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Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA

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Abstract

A major component of the cellular antiviral system is the latent protein kinase PKR, which is activated by binding to either double-stranded RNA (dsRNA) or the cellular PACT protein. Activated PKR phosphorylates the translation initiation factor eIF2, thereby inhibiting viral and cellular protein synthesis and virus replication. To evade the antiviral effects of PKR, many viruses, including influenza A virus, have evolved multiple mechanisms. For influenza A virus, the non-structural (NS1A) protein plays a major role in blocking activation of PKR during virus infection. The mechanism by which the NS1A protein inhibits PKR activation in infected cells has not been established. In the present study, we first carried out a series of in vitro experiments to determine whether the NS1A protein could utilize a common mechanism to inhibit PKR activation by both PACT and dsRNA, despite their different modes of activation. We demonstrated that the direct binding of the NS1A protein to the N-terminal 230 amino acid region of PKR can serve as such a common mechanism and that this binding does not require the RNA-binding activity of the NS1A protein. The lack of requirement for NS1A RNA-binding activity for the inhibition of PKR activation in vivo was established by two approaches. First, we showed that an NS1A protein lacking RNA-binding activity, like the wild-type (wt) protein, blocked PKR activation by PACT in vivo, as well as the downstream effects of PKR activation in cells, namely, eIF2 phosphorylation and apoptosis. In addition, we demonstrated that PKR activation is inhibited in cells infected with a recombinant influenza A virus expressing NS1A mutant protein that cannot bind RNA, as is the case in cells infected with wild-type influenza A virus.

Keywords: PKR; Influenza virus; Double-stranded RNA; PACT; Viral evasion; Interferon action

Introduction

The Ser/Thr protein kinase, PKR, is constitutively expressed in mammalian cells and is further increased by interferon treatment (Hovanessian, 1989; Meurs et al., 1990). PKR needs to be activated via a conformational change that is brought about by binding to either of its activators, double-stranded RNA (dsRNA) or the PACT protein (Galabru and Hovanessian, 1987; Ito et al., 1999; Patel and Sen, 1998; Williams, 1999). Activation results in PKR autophosphorylation, and the activated PKR then phosphorylates specific target proteins (see below). Recent evidence indicates that the modes of activation by dsRNA and PACT differ. The current model for dsRNA activation is that an intramolecular interaction between the N-terminal and C-terminal domains of PKR keeps the protein in the inactive conformation and that the binding of dsRNA to the two dsRNA-binding motifs (dsRBM1 and dsRBM2) in the N-terminal domain of PKR releases the C-terminal kinase domain, thus activating the enzyme (Nanduri et al., 2000). In contrast, in PACT activation, one of its domains (domain 3) binds to the C-terminal domain of PKR, releases it from its interaction with the N-terminal domain and thereby activates its kinase domain (S. Li, G. Peters, K. Ding, X. Zhang, J. Qin and G. C. Sen, unpublished results). Although PACT is a dsRNA-binding protein, its dsRNA-binding activity, which is possessed by each of two domains (domains 1 and 2), is not required for PKR activation. Rather, domain 3 of PACT, which

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does not bind dsRNA, is necessary and sufficient for activating PKR (Peters et al., 2001).

Activated PKR blocks cellular and viral protein synthesis by phosphorylating the α subunit of the eIF2 translation initiation factor (eIF2α), thereby inhibiting viral replication in infected cells (Gale and Katze, 1998; Samuel, 1993). Thus, PKR plays a central role in host defense against viral infection. To evade the antiviral effects of PKR, viruses have evolved multiple strategies to block PKR activation or action (Gale and Katze, 1998). It has been reported that influenza A virus uses two such strategies: its non-structural (NS1A) protein blocks PKR activation; and viral infection activates a cellular inhibitor of PKR, $p58^{IPK}$ (Lu et al., 1995; Melville et al., 1997; Tan et al., 1998). The mechanism by which the NS1A protein inhibits PKR activation in infected cells has not been established. The NS1A protein contains an RNA-binding domain, which is comprised of its N-terminal 73 amino acids and which binds dsRNA with low affinity (Chien et al., 1997, 2004; Hatada and Fukuda, 1992; Liu et al., 1997; Qian et al., 1995). The NS1A protein via its RNA-binding domain can block PKR activation by dsRNA in vitro (Hatada et al., 1999; Lu et al., 1995). However, because the NS1A RNA-binding domain has a much lower affinity for dsRNA than the dsRNA-binding domains of PKR (Chien et al., 2004), it is not clear that the NS1A protein can inhibit PKR activation by effectively competing with PKR for dsRNA in vivo. Experiments using mutant influenza A viruses have not established that the RNA-binding domain of the NS1A protein is required for the inhibition of PKR activation in infected cells (Donelan et al., 2004; Hatada et al., 1999). Consistent with a different mechanism, namely that direct interaction of the NS1A protein with PKR blocks its activation, it has been reported that the NS1A protein binds PKR in infected cells (Tan and Katze, 1998). However, such an NS1A–PKR interaction in infected cells was not found by another group of investigators (Falcon et al., 1999).

To gain insights into the mechanism of NS1A-mediated inhibition of PKR activation, we first carried out a series of in vitro experiments to determine whether the NS1A protein could utilize a common mechanism to inhibit PKR activation by both PACT and dsRNA, despite their different modes of activation. We demonstrate that the direct binding of the NS1A protein to PKR can serve as such a common mechanism. Thus, although the NS1A protein can bind to PACT, we show that binding of NS1A to PKR, but not to PACT, is responsible for blocking PKR activation by PACT in vitro. Furthermore, we show that PKR binding by the NS1A protein, as well as its mutant that is defective in binding dsRNA, inhibits PKR activation by dsRNA as well as by PACT. We then show that the NS1A protein and its mutant defective in dsRNA-binding block PACT-mediated PKR activation and the resultant eIF2α phosphorylation in vivo. Finally, we demonstrate that PKR activation is inhibited in cells infected with a recombinant influenza A virus expressing NS1 mutant protein that cannot bind RNA, as is the case in cells infected with wild-type virus. These results, which establish that the RNA-binding domain of the NS1A protein is not required for blocking PKR activation in vitro and in vivo, provide a rationale for determining whether the direct interaction of the NS1A protein with PKR plays an important role in the blocking of PKR activation during influenza A virus infection.

Results

The NS1A protein inhibits PKR activation mediated by PACT

PKR can be activated by either dsRNA or the cellular PACT protein (Clemens and Elia, 1997; Galabru and Hovanessian, 1987; Patel and Sen, 1998). To determine whether the influenza virus NS1A protein inhibits PKR activation mediated by PACT, increasing amounts of glutathionetransferase (GST)-NS1A protein were added to PKR in the presence of PACT (Fig. 1). In this in vitro assay, we used PKR that had been purified from human cell extracts and an equal amount (15 nM) of purified recombinant PACT. Activation of PKR was measured by its autophosphorylation. Without added PACT, there was only a low level of phosphorylation of PKR (lane 1, Fig. 1, top), which was substantially increased upon addition of the PACT activator to the reaction mixture (lane 2). These results demonstrated that the PKR preparation was virtually free of any co-purified activator, such as dsRNA, and that the PACT preparation was highly active. When the GST-NS1A protein was added to the reaction, we observed a dose-dependent inhibition of PKR autophosphorylation (lanes 3–5). The same amounts of GST protein had little effect (lanes 6–8), indicating that the observed inhibition of PKR activation by GST-NS1A was due to the NS1A part of the fusion protein. Quantitation of the intensities

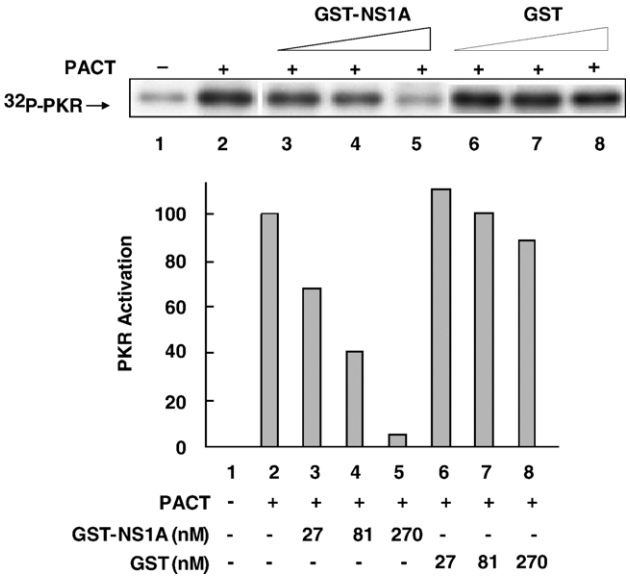

Fig. 1. NS1A blocks activation of PKR by PACT in vitro in a dose-dependent manner. The effects of increasing concentrations of purified NS1A protein on PRK activation by wt PACT were tested. PKR activation assays were performed, and PKR activation levels were quantified by PhosphorImager analysis and presented in the bottom chart. PKR was incubated with purified PACT (15 nM) as an activator in the absence of added protein (lane 2), in the presence of the indicated concentrations of purified GST-NS1A protein (lanes 3 to 5) or in the presence of the indicated concentrations of purified GST protein (lanes 6 to 8), as described in Materials and methods. As a control, buffer was added to the PKR activation reaction (lane 1). Arrow indicates ^{32}P-labeled PKR.

of the bands demonstrated that NS1A, at the highest dose, almost completely blocked PKR activation by PACT (Fig. 1, bottom).

The binding of the NS1A protein to PKR residues 1–230 is responsible for the inhibition of PACT-mediated activation of PKR

To identify the protein–protein interactions that are responsible for the inhibition of PACT-mediated inhibition of PKR activation, GST-NS1A was used in a GST pulldown assay with PKR (Fig. 2A) and PACT (Fig. 2B). The GST-NS1A protein, but not GST, bound to PKR (Fig. 2A, lanes 1 and 5). To determine the region of PKR to which the NS1A protein binds, we tested a series of C-terminal deletion mutants of PKR for binding to GST-NS1A. The GST-NS1A protein bound to PKR (1–300) and full-length PKR strongly (lanes 1 and 2), and also bound to PKR (1–230) (lane 3), but not to PKR (1–170) (lane 4). These results demonstrate that the NS1A protein requires residues 1–230 of PKR for binding. In contrast, dsRNA requires only residues 1–170 of PKR for binding (Feng et al., 1992; Green and Mathews, 1992; Katze et al., 1991; McCormack et al., 1992; Patel and Sen, 1992).

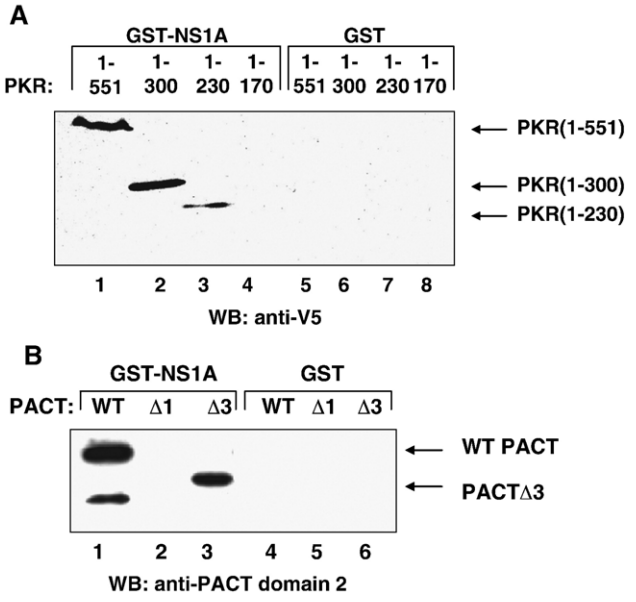

Fig. 2. NS1A binds to both PKR and PACT. (A) NS1A binds to PKR residues 1 to 230. The binding of GST-NS1A to PKR and truncated PKR mutants was tested in GST pulldown assays. 0.5 μg of purified GST or 1 μg of purified GST-NS1A protein was incubated with cell extracts containing V5-tagged PKR proteins in binding buffer containing glutathione–Sepharose 4B. After binding, the PKR protein constructs interacting with GST-NS1A protein were analyzed by Western blotting with V5 antibody. 1–551, PKR(1–551); 1–300, PKR(1–300); 1–230, PKR(1–230); 1–170, PKR(1–170). (B) PACT binds to NS1A, and its domain 1 is necessary for this binding. One microgram of bacterially purified wt PACT, PACTΔ1 missing domain 1 and PACTΔ3 lacking domain 3 was incubated with 1 μg of purified GST-NS1A or 0.5 μg of GST protein, and GST pulldown assays were performed. Western blot analysis was performed to detect PACT proteins interacting with NS1A using anti-PACT domain 2 antibody. WT, wt PACT, the bottom band in the lane for wt PACT is degraded product; Δ1, PACT mutant missing domain 1; Δ3, PACT mutant missing domain 3.

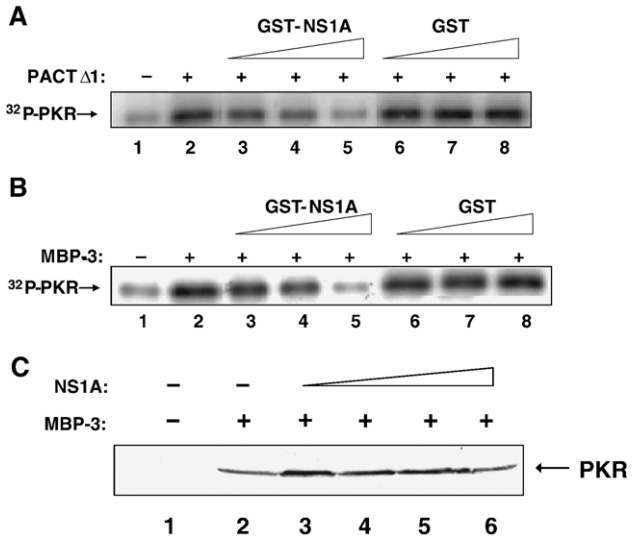

Fig. 3. NS1A blocks PKR activation by binding to PKR, not PACT. (A) NS1A blocks PKR activation by PACTΔ1. PKR activation assays in vitro were performed to test the effects of increasing concentrations of NS1A protein on PACTΔ1-mediated PKR activation. PACTΔ1 was used at 15 nM concentration. Lane 1, buffer control; lane 2, PACTΔ1 alone; lanes 3 and 6, 27 nM GST proteins; lanes 4 and 7, 81 nM GST proteins; lanes 5 and 8, 270 nM GST proteins; (B) NS1A blocks PKR activation by PACT domain 3. PKR kinase assays were done as above, except that 10 nM of MBP-3 was used as an activator. (C) NS1A does not disrupt binding of PACT domain 3 to PKR. Binding of MBP-3 to purified FLAG-tagged PKR was tested in the presence of 0.1 μg (lane 3), 0.3 μg (lane 4), 1 μg (lane 5) or 2 μg (lane 6) of purified GST-NS1A protein. As controls, PKR was incubated with either immobilized MBP (lane 1) or MBP-3 (lane 2) in the absence of NS1A protein. The PKR protein interacting with MBP-3 was Western blotted with FLAG antibody.

Similar pulldown assays demonstrated that the NS1A protein also binds to PACT (lane 1, Fig. 2B). PACT has three identifiable domains, all of which bind to PKR, but only the binding of domain 3 leads to activation of PKR. In contrast, only domain 1 of PACT is required for binding to the NS1A protein. Thus, GST-NS1A bound strongly to PACTΔ3, missing domain 3 (lane 3), but did not bind to PACTΔ1, missing domain 1 (lane 2). The latter result showed that domains 2 and 3, both present in PACTΔ1, were not sufficient for binding to GST-NS1A.

To determine whether the interaction with PKR or the interaction with PACT was required for the observed NS1A protein-mediated inhibition of PKR activation, we took advantage of the fact that the NS1A protein did not bind to either PACTΔ1 or MBP-3 (a fusion protein of PACT domain 3 with the maltose binding protein), each of which can activate PKR. If the NS1A protein inhibited PKR activation caused by PACTΔ1 or MBP-3, then such inhibition could only result from the binding of the NS1A protein to PKR. In fact, this was the case: NS1A inhibited activation of PKR by PACTΔ1 (Fig. 3A) and MBP-3 (Fig. 3B) in a dose-dependent fashion. We conclude that the binding of the NS1A protein to residues 1–230 of PKR, but not to PACT, is responsible for the inhibition of PACT-mediated PKR activation.

One possible mechanism by which the NS1A protein might inhibit PKR activation by PACT would be by blocking the

binding of PACT domain 3 to PKR. The results in Fig. 3C demonstrate that this is not the case. Increasing amounts of the GST-NS1A protein did not inhibit binding of MBP-3 to PKR (lanes 3–6). These results indicate that the observed inhibitory effect of the NS1A protein on PKR activation is not mediated by impairing the interaction of PACT domain 3 with PKR.

The binding of the NS1A protein to PKR blocks its activation by dsRNA as well as by PACT and does not require dsRNA binding by the NS1A protein

The NS1A protein binds dsRNA, albeit with a low affinity. Previous experiments have shown that the NS1A protein via its RNA-binding domain can inhibit PKR activation in vitro by binding dsRNA. To determine whether the NS1A protein can inhibit PKR activation independently of its dsRNA-binding activity, when activation is mediated by either PACT or dsRNA, we used a mutant NS1A protein in which the arginine (R) at position 38 has been replaced by alanine (A). This R38A mutant NS1A protein has little or no dsRNA-binding activity (Wang et al., 1999). Like the wild-type NS1A protein, the R38A mutant protein binds PKR strongly (Fig. 4A). The R38A mutant NS1A protein inhibits PKR activation in a dose-dependent manner, whether PKR is activated by PACT or by dsRNA (Fig. 4B). Consequently, although the modes of PKR activation by dsRNA and PACT differ, the binding of the R38A NS1A

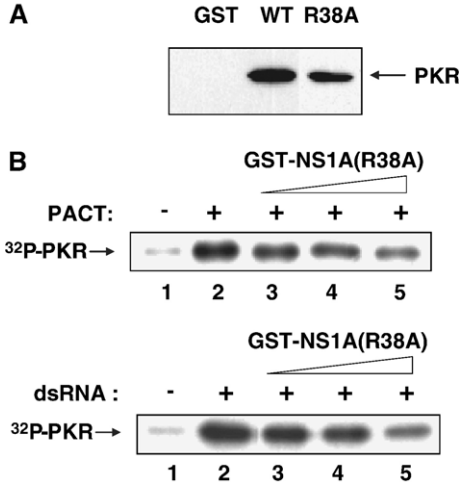

Fig. 4. RNA-binding property of NS1A is not required for inhibition of PKR activation by PACT and dsRNA. (A) Like wt NS1A, an RNA-binding deficient NS1A mutant, NS1A(R38A), binds to PKR. The binding of wt NS1A and NS1A(R38A) to FLAG-tagged PKR expressed from HT1080 cells was tested by GST pulldown assays. 0.5 μg of purified GST (lane 1), 1 μg of purified wt NS1A protein (lane 2) or 1 μg of purified NS1A (R38A) (lane 3) was incubated with cell extracts containing FLAG-tagged PKR and then precipitated by using glutathione–Sepharose 4B. The FLAG-tagged PKR interacting with NS1A proteins was analyzed by Western blotting with FLAG antibody. WT, GST-wt NS1A; R38A, GST-NS1A (R38A). (B) NS1A (R38A) inhibits PKR activation by PACT and dsRNA. In vitro kinase activation assays were performed using PACT (upper panel) or dsRNA (lower panel) as the activator, in the presence of increasing concentrations of GST-tagged NS1A(R38A) protein. Lane 1, buffer control; lanes 2 to 5, 15 nM PACT (upper panel) or 1 μg/ml dsRNA (lower panel); lane 3, 27 nM NS1A(R38A) protein; lane 4, 81 nM NS1A(R38A) protein; lane 5, 270 nM NS1A(R38A) protein.

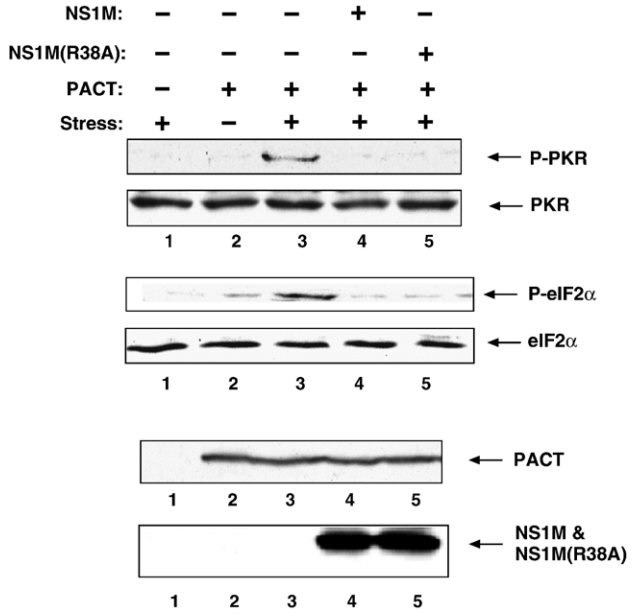

Fig. 5. NS1A blocks PACT-activated phosphorylation of PKR and eIF2α in stressed cells. HT1080 cells were cotransfected with FLAG-tagged PACT and NS1A constructs, NS1M and NS1M (R38A), at 1:1 ratio and treated with 50 ng/ml of actinomycin D for 1 h. As controls, cells were transfected with empty vector or FLAG-PACT vector alone and left treated or untreated. Cell extracts were then prepared and analyzed by SDS-PAGE and Western blotting. The top panel shows phosphorylation level and protein level of PKR detected by anti-phospho-PKR (P-PKR) and anti-PKR antibodies (PKR). The second panel shows phosphorylation level and protein level of eIF2α detected by antibodies against phospho-eIF2α (P-eIF2α) and eIF2α. The third and the bottom panels show expression of PACT and NS1A proteins detected by Western blot analysis with anti-FLAG antibody and anti-NS1A antibody, respectively. NS1M, NS1A missing residues 184–188 required for CPSF binding and inhibition of cellular gene expression; NS1M (R38A), NS1A with R38A mutation and with deletion of residues 184–188.

mutant protein to PKR in vitro inhibits PKR activation mediated by both these activators.

The NS1A protein inhibits PKR activation by PACT in vivo

When cells expressing PACT are subjected to extracellular stress, by treatment with a very low dose of actinomycin D, PKR is phosphorylated (activated), and, as a consequence, eIF2α is phosphorylated (Patel et al., 2000; Peters et al., 2001). We determined whether the NS1A protein inhibits both PACT-mediated PKR activation and the resulting eIF2α phosphorylation. HT1080 cells, which express very little PACT (Patel and Sen, 1998; Peters et al., 2001), were transfected with a vector expressing PACT. Treatment of these cells with actinomycin D activated PKR, as measured by its phosphorylation (Fig. 5, top panel, lane 3) and the consequent phosphorylation of eIF2α, a major target of PKR (Fig. 5, second panel, lane 3). These phosphorylations were dependent on PACT expression because they did not take place after actinomycin D treatment of cells transfected with an empty vector (Fig. 5, lane 1). To determine whether the NS1A protein inhibited these two phosphorylations, a plasmid expressing the NS1A protein was cotransfected with the PACT expression

vector. Because wild-type NS1A protein inhibits the 3′ end processing of cellular pre-mRNAs, including pre-mRNAs expressed from plasmids under the control of polymerase II promoters, we expressed a mutant form of the NS1A protein (NS1M) that lacks this function (Noah et al., 2003). Both the NS1M protein and its R38A mutant (NS1M(R38A)) blocked the phosphorylation of both PKR and eIF2α in vivo (Fig. 5, lanes 4 and 5). PACT was expressed at similar levels in all transfected cells (Fig. 5, third panel), and the NS1M and NS1M(R38A) proteins were expressed equally well (Fig. 5, bottom panel), thus validating these results.

We have developed a quantitative cell survival assay to measure apoptosis resulting from PACT-mediated activation of PKR. In this assay, an expression vector of PACT is cotransfected with a GFP expression vector. After treatment with a low level of actinomycin D, live transfected cells are identified by their GFP expression and counted by FACS. Almost all (approximately 90%) of the PACT-expressing cells are killed upon actinomycin D treatment (Fig. 6, lane 2). When cells are transfected with a vector for expressing only GFP, but not PACT, they are not killed by the actinomycin D treatment (data not shown). Co-expression of the NS1M or the NS1M (R38A) protein efficiently protected the cells from PACT-mediated apoptosis (Fig. 6, lanes 3 and 4). The results presented in Figs. 5 and 6 demonstrate that, in vivo, the NS1A protein blocks PACT-mediated PKR activation and the consequent phosphorylation of eIF2α and cellular apoptosis. Moreover, the RNA-binding activity of the NS1A protein is not required for this protective effect.

The RNA-binding activity of the NS1 protein is not required for inhibiting PKR activation in virus-infected cells

Finally, we confirmed that the above conclusions were true for influenza-A-virus-infected cells as well. For this purpose, a mutant influenza A virus that encodes the R38A mutant of the NS1A protein was generated. Cells were infected with the wt or the mutant virus, and PKR activation was monitored by measuring the levels of PKR and eIF2α phosphorylations

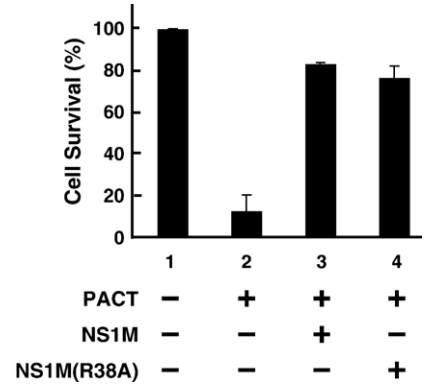

Fig. 6. NS1A blocks stress-induced PACT-mediated cell-killing. Cell survival assays were performed to test the effects of NS1A proteins on PACT-mediated cell-killing by monitoring the loss of EGFP-expressing cells after cotransfection of indicated expression constructs with an EGFP expression vector. The error bars represent the standard error calculated from three independent experiments.

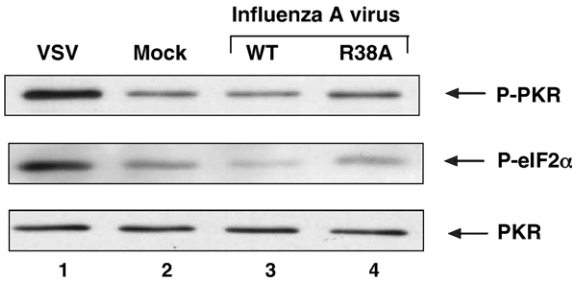

Fig. 7. Both wt and R38A mutant influenza A viruses inhibit PKR activation in infected cells. Recombinant influenza A viruses encoding wt NS1 and dsRNA-binding deficient NS1 mutant NS1(R38A) were generated using the 12-plasmid transfection system (Takeda et al., 2002). A549 cells were mock-infected or infected with the recombinant viruses for 7 h. VSV infection of A549 cells can activate PKR and thus was used as a PKR activation control. Cell extracts were prepared and subjected to SDS-PAGE and Western blotting analysis. The top panel shows phosphorylation level of PKR detected by anti-phospho-PKR antibody. The middle panel shows phosphorylation level of eIF2α detected by anti-phospho-eIF2α. The bottom panel shows total PKR protein detected by anti-PKR antibody.

(Fig. 7). As controls of this experiment, we verified that both proteins were highly phosphorylated in cells infected with VSV (Fig. 7, lane 1), but not in mock-infected cells (Fig. 7, lane 2). There was little PKR activation in cells infected with the wt influenza A virus (Fig. 7, lane 3). The same was true for the mutant virus expressing the R38A mutant of the NS1A protein (Fig. 7, lane 4). These results conclusively demonstrate that the RNA-binding property of the NS1 protein is not required for its ability to block PKR activation in influenza-A-virus-infected cells.

Discussion

Both dsRNA and PACT activate PKR, but via different mechanisms. Although traditionally it has been thought that viral dsRNA is the activator of PKR in virus-infected cells, there is no compelling evidence in the literature to support this notion. It is possible that viruses activate PACT, which in turn activates PKR. Based on the concept that viruses would need to block PKR activation by both dsRNA and PACT, we carried out in vitro experiments to determine whether the NS1A protein of influenza A virus, the viral protein that has been implicated in the inhibition of PKR activation during virus infection (Bergmann et al., 2000), can block PKR activation by both activators. We established that this was indeed the case by demonstrating that the binding of the NS1A protein to the N-terminal 230 amino acid region of PKR is necessary and sufficient to block PKR activation by both PACT and dsRNA in vitro. The ability of the NS1A protein to bind to PKR and block its activation by both activators does not require the RNA-binding activity of the NS1A protein. Thus, the R38A mutant NS1A protein, which lacks RNA-binding activity, effectively binds to PKR and blocks its activation by both dsRNA and PACT. Furthermore, we demonstrated that the RNA-binding activity of the NS1A protein is not required for its ability to inhibit PKR activation in vivo. Thus, we showed that the R38A mutant NS1A protein, like the wild-type (wt) protein, blocks

PKR activation by PACT in vivo, as well as the downstream effects of PKR activation in cells, namely, eIF2α phosphorylation and apoptosis. In addition, the activation of PKR was inhibited in cells infected by a recombinant influenza A virus expressing an NS1A protein lacking RNA-binding activity.

These results raise two important issues. First, how does the binding of the NS1A protein to PKR block the activation of PKR by two activators (dsRNA and PACT) whose modes of activation are quite distinct? This issue is also relevant for the HSV-1 protein US11, which has little sequence homology with NS1A, but can similarly block PKR activation by either dsRNA or PACT (Peters et al., 2002). It is curious to note that both NS1A and US11 proteins are RNA-binding proteins, but this property of neither protein is required for inhibiting PKR activation. Instead, what is required is the ability to bind PKR directly. Although both proteins bind to the N-terminal region of PKR, the exact binding sites are different. Residues 1–170 of PKR are sufficient to bind US11, but not NS1A, which requires residues 170–230 as well. Current information (S. Li et al., unpublished results) suggests that an intramolecular interaction between the N-terminal region and the kinase domain of PKR is responsible for keeping the protein in an inactive conformation and the binding of dsRNA to the N-terminal region or the binding of PACT domain 3 (PACTd3) to the kinase domain causes the conformational change that activates PKR (Fig. 8A). Results presented here indicate that the binding of NS1A to the linker region of PKR, between the dsRNA-binding domain and the kinase domain, blocks this conformational change (Fig. 8B). The block is not mediated by interfering with the PACTd3/PKR interaction (Fig. 3C) but by strengthening the intramolecular interaction between the two domains of PKR and freezing it in an inactive state. This model is valid for the inhibitory action of US11 as well (Peters et al., 2002). We believe that this mechanism also holds true for PKR activation by dsRNA (Fig. 8B), in which case the PKR/dsRNA/NS1A complex remains frozen in the inactive conformation, a postulate that can be experimentally tested in the future. Note that the model does not require an interaction between NS1A and dsRNA, which is supported by our experimental results.

The second issue is: does the NS1A protein in influenza-A-virus-infected cells utilize the mechanism described here, namely, binding to the N-terminal region of PKR, to block PKR activation? This mechanism would enable influenza A virus to block activation by both dsRNA and PACT. However, previous studies have provided conflicting results concerning whether the NS1A protein interacts directly with PKR in infected cells. An alternative mechanism for blocking PKR activation is that the NS1A protein via its RNA-binding domain sequesters dsRNA, rendering it unavailable for PKR activation. This mechanism would inhibit only dsRNA activation, but not PACT activation, of PKR. In addition, it is not clear whether the NS1A RNA-binding domain is capable of sequestering dsRNA away from PKR because the NS1A RNA-binding domain has a much lower affinity for dsRNA than the two dsRNA-binding domains in PKR (Chien et al., 2004; Green and Mathews, 1992). The results presented in Fig. 7 clearly show that this

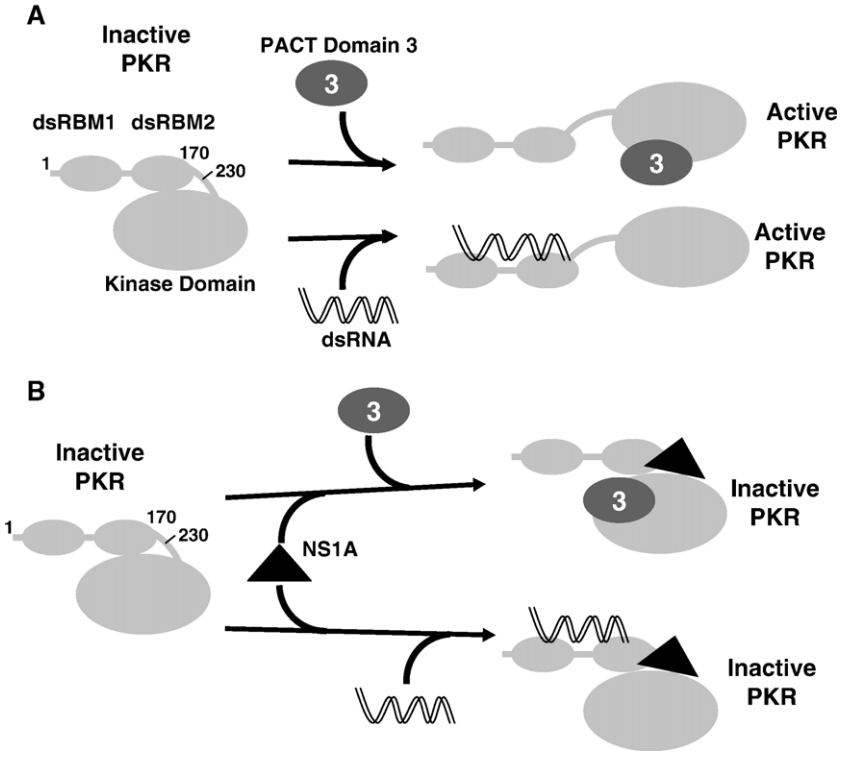

Fig. 8. Model for NS1A-mediated inhibition of PKR activation. (A) Binding of dsRNA to PKR dsRBM1 and dsRBM2 or binding of PACT domain 3 to PKR kinase domain disrupts the intramolecular interaction between dsRBM2 and the kinase domain of inactive PKR and induces similar conformational changes leading to its activation. (B) Binding of NS1A protein to residues to the linker region of PKR prevents PKR activation by PACT domain 3, even though PACT domain 3 can still bind to the kinase domain of PKR. Such a binding also blocks dsRNA-mediated PKR activation.

alternative mechanism does not operate in influenza-A-virus-infected cells: PKR activation is blocked equally well by the wt NS1 protein and the mutant NS1 protein that cannot bind RNA. Hence, the results presented here provide a rationale for determining whether the interaction of the NS1A protein with PKR plays an important role in the blocking of PKR activation during influenza A virus infection. These experiments have the potential of determining the contribution of an additional mechanism for the inhibition of PKR activation in influenza-A-virus-infected cells by the cellular $p58^{IPK}$ protein.

Materials and methods

Cell lines

HEK293T, human embryonic kidney cells, HT1080 cells, human fibrosarcoma cells and A549, human lung carcinoma epithelial cells, were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics at 37 °C under a 5% CO_2 atmosphere.

Antibodies and other reagents

Actinomycin D, FLAG peptide, anti-FLAG monoclonal M2 antibody and M2-agarose were obtained from Sigma-Aldrich Corporation. Lipofectamine 2000 and anti-V5 monoclonal antibody were purchased from Invitrogen Corporation. Anti-PKR antibody was from Santa Cruz. Phospho-specific anti-eIF2α, phospho-specific anti-PKR (against phosphorylated threonine 445) and anti-eIF2α antibodies were purchased from Cell Signaling Technology. PACT anti-domain 2 and anti-domain 3 peptide antibodies (Peters et al., 2001, 2002) were custom-produced by Bio-Synthesis, Inc. FuGENE 6 transfection reagent was from Roche. $CaPO_4$ transfection reagents were prepared according to *Current Protocols in Molecular Biology.*

Expression vectors

The generation of FLAG-tagged and V5-tagged PKR constructs, pcDNA3-PKR(K296R)-FLAG and pV5-PKR, and FLAG-tagged PACT construct pcDNA3-FLAG-PACT as well have been described previously (Goh et al., 2000; Peters et al., 2001, 2002). pEGFP-C1 containing the cDNA coding for enhanced green fluorescent protein was from Promega. To generate V5-tagged PKR deletion mutants, DNA fragments corresponding to residues 1–170, 1–230 and 1–300 of PKR were amplified by PCR with primers containing restriction sites *Bam*HI and *Xba*I. The PCR products were ligated in-frame into a V5-tagged vector, Vet (Goh KC, EMBO 2000). To generate bacterial expression vectors containing GST-tagged wt NS1A, the A/Udorn/72 NS1 full-length open reading frame was PCR-amplified and cloned into the unique *Bam*HI and *Eco*RI sites of the pGEX-3X plasmid to allow expression as GST fusion proteins. GST-tagged NS1A mutants with an alanine substitution at arginine 38 (R38A) were generated by using two rounds of PCR and specific oligonucleotide primers. The resultant DNA was cloned into *Bam*HI/*Eco*RI-digested pGEX-3X. To generate NS1A mammalian expression vector containing a mutated binding site for the 30 kDa subunit of the cleavage and polyadenylation-specific factor (CPSF), amino acids 184 to 188 of the influenza A/Udorn/72 NS1A protein were changed from GLEWN to RFLRY (NS1M) as described previously (Noah et al., 2003), and the resulting DNA was cloned into pcDNA3 vector. Its derived mutant containing R38A mutation, NS1M(R38A), in pcDNA3 was also made as described above. All the resulting DNA clones with internal mutations were sequenced to verify the introduction of desired mutations.

Purification of NS1A, PACT and their derivatives

GST-NS1A fusion proteins were purified as previously described (Qiu and Krug, 1994). The purity of GST-fusion protein was established by SDS-PAGE followed by coomassie blue staining and Western blotting with anti-GST antibody (Amersham). Bacterial expression and purification of wt PACT, PACTΔ1, PACTΔ3, and the fusion protein of maltose binding protein (MBP) and PACT domain 3, MBP-3, were described previously (Peters et al., 2001, 2002).

PKR kinase assay in vitro

PKR activation assays were performed using ectopically expressed V5-tagged PKR (Yin et al., 2003). V5-PKR was expressed in HEK293T cells by $CaPO_4$ transfection and immunoprecipitated with anti-V5 monoclonal antibody. Kinase assay was done in activity buffer containing: immobilized PKR, an activator (dsRNA, purified wt PACT, PACTΔ1 or MBP-3) and 1 μCi of [γ-^{32}P]ATP at 30 °C for 30 min. To test the effects of purified GST-wt NS1A or GST-NS1A(R38A) mutant on PKR activation, PKR immunoprecipitate was incubated with purified GST-NS1A proteins or GST protein control at 4 °C for 5 min followed by addition of a purified PKR activator and [γ-^{32}P]ATP. As a control, neither PKR activator nor GST proteins was added to the PKR activation reaction. Autophosphorylated PKR was analyzed by SDS-PAGE and visualized by auto-radiography. PKR activation levels were quantified by PhosphorImager analysis and presented as a chart. The amount of radioactivity in PKR in the absence of any added purified protein was regarded as background and subtracted from all other values. The amount of radioactivity in PKR activated by an activator alone was considered 100%, and the values for GST proteins are presented as percentages of that value.

Assay for binding of PACT domain 3 to PKR

Purification of PKR(K296R)-FLAG protein and in vitro PACT domain 3-PKR binding assay were described previously (Peters et al., 2002). To test the effect of NS1A protein on binding of MBP-3 to PKR, increasing amounts of GST-NS1A protein were pre-incubated with 0.5 μg resin-bound MBP or MBP-3 in binding buffer (20 mM Tris–HCl, 10 mM β-mercaptoethanol, 1 mM EDTA and 10% glycerol, pH 7.5) at 4

°C for 5 min, and then 1 μg purified PKR(K296R)-FLAG protein was added to the mixture and incubated at 4 °C for 1 h. Proteins bound to the beads were analyzed by SDS-PAGE followed by Western blot analysis with anti-FLAG antibody for detecting PKR(K296R)-FLAG and anti-PACT domain 3 antibody for detecting MBP-3.

GST pulldown assay

To analyze protein interactions between NS1A proteins and PKR or PACT proteins, GST or GST-NS1A proteins were mixed with cell extracts containing V5-tagged or FLAG-tagged PKR proteins in binding buffer A (20 mM Tris–HCl, 50 mM NaCl, 0.4% Triton X-100, 20% glycerol, 100 units/ml aprotinin, 0.2 mM PMSF, pH 8.0) or with bacterially purified PACT proteins in binding buffer B (20 mM Tris–HCl, 200 mM NaCl, 1% Triton X-100, 20% glycerol, 100 units/ml aprotinin, 0.2 mM PMSF, pH 8.0). After 1 h at 4 °C on a rotating wheel, 30 μl of glutathione–Sepharose 4B (Amersham Pharmacia) (50% solution in binding buffer) was added, and the mixtures were spun for 2 h. The beads were precipitated and washed three times with 500 μl of binding buffer. The proteins binding to NS1A proteins were analyzed by SDS-PAGE followed by Western blot analysis with anti-FLAG antibody, anti-V5 antibody or anti-PACT domain 2 antibody. GST proteins were detected with anti-GST antibody.

Generation of recombinant influenza A/Udorn/72 virus from cloned DNA

pHH21 plasmids containing the full-length cDNAs for each of the eight influenza A/Udorn/72 genomic RNA segments and four pcDNA plasmids encoding PB1, PB2, PA and NP of influenza A/Udorn/72 were provided by Makoto Takeda (Takeda et al., 2002). Alteration of Udorn NS1A residue 38 from R to A (R38A mutant) was performed by using two rounds of PCR. The resulting DNA was sequenced and cloned into pHH21. Viruses encoding the wild-type and R38A mutant NS1A proteins were generated by cotransfecting 293T cells with eight plasmids encoding the vRNA segments and four plasmids expressing the PB1, PB2, PA and NP proteins (Takeda et al., 2002). At various times posttransfection, culture supernatants were collected. Viruses were titered by plaque assay on MDCK cells, and individual plaques were amplified in 10-day-old embryonic chicken eggs at 34 °C. Amplified virus was titered by plaque assay on MDCK cells, and all the genomic RNA segments were sequenced.

Analysis of the phosphorylations of PKR and eIF2α in vivo

To measure the phosphorylation levels of PKR and eIF2α in transfected cells, HT1080 cells were transfected with pcDNA3, or pcDNA3-FLAG-PACT alone, or pcDNA3-FLAG-PACT together with NS1A constructs using Lipofectamine 2000 reagent. Wt NS1A binds to the 30 kDa subunit of CPSF, thereby inhibiting 3′-end processing of pre-mRNA of cellular or transfected genes. To co-express PACT and NS1A in cells, NS1A mutants [NS1M and NS1M(R38A)] deficient in CPSF binding were used (Noah et al., 2003). After 47 h, cells were treated with 50 ng/ml of actinomycin D for 1 h, harvested and lysed in lysis buffer (20 mM Tris, 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1 M phenylmethylsulfonyl fluoride, 200 units/ml aprotinin, 100 μM vanadate, 1 mM NaF, 2 nM okadaic acid, 25 mM β-glycerophosphate, pH 8.0). Protein concentrations of cell lysates were measured by the Bradford method (BioRad, CA). Cell extracts containing 50 μg of protein were resolved on SDS polyacrylamide gels. FLAG-PACT and NS1A proteins were detected using anti-FLAG antibody and NS1A antibody, independently. Phospho-specific anti-eIF2α and phospho-specific anti-PKR antibodies were used to detect phosphorylated proteins, and anti-eIF2α and anti-PKR antibodies were used to detect PKR and eIF2α protein levels.

To measure the phosphorylation levels of PKR and eIF2α in virus-infected cells, confluent A549 cells in 35 mm dishes were infected with recombinant wild-type influenza A virus or R38A mutant virus at an moi of 5 pfu/cell. As a positive control, A549 cells were infected with Vesicular Stomatitis Virus (VSV). At 7 h post-infection, cells were treated with 25 μM protein phosphatase inhibitor, Calyculin A, for 30 min. Cells were then washed with cold PBS, and RIPA buffer (50 mM Tris–Cl pH 7.5, 1% NP-40, 0.5% sodium deoxycolate, 0.05% SDS, 1 mM EDTA, 150 mM NaCl) containing protease inhibitor was added to lyse the cell. Total cell lysates were analyzed for PKR phosphorylation and eIF2α phosphorylation as described above.

Cell survival assay

HT1080 cells were cotransfected with pcDNA3, or pcDNA3-FLAG-PACT, or pcDNA3-FLAG-PACT with NS1M or NS1M(R38A) construct, as well as pEGFP-C1 using Lipofectamine 2000 transfection reagent. The total amount of DNA used was 8.5 μg, and the ratio of FLAG-PACT:NS1A consruct:pEGFP-C1 was 3.75:3.75:1. At 6 h after transfection, cells were treated with 50 ng/ml of actinomycin D for inducing PACT-mediated apoptosis (Peters et al., 2001). At 48 h after transfection, the media containing dead cells was removed and cells remaining on the plates were washed with PBS three times and collected. By using flow cytometry, effects of NS1A proteins on cell-killing by PACT were analyzed by comparing the percentages of EGFP-expressing cells for transfection with vector control, transfection with PACT alone and transfections with PACT and NS1A constructs. The cell survival value for vector transfection is considered 100%, and the values for other transfections are presented as percentage of that value.

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