Influenza B Virus Ribonucleoprotein Is a Potent Activator of the Antiviral Kinase PKR

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Abstract

Activation of the latent kinase PKR is a potent innate defense reaction of vertebrate cells towards viral infections, which is triggered by recognition of viral double-stranded (ds) RNA and results in a translational shutdown. A major gap in our understanding of PKR’s antiviral properties concerns the nature of the kinase activating molecules expressed by influenza and other viruses with a negative strand RNA genome, as these pathogens produce little or no detectable amounts of dsRNA. Here we systematically investigated PKR activation by influenza B virus and its impact on viral pathogenicity. Biochemical analysis revealed that PKR is activated by viral ribonucleoprotein (vRNP) complexes known to contain single-stranded RNA with a 5’-triphosphate group. Cell biological examination of recombinant viruses showed that the nucleocytoplasmic transport of vRNP late in infection is a strong trigger for PKR activation. In addition, our analysis provides a mechanistic explanation for the previously observed suppression of PKR activation by the influenza B virus NS1 protein, which we show here to rely on complex formation between PKR and NS1’s dsRNA binding domain. The high significance of this interaction for pathogenesis was revealed by the finding that attenuated influenza viruses expressing dsRNA binding-deficient NS1 proteins were rescued for high replication and virulence in PKR-deficient cells and mice, respectively. Collectively, our study provides new insights into an important antiviral defense mechanism of vertebrates and leads us to suggest a new model of PKR activation by cytosolic vRNP complexes, a model that may also be applicable to other negative strand RNA viruses.

Introduction

The presence and replication of viral nucleic acids in vertebrate cells triggers innate immune responses by the activation of antiviral enzymes and induction of type I interferon (IFN) genes [1]. The double-stranded (ds) RNA-dependent protein kinase PKR is a key mediator of this innate immune defense functioning as a signal transducer in a variety of cellular processes [2,3]. Human PKR is a latent serine/threonine kinase of 551 amino acids with two consecutive N-terminal double-stranded (ds) RNA-binding motifs, a linker domain, and a C-terminal kinase domain [4]. PKR is present in non-stimulated cells at basal levels, but its expression is upregulated by type I IFN, which allows a robust response to viral infection [5]. Activation of PKR during infection involves recognition of viral nucleic acids, which induces a structural rearrangement leading to dimerization and autophosphorylation of the kinase at threonine residues 446 and 451 [2]. The best-studied natural target site of activated PKR is serine 51 of the alpha subunit of the eukaryotic translation initiation factor 2 (eIF2α). Its phosphorylation brings about a translational block of cellular and mRNAs and hence, a strong impairment of viral replication [2,3]. In addition, PKR controls transcriptional activation of the nuclear factor-kappa B (NF-κB) pathway and was also shown to mediate apoptosis and to function as a tumour suppressor [3].

Many virus families have evolved gene products targeting PKR, illustrating the high relevance of this kinase in antiviral defense [3,6]. The inhibitory mechanisms include PKR degradation, sequestration of viral dsRNA by a viral protein, preventing PKR activation through inhibitory viral proteins or viral decoy RNA, and regulating the phosphorylation of eIF2α through a viral pseudosubstrate or recruitment of a cellular phosphatase [3]. Early studies identified dsRNA with a minimum length of 34 base pairs as a prototypical activator of PKR [7] and the sources of these kinase-inducing nucleic acids have been well recognized for several classes of viruses: Complex DNA viruses such as vaccinia virus, adenovirus or herpes simplex virus transcribe open reading frames in opposite orientations leading to formation of duplex RNAs [8].
In reovirus-infected cells, the viral genome consists of dsRNA and also the genomes of many plus-strand RNA viruses contain long stretches of extensively base-paired secondary structure elements [8]. Surprisingly, there is little knowledge about the specific nucleic acids of influenza and other negative-sense RNA viruses that trigger PKR activation, as earlier attempts failed to detect dsRNA in cells infected with such viruses [9,10]. Progress in this area has been hampered in part by viral suppressors of PKR, which inhibits PKR activation. This interaction seems to be crucial for viral pathogenicity, as a strong attenuation of NS1 mutant viruses was largely rescued in PKR-deficient mice and cells. Taken together, these findings suggest a new model for the induction and inhibition of PKR by influenza virus that may also apply to viruses with a similar genome structure.

The influenza A and B viruses are globally distributed pathogens of the Orthomyxoviridae family that cause acute severe respiratory disease and around 40,000 deaths each year in the European Union alone (http://ecdc.europa.eu/Health_topics/respiratory_disease and around 40,000 deaths each year in the European Union alone (http://ecdc.europa.eu/Health_topics/respiratory_disease and around 40,000 deaths each year in the European Union alone (http://ecdc.europa.eu/Health_topics/respiratory_disease and around 40,000 deaths each year in the European Union alone (http://ecdc.europa.eu/Health_topics/respiratory_disease). These viruses have a segmented genome necessitates the application of reverse genetic procedures for such mutant viruses was largely rescued in PKR-deficient mice and cells. Taken together, these findings suggest a new model for the induction and inhibition of PKR by influenza virus that may also apply to viruses with a similar genome structure.

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PKR deficiency rescues attenuated replication and virulence of influenza B mutant viruses with dsRNA-binding deficient NS1 proteins

Our previous analyses revealed three basic amino acid clusters at positions 47/50, 58/60/64 and 77/78 in the N-terminal region of the influenza B virus NS1 protein to be essential for dsRNA binding as well as for inhibition of PKR and eIF2α phosphorylation [16,21] (Fig. 1A). Hence, loss-of-function viruses expressing NS1 proteins with alanine replacements at those essential positions (mutants #2, #4, #6) or at amino acids 33/38 (mutant #1, dsRNA binding reduced) were attenuated for replication in IFN-competent hosts by several orders of magnitude [16]. In contrast, viruses expressing NS1 proteins with alanine replacements at positions 52/53/54 or 83/86 (mutants #3 and #7), which retained dsRNA binding, inhibited PKR activation and replicated to high titers [16].

To address the question of whether PKR or another antiviral factor was mainly responsible for the attenuation of viruses with dsRNA binding-defective NS1 protein, we conducted a growth curve analysis in embryonic fibroblasts from PKR−/− and PKR−/− mice (Fig. 1B). The WT and control mutant virus #3 replicated
equally well in PKR+/- and PKR-/- cells reaching titers of 1–2×10⁶ FFU/ml. Replication of mutant virus #7 was slightly lower, but this characteristic was independent of the PKR status.

In contrast, replication of mutant viruses #2, #4 and #6 expressing dsRNA-binding defective NS1 proteins was reduced in normal mouse embryonic fibroblasts (MEF) by about three orders of magnitude (1.5–7.1×10³ FFU/ml) in comparison to WT. The mutant virus #1 replicated to a slightly higher titer (4.9×10⁴ FFU/ml). Significantly, the absence of PKR strongly boosted replication of all four mutant viruses (#1, #2, #4 and #6) to titers between 5.4×10⁵ and 1.1×10⁶ FFU/ml. Interestingly, minimal replication of the isogenic virus with a complete deletion of the NS1 gene (delNS1 virus) was observed both in the absence and presence of PKR, pointing to a vital NS1 function beyond the inhibition of PKR.

To evaluate NS1 dsRNA binding activity in vivo, we compared the growth of WT and selected mutant viruses in the lungs of wild-type and PKR-/- mice [39] three and six days after intranasal infection of PKR+/- or PKR-/- MEFs were infected with WT virus, delNS1 virus or NS1 mutant viruses #1, #2, #3, #4, #6 or #7 at an MOI of 0.1. Virus titers were determined at the indicated time points and represent the average of two independent experiments performed as duplicates. Error bars indicate the standard deviation. (C) For infection studies in mice the indicated representative recombinant influenza B/Lee viruses were chosen according to their ability to block PKR activation. Groups of eight-week-old female PKR-/- and wild type C57BL6 mice were anesthetized and infected intranasally with 1×10⁵ ffu of the indicated recombinant influenza B/Lee virus. For viral lung titrations, three mice were sacrificed at day 3 and at day 6 post-infection and virus titers were determined in lung homogenates. Error bars indicate the standard deviation. Statistical analysis indicated significant differences between WT and mutant virus titers. *, p<0.05; **, p<0.01; n.d., not detectable. Other recombinant viruses were not tested in this setting.

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infection with $1 \times 10^5$ FFU (Fig. 1C). This dose was the highest applicable amount possible, due to the low growth of some of the mutant viruses. Wild-type mice had strongly reduced lung titers (by about two orders of magnitude) of the mutants expressing dsRNA-binding deficient NS1 proteins (#2 and #4), in comparison to WT virus and control mutant #7; even so, all three mutant viruses replicated in PKR−/− mice to a similar extent as the WT. The delNS1 virus was not detected at all in normal mice. However, visible delNS1 virus titers were determined in the lungs of PKR−/− animals, which were, however, considerably reduced compared to WT virus. A similar strong attenuation of replication in PKR−/− mice was recently reported for an influenza B/Yamagata/88 mutant virus expressing a severely truncated NS1 protein of 18 amino acids [40]. The differences in delNS1 virus growth in vitro and in vivo might be due to a higher permissiveness of the murine respiratory tract in the absence of PKR compared to fibroblasts, or the slightly different genetic backgrounds of the PKR-deficient animals and cells studied. All WT mice survived the challenge with each virus, but there were marked differences in the body weights of infected animals (Fig. S1A). WT and the control mutant virus #7 caused a transient weight loss of up to 15%, whereas there was little or no weight reduction in mice infected with the mutants #2, #4 or delNS1 virus. Interestingly, PKR−/− mice rapidly lost weight and succumbed to infection between day 7 and 9 after challenge with the WT and all four viruses expressing mutant NS1 proteins, but there was little weight loss in PKR-deficient mice after delNS1 infection (Fig. S1B). These findings demonstrate that PKR can strongly inhibit replication of influenza B virus; however, PKR can be impeded by the dsRNA binding function of the viral NS1 protein.

DsRNA-binding NS1 proteins form an RNase-sensitive complex with PKR in infected cells

To test the hypothesis that NS1 forms a complex with PKR involving dsRNA, we first immunoprecipitated lysates of WT and mutant virus-infected human A549 cells with anti-NS1 serum (Fig. 2A). Immunoblot analysis showed that PKR was specifically coprecipitated with dsRNA binding NS1 proteins expressed by the WT and mutant viruses #1, #3 and #7. In contrast, very little or no PKR was detected in precipitates containing the dsRNA binding-deficient NS1 proteins of the mutant viruses #2, #4 and #6, that fail to prevent activation of PKR [16,21]. Further analysis showed that pre-treatment of lysate with dsRNA-specific RNase III eliminated the detection of WT NS1-PKR complexes in a dose-dependent manner (Fig. 2B). These results suggest that dsRNA-binding of the NS1 protein is a prerequisite for complex formation with PKR.

Wild-type but not dsRNA binding-deficient NS1 protein co-sediments with PKR and viral RNP

To characterize the intracellular complexes containing PKR and NS1 protein, we fractionated lysates of A549 cells infected with the WT or NS1 mutant #4 virus by sucrose density gradient...
centrifugation (Fig. 3A). Immunoblot analysis of fractions 1 to 9 (top to bottom) showed that the NS1 WT, but not the dsRNA binding-deficient mutant protein co-sedimented with PKR and NP in higher order complexes in fractions 7 to 9 (Fig. 3A). Dot-blot analysis detected genomic viral RNA of the HA and NS segments in the same fractions, indicating the presence of vRNPs (Fig. 3A, panels “HA and NS vRNA”). As expected, control blots showed in lysates of mutant virus-infected cells the presence of phospho-PKR, whereas little activation occurred with WT virus (Fig. 3B, right panel, “lysate”). Since PKR co-sedimented with vRNP the question arose whether these factors interact and if this interaction could be involved in PKR activation. Therefore, we immunoprecipitated lysates of WT and mutant virus-infected cells with PKR-specific antibody and examined the precipitates by immunoblotting and RNA hybridization (Fig. 3B). Efficient co-precipitation of the wild-type NS1, but not the mutant protein was observed (Fig. 3B). We also detected the negative-stranded viral RNA, a major component of the viral RNP, in the precipitates without difficulty. Hence, these results indicate that PKR associates with viral RNP and, depending on intact dsRNA binding, also with the NS1 protein.

**Activation of PKR correlates with cytoplasmic accumulation of vRNPs**

Next, we compared the activation of PKR after infection of cells with the NS1 loss-of-function mutant #4 and WT virus in relation to the intracellular localization of vRNP complexes (Fig. 4). Confocal microscopy detected vRNPs as stained by NP-specific antibody in the nucleus at 8 hours post infection (p.i.), but increasing cytosolic signals appeared at 12 and 16 hours p.i. for both viruses (Fig. 4A). At the later time-points, the NS1 proteins were detected in the nuclear and the cytoplasmic compartments, although we noted a slightly stronger nuclear signal for the mutant protein (Fig. 4A). Starting at 12 hours p.i., the cytosolic appearance of vRNP was paralleled by detection of activated PKR in cells infected with the mutant, but not the WT virus (Fig. 4B). A similar picture was observed in cells infected with the delNS1 mutant virus (data not shown). Intriguingly, PKR activation by the mutant #4 virus was strongly reduced in the presence of leptomycin B (LMB), a fungal drug that specifically inhibits the CRM1 pathway and thus the nuclear export of vRNP [41] (Fig. 4A). Immunoblotting for NP, NS1 and tubulin showed comparable viral protein synthesis in the presence of LMB (Fig. 4B) and cell fraction analysis confirmed that

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**Figure 3.** NS1 WT but not dsRNA-binding deficient NS1 protein co-sediments with PKR and vRNP upon density gradient centrifugation. (A) A549 cells were infected with WT virus or mutant virus #4 at an MOI of 1. Cells were lysed 12 hours p.i. and subjected to centrifugation through a continuous 5 to 50% sucrose gradient. 16 fractions were taken from top to bottom. Fractions 1 to 9 were analyzed by immunoblotting with antibodies specific for PKR and the viral NP and NS1 proteins. Also, RNA was extracted from gradient fractions 1 to 9 and was subjected to dot blot hybridization with probes specific for HA vRNA and NS vRNA, respectively (panels “HA and NS vRNA”). Whole cell lysates were analyzed by immunoblotting with antibodies specific for phospho-PKR, total PKR, viral NP, viral NS1 and tubulin as indicated (right panel, “lysate”). (B) A549 cells were mock treated or infected with WT virus or virus mutant #4 as described in panel A. Lysates were prepared and subjected to immunoprecipitation with anti-PKR (α) or control antibody (ctrl). The precipitated complexes were analyzed by immunoblotting for PKR and NS1 proteins. RNA was isolated from an identical set of PKR immunoprecipitates of cells infected with the mutant virus and subjected to dot blot analysis with an RNA-probe specific for HA vRNA.

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Figure 4. The nuclear egress of vRNPs late in infection leads to PKR activation.

(A) A549 cells grown on glass cover slips were infected with WT or mutant virus #4 at an MOI of 1. Cells were mock treated or complemented with LMB starting at 3 hours p.i. At 8, 12 and 16 hours p.i., cells were fixed and stained for NP (shown in red color), and also for the NS1 protein at the 16 h time-point (shown in green color). Microscopic sample analysis was conducted by confocal laser scanning microscopy. Scale bar, 10 μm. (B) A549 cells grown in culture dishes were infected with WT virus or mutant virus #4 at an MOI of 1. Cells were mock treated or complemented with LMB starting 3 hours p.i. and lysed 8, 12 and 16 hours p.i. Whole cell lysates were analyzed by immunoblotting with antibodies specific for phospho-PKR, total PKR, NP, NS1 and tubulin as indicated.

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LMB strongly reduced cytosolic NP levels under these condition (Fig. S3). These findings suggest that activation of PKR is associated with the cytosolic appearance of vRNPs late in infection.

Purified viral RNPs trigger PKR kinase activity in vitro
To test the hypothesis that influenza virus RNP activates PKR directly, we purified vRNPs from influenza B/Lee virions (Fig. S2) and tested them for stimulation of PKR autophosphorylation. V5 epitope-tagged PKR was expressed in 293T cells and precipitated from cell lysate with tag-specific antibody. Activation of the precipitated kinase in the presence of vRNP was assessed by incubation with [γ-32P]-ATP followed by analysis on SDS gels and autoradiography. Fig. 5A shows an exposure of autophosphorylated PKR (lanes “32P-PKR”) and the same region of the gel after staining with Coomassie Blue (Lanes “PKR-V5”, “NP” and “IgG”). Interestingly, the purified vRNP stimulated PKR autophosphorylation in a dose-dependent manner similar to the synthetic dsRNA polyriboinosinic: polyribocytidylic acid (pI:C) (Fig. 5A). Pre-treatment of vRNP with either dsRNA-specific RNase I or ssRNA-specific RNase F prior to the kinase reaction abolished PKR activation, which argues against the presence of a protein activator of PKR in the reaction (Fig. 5A). Significantly, influenza virus RNP hardly activated the dsRNA binding-deficient PKR-K60A mutant, further confirming the dsRNA-dependency of vRNP-mediated kinase stimulation (Fig. 5B). Control reactions showed that the PKR-K60A mutant enzyme was activated by heparin, a common trigger for PKR autophosphorylation [3], excluding the possibility that the mutation affected general kinase activity. We note that influenza virions package only RNPs associated with the cytosolic appearance of vRNPs late in infection. These findings suggest that activation of PKR is associated with the cytosolic appearance of vRNPs late in infection. For technical reasons, we focused our experiments on influenza B rather than A virus. Although both influenza A and B viruses can trigger PKR activation, influenza B virus NS1 protein in targeting the antiviral 2′-5′-OAS/RNase L system [32]. Our study revealed a further distinction between type A and B influenza viruses since the B/deINS1 virus behaved benignly in both PKR+/− and PKR−/− mice, whereas a comparable A/deINS1 virus regained virulence in PKR null mice [15]. This finding points to the existence of an additional important function of the B/NS1 protein during viral replication, which is possibly related to its recently reported activity in modifying the nuclear speckle compartment of the host cell [38].

The interactions of PKR with its RNA effectors and viral inhibitors are complex and incompletely understood. Our analysis of defined loss-of-inhibition mutant viruses offers new insights into the conundrum of how dsRNA-dependent PKR is activated during infection by influenza virus that does not generate long duplex RNA [10]. Based on several lines of evidence, we put forward the hypothesis that influenza virus RNP functions as a non-canonical activator of PKR in the cytosol: First, PKR autophosphorylation in infected cells occurred concomitantly with the cytosolic appearance of vRNP, when a functional NS1 protein was absent. Second, PKR activation was largely abolished when the vRNA CYtoplasmic export of vRNP was blocked by LMB treatment. Third, biochemical analysis demonstrated that purified vRNP activates PKR in an in vitro kinase assay directly. The RNA-dependency of this stimulation was shown by its sensitivity to pretreatment with single- and double-strand-specific RNases and the failure of the vRNP to activate a dsRNA binding-defective PKR. We favour the explanation that the vRNA promoter structure formed by the partially complementary 5′- and 3′-ends within vRNP is an important determinant for PKR activation. Synthetic A-form RNA consisting of a short stem region flanked by single-stranded nucleotides was previously shown to efficiently stimulate PKR in vitro [44]. There is now ample evidence from structural, biochemical and functional studies that the 14–16 nucleotides of the vRNA termini engage in base-pairing interactions and exist in form of a panhandle and/or a related corkscrew structure [45–47] (summarized in [11]). Intriguingly, the 5′-ends of influenza virus RNAs carry a tripophosphate structure [11] and this chemical group was recently reported to support activation of PKR by stem-loop RNA [43]. In fact, the present study is the first characterization of a natural viral RNA/RNP with a 5′-tripophosphate group that triggers PKR, as the studies mentioned above tested exclusively synthetic model RNAs. Our mechanistic model suggests PKR can contact RNA residues within the vRNP despite their association with the viral NP and polymerase proteins.

A second novel aspect of our study centres on the mode by which the influenza B virus NS1 protein inhibits PKR. Previously, we showed that dsRNA binding mutations within NS1 prevented PKR inhibition, but did not eliminate the suppression of IFN induction that is triggered through the RNA helicase RIG-I [9,16,48]. Our new study explains the former observation by showing that the NS1 protein entangles PKR into an immuno-precipitable complex in infected cells. These PKR-NS1 complexes were sensitive to treatment with dsRNA-specific RNase and the interaction required a functional NS1 dsRNA binding domain. The most likely interpretation of these findings is that RNA to which both proteins bind facilitates the interaction of PKR and NS1. Interestingly, the association with PKR appears to be necessary but not sufficient to preclude its activation. In fact, all NS1 mutants that were not associated with PKR failed to block kinase activation (this study; [9,16,48]), but the NS1 mutant #1 did not prevent PKR activation even though it interacted with the range of wild-type virus in the lungs of PKR−/− mice and fibroblasts. Interestingly, this is slightly different to a reported main function of the dsRNA binding domain of the influenza A virus NS1 protein in targeting the antiviral 2′-5′-OAS/RNase L system [32]. Our study revealed a further distinction between type A and B influenza viruses since the B/deINS1 virus behaved benignly in both PKR+/− and PKR−/− mice, whereas a comparable A/deINS1 virus regained virulence in PKR null mice [15]. This finding points to the existence of an additional important function of the B/NS1 protein during viral replication, which is possibly related to its recently reported activity in modifying the nuclear speckle compartment of the host cell [38].

Discussion
The present study showed that PKR is a major factor restricting the growth of influenza B virus in vitro and in vivo and revealed mechanisms for its activation and subversion. We previously reported that mutational inactivation of the dsRNA binding domain of the B/NS1 protein attenuated viral replication and ablated the control of PKR and eIF2α phosphorylation, but only slightly affected IFN suppression [16]. Our new analysis firmly establishes that the key activity of the B/NS1 dsRNA binding domain is to silence PKR and excludes the possibility that another antiviral factor(s) is chiefly responsible for the observed growth defects of the mutants: The absence of PKR restored a virulent phenotype of the mutant viruses expressing dsRNA binding-deficient NS1 protein in mice and viral titers were elevated to the
Figure 5. Purified viral RNP activates PKR autophosphorylation in vitro. (A) V5-tagged PKR was expressed in 293T cells and precipitated from lysate by anti-V5 antibody. Kinase activation was assayed on precipitated PKR in the presence of 10 μCi [γ-32P]ATP for 30 min at 30°C. PKR was activated as indicated by 2.5, 5 or 7.5 μl vRNP, or 2.5 ng/μl pI:C. In further reactions, 5 μl vRNP were pre-treated with 5 U RNase III or 5 U RNase I, respectively. Kinase reactions were analyzed by SDS gel electrophoresis and autoradiography (upper panel). Autophosphorylation of PKR was evaluated using the AIDA software and is given as fold activation compared to mock treated PKR. The values represent the average of three independent experiments. Error bars indicate the standard deviation. After exposure the SDS gel was stained with Coomassie blue to visualize precipitated V5-PKR, NP and the IgG heavy chain (lower panel). (B) V5-tagged PKR and V5-tagged mutant PKR-K60A that does not bind dsRNA were expressed in 293T cells, precipitated and stimulated in the autophosphorylation assay by vRNP or treatment with heparin as indicated. Quantification was done as described in panel A. (C) V5-tagged PKR was expressed in 293T cells, precipitated and subjected to the kinase activation assay as described above. PKR was incubated as indicated without or with 0.3, 1.0 or 3.0 μM of a transcribed model vRNA of 53 nucleotides, which was mock-treated or dephosphorylated by phosphatase, respectively, as indicated. doi:10.1371/journal.ppat.1000473.g005
Propagation of virus infection. Viral RNP is located in the nucleus early in infection and is not accessible for cytosolic PKR (panel A). Upon nuclear export a tRNA/RNP complexes with structured 5' and 3' ends together with the phosphorylation status of PKR (Fig. 6A) of influenza virus. Viral RNP complexes are recognized by PKR and provide the major stimulus for its activation. We detected both vRNA and the NS1 protein in PKR immunoprecipitates from infected cell lysate raising the possibility that NS1 inhibits activation of PKR by cytosolic vRNP through the formation of a hetero-trimeric complex. However, we cannot rule out the option that binding of NS1 to PKR in infected cells is mediated by a yet undetermined viral or host-derived nucleic acid; for that reason more work will be required to elucidate the specific components involved.

The influenza B virus non-structural NS1 protein has apparently evolved in a way that suits the specific needs of the pathogen to inhibit PKR (Fig. 6). During the first hours of infection viral RNPs are confined to the nucleus and not accessible for the cytosolic sensor (Fig. 6A). At later stages, the vRNPs are exported to the cytoplasm where they assemble into progeny virions at the plasma membrane. Cytoplasmic vRNPs act as PKR activators in the absence of functional NS1 protein, thus reducing protein synthesis and virus growth (Fig. 6B). However, in the wild-type situation NS1 protein is expressed and prevents activation of PKR by vRNPs, thereby facilitating full-level synthesis of structural viral proteins and efficient viral propagation (Fig. 6C). This scenario is supported by the dynamic intracellular trafficking of the B/NS1 protein showing that it migrates to nuclear speckles early in infection, but relocates to the cytoplasm at later time-points [38]. The model may also explain phenotypes of mutant influenza viruses with lesions in the NS1 protein that activate PKR and show a selective reduction in the synthesis of late viral gene products [16,17,51].

Finally, we suggest that activation of PKR by other negative strand RNA viruses that generate little or no detectable long duplex RNA may occur in a similar way. The genomes of many of these viruses enter the cytoplasm as part of an RNP structure and carry a 5'-triphosphate group. In fact, the conserved ends of the genomic RNAs of several members of the Bunyaviridae family also form a structured panhandle [52] and the level of phosphorylated eIF2α increased during infection with the prototypic Bunyamwera virus [53]. Further observations suggest that also non-segmented negative-strand RNA viruses may induce PKR by a ribonucleoprotein structure; for instance, the Ebola virus VP35 protein counteracts stimulation of PKR, indicating indirectly that members of the Filoviridae family activate and prevent induction of the kinase [54]. In addition, replication and virulence of vesicular stomatitis virus, a member of the Rhabdoviridae, is strongly enhanced in PKR-deficient mice [55]. Exploring the methods used by viral pathogens to counteract PKR not only promises an improved understanding of the innate immune defense against viral infections, but may also foster the development of novel therapeutic substances. Our study suggests that it should be possible to shift the course of influenza virus infection from efficient pathogen replication towards its elimination via the body’s innate defense if the NS1-mediated blockade of PKR could be suspended. The recent description of substances capable to modulate NS1’s activity in IFN suppression supports the concept that this viral protein is indeed amenable to control by therapeutic substances [56].

Materials and Methods

Cells and viruses

293T and A549 cells were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM l-glutamine, and antibiotics. Madin-Darby canine kidney (MDCK) type II cells were grown in minimal essential medium (MEM) with the same additives. MEFs were prepared from C57BL/6 mice (PKR−/−) or mice devoid of functional PKR (PKR−/−) [39] and cultured in DMEM supplemented with 10% FCS, 2 mM l-glutamine and 50 μM β-mercaptoethanol. All cells were maintained at 37°C and 5% CO2. The recombinant influenza B/Lee WT and mutant viruses ΔNS1, NS1 33/38 (#1), NS1 47/50 (#2), NS1 52/53/54 (#5), NS1 58/60/64 (#4), NS1 77/78 (#6), and NS1 83/86 (#7) have been described elsewhere [16,20]. To analyze viral replication, monolayer cultures of PKR+/+ or PKR−/− MEFs were infected at a multiplicity of infection (MOI) of 0.1 and incubated at 33°C in DMEM containing 0.2% bovine albumin and 0.5 μg/ml trypsin. Virus titers were determined by indirect immunofluorescence staining and were expressed as fluorescence-forming units (ffu)/ml as described before [20].

Figure 6. Model for the induction and inhibition of antiviral PKR by influenza virus. The three panels show the localization of the genomic vRNA/RNP complexes with structured 5' and 3' ends together with the phosphorylation status of PKR in early (panel A) and late phases (panels B and C) of virus infection. Viral RNP is located in the nucleus early in infection and is not accessible for cytosolic PKR (panel A). Upon nuclear export at later stages, vRNP complexes stimulate PKR activation in the absence of functional NS1 protein, leading to an inhibition of viral proteins synthesis and replication (panel B). However, the presence of functional viral NS1 protein suppresses this antiviral reaction thereby supporting high-level viral propagation (panel C).

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Infections of mice
Eight-week-old female wild type C57BL6 PKR+/− and PKR−/− mice with the mixed 129/Sv(ev)×C57BL/6j background [39] were anesthetized and infected intranasally with 50 μl of phosphate-buffered saline (PBS) containing 1×10^6 ffu of the indicated recombinant influenza B/Lee viruses. For viral lung titrations, three mice were sacrificed at day 3 and at day 6 post-infection. Mice lungs were homogenized in 1 ml PBS, and titrated by immunofluorescence using a monoclonal antibody against influenza B virus nucleoprotein (Abcam; ab20711-100). Comparison of viral lung titers in WT and mutant virus-infected mice at each time-point was done using the Student’s t test for pairwise comparisons. For monitoring of viral disease, 8 animals were weighed daily for two weeks and euthanized when observed in extremis. Mice were bred and maintained at the Mount Sinai School of Medicine in accordance with Federal and university guidelines.

Plasmids
pcDNA3.1-PKR-V5/His was constructed by integration of the human PKR cDNA into the vector pcDNA3.1-V5/His (Invitrogen). The derived pcDNA3.1-PKR-K60A-V5/His plasmid was constructed with the QuikChange mutagenesis kit (Stratagene). The integrity of the constructs was confirmed by DNA cycle sequencing.

Immunoprecipitation and immunoblot analysis
2.5×10^6 A549 cells were mock-treated or infected with WT, ΔNS1 or NS1 mutant virus at an MOI of 1. Cell extracts were prepared 8 hours p.i. in IP lysis buffer (1% Igepal-CA 630, 150 mM NaCl, 20 mM Tris HCl, pH 7.5, 1 mM EDTA, 10 mM Na-b-glycerophosphate, 2 mM Na3VO4, 1 mM Pefabloc). When indicated, lysates were pre-incubated with RNase III (Ambion) for 10 min at 33°C before incubation with B/NS1-specific rabbit antiserum [16]. Immune complexes were collected on protein-G-agarose beads (Roche), washed and the precipitated proteins were dissolved in SDS sample buffer. For PKR immunoprecipitation, cells were lysed in kinase binding buffer (20 mM Hepes pH 7.5, 300 mM NaCl, 5 mM Mg(OAc)2, 10% glycerol, 25 mM Na-b-glycerophosphate, 2 mM Na3VO4, 1 mM Pefabloc) containing 0.5% Igepal-CA 630. PKR was precipitated with rabbit anti-PKR antibody (Epitomics) and immunocomplexes were collected as described above. The precipitated proteins were analyzed as indicated by SDS gel electrophoresis and immunoblotting using the primary B/NS1-specific rabbit antiserum, mouse anti-PKR antibody 71/10 (Ribogene) or rabbit anti-PKR antibody (Epitomics), mouse anti-NP antibody (AbD Serotec), rabbit antiphospho-PKR (Thr446) antibody (Cell Signaling), mouse anti-tubulin antibody (Sigma) and suitable secondary horseradish peroxidase (HRP)-conjugated IgG together with an enhanced chemiluminescence protocol (Pierce). To detect PKR-associated viral nucleic acids, the PKR immunoprecipitate was subjected to proteinase K digestion and phenol extraction. Purified RNA was subjected to dot blot analysis with probes specific for HA vRNA as described below.

Density gradient centrifugation
2.5×10^6 A549 cells were mock-treated or infected with B/Lee WT or NS1 58/60/64 (#4) mutant virus at an MOI of 1 at 33°C. Cells were lysed 12 hours p.i. in Lyp-P100 buffer (25 mM Hepes pH 7.5, 100 mM NaCl, 2.5 mM MgCl2, 0.1% Igepal-CA 630, 10 mM β-glycerophosphate, 2 mM Na3VO4, 1 mM Pefabloc, 5 μl/ml RNasin). Lysates were loaded onto a continuous sucrose gradient (5 to 50% in Lyp-P100 buffer) and centrifuged in a SW60Ti rotor (Beckman) for 150 minutes at 30,000 rpm and 4°C. Sixteen 250 μl fractions were taken from top to bottom. Fractions 1 to 9 were halved and analyzed by immunoblotting or subjected to RNA extraction (RNeasy, Qiagen), respectively. Denatured extracted RNA in 7.5% formamide and 10×SSC were dotted onto Nylon transfer membrane and NS and HA vRNAs were detected with segment-specific DIG-labeled probes. DIG-labelled probes were generated from linearized template DNA by T7 RNA polymerase-mediated transcription using the DIG Northern Starter Kit (Roche).

Leptomycin B (LMB) treatment of infected cells
1×10^6 A549 cells were either mock-treated of infected with B/Lee WT or NS1 58/60/64 (#4) mutant virus at an MOI of 1. 7.5 ng/ml LMB (Sigma) or an equal volume of 70% methanol as a solvent control was added to the supernatant at 3 hours p.i.. At 6, 12 and 16 hours p.i. cells were lysed in kinase binding buffer containing 0.5% Igepal-CA 630 and were analyzed by immunoblotting, as described above. The fractionation of cell lysates is described in the supporting materials and methods section (Text S1). In parallel, A549 cells grown on glass coverslips were infected and treated with LMB as described above. The cells were fixed with 2.5% paraformaldehyde and permeabilized with 0.2% Triton X-100 at 8, 12 or 16 hours. Viral NP was stained with primary mouse anti-NP antibody (AbD Serotec) and secondary goat anti-mouse Alexa 594 antibody (Molecular Probes). Cells were not stained for PKR as the available antibody appeared to be not suitable. Cells were analyzed with a LSM510 Meta confocal laser scanning microscope (Zeiss, Jena, Germany) equipped with a 63x/1.2 water objective lens. Data were analyzed and processed by the LSM Image Browser 3.5 and Adobe Photoshop 4.0 software packages.

PKR autophosphorylation assay
5×10^6 293T cells were transfected with pcDNA3.1-PKR-V5/His or pcDNA3.1-PKR-K60A-V5/His, respectively. Cells were lysed in kinase binding buffer containing 0.5% Igepal-CA 630, the cleared lysate was incubated with mouse anti-V5 antibody (AbD Serotec) and immunocomplexes were collected on protein-G-agarose beads (Roche), washed and the precipitated proteins were dissolved in SDS sample buffer. For PKR immunoprecipitation, cells were lysed in kinase binding buffer (20 mM Hepes pH 7.5, 300 mM NaCl, 5 mM Mg(OAc)2, 10% glycerol, 25 mM Na-b-glycerophosphate, 2 mM Na3VO4, 1 mM Pefabloc) containing 0.5% Igepal-CA 630. PKR was precipitated with rabbit anti-PKR antibody (Epitomics) and immunocomplexes were collected as described above. The precipitated proteins were analyzed as indicated by SDS gel electrophoresis and immunoblotting using the primary B/NS1-specific rabbit antiserum, mouse anti-PKR antibody 71/10 (Ribogene) or rabbit anti-PKR antibody (Epitomics), mouse anti-NP antibody (AbD Serotec), rabbit antiphospho-PKR (Thr446) antibody (Cell Signaling), mouse anti-tubulin antibody (Sigma) and suitable secondary horseradish peroxidase (HRP)-conjugated IgG together with an enhanced chemiluminescence protocol (Pierce). To detect PKR-associated viral nucleic acids, the PKR immunoprecipitate was subjected to proteinase K digestion and phenol extraction. Purified RNA was subjected to dot blot analysis with probes specific for HA vRNA as described below.

Activation of PKR by Influenza Virus
The phosphatase was inactivated at 65°C for 15 min. vRNAs were purified by phenol/chloroform extraction and ethanol precipitation and concentrations were determined by UV spectroscopy. Precipitated PKR was incubated with mock-treated or phosphatase-treated vRNA for 30 min at 30°C. Autophosphorylation of PKR and densitometric analyses were done as described above.

**Supporting Information**

**Figure S1** Pathogenicity of influenza B viruses expressing dsRNA-binding defective NS1 proteins is enhanced in PKR null mice. (A) Groups of eight-week-old female PKR−/− and wild type C57B6 mice were anesthetized and infected intranasally with 2×10^1 pfu of the indicated recombinant influenza B/Lee virus. For monitoring of viral disease, 8 animals were weighed daily for two weeks, and mean percentage weight loss of each group was compared with the weight immediately prior to infection. Mice were euthanized when observed in extremis. (B) Survival rate of PKR−/− mice after infection with recombinant influenza B viruses. Dead animals were scored daily and represented as the percentage of surviving animals. Found at: doi:10.1371/journal.ppat.1000473.s001 (0.18 MB TIF)

**Figure S2** Preparation of vRNPs from recombinant influenza B/Lee virus. Virus was grown in embryonated chicken eggs and purified by phenol/chloroform extraction and ethanol precipitation. Viral NP and the marker antigens for the nuclear and cytoplasmic fractions were analyzed by immunoblotting for the vRNA of the NS segment (lower panel). RNA was extracted from the fractions and subjected to reverse transcription (RT)-PCR with primers specific for the vRNA of the NS segment (lower panel). Found at: doi:10.1371/journal.ppat.1000473.s002 (0.58 MB TIF)

**Figure S3** Treatment of infected cells with LMB inhibits the nuclear export of NP. A549 cells were infected with influenza B/Lee WT virus at an MOI of 1. Cells were mock treated or complemented with LMB starting at 3 hrs p.i. Cells were lysed at 15 hrs p.i. and cytoplasmic (C.) and nuclear fractions (N.) were generated. The fractions were analyzed by immunoblotting for viral NP and the marker antigens for the nuclear and cytoplasmic fractions, PARP and tubulin, respectively. Found at: doi:10.1371/journal.ppat.1000473.s003 (0.11 MB TIF)

**Text S1** This file contains supporting materials and methods. Found at: doi:10.1371/journal.ppat.1000473.s004 (0.03 MB DOC)

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**Author Contributions**

Conceived and designed the experiments: BD AGS TW. Performed the experiments: BD LMS JS RH. Analyzed the data: BD LMS JS RH AGS TW. Contributed reagents/materials/analysis tools: ZW UK. Wrote the paper: BD TW.

**References**

1. Garcia-Sastre A, Biron CA (2006) Type 1 interferons and the virus-host relationship: a lesson in detente. Science 312: 879–882.
2. Sadler AJ, Williams BR (2007) Structure and function of the protein kinase R. Curr Top Microbiol Immunol 316: 253–292.
3. Garcia MA, Gil J, Venuto I, Guerra S, Domingo E, et al. (2006) Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action. Microbiol Mol Biol Rev 70: 1032–1060.
4. Meurs E, Chong K, Galabru J, Thomas NS, Kerr JM, et al. (1999) Molar cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. Cell 62: 379–390.
5. Kuhlen KL, Samuel CE (1999) Mechanism of interferon action: functional characterization of positive and negative regulatory domains that modulate transcriptional activation of the human RNA-dependent protein kinase Pkr promoter. Virology 254: 162–195.
6. Gale MJ, Katze MG (1998) Molecular mechanisms of interferon resistance mediated by viral-directed inhibition of PKR, the interferon-induced protein kinase. Pharmacol Ther 79: 29–46.
7. Manche L, Green SR, Schmedt C, Mathews MB (1992) Interactions between the double-stranded RNA-binding motifs of the protein kinase PKR. Virology 219: 339–349.
8. Jacobs BL, Langland JO (1996) When two strands are better than one: the mediators and modulators of the cellular response to double-stranded RNA. Virology 219: 339–349.
9. Pichlmair A, Schulz O, Tan CP, Naslund TL, Liljestrom P, et al. (2006) RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. Science 314: 997–1001.
10. Weber F, Wagner V, Rasmussen SB, Hartmann R, Pahalan SR (2006) Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. J Virol 80: 5059–5064.
11. Palese P, Shaw M (2007) Orthomyxoviridae: The viruses and their replication. In: Knipe DM, Howley PM, eds. Fields - Virology. 5th ed. Philadelphia: Lippincott Williams & Wilkins. pp 1647–1690.
12. Majde JA (2000) Viral double-stranded RNA, cytokines, and the flu. J Interferon Cytokine Res 20: 259–272.
13. Tian B, Mathews MB (2001) Functional characterization of and cooperation between the double-stranded RNA-binding motifs of the protein kinase PKR. J Biol Chem 276: 9936–9944.
14. Li S, Min JY, Krug RM, Sen GC (2006) Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or dsDNA. J Virol 80: 15–21.
15. Bergmann M, Garcia-Sastre A, Camero E, Pehamberger H, Wolf K, et al. (2000) Influenza virus NS1 protein counteracts PKR-mediated inhibition of replication. J Virol 74: 6203–6206.
30. Kochs G, Garcia-Sastre A, Martinez-Sobrido L (2007) Multi-antigen interferon actions of the influenza A virus NS1 protein. J Virol 81: 7011–7021.

31. Lu Y, Qian XY, Krug RM (1994) The influenza virus NS1 protein: a novel inhibitor of pre-mRNA splicing. Genes Dev 8: 1817–1828.

32. Min JY, Krug RM (2006) The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2′–5′ oligo (A) synthetase/RNAse L pathway. Proc Natl Acad Sci U S A 103: 7100–7105.

33. Park YW, Katze MG (1995) Translational control by influenza virus. Identification of cis-acting sequences and trans-acting factors which may regulate selective viral mRNA translation. J Biol Chem 270: 28433–28439.

34. Qiu Y, Krug RM (1994) The influenza virus NS1 protein is a poly(A)-binding protein that inhibits nuclear export of mRNAs containing poly(A). J Virol 68: 2425–2432.

35. Satterly N, Tsai PL, van Deursen J, Nussenzveig DR, Wang Y, et al. (2007) Influenza virus targets the mRNA export machinery and the nuclear pore complex. Proc Natl Acad Sci U S A 104: 1053–1058.

36. Lenschow DJ, Lai C, Frias-Staheli N, Giannakopoulos NV, Lutz A, et al. (2007) IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses. Proc Natl Acad Sci U S A 104: 1371–1376.

37. Yuan W, Krug RM (2001) Influenza B virus NS1 protein inhibits conjugation of the interferon (IFN)–induced ubiquitin–like ISG15 protein. Embo J 20: 362–371.

38. Schnieder J, Dauber B, Melen K, Jullkunen I, Wolff T (2008) Analysis of influenza B–virus NS1 protein trafficking reveals a novel interaction with nuclear speckle domains. J Virol 83: 701–711.

39. Yang YL, Reis LF, Pavlovic J, Aguzzi A, Schafer R, et al. (1995) Deficient RNA polymerase synthesis due to a mutation in the polymerase subunit PA. J Virol 69: 2941–2945.

40. Zheng X, Bevilacqua PC (2004) Activation of the protein kinase PKR by short double-stranded RNAs with single-stranded tails. Rna 10: 1934–1945.

41. Hsu MT, Parvin JD, Gupta S, Krystal M, Palese P (1987) Genomic RNAs of influenza viruses are held in a circular conformation in virions and in infected cells by a terminal panhandle. Proc Natl Acad Sci U S A 84: 8140–8144.

42. Hornung V, Ellegast J, Kim S, Brezouza K, Jiang A, et al. (2006) 5′-Triphosphate RNA is the ligand for RIG-I. Science 314: 994–997.

43. Min JY, Li S, Sen CC, Krug RM (2007) A site on the influenza A virus NS1 protein mediates both inhibition of PKR activation and temporal regulation of viral RNA synthesis. Virology 363: 236–243.

44. Zheng X, Bevlacqua PC (2004) Activation of the protein kinase PKR by short double-stranded RNAs with single-stranded tails. Rna 10: 1934–1945.

45. Min JY, Li S, Sen CC, Krug RM (2007) A site on the influenza A virus NS1 protein mediates both inhibition of PKR activation and temporal regulation of viral RNA synthesis. Virology 363: 236–243.

46. Feng Z, Cerveny M, Yan Z, He B (2007) The VP35 protein of Ebola virus is an interferon antagonist NSs. J Virol 77: 5507–5511.

47. Zheng X, Bevlacqua PC (2004) Activation of the protein kinase PKR by short double-stranded RNAs with single-stranded tails. Rna 10: 1934–1945.

48. Klumpp K, Kaigroek RW, Baudin F (1997) Roles of the influenza virus polymerase and nucleoprotein in forming a functional RNP structure. Embo J 16: 1248–1257.

49. Bourgoin V, Trotlage S, Brezouza K, Jung A, et al. (2006) 5′-Triphosphate RNA is the ligand for RIG-I. Science 314: 994–997.

50. Min JY, Li S, Sen CC, Krug RM (2007) A site on the influenza A virus NS1 protein mediates both inhibition of PKR activation and temporal regulation of viral RNA synthesis. Virology 363: 236–243.

51. Schmaljohn C, Nichol S (2007) Bunyaviridae: The viruses and their replication. In: Knipe DM, Howley PM, eds. Fields-Virology. Philadelphia: Lippincott Williams & Wilkins.

52. Streiterfeld H, Boyd A, Fazakerley JK, Bridgen A, Elliott RM, et al. (2003) Activation of PKR by Bunyamwera virus is independent of the viral interferon antagonist NSs. J Virol 77: 5507–5511.

53. Seong YH, Bae SH, Cheong HK, Lee JH, Cheong C, Kainoshi M, et al. (2001) Structural features of an influenza virus promoter and their implications for viral RNA synthesis. J Virol 81: 10662–10667.

54. Stojdl DF, Abraham N, Knowles S, Marius R, Brasey A, et al. (2000) The interferon-induced ubiquitin-like ISG15 protein. Embo J 19: 362–371.

55. Streiterfeld H, Boyd A, Fazakerley JK, Bridgen A, Elliott RM, et al. (2003) Activation of PKR by Bunyamwera virus is independent of the viral interferon antagonist NSs. J Virol 77: 5507–5511.

56. Schmaljohn C, Nichol S (2007) Bunyaviridae: The viruses and their replication. In: Knipe DM, Howley PM, eds. Fields-Virology. Philadelphia: Lippincott Williams & Wilkins.

57. Schmaljohn C, Nichol S (2007) Bunyaviridae: The viruses and their replication. In: Knipe DM, Howley PM, eds. Fields-Virology. Philadelphia: Lippincott Williams & Wilkins.

58. Takahashi K, Katze MG (1996) Biochemical and genetic evidence for complex formation between the influenza A virus NS1 protein and the interferon-induced PKR protein kinase. J Interferon Cytokine Res 16: 757–766.

59. Schmaljohn C, Nichol S (2007) Bunyaviridae: The viruses and their replication. In: Knipe DM, Howley PM, eds. Fields-Virology. Philadelphia: Lippincott Williams & Wilkins.

60. Streiterfeld H, Boyd A, Fazakerley JK, Bridgen A, Elliott RM, et al. (2003) Activation of PKR by Bunyamwera virus is independent of the viral interferon antagonist NSs. J Virol 77: 5507–5511.

61. Feng Z, Cereveny M, Yan Z, He B (2007) The VP35 protein of Ebola virus is an interferon antagonist NSs. J Virol 81: 182–192.

62. Streiterfeld H, Boyd A, Fazakerley JK, Bridgen A, Elliott RM, et al. (2003) Activation of PKR by Bunyamwera virus is independent of the viral interferon antagonist NSs. J Virol 77: 5507–5511.

63. Schmaljohn C, Nichol S (2007) Bunyaviridae: The viruses and their replication. In: Knipe DM, Howley PM, eds. Fields-Virology. Philadelphia: Lippincott Williams & Wilkins.

64. Stojdl DF, Abraham N, Knowles S, Marius R, Brasey A, et al. (2000) The murine double-stranded RNA-dependent protein kinase PKR is required for resistance to vesicular stomatitis virus. J Virol 74: 9580–9585.

65. Schmaljohn C, Nichol S (2007) Bunyaviridae: The viruses and their replication. In: Knipe DM, Howley PM, eds. Fields-Virology. Philadelphia: Lippincott Williams & Wilkins.

66. Tao SJ, Vogel U, Scholtissek C (1995) Amino acid replacements leading to temperature-sensitive defects of the NS1 protein of influenza A virus. Arch Virol 140: 945–950.

67. Schmaljohn C, Nichol S (2007) Bunyaviridae: The viruses and their replication. In: Knipe DM, Howley PM, eds. Fields-Virology. Philadelphia: Lippincott Williams & Wilkins.

68. Schmaljohn C, Nichol S (2007) Bunyaviridae: The viruses and their replication. In: Knipe DM, Howley PM, eds. Fields-Virology. Philadelphia: Lippincott Williams & Wilkins.

69. Schmaljohn C, Nichol S (2007) Bunyaviridae: The viruses and their replication. In: Knipe DM, Howley PM, eds. Fields-Virology. Philadelphia: Lippincott Williams & Wilkins.

70. Schmaljohn C, Nichol S (2007) Bunyaviridae: The viruses and their replication. In: Knipe DM, Howley PM, eds. Fields-Virology. Philadelphia: Lippincott Williams & Wilkins.