS

High IFN- β induction early after infection of human primary lung endothelial cells with PHV but not ANDV. Viral infections generally result in activation of a type I interferon response with resulting production of IFN- β . Here we precisely examined the induction of IFN- β mRNA in human primary lung endothelial cells (the main target cell in HPS) infected with pathogenic ANDV or nonpathogenic PHV. HMVEC-L cells were infected with ANDV or PHV at an MOI of 1, total RNA was purified at 24 h postinfection, and the induction of IFN- β mRNA was analyzed by RPA. ANDV failed to significantly up-regulate IFN- β mRNA, whereas high levels of IFN- β mRNA were induced in PHV-infected cells (Fig. 1A). Equivalent levels of total RNA were loaded in each lane, as confirmed by glyceraldehyde-3-phosphate dehydrogenase mRNA levels (Fig. 1A). Cells contained comparable amounts of virus, as indicated by accumulation of virus nucleocapsid protein (Fig. 1B). As an additional control, cells were treated with equivalent

amounts of ANDV (Fig. 1A) or PHV (data not shown) inactivated by gamma irradiation. No induction of the IFN- β mRNA was seen with these treated cells, indicating that IFN- β induction was dependent on viral replication. These observed differences in IFN- β mRNA levels induced in ANDV- and PHV-infected cells correlated with differences in the amount of IFN- β detected in cell supernatants (Fig. 1C).

IRF-3 activation is strongly induced by PHV but not ANDV in primary lung endothelial cells. Activation of IRF-3 plays an important role in the induction of IFN- β mRNA by viruses (16). RNA viral infections frequently initiate a cascade of IRF-3 phosphorylation, dimerization, and nuclear translocation, thereby inducing a number of antiviral responses, including IFN- β . For this reason we examined whether the differences in IFN- β mRNA induction observed between ANDV and PHV infections correlated with IRF-3 activation. HMVEC-L cells were infected with ANDV or PHV, and IRF-3 homodimerization was assayed at 12 h and 24 h postinfection (Fig. 2A). The activation of IRF-3 was measured by Western blot detection of dimers resolved by native gel electrophoresis. IRF-3 dimers were evident in PHV-infected cells as early as 12 h postinfection (lane 4). In contrast, no IRF-3 dimer formation could be detected in the ANDV-infected cells at the same time point (lane 3). By 24 h postinfection, IRF-3 dimer formation in PHV-infected cells was highly prominent (lane 9), comparable to the positive control Sendai virus-infected cells (lane 10), whereas a barely detectable dimer band could be seen in the ANDV-infected cells (lane 8). No IRF-3 dimer formation was detected at any time in the presence of inactivated virus (lanes 1 and 6) and PHV (lanes 2 and 7), showing that active virus replication was required for activation.

Next, the nuclear translocation of IRF-3 was examined by immunofluorescence staining of PHV- or ANDV-infected cells using anti-IRF-3 and anti-N antibodies, to test whether IRF-3 dimers would be detected in the nucleus of PHV-infected cells but not in those infected with ANDV. IRF-3 nuclear translocation could be detected in PHV-infected HMVEC-Ls at 24 h postinfection but was barely detectable in ANDV HMVEC-Ls at the same time point (Fig. 2B). No IRF-3 nuclear translocation could be detected in the presence of inactivated virus or in the uninfected control cells. These results confirm that there is considerably less IRF-3 activation and resulting IFN- β mRNA in ANDV-infected lung endothelial cells than that induced in PHV-infected cells.

High levels of Stat-1 and Stat-2 activation in response to infection with PHV but not ANDV. Having found clear differences between ANDV and PHV in activation of the antiviral response, we were interested in examining whether differences could be found in the interferon signaling pathway, particularly Stat-1 and Stat-2 activation. HMVEC-L cells were infected with ANDV or PHV at an MOI of 1, and cell lysates were analyzed by SDS-PAGE followed by Western blotting. Primary antibodies used in the Western blotting detected total Stat-1, Stat-2, IRF-9, and viral nucleocapsid protein, in addition to the phosphorylated forms of Stat-1 and Stat-2. Similar levels of total Stat-1 and Stat-2 mRNAs (data not shown) and proteins (Fig. 3) were induced by ANDV and PHV. However, only PHV induced detectable phosphorylation of Stat-1 and Stat-2 as early as 12 h postinfection, and significantly increased levels

FIG. 3. Phosphorylation of Stat-1 and Stat-2 by hantavirus infection. HMVEC-L cells were infected with ANDV or PHV at an MOI of 1 and lysed at 24 h postinfection. Cell lysates were analyzed by SDS-PAGE followed by Western blotting. Primary antibodies used in the Western blotting detected total Stat-1, Stat-2, IRF-9, and viral nucleocapsid protein, in addition to the phosphorylated forms of Stat-1 and Stat-2, as indicated. Although both ANDV and PHV similarly induced total Stat-1 and Stat-2, there was a significant difference between them in the induction of the phosphorylated forms of Stat-1 and Stat-2. Only PHV induced phosphorylation of Stat-1 and Stat-2 as early as 12 h postinfection.

of phosphorylated Stat1/2 were detected at 24 h postinfection (lanes 5 and 9). In contrast, in ANDV-infected HMVEC-Ls, phosphorylated forms of Stat-1 and Stat-2 were undetectable at 12 h postinfection and barely detectable at 24 h postinfection (lanes 3 and 7). The finding of considerably less phosphorylated Stat-1 and Stat-2 in ANDV- versus PHV-infected lung endothelial cells may simply mirror the much lower IRF-3 activation by ANDV relative to PHV, as documented above, or may indicate that ANDV has additional properties to specifically downregulate interferon signaling.

ANDV and PHV both downregulate Stat-1- and Stat-2-induced phosphorylation in response to exogenous IFN- β . To examine whether the difference seen in Stat-1/2 phosphorylation during infection of HMVEC-Ls with ANDV and PHV reflects a difference in interferon signaling between the two viruses (in addition to the differences in initial interferon induction), a set of experiments was performed in Vero-E6 cells. Vero-E6 cells are defective in endogenous IFN- β production but retain the capacity to respond to exogenous interferon. These cells are readily infectible with hantaviruses, allowing us to evaluate the interferon signaling pathway during ANDV and PHV infection. Vero-E6 cells were infected with ANDV (Fig. 4A) or PHV (Fig. 4B) at an MOI of 1 and at 48 h postinfection were either treated with 1,000 U of IFN- β for 20 min or left untreated. Cell lysates were analyzed by SDS-PAGE followed by Western blotting using antibodies against

FIG. 4. Virus downregulation of Stat-1 and Stat-2 tyrosine phosphorylation in response to IFN- β . Vero-E6 cells were infected with ANDV (A) or PHV (B) at an MOI of 1. At 48 h postinfection, uninfected, inactivated virus and infected cells were either treated with 1,000 U of IFN- β for 20 min or left untreated. Cell lysates were analyzed by SDS-PAGE followed by Western blotting using antibodies against phospho-Stat-1, total Stat-1, phospho-Stat-2, total Stat-2, actin, or viral nucleocapsid protein as indicated.

phospho-Stat-1, total Stat-1, phospho-Stat-2, total Stat-2, actin, or viral nucleocapsid protein as indicated. Surprisingly, both ANDV (Fig. 4A, lane 6 compared to lanes 4 and 5) and PHV (Fig. 4B, lane 6 compared to lanes 4 and 5) had the ability to downregulate the interferon signaling pathway by blocking the tyrosine phosphorylation of Stat-1 and Stat-2. Uninfected cells (lane 4) and cells treated with gamma-irradiated inactivated virus (lane 5) were included as controls. The inhibition of interferon signaling by both ANDV and PHV required productive viral replication and viral protein synthesis, as cell treatment with gamma-irradiated virus had no effect.

Both ANDV and PHV infections inhibit Stat-1 nuclear translocation in response to IFN- β . To further examine the viral inhibition of Stat-1/2 signaling, the effects of ANDV and PHV infection on the nuclear translocation of Stat-1 were also tested in virus-infected Vero-E6 cells. At 48 h postinfection, cells were treated with 1,000 U of IFN- β for 20 min, fixed, and double stained with anti-Stat-1 antibodies and either ANDV- or PHV-specific antibodies. ANDV and PHV clearly inhibited the Stat-1 nuclear translocation in response to interferon (Fig. 5), consistent with the abilities of both viruses to downregulate Stat-1 and Stat-2 tyrosine phosphorylation (Fig. 4).

Stat-1 nuclear translocation in response to IFN- β is inhibited by ANDV and PHV glycoproteins. As hantavirus Gn glycoprotein cytoplasmic tails had previously been shown to be able to modulate immunoreceptor cell signaling (7), we examined the potential role of the viral glycoproteins in the inhibition of Stat-1 nuclear translocation in response to IFN- β .

Vero-E6 cells were transfected with plasmids expressing ANDV GPC, PHV GPC, and Ebola virus VP35. Ebola virus VP35 was used as a negative control, as although it is an interferon antagonist, it has been shown not to inhibit Stat-1 nuclear translocation (22). At 48 h posttransfection, cells were treated with 1,000 U of IFN- β for 20 min, fixed, and double stained with antibodies detecting ANDV glycoproteins, PHV glycoproteins, Ebola virus VP35, or Stat-1 (Fig. 6A). Ninety percent of cells expressing either ANDV GPC (row 1) or PHV GPC (row 2) showed no evidence of Stat-1 nuclear translocation. Strong Stat-1 nuclear staining was observed in neighboring cells which were not expressing the ANDV or PHV glycoproteins, clearly confirming the IFN signaling activation of these cell monolayers (Fig. 6A, rows 1 and 2). In addition, control cells transfected with the VP35 plasmid also exhibited extensive Stat-1 nuclear translocation (Fig. 6A, row 3).

To examine whether the inhibition of Stat-1 nuclear translocation by the viral glycoproteins merely reflected a general inhibition of cellular protein nuclear translocation, IRF-3 localization following Sendai virus infection in the presence of expressed ANDV glycoproteins was examined by immunofluorescence staining. Sendai virus infection is known to result in robust IRF-3 activation and nuclear translocation (16). No inhibition of Sendai virus-induced IRF-3 nuclear translocation was observed in the ANDV glycoprotein-expressing cells (Fig. 6B, row 2). In addition, the expression of ANDV glycoproteins by themselves did not alter the localization of IRF-3 (row 1). These results clearly show that the glycoproteins of both patho-

FIG. 5. Nuclear translocation of Stat-1 is inhibited by ANDV and PHV infection. Vero-E6 cells were infected with ANDV or PHV. At 48 h postinfection cells were treated with 1,000 U of IFN- β for 20 min, fixed, and double stained using anti-ANDV serum, anti-PHV serum (middle panels), or anti-Stat-1 (left panels) antibodies. Right panels show the merged images.

genic (ANDV) and nonpathogenic (PHV) hantaviruses function as antagonists of the interferon signaling pathway and that their inhibition of Stat-1 nuclear translocation is not the result of a generalized inhibition of nuclear translocation of cellular proteins. Despite PHV Gn inhibiting Stat-1/2 activation, Stat-1/2 phosphorylation was seen early post-virus infection of cells (Fig. 3). Presumably, this reflects the fact that not all cells were infected, and Stat activation will take place in the uninfected cells in response to interferon secreted by the infected cells.

DISCUSSION

In this study we demonstrated that PHV, but not ANDV, was able to induce a robust IFN- β response early after infection (24 h postinfection) of primary lung endothelial cells and that these differences correlated with strong IRF-3 activation by PHV but not ANDV. While total Stat-1/2 levels induced by these viruses were similar, considerably more phosphorylated Stat-1/2 was detected in cells infected with PHV relative to ANDV infection. To more precisely dissect whether this merely reflected the difference in initial IRF-3 activation (and IFN production) or whether ANDV impairs IFN signaling, infection of cells deficient in IFN production (Vero-E6 cells) was examined. Surprisingly, both viruses were found to downregulate the level of Stat-1/2 activation induced by exogenous IFN- β added to Vero-E6 cells, and this inhibition of interferon signaling could be achieved solely by transient expression of the hantavirus glycoproteins. Thus, while both viruses could interfere with IFN signaling, there was a major difference in the initial interferon induction via IRF-3 activation between ANDV and PHV in infected primary lung endothelial cells, and this correlates with the reported differences in pathogenicity of these viruses.

The ability of hantavirus to induce interferon or up-regulate interferon-inducible genes has been described previously, but these studies have reported contradictory findings (6, 13, 14, 21). These differences may be the result of the inherent variability of microarray assays used in some of the studies or differences in the analysis methods, virus strains, or multiplicities of infection utilized. The results presented here are consistent with the report documenting that early cellular responses induced by nonpathogenic hantaviruses are strikingly

higher than those induced by a pathogenic hantavirus (6). However, while those authors found no direct induction of IFN- β at early times postinfection with any of the hantaviruses analyzed, we clearly demonstrated that IFN- β is induced by PHV 24 h postinfection in human primary lung endothelial cells. In addition, we did not detect any activation of interferon or interferon signaling by inactivated virus, as has been previously reported (21).

Despite the number of studies reporting total cellular responses to hantavirus infection, the mechanisms of regulation have not been studied. IRF-3 is one of the major players in the induction of the innate immune response by viruses. Focusing on activation of IRF-3 by ANDV and PHV led us to the conclusion that PHV strongly activates IRF-3, whereas very little IRF-3 activation was seen with ANDV. Many viruses have the ability to interfere with the induction of interferon by inhibiting IRF-3 activation. Viral proteins can inhibit IRF-3 activation at different levels, including IRF-3 phosphorylation, dimerization, and nuclear translocation, or its interaction with its transcriptional coactivator, Creb-binding protein (2–4, 8, 17). What causes the downregulation of IRF-3 activation by ANDV in comparison to PHV is not known yet. Although multiple early time points post-ANDV infection were examined in our study, we did not observe the two-step IRF-3 activation seen previously in severe acute respiratory syndrome coronavirus infections (25). Further studies are needed to test if ANDV acts upstream of IRF-3 activation. For instance, ANDV could decrease IRF-3 activation either directly or indirectly by inhibiting earlier activation steps, which include TBK1 or IKK ϵ kinases. The block may even lie further upstream at the step involving TRAF3 or MAVS activation of TBK1 or IKK ϵ , or at the RIG-I/Mda-5 level (27, 28). Of significance is the very recent finding that expressed peptides representing the cytoplasmic tail of Gn of the pathogenic NY-1 hantavirus can inhibit RIG-I- and TBK-1-directed immune responses but do not block transcriptional responses directed by activated IRF-3, indicating that virus blockage of interferon activation may lie upstream of IRF-3 phosphorylation (1). These results, using peptides from the HPS-associated NY-1 virus, differ from the findings presented here obtained by ex-

FIG. 6. A. ANDV and PHV glycoproteins inhibit IFN- β -induced Stat-1 nuclear translocation. Vero-E6 cells were transfected with plasmids expressing ANDV GPC, PHV GPC, or Ebola virus VP35 (as a control). At 48 h posttransfection, cells were treated with 1,000 U of IFN- β for 20 min, fixed, and double stained using antibodies detecting Stat-1 (left panels), ANDV glycoproteins, PHV glycoproteins, and Ebola virus VP35 (middle panels). Right panels show the merged images. B. ANDV glycoprotein expression does not result in a general block of cellular protein nuclear translocation. Vero-E6 cells were transfected with plasmids expressing HA-tagged IRF-3 and ANDV GPC. At 24 h posttransfection cells were infected with Sendai virus (row 2) or left uninfected (row 1). The cells were fixed at 40 h posttransfection and double stained using anti-HA monoclonal antibody detecting expressed IRF-3 (left panels) or rabbit anti-ANDV polyclonal sera (middle panels). Right panels show the merged images.

pression of full-length authentic glycoproteins of the HPS-associated ANDV. Contrary to the peptide findings of Alff et al. (1), no effect of Gn protein on IRF-3 activation by Sendai virus was observed, although IRF-3 is downstream of RIG-I and TBK-1, which Alff and colleagues found to be inhibited (Fig. 6B). The results may reflect differences in pathogenic mechanisms employed by NY-1 versus ANDV or may result from use of peptides versus authentic viral glycoproteins. Further studies will be necessary to more precisely map the

viral determinants down-regulating interferon induction and compare mechanisms employed by different pathogenic hantaviruses.

RNA viruses have also been known to interfere with interferon production by specifically inhibiting the Jak/Stat interferon signaling pathway (5, 10). In general, in order for Stat proteins to have a biological effect on transcriptional activation of antiviral responses, they must be phosphorylated and translocate to the cell nucleus (15). Here we have shown that Stat

phosphorylation was observed in PHV but not in ANDV infection of primary microvascular lung cells. It was possible that this may have simply reflected the difference in the initial interferon induction by these two viruses or, in addition, that signaling through the Jak/Stat pathway is blocked by ANDV infection. However, despite their differences in interferon induction, both ANDV and PHV were found to inhibit the interferon signaling pathway by down-regulating phosphorylation and nuclear translocation of Stats. Further, the viral glycoproteins were shown to be involved in this blockage of interferon signaling. This result was somewhat unexpected, as RNA virus nonstructural proteins, rather than glycoproteins, have been more frequently implicated in modulation of the innate immunity induced by virus infection (8). Although a few reports have implicated RNA virus nucleocapsid proteins, no negative-stranded RNA virus glycoproteins have been shown to be involved in modulation of virus-induced cell immunity (8, 17). The somewhat unusual finding with the hantavirus glycoproteins may be related to a rather unique characteristic of hantavirus Gn glycoproteins, which is their possession of a long cytoplasmic tail (152 amino acids in the case of ANDV). Conserved signals such as those for nitrosylation and possible redox regulation are present in the hantavirus Gn cytoplasmic tails. Most importantly, a conserved YRTL motif, predicted to be a tyrosine kinase motif and a glycoprotein trafficking signal, is present within all hantavirus Gn cytoplasmic tails (26). The YRTL motif is also part of a possible ITAM (with the consensus sequence YxxL/Ix 6-8YxxL/I), which is conserved among some, but not all, hantaviruses (7). Further studies will be required to precisely map which hantavirus glycoprotein regions are directly involved in downregulation of interferon signaling.

In conclusion, our results show that, unlike PHV, ANDV is unable to elicit strong induction of an IFN- β response in human primary lung endothelial cells. This was linked to the absence of IRF-3 activation by ANDV at early times postinfection. However, both viruses possess the ability to downregulate the interferon signaling pathway and, unexpectedly, the viral glycoproteins were found to contribute to this downregulation.

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