Respiratory Syncytial Virus Limits α Subunit of Eukaryotic Translation Initiation Factor 2 (eIF2α) Phosphorylation to Maintain Translation and Viral Replication*

Dayna J. Groskreutz‡§, Ellen C. Babor†, Martha M. Monick‡, Steven M. Varga§, and Gary W. Hunninghake‡§

From the ‡Division of Pulmonary, Critical Care, and Occupational Medicine, §Institute for Clinical and Translational Science, and ¶Department of Microbiology and Interdisciplinary Graduate Program in Immunology, University of Iowa Roy J. and Lucille A. Carver College of Medicine and Veterans Affairs Medical Center, Iowa City, Iowa 52242

The impact of respiratory syncytial virus (RSV) on morbidity and mortality is significant in that it causes bronchiolitis in infants, exacerbations in patients with obstructive lung disease, and pneumonia in immunocompromised hosts. RSV activates protein kinase R (PKR), a cellular kinase relevant to limiting viral replication (Groskreutz, D. J., Monick, M. M., Powers, L. S., Yarovinsky, T. O., Look, D. C., and Hunninghake, G. W. (2006) J. Immunol. 176, 1733–1740). It is activated by autophosphorylation, likely triggered by a double-stranded RNA intermediate during replication of the virus. In most instances, ph-PKR targets the α subunit of eukaryotic translation initiation factor 2 (eIF2α) protein via phosphorylation, leading to an inhibition of translation of cellular and viral protein. However, we found that although ph-PKR increases in RSV infection, significant eIF2α phosphorylation is not observed, and inhibition of protein translation does not occur. RSV infection attenuates eIF2α phosphorylation by favoring phosphatase rather than kinase activity. Although PKR is activated, RSV sequesters PKR away from eIF2α by binding of the kinase to the RSV N protein. This occurs in conjunction with an increase in the association of the phosphatase, PP2A, with eIF2α following PKR activation. The result is limited phosphorylation of eIF2α and continued translation of cellular and viral proteins.

Respiratory syncytial virus (RSV) is a ubiquitous pathogen that causes upper respiratory infections in healthy adults, bronchiolitis and pneumonia in young children, exacerbations in patients with obstructive lung disease, and life-threatening pneumonia in immunosuppressed patients. RSV infection early in life has been associated with the subsequent development of asthma (2–9). RSV is a member of the Paramyxoviridae family and consists of a negative strand RNA genome in a nucleocapsid surrounded by an envelope (10). Production of dsRNA is part of the replicative cycle for RNA viruses like RSV, and RNA serves as a template for both transcription and replication (11). Entry into the host respiratory epithelium is by cell surface fusion, and infection leads to viral replication and subsequent host inflammatory responses (12–17).

We previously showed that RSV increases the amount of protein kinase R (PKR) in airway epithelial cells (1). When activated by phosphorylation, PKR inhibits cellular translation through its ability to phosphorylate eIF2α on the Ser-51 regulatory site (18). eIF2α is a GTP-binding protein that delivers the initiator methionyl-tRNA to the small ribosomal subunit in translation initiation. Phosphorylation of eIF2α converts eIF2 from a substrate to an inhibitor of its GDP-GTP exchange factor eIF2B, blocking protein synthesis (19). If the phosphorylation of PKR during RSV infection triggers activation of eIF2α, then cellular and viral protein translation should markedly decrease. However, viral protein translation occurs in a robust fashion during RSV infection.

Viruses have different strategies to maintain viral protein translation, and PKR plays a limited role with some viruses and cell types. Subgenomic hepatitis C virus RNA replicates more efficiently in PKR knock-out mouse embryonic fibroblasts than in wild type mouse embryonic fibroblasts. The suppression of PKR activity by small interfering RNA enhances the level of hepatitis C virus RNA replication, suggesting PKR controls hepatitis C virus replication, likely via eIF2α phosphorylation (20). These findings were confirmed in PKR knockdown cells (21). However, inhibition of PKR by antisense peptide-conjugated phosphorodiamidate morpholino oligomers has no effect on severe acute respiratory syndrome virus titers nor does it affect the severe acute respiratory syndrome-induced phosphorylation of eIF2α (22). These observations suggest that another eIF2α kinase may regulate its activation or the severe acute respiratory syndrome virus has direct inhibitory effects on eIF2α. Additionally, cells void of PKR protein by RNA interference do not alter the growth of adenovirus, reovirus, or measles virus (22, 23). Studies using alphanivirus demonstrate a decrease in viral titers in PKR−/− cells (24). The role of PKR in viral replication is virus- and cell-specific.

PKR activation usually results in eIF2α phosphorylation, and this activity is balanced by phosphatases that dephosphorylate eIF2α (25–28). Two well studied phosphatases of eIF2α are

*This work was supported, in whole or in part, by National Institutes of Health Grants HL089392-02, HL079901-01A1, AI 063520, and RR00059 from the General Clinical Research Centers Program and Grant U1R024979 from the NCRR. This work was also supported by a Veterans Affairs Merit Review grant.

1 To whom correspondence should be addressed: Division of Pulmonary, Critical Care, and Occupational Medicine, 100 EMRB, Iowa City, IA 52242. Tel.: 319-356-1302; Fax: 319-353-6406; E-mail: dayna-groskreutz@uiowa.edu.

2 The abbreviations used are: RSV, respiratory syncytial virus; m.o.i., multiplicity of infection; hTBE, human tracheobronchial epithelial; PKR, protein kinase R; dsRNA, double-stranded ribonucleic acid; eIF2α, α subunit of translation initiation factor 2; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; poly(I:C), polyinosinic-polycytidylic acid; rATP, recombinant ATP.
protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A). PP1 consists of a catalytic subunit of 37 kDa, which is bound to a number of different regulatory or targeting subunits. The formation of these complexes converts PP1 into different forms that have different substrate specificities. The PP1 catalytic subunit (PP1c) has four isoforms (PP1α, PP1β, and the splice variants PP1γ1 and PP1γ2). PP1 contributes to cellular recovery from stress by acting as an elf2α phosphatase. PP1 regulators inhibitor-1 (I-1), growth arrest and DNA damage-inducible protein 34 (GADD34), and constitutive repressor of regulators inhibitor-1 (I-1), growth arrest and DNA damage-inducible protein 34 (GADD34), and constitutive repressor of PP2A to promote inhibition of PP2A and protein synthesis (33). The roles of various elf2α kinases and phosphatases play in regulating RSV infection have not been evaluated.

The results of our study reveal that RSV limits PKR activity and elf2α phosphorylation by favoring phosphatase rather than kinase activity. RSV sequesters active PKR away from elf2α by binding of the kinase to the RSV N protein. At the same time, recruitment of PP2A to elf2α following PKR activation still occurs. The net effect is limited elf2α phosphorylation and maintenance of cellular and viral protein translation.

**EXPERIMENTAL PROCEDURES**

*Materials*—Chemicals were obtained from Sigma and Calbiochem. Protease inhibitors were obtained from Roche Applied Science. 0.4% trypan blue solution and mouse monoclonal antibody to β-actin were obtained from Sigma. Polyinosinic-polycytidylic acid (poly(I-C)) was obtained from Inovigen (San Diego, CA). Thapsigargin was obtained from Calbiochem. RNase III (AM2290) was obtained from Ambion (Austin, TX). Rabbit polyclonal antibodies to PKR (Thr-451) 3075, ph-PKR (Thr-451) 3072, ph-PKR (Thr-451) 3075, and ph-elf2α (Ser-51) 9721 and rabbit monoclonal antibody to PP2A (C subunit) 2259 were obtained from Cell Signaling Biotechnology (Beverly, MA). Goat polyclonal antibodies elf2α (K-17) sc-30882 and PP1α (N-19) sc-6105, rabbit polyclonal antibody to ph-PKR (Thr-446) (sc-101783); isotype control IgG antibodies rabbit (sc-2027), mouse (sc-2025), and goat (sc-2028); and mouse monoclonal antibodies to PP1c (E-9) sc-7482 and PKR (B-10) sc-6282 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Goat monoclonal antibody to RSV (B65860G) was obtained from Biodesign International (Saco, ME). Immunofluorescent AlexaFluor 488 goat antibody (A11055) was purchased from Invitrogen.

*Human Epithelial Cells*—A549 lung epithelial cells were obtained from the American Type Cell Collection (ATCC) (Manassas, VA) and cultured in minimum essential medium (Invitrogen) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS) and gentamicin. Human tracheobronchial epithelial (hTBE) cells were obtained from Dr. Joseph Zabner and the Cell Culture Core under a protocol approved by the University of Iowa Institutional Review Board. Epithelial cells were isolated from tracheal and bronchial mucosa by enzymatic dissociation and cultured in Laboratory of Human Carcinogenesis (LHC)-8e medium on plates coated with collagen/albumin for study up to passage 10 as described previously (34). Cells at 50–80% confluency were treated with human RSV strain A2 (m.o.i. 2). Viral stocks were obtained from Advanced Biotechnologies, Inc. (Columbia, MD). The initial stock (1.1 × 10⁹ TCID₅₀) was aliquoted and kept frozen at −135 °F, and a fresh aliquot was thawed for each experiment. Recombinant vaccinia viruses were a gift from G. W. Wertz and T. J. Braciale (University of Virginia, Charlottesville) and J. L. Beeler (United States Food and Drug Administration, Bethesda) and were prepared as described previously (35–38), and they were propagated in BSC-40 cells (ATCC).

*Cell Protein Isolation and Western Blot Analysis*—20 μg of protein was mixed 1:1 with 2× sample buffer, and Western blot analysis was performed as described previously (39).

*Immunoprecipitation*—Immunoprecipitation of cellular protein was performed using the protein G Dynabead immunoprecipitation kit (Invitrogen) according to the manufacturer’s instructions. Immunoprecipitation was followed by Western blot as referenced above to determine association between cellular proteins.

*PKR Activity Assay*—PKR protein was immunoprecipitated as described above, and PKR activity was determined by performing a PKR activity assay using the Kinase-Glo luminescent kinase assay (V6711) and recombinant ATP (rATP) (PI132) from Promega (Madison, WI) according to the manufacturer’s instructions. Briefly, the Kinase-Glo reagent uses a proprietary luciferase that generates a stable luminescent signal. An equal volume of Kinase-Glo reagent is added to rATP and purified kinase/substrate (PKR) to measure PKR autophosphorylation. The Kinase-Glo reagent produces luminescence that is directly proportional to the amount of ATP present in the kinase reaction and inversely proportional to the amount of kinase activity. Thus, a decrease in luminescent signal corresponds to an increase in kinase activity. Next, PKR activity was verified using a standard autophosphorylation assay. Immunoprecipitated PKR protein was washed twice with kinase buffer containing 50 mM Trıs, pH 7.4, 150 mM NaCl, and 1% Nonidet P-40. Washed protein was resuspended in kinase buffer (50 mM K₃PO₄, pH 6.8, 0.1 mg/ml bovine serum albumin, 15 mM MgCl₂ with 6 μM [³²P]ATP (35 μCi) and incubated at 30 °C for 30 min. The kinase reaction was stopped by the addition of 65 μL of 2× SDS-PAGE loading buffer and was boiled for 5 min before separation by 10% SDS-PAGE. The gel was transferred to a nitrocellulose membrane, and [³²P]-labeled protein was quantified by autoradiography. The total PKR was determined by Western blot with a mouse monoclonal antibody to PKR (Santa Cruz Biotechnology).

*Nascent Protein Synthesis*—New protein synthesis was measured using the Click iT protein reaction buffer kit, Click iT metabolic labeling reagents for proteins (1-azidohomoalanine), and Click IT chemistry reagents (tetramethylrhodamine alkyne) from Invitrogen according to the manufacturer’s instructions. Briefly, an azide-containing biomolecule (1-azidohomoalanine) was incorporated into the newly synthesized protein. Next, the modified protein was detected with a corresponding alkyne-containing dye or hapten (tetrameth-
There was a chemoselective ligation or “click” reaction between the azide (L-azidohomoalanine) and alkyne (tetramethylrhodamine). Gel electrophoresis was performed, and newly synthesized proteins labeled with the Click iT reaction were detected using the Click iT protein reaction buffer kit. To directly detect tetramethylrhodamine detection reagent, the lanes were imaged with a Versadoc imager (Bio-Rad).

**Statistical Analysis**—Statistical analysis was performed on the PKR activity assay data. Significance between the groups was confirmed by one-way analysis of variance with a Bonferroni’s test for multiple comparisons (GraphPad statistical analysis software). Variability is expressed by standard error of the mean.

**RESULTS**

Active PKR Increases in RSV Infection—In a previous study, we showed that RSV increases the amount of total PKR (1), and in Fig. 1A, after 24 h of RSV infection (m.o.i. 2) of A549 airway epithelial cells, there is phosphorylation of PKR at both sites of autophosphorylation (threonine 451 and threonine 446) indicating activation of PKR. We performed PKR activity assays to confirm PKR activation as measured by autophosphorylation in RSV infection (Fig. 1B). A549 cells were exposed to 24 h of control media alone, 24 h of RSV (m.o.i. 2), or 6 h of 20 µg/ml transfected poly(I-C), a nonspecific synthetic double-stranded RNA known to activate PKR. Cells were harvested, and PKR protein was obtained by immunoprecipitation. The PKR protein was incubated with 1 µM luminescent rATP for 30 min. KinaseGlo reaction buffer was added, and relative luminescence was used to determine kinase activity. In this assay, a decrease in relative luminescence indicates a decrease in measured free ATP in the supernatant and an increase in kinase activity and autophosphorylation of PKR. Fig. 1B shows a significant decrease in relative luminescence of rATP in the supernatant (indicating an increase in PKR autophosphorylation) in both RSV infection and poly(I-C) transfection conditions. We repeated the PKR kinase assay using a standard 32P-labeled ATP method. A549 cells were exposed to 24 h of control media alone, 24 h of RSV (m.o.i. 2), or 6 h of 20 µg/ml transfected poly(I-C). Cells were harvested, and PKR protein was immunoprecipitated. PKR protein was incubated with [32P]ATP to measure PKR autophosphorylation, and the products were run on a gel. Autoradiography shows that both RSV and poly(I-C) transfection result in PKR activation. Densitometry shows relative amounts of protein. Western blot (WB) confirms the presence of PKR protein on the membrane. Asterisk corresponds to the statistical significance p <0.0001 noted at the bottom of the figure.
Because ph-PKR normally phosphorylates eIF2α resulting in inhibition of cellular translation, we expected the increased ph-eIF2α seen in RSV infection to result in increased ph-eIF2α. Surprisingly, the increase in ph-eIF2α in RSV infection was minimal (Fig. 2A). We measured ph-eIF2α after transfection with poly(I-C) to determine whether significant eIF2α phosphorylation can occur in our cell system. We also measured ph-eIF2α after treatment with thapsigargin, an activator of another eIF2α kinase, PKR-like endoplasmic reticulum kinase. No significant change in ph-eIF2α is observed when cells are exposed to control media alone, whereas thapsigargin maximally increases ph-eIF2α within 1 h of treatment, and transfected poly(I-C) increases ph-eIF2α by 6 h post-transfection (Fig. 2B). Phosphorylation of PKR (Thr-446) also occurred with transfection of poly(I-C). The expected increase in ph-eIF2α occurred with thapsigargin and poly(I-C) but did not occur with RSV infection, suggesting that the blunted activation of eIF2α was unique to RSV.

**RSV Decreases Association of PKR with eIF2α**—If PKR is activated in RSV infection, but eIF2α phosphorylation does not occur, then either the active kinase, PKR, is unable to effectively phosphorylate its substrate eIF2α, or the counter-regulatory phosphatase activity exceeds that of the PKR kinase activity. In evaluation of these possibilities, we performed immunoprecipitation experiments to determine the relative association of kinases and phosphatases with eIF2α. As shown in Fig. 3A, eIF2α co-immunoprecipitates with PKR under base-line conditions, but after exposure to RSV for 24 h, there is less association of PKR with eIF2α. Densitometry shows relative amounts of protein in each group. Immunoprecipitation with an isotype control antibody does not reveal any nonspecific immunoprecipitation of eIF2α. The upper blot in Fig. 3A confirms that the PKR antibody specifically pulls down the PKR protein in equal amounts in control and RSV conditions.

**Poly(I-C) Increases Association of PKR and eIF2α**—Because our previous experiments demonstrated phosphorylation of
PP2A in RSV infection. The more specific activity of RSV may be on limiting the PKR kinase activity, and PP2A may be recruited to eIF2α to some extent under any dsRNA conditions.

RSV Proteins Bind to PKR—We considered whether RSV is binding PKR or eIF2α and changing their relative association. We performed an immunoprecipitation for PKR protein followed by a Western blot for RSV using an RSV antibody that detects multiple RSV proteins, including G, F1, N, P, and M2-2. Fig. 5A (left panel) shows that when immunoprecipitation for PKR protein is performed in A549 cells, Western blot for RSV proteins shows a clear association of the RSV N protein with PKR. This finding is confirmed in primary airway epithelial (hTBE) cells (Fig. 5A, right panel). A similar association of eIF2α protein with RSV proteins was not demonstrated (data not shown). Similarly, immunoprecipitation with the same RSV antibody in A549 cells shows an association of RSV proteins with PKR but not with eIF2α (Fig. 5B). To clarify whether the N protein is binding directly to PKR or whether PKR and N protein approximate each other because their functions both require binding to viral RNA, we repeated the experiment but treated RSV-infected protein lysates with RNase III as described previously (40). We then performed immunoprecipitation for PKR protein. Fig. 5C shows that the co-immunoprecipitation of PKR and RSV N protein is not decreased with RNase III incubation. These findings suggest that PKR and RSV N protein are directly binding in an RNA-independent manner. This finding is consistent with other literature demonstrating PKR binding to other protein substrates in both RNA-dependent and RNA-independent manners (40).

To more specifically determine the role of the RSV N protein, we infected A549 cells with a vaccinia virus construct with an RSV N gene inserted (vaccN) and used a vaccinia virus construct with a β-galactosidase gene inserted (vaccβ-gal) as a control. As shown in Fig. 6A, eIF2α co-immunoprecipitates with PKR after exposure to vaccinia virus with the β-galactosidase gene (vaccβ-gal) exposure (top panel), but after exposure to vaccinia virus expressing the RSV N gene (vaccN) for 24 h (top panel), there is minimal association of PKR with eIF2α. The RSV N protein is pivotal in prohibiting the association of PKR and eIF2α. To confirm a physical association between PKR and the RSV N protein, we performed an immunoprecipitation for PKR and subsequent Western blot for multiple RSV proteins

**FIGURE 3.** RSV decreases the association of PKR with eIF2α. A, A549 cells were exposed to control media or RSV (m.o.i. 2) for 24 h. Whole cell lysates were obtained, and immunoprecipitation (IP) for PKR protein was performed followed by Western blot (WB) for PKR and eIF2α. Densitometry was performed (right panel). These data are representative of three experiments (n = 3). B, iso, isotype. 8, A549 cells were exposed to control media or RSV (m.o.i. 2) for 24 h. Whole cell lysates were obtained, and immunoprecipitations for eIF2α or PP2A protein were performed followed by Western blot for PP2A or eIF2α. C, A549 cells were exposed to control media or RSV (m.o.i. 2) for 24 h. Whole cell lysates were obtained, and immunoprecipitations for PP1 or PP1c protein (catalytic portion) were performed followed by Western blot for PP1, PP1c protein (catalytic portion), or eIF2α.

**FIGURE 4.** Poly(I-C) increases the association of PKR with eIF2α. A549 cells were exposed to control media or transfected poly(I-C) (20 μg/ml) for 6 h. Whole cell lysates were obtained, and immunoprecipitation (IP) for PKR or eIF2α protein was performed followed by Western blot (WB) for eIF2α and PP2A (n = 3).
RSV and Translation

**FIGURE 5. RSV proteins bind PKR.** A, A549 cells (left panel) or hTBE cells (right panel) were exposed to control media or RSV (m.o.i. 2) for 24 h. Whole cell lysates were obtained, and immunoprecipitation (IP) for PKR protein was performed followed by Western blot (WB) for RSV proteins (n = 3). Iso, isotype. B, A549 cells were exposed to control media or RSV (m.o.i. 2) for 24 h. Whole cell lysates were obtained, and immunoprecipitation for PKR protein was performed followed by Western blot for PKR and eIF2α (n = 3). C, A549 cells were exposed to control media or RSV (m.o.i. 2) for 24 h. Whole cell lysates were obtained and incubated with or without RNase III. Immunoprecipitation for PKR protein was performed followed Western blot for PKR and RSV proteins.

after exposure to vaccinia virus with the β-galactosidase gene (vacvβ-gal) or vaccinia virus construct with an RSV N gene inserted (vacvN). Fig. 6A (bottom panel) demonstrates that PKR associates with the RSV N protein and that the vaccinia virus construct specifically expresses the RSV N protein. There is no significant binding of the RSV N protein to eIF2α or PP2A (data not shown). A light band corresponding to the RSV P protein was seen in Fig. 5, so we repeated the experiment with a vaccinia virus expressing the RSV P protein (vacvP). No immunoprecipitation between PKR and the RSV P protein was observed (data not shown).

We have shown that there is minimal eIF2α phosphorylation in RSV-infected cells (Fig. 2A). We have also shown that RSV N protein binds directly to PKR (Fig. 5). We next determined whether binding of the N protein to PKR directly affects eIF2α phosphorylation. We exposed A549 cells to control media for 24 h, a vaccinia virus construct with a β-galactosidase gene inserted (vacvβ-gal) for 24 h, or a vaccinia virus construct with an RSV N gene inserted (vacvN) for 24 h. We transfected the infected cells with 20 μg/ml of poly(I-C). After harvesting the cells, we performed a Western blot for ph-eIF2α. Fig. 6B shows that there is less ph-eIF2α protein when the cells are exposed to vaccinia virus with the RSV N gene inserted as compared with vaccinia virus with the β-galactosidase gene. These data provide further evidence that the N protein directly inhibits PKR phosphorylation of eIF2α.

**DISCUSSION**

In this study, we demonstrate that RSV increases PKR activation but does not increase the phosphorylation of the PKR target, eIF2α, maintaining protein translation. RSV prevents eIF2α phosphorylation by specific binding of the RSV N protein to the active kinase, PKR, limiting the association of PKR with eIF2α. This occurs in the setting of the normal increase in the binding of the phosphatase, PP2A, with eIF2α following PKR activation. These events of decreased kinase association with eIF2α (specific to RSV infection) and increased phosphatase association with eIF2α (seen with nonspecific dsRNA) together limit its phosphorylation. By limiting eIF2α phosphorylation, RSV maintains translation of nascent viral and cellular proteins (Fig. 8).

Viruses do not possess the necessary components for autonomous translation of viral proteins and subsequent replication, and they need to maintain host protein synthesis machinery to...
produce viral proteins. Although recent evidence indicates that PKR can undergo adaptive changes at sites associated with eIF2α/H9251 recognition and thus overcome viral mimicry (41), viruses have evolved various counter methods for maintaining cellular translation. Herpes simplex virus (type-1) makes a viral gene product, Us11, that associates with ribosomes to prevent eIF2α phosphorylation.

FIGURE 6. RSV N protein binds PKR in vaccinia virus construct and limits eIF2α phosphorylation. A, A549 cells were exposed to vaccinia virus expressing the RSV N gene (m.o.i. 2) or vaccinia virus expressing β-galactosidase for 24 h. Whole cell lysates were obtained, and immunoprecipitation (IP) for PKR protein was performed followed by Western blot (WB) for eIF2α or RSV (n = 3), iso, isotype. B, A549 cells were exposed to vaccinia virus expressing β-galactosidase (β-gal) gene or vaccinia virus expressing the RSV N gene (m.o.i. 2) for 24 h. Infected cells were transfected with 20 μg/ml poly(I-C) for 6 h. Whole cell lysates were obtained, and Western blot for ph-eIF2α was performed. Densitometry shows relative amounts of ph-eIF2α protein.

FIGURE 7. RSV does not alter nascent protein synthesis. A, A549 cells were exposed to control media alone, RSV (m.o.i. 2) for 24 h, thapsigargin (50 μg/ml) for 1 h, or poly(I-C) (20 μg/ml) for 6 h. Cells were harvested, and nascent protein synthesis was detected. B, A549 cells were exposed to control media or RSV (m.o.i. 2) for 24 h. Immunofluorescence confirms significant RSV infection. Aby, antibody.
PKR activation and premature cessation of protein synthesis, and additionally, it masks or sequesters dsRNA (42). Herpes simplex virus, type-1, also maintains eIF2α function by making the γ134.5 protein that interacts with PP1 to activate it and dephosphorylate eIF2α (43). The Myd116 African swine fever virus also interacts with the catalytic subunit of PP1 and increases its phosphatase activity on eIF2α (44). Vaccinia virus manufactures a K3L protein that acts as a direct inhibitor of eIF2α phosphorylation by PKR (45). Additionally, the vaccinia virus makes an E3L protein that inhibits PKR activation by dsRNA masking or sequestering (46). The empty vaccinia virus constructs used on our experiments (vacvβ-gal) did not seem to alter the relative association of PKR and eIF2α as RSV did, although other independent vaccinia virus effects on our cells cannot be excluded. Regardless, there is clearly an evolutionary advantage for viruses to maintain control of cellular translation.

The effect of RSV on protein translation has not been described, and the activity of the RSV N gene in regulation of cellular proteins is also novel. The other functions of the N protein have previously been described. The nucleoprotein (N protein) binds to genomic and anti-genomic RNA to form an RNase-resistant nucleocapsid (10). The phosphoprotein (P protein) is believed to function as a chaperonin for soluble N protein and as a polymerase cofactor (10). It is intriguing that a viral protein important for viral mRNA production like the N protein as RNase III does not decrease the binding of PKR to the N protein. The binding of PKR to N protein is likely a protein-protein binding. Both RNA-dependent and -independent associations of PKR with specific proteins have been demonstrated (40).

Our data indicate that the interaction between PKR and the RSV N protein is not due to RNA. In theory, RNA might partially bridge the PKR and N proteins; however, it is unlikely that RNA is responsible for the physical association of PKR, and the N protein as RNase III does not decrease the binding of PKR to the N protein. The binding of PKR to N protein is likely a protein-protein binding. Both RNA-dependent and -independent associations of PKR with specific proteins have been demonstrated (40).

It has been shown that PKR activation and dimerization compose a key switch that regulates phosphorylation of eIF2α (47). Other evidence suggests that PKR is found with its substrates, even in absence of activation, and this association may not be transient (48, 49). These data and our data suggest the PKR is already bound to eIF2α prior to activation and that activation of the kinase results in phosphorylation of its substrate. Consistent with this, others in our laboratory have shown that kinases and their substrates associate with one another in the absence of stimulation (50). Furthermore, our data show that our proteins are not nonspecifically co-immunoprecipitating as eIF2α and PP2A do not pull down with other RSV proteins. Our data show that PKR is activated in RSV infection (1), but it is no longer in proximity to associate with eIF2α because binding of PKR to the N protein of RSV prohibits this interaction.

The respective roles of PP1 and PP2A phosphatases with regard to eIF2α and its kinases independent of viral infection have been described. Both PP1 and PP2A have been reported to dephosphorylate PKR and eIF2α (25–28, 51), and PP1 co-immunoprecipitates with PKR and eIF2α (25, 27) Phosphorylation of the B56α subunit of PP2A by PKR under conditions of PKR activation has been described (33). Others have investigated phosphatase activity specifically in viral infection. Hakki and Geballe (52) described an increase in the levels of PP1 and PP2A phosphatases and phosphatase activity limiting eIF2α phosphorylation in human cytomegalovirus infection.

Others have reported increased PP2A levels and activity in cells infected with hepatitis and human immunodeficiency virus (53–57). Other studies indicate that simian virus 40 or polyomavirus inhibition of PP2A is important for cellular transformation in malignancy (58, 59). The fact that RSV or nonspecific dsRNA may be affecting PP2A location, levels, or activity is consistent with these published data. We find no definitive role of PP1 association with eIF2α or its kinases in RSV infection in our model system. It appears that PP2A is the active phosphatase both in RSV infection of airway epithelial cells and upon activation by dsRNA.

RSV is a clinically important pathogen, particularly for infants, patients with obstructive lung disease, and the immunosuppressed. Our study demonstrates that RSV maintains cellular translation to allow for continued viral replication. These observations suggest that PKR, eIF2α, and PP2A may be important cellular targets for therapy in RSV infection, and the RSV N protein may be instrumental in maintaining viral translation and replication.

Acknowledgments—We thank Alan Ryan, Lynda Ostedgaard, and Yuping Zhang for their technical assistance and Joseph Zabner for review of the manuscript.
