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Virology 363 (2007) 236–243
VIROLOGY
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A site on the influenza A virus NS1 protein mediates both inhibition of PKR activation and temporal regulation of viral RNA synthesis

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Received 22 January 2007; accepted 30 January 2007
Available online 22 February 2007

Abstract

It is not known how influenza A viruses, important human pathogens, counter PKR activation, a crucial host antiviral response. Here we elucidate this mechanism. We show that the direct binding of PKR to the NS1 protein *in vitro* that results in inhibition of PKR activation requires the NS1 123–127 amino acid sequence. To establish whether such direct binding of PKR to the NS1 protein is responsible for inhibiting PKR activation in infected cells, we generated recombinant influenza A/Udorn/72 viruses expressing NS1 proteins in which amino acids 123/124 or 126/127 are changed to alanines. In cells infected with these mutant viruses, PKR is activated, eIF-2α is phosphorylated and viral protein synthesis is inhibited, indicating that direct binding of PKR to the 123–127 sequence of the NS1 protein is necessary and sufficient to block PKR activation in influenza A virus-infected cells. Unexpectedly, the 123/124 mutant virus is not attenuated because reduced viral protein synthesis is offset by enhanced viral RNA synthesis at very early times of infection. These early viral RNAs include those synthesized predominantly at later times during wild-type virus infection, demonstrating that wild-type temporal regulation of viral RNA synthesis is absent in 123/124 virus-infected cells. Enhanced early viral RNA synthesis after 123/124 virus infection also occurs in mouse PKR−/− cells, demonstrating that PKR activation and deregulation of the time course of viral RNA synthesis are not coupled. These results indicate that the 123/124 site of the NS1A protein most likely functionally interacts with the viral polymerase to mediate temporal regulation of viral RNA synthesis. This interaction would occur in the nucleus, whereas PKR would bind to NS1A proteins in the cytoplasm prior to their import into the nucleus.

Keywords: Influenza A virus; NS1 protein; PKR activation; Viral RNA synthesis

Introduction

The protein kinase PKR is constitutively expressed in mammalian cells and is further increased by interferon (IFN) treatment (Hovanessian, 1989; Meurs et al., 1990). After activation by binding either double-stranded RNA (dsRNA) or the cellular PACT protein (Galabru and Hovanessian, 1987; Patel and Sen, 1998), PKR phosphorylates the α subunit of the eIF2 translation initiation factor, resulting in the inhibition of cellular and viral protein synthesis and viral replication (Gale and Katze, 1998; Samuel, 1993). Hence, PKR is a major component of the cellular antiviral system, and it is crucial for viruses to block PKR activation or the downstream effects resulting from its activation.

Many viruses use different strategies to inhibit the antiviral actions of PKR (Gale and Katze, 1998). Here we focus on the strategy employed by influenza A viruses, which are important human pathogens that are responsible for seasonal epidemics as well as for periodic pandemics that result in high mortality rates (Wright and Webster, 2001). PKR is not activated after infection by influenza A virus (Katze et al., 1986), but the mechanism by which the virus inhibits PKR activation has not been established. Two mechanisms that have been postulated involve the viral NS1 protein (NS1A protein), a multi-functional protein that participates in both protein–RNA and protein–protein interactions. The N-terminal 73-amino acid RNA-binding domain of the NS1A protein specifically binds A-form dsRNA, albeit with low affinity (Chien et al., 2004; Hatada

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doi:10.1016/j.virol.2007.01.038

and Fukuda, 1992; Lu et al., 1995; Wang and Krug, 1996a, 1996b). The rest of the NS1A protein, which is denoted as the effector domain, has binding sites for several cellular proteins. It binds the 30-kDa subunit of the cellular cleavage and polyadenylation specificity factor (CPSF30), resulting in the inhibition of the 3′ end processing of cellular pre-mRNAs (Li et al., 2001; Nemeroff et al., 1998; Noah et al., 2003; Twu et al., 2006), and also binds p85β, resulting in the activation of phosphatidylinositol-3-kinase (PI13K) signaling (Hale et al., 2006). It has been proposed that: (i) the N-terminal RNA-binding domain of the NS1A protein sequesters dsRNA away from PKR (Garcia-Sastre, 2001; Hatada et al., 1999; Lu et al., 1995); or (ii) inhibition of PKR activation results from the direct binding of PKR to the NS1A protein (Li et al., 2006a). In addition, it has been proposed that a cellular protein is involved in inhibiting PKR activation, specifically that influenza A virus infection activates a cellular PKR inhibitor, $p58^{IPK}$ (Melville et al., 1999). It was possible that both the viral NS1A protein and the cellular $p58^{IPK}$ protein participate in the inhibition of PKR activation in infected cells.

The role of the dsRNA-binding activity of the NS1A protein in inhibiting PKR activation has been assessed using a recombinant influenza A/Udorn/72 virus expressing a NS1A protein lacking only dsRNA-binding activity (Min and Krug, 2006). PKR is not activated in cells infected by this mutant virus, indicating that sequestering of dsRNA by the NS1A protein is not required for the inhibition of PKR activation in infected cells. However, this result did not eliminate the possibility that activated cellular $p58^{IPK}$ protein by itself can block PKR activation in the absence of NS1A-mediated dsRNA binding. *In vitro* binding assays provided support for the role of direct binding of the NS1A protein to PKR in the inhibition of PKR activation (Li et al., 2006a). Direct binding of the NS1A protein *in vitro* to the N-terminal 230-amino acid region of PKR inhibited PKR activation, and both PKR binding and the inhibition of its activation did not require the dsRNA-binding activity of the NS1A protein. It is not known whether such direct binding is responsible for the inhibition of PKR binding in influenza A virus-infected cells, particularly as co-immunoprecipitation experiments to identify NS1A–PKR complexes have given conflicting results (Falcon et al., 1999; Tan and Katze, 1998).

In the present study we use a genetic approach to elucidate the mechanism by which PKR activation is inhibited in cells infected by influenza A virus. Using recombinant influenza A/Udorn/72 viruses with mutations in the NS gene, we demonstrate that binding of PKR to a specific site in the NS1A protein, its 123–127 amino acid sequence, is necessary and sufficient for the inhibition of PKR activation in virus-infected cells. These experiments also yielded an unanticipated insight into the mechanism of another important feature of influenza A virus infection, the temporal regulation of viral RNA synthesis. Previous experiments have established that the transcription and replication of viral genes in influenza A virus-infected cells is divided into an early and a late phase (Krug et al., 1989; Shapiro et al., 1987; Skehel, 1973), but the mechanism of this temporal regulation has remained a mystery for approximately 20 years. We show that changes in amino acids 123 and 124 of the NS1A protein result in the deregulation of the time course of viral RNA synthesis, leading to the enhanced synthesis of most, if not all, viral RNAs at very early times of infection. Further, we show that this deregulation is independent of PKR activation, indicating that the 123/124 amino acid site of the NS1A protein most likely functionally interacts with the viral polymerase to mediate temporal regulation of viral RNA synthesis.

Results

Identification of the binding site for PKR on the NS1A protein in vitro

We showed previously that direct binding of the influenza A virus NS1 protein (NS1A protein) *in vitro* to the N-terminal 230-amino acid region of PKR inhibits PKR activation (Li et al., 2006a). We employed these *in vitro* assays to identify the specific binding site for PKR on the NS1A protein. In the first set of mapping experiments, we used NS1A proteins containing C-terminal truncations of various lengths (Fig. 1A). The N-terminal region that contains only the dsRNA-binding domain, the N-terminal 1–73 amino acid fragment (Wang and Krug, 1996a, 1996b), does not bind PKR nor inhibit its activation, verifying that the dsRNA-binding activity of the NS1A protein is not sufficient to inhibit PKR activation. The PKR activation assay showed that the 73–150 amino acid region of NS1A is required, and the PKR binding assay showed that the required region is shorter, amino acids 125–150. Based on these results, we generated a series of mutant NS1A proteins in which various pairs of amino acids were replaced with two other amino acids, usually two alanines. Fig. 1B shows the mutant NS1A proteins that identified the PKR binding site. The mutant NS1A protein containing alanines at positions 123 and 124 (123/124 mutant) or at positions 126 and 127 (126/127 mutant) do not bind PKR, indicating that amino acids 123–127 of the NS1A protein are required for PKR binding *in vitro*.

The PKR binding site on the NS1A protein is required for the inhibition of PKR activation in virus-infected cells

To establish whether direct binding of PKR to the NS1 protein is responsible for inhibiting PKR activation in infected cells, we generated recombinant influenza A/Udorn/72 viruses that encode NS1A proteins with either the 123/124 or the 126/127 mutation, or as a control, the 120/121 mutation shown in Fig. 1. It should be emphasized that the bases that were mutated to produce these amino acid changes are in the intron that is removed by splicing to produce NS2 mRNA and hence do not change the amino acid sequence of the NS2 protein (Lamb and Krug, 2001). Human A549 cells were infected at a high multiplicity (5 plaque-forming units (pfu)/cell) with these mutant Udorn viruses or with wild-type (wt) Udorn virus. Mock-infected cells served as an additional control. Six hours after infection or mock-infection, cell extracts were analyzed by immunoblots using antibody specific for PKR that

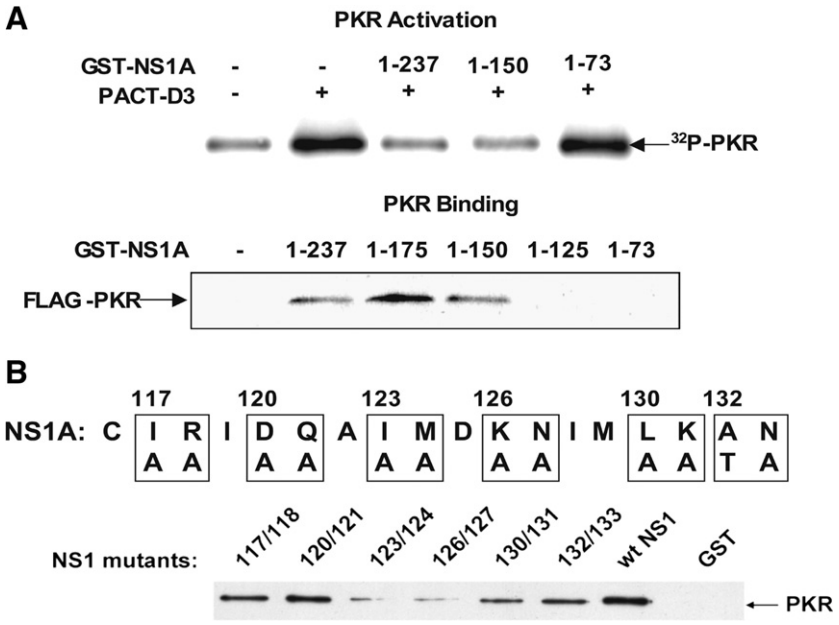

Fig. 1. Binding of PKR to the NS1A protein *in vitro* that results in inhibition of PKR activation requires the NS1A 123–127 amino acid sequence. (A) Top: The effect of GST-NS1A proteins containing the indicated N-terminal sequences on PKR activation *in vitro*. Bottom: The binding of FLAG-tagged PKR to GST-NS1A proteins containing the indicated N-terminal sequences. (B) The binding of FLAG-tagged PKR to GST-NS1A proteins with the indicated amino acid substitutions.

is phosphorylated at threonine 451, the activated form of PKR (Fig. 2A). PKR was activated in cells infected with either the 123/124 or the 126/127 mutant virus, but not in cells infected with the wt or 120/121 mutant virus. Hence, mutation of the NS1A amino acids (123–127) that constitute its PKR binding site results in the activation of PKR during infection. We conclude that the binding of PKR to this NS1A site is necessary and sufficient for the inhibition of PKR activation in influenza A virus-infected cells.

PKR activation in the mutant virus-infected cells results in predicted downstream effects. Immunoblot analysis demonstrated that eIF2-α is phosphorylated at serine 51 in cells infected with either the 123/124 or 126/127 mutant virus (Fig. 2A). To determine the effect of PKR activation on viral protein synthesis, cells infected with the 123/124 mutant virus were labeled with (^{35}S)-methionine and cysteine for 30 min at 4, 6 and 8 h post-infection (Fig. 2B). The rate of viral protein synthesis dramatically decreases after 4 h post-infection, which is not the case in cells infected with wt virus (see below). There was only a slight decrease in the amount of two representative viral mRNAs (M1 and HA mRNAs) after 4 h post-infection, as measured by quantitative RT-PCR (Fig. 2C), indicating that the dramatic decrease in viral protein synthesis after 4 h is not caused by a reduction in viral mRNAs, but rather is at the level of translation. As shown below, the inhibition of translation is due solely to PKR activation.

Mutation of NS1A amino acids 123 and 124 results in a second phenotype, the deregulation of the temporal regulation of viral RNA synthesis

Unexpectedly, despite the dramatic decrease in the rate of viral protein synthesis after 4 h of infection, the 123/124 virus is not attenuated (Fig. 3). The plaques formed by wild-type and 123/124 mutant virus are similar in size, and during multiple cycle growth, i. e., after infection with 0.001 pfu/cell, the 123/124 virus actually replicated slightly faster than the wt virus.

We identified the basis for the lack of attenuation of the 123/124 mutant virus: the 123/124 mutation also adversely affects a previously undocumented function of the NS1A protein, the temporal regulation of viral RNA synthesis. The first evidence for this conclusion came from the difference in the time course of viral protein synthesis in 123/124 mutant virus-infected cells compared to that in wt virus-infected cells (Fig. 4A). The rate of viral protein synthesis at 4 h post-infection was approximately 10-times greater in 123/124 virus-infected cells than in wt virus-infected cells. All viral proteins identifiable by gel electrophoresis were synthesized at high levels at 4 h in 123/124 virus-infected cells. Subsequent to 4 h post-infection, viral protein synthesis in 123/124 virus-infected cells decreased due to PKR activation. In contrast, in wt virus-infected cells the rate of viral protein synthesis gradually increased from 4 to 8 h post-infection, but did not achieve the high rate of viral protein synthesis observed in 123/124 virus-infected cells at 4 h.

As shown in Fig. 4B, the enhanced rate of viral protein synthesis at 4 h after infection with the 123/124 mutant virus is coupled with enhanced synthesis of viral mRNAs and virion RNAs (vRNAs) at times prior to 4 h post-infection. As early as 0.75 h post-infection, the amounts of the M1, HA and NP mRNAs in 123/124 virus-infected cells were significantly higher than those in wt virus-infected cells. From 0.75 to 2 h post-infection the levels of viral mRNAs and vRNAs in 123/124 virus-infected cells increased to approximately 4–6 fold the levels in wt virus-infected cells, and at 3 h was 3–5-fold higher.

Subsequently, similar levels of these virus-specific RNAs were present in 123/124- and wt virus-infected cells because virus-specific RNA synthesis increased at these later times in wt virus-infected cells. The enhanced synthesis of the M and HA vRNAs, and their encoded viral mRNAs and proteins, M1 and HA, at very early times in 123/124 virus-infected cells indicates that the early-to-late switch in viral gene expression that occurs in wt virus-infected cells (Krug et al., 1989; Shapiro et al., 1987; Skehel, 1973) no longer operates in the 123/124 mutant virus-infected cells. In wt virus-infected cells the synthesis of M1 and HA, which are major structural proteins, are synthesized predominately at later times after infection as a result of a later onset in the synthesis of their vRNAs and mRNAs (Krug et al., 1989; Shapiro et al., 1987). In contrast, both early and late vRNAs, viral mRNAs and proteins are synthesized at early times in 123/124 virus-infected cells.

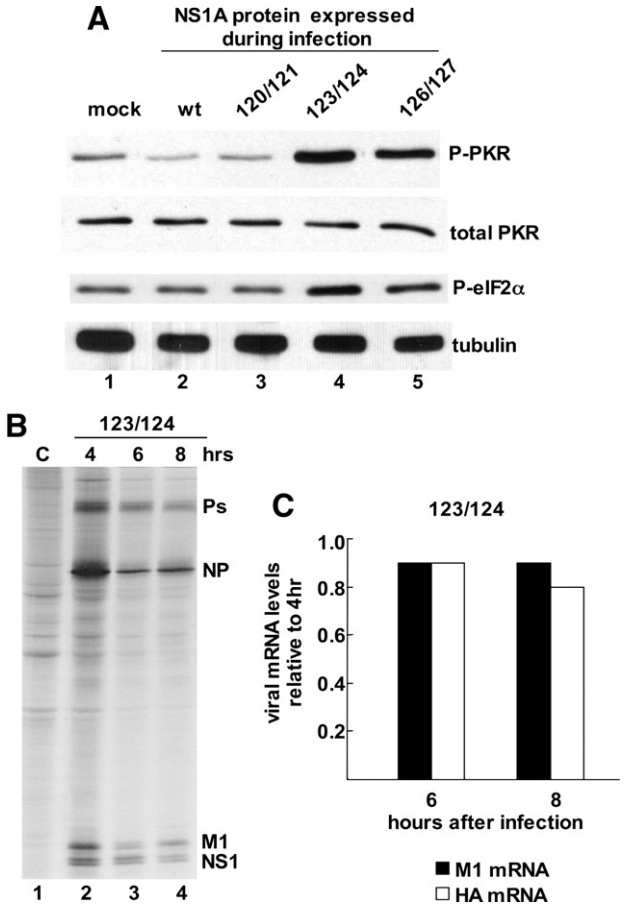

Fig. 2. PKR is activated in cells infected by recombinant influenza A/Udorn/72 viruses expressing NS1A proteins in which amino acids 123/124 or 126/127 are changed to alanines. (A) At 6 h after mock infection or infection with the wt or indicated mutant virus, cell extracts were analyzed by immunoblots using antibody specific for either phosphorylated PKR, total PKR, phosphorylated eIF2α, or tubulin (gel loading control). Antibodies specific for PKR phosphorylated at threonine 445 and for eIF2α phosphorylated at serine 51 were purchased from Cell Signaling. (B) The rate of viral protein synthesis at the indicated times after infection with the 123/124 mutant virus. ^{35}S-labeled proteins were resolved by SDS-polyacrylamide gel electrophoresis. (C) The relative amounts of the M1 and HA viral mRNAs at 4, 6 and 8 h after infection with the 123/124 mutant virus.

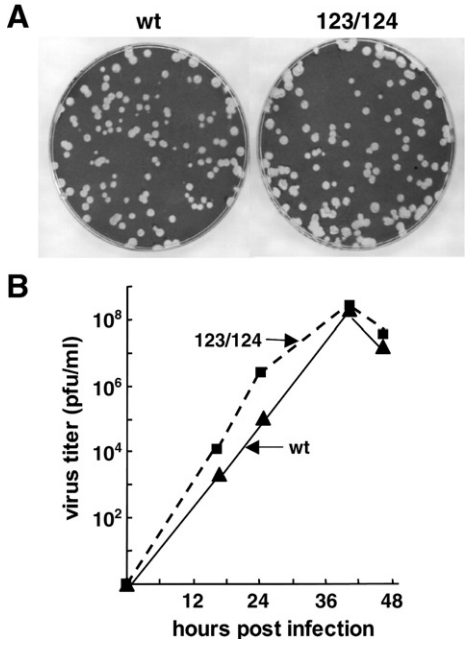

Fig. 3. The 123/124 mutant virus is not attenuated. (A) Plaque sizes of the wt and 123/124 mutant virus. (B) Multiple cycle growth curves of the wt and 123/124 mutant virus.

Deregulation of temporal regulation of viral RNA synthesis also occurs in mouse PKR knockout cells infected with the 123/124 mutant virus

To determine whether PKR activation and the deregulation of the time course of viral RNA synthesis are coupled, we used mouse cells lacking PKR (Yang et al., 1995). In mouse PKR−/− cells, inhibition of viral protein synthesis no longer occurs in cells infected with the 123/124 mutant virus (Fig. 5A, compare lanes 15 and 16 to lanes 8 and 9), verifying that protein synthesis inhibition in 123/124 virus-infected cells is caused solely by PKR activation. In addition, in PKR−/− cells the synthesis of viral proteins, including late proteins, occurs at a substantially greater rate in 123/124 virus-infected cells than in wt virus-infected cells (compare lanes 14–16 to lanes 11–13). For example, viral protein synthesis, including the synthesis of the late M1 protein, is readily detected at 2 h after infection in 123/124 virus-infected cells, whereas viral protein synthesis is barely detectable at 2 h in wt virus-infected cells. Enhanced viral protein synthesis in 123/124 virus-infected PKR−/− cells is coupled to enhanced viral mRNA synthesis at early times after infection: the amounts of the M1 and NP mRNAs in 123/124 virus-infected cells at 2 h post-infection were 5–6 times higher than in wt virus-infected cells (Fig. 5B). These results demonstrate that the two phenotypes resulting from the 123/124 mutation in the NS1A protein, PKR activation and deregulation of the time course of viral RNA synthesis, are not coupled.

Discussion

In this study we use a genetic approach to elucidate the mechanism by which PKR activation is inhibited in influenza A

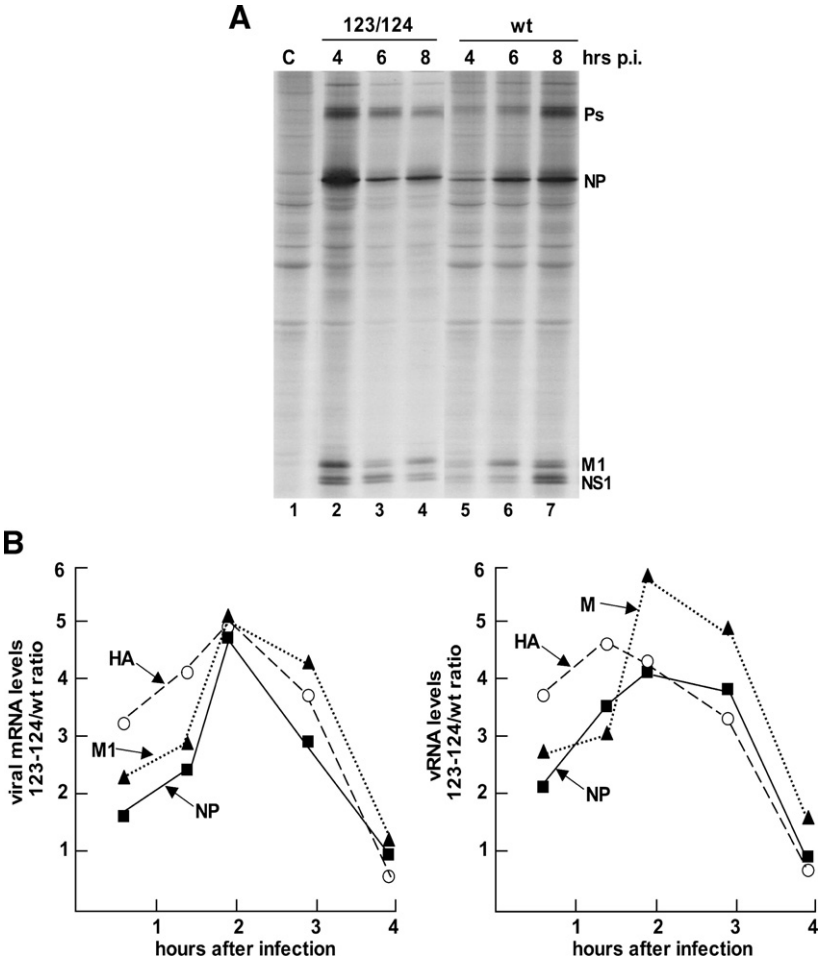

Fig. 4. The wt temporal regulation of viral RNA synthesis is absent in cells infected by the 123/124 mutant virus. (A) Comparison of the rate of viral protein synthesis at the indicated times after 123/124 virus infection with the rate at these time points after wt virus infection. The results of lanes 2–4 of Fig. 2B are reshown here in lanes 5–7. (B) Left: The amounts of the viral HA, M1 and NP mRNAs during the first 4 h after 123/124 virus infection relative to the amounts of these mRNAs during this time period after wt virus infection. Right: The amounts of the HA, M and NP vRNAs during the first 4 h after 123/124 virus infection relative to the amounts of these vRNAs during this time period after wt virus infection. M vRNA encodes both the M1 and M2 viral proteins.

virus-infected cells. We show that direct binding of PKR to the NS1A protein *in vitro* that results in inhibition of PKR activation requires the NS1A 123–127 amino acid sequence, and that PKR is activated in cells infected with a recombinant virus expressing a NS1A protein in which amino acids 123/124 or 126/127 are mutated. We conclude that binding of PKR to the 123–127 sequence of the NS1A protein is necessary and sufficient for the inhibition of PKR activation in influenza A virus-infected cells. We did not find any evidence that a host cell factor, such as $p58^{IPK}$ (Melville et al., 1999), protects PKR from activation in cells infected by these two influenza A mutant viruses. It is therefore likely that inhibition of PKR activation in influenza A virus-infected cells is mediated solely by the viral NS1A protein. The binding of the NS1A protein to PKR, specifically to its N-terminal 230 amino acid region (Li et al., 2006a), would be expected to prevent PKR from undergoing the conformational change that is required for its activation (Li et al., 2006b). In addition, it is possible that the NS1A protein imports the bound PKR to the nucleus, thereby sequestering it from its eIF2-α substrate, as has recently been reported for the TRS1 protein of human cytomegalovirus (Hakki et al., 2006).

Other viruses employ different strategies to inhibit the antiviral action of PKR. For example, the ICP34.5 protein of herpes simplex-1 binds to a specific cellular protein phosphatase, thereby promoting its ability to dephosphorylate eIF2-α (Leib et al., 2000). Other viruses encode proteins that serve as pseudo substrates for activated PKR, and thus spare eIF2-α from phosphorylation (Davies et al., 1993). Some viruses target the activation of PKR by dsRNA by several mechanisms. For example, adenovirus encodes a small RNA, VAI RNA, which binds to the dsRNA-binding domain of PKR without activating it, thereby blocking activation by dsRNA (Mathews and Shenk, 1991). Several viruses encode RNA-binding proteins that sequester dsRNA away from PKR (Chang et al., 1992; Imani and Jacobs, 1988). In contrast, the RNA-binding domain of the NS1A protein of influenza A virus, which has a low affinity for dsRNA (Chien et al., 2004), is not able to sequester dsRNA away from PKR in infected cells (Li et al., 2006b; Min and Krug, 2006).

Our results also reveal that the NS1A protein mediates temporal control of viral RNA synthesis in influenza A virus-infected cells. Gene expression in influenza A virus-infected

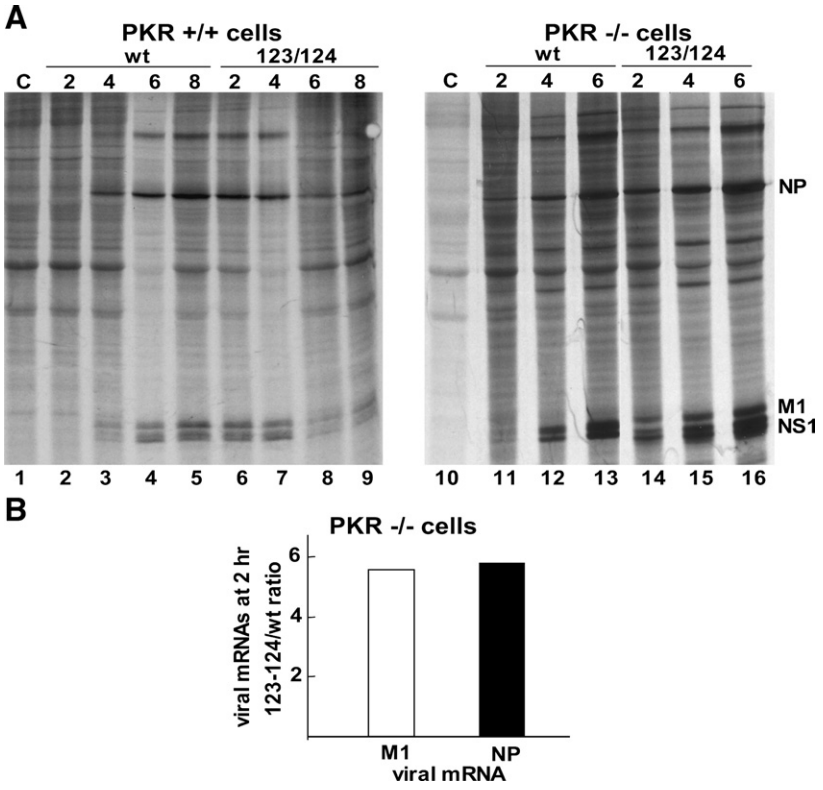

Fig. 5. PKR activation and deregulation of the time course of viral RNA synthesis are not coupled. (A) The time course of viral protein synthesis in mouse PKR+/+ and PKR−/− cells after infection with either wt virus or 123/124 virus at a multiplicity of infection of 5 pfu/cell. (B) The amounts of the M1 and NP mRNAs at 2 h after infection of PKR−/− cells with the 123/124 virus relative to the amounts of these two mRNAs at 2 h after wt virus infection.

cells has been shown to be divided into an early and a late phase (Krug et al., 1989; Shapiro et al., 1987; Skehel, 1973). During the early phase the NS and NP vRNAs are selectively replicated and transcribed, whereas during the late phase all vRNAs, including the NS and NP vRNAs, are replicated and transcribed at a high rate. The mechanism of this temporal regulation of influenza virus RNA synthesis has remained a mystery for approximately 20 years (Krug et al., 1989; Shapiro et al., 1987). Our results provide the first insight into this mechanism by demonstrating that amino acid changes at a specific position in the NS1A protein (amino acids 123 and 124) result in the deregulation of the time course of viral RNA synthesis: both early and late vRNAs, viral mRNAs and proteins are synthesized at high levels at very early times after infection. Deregulation also occurs in mouse PKR−/− cells, demonstrating that this phenotype is independent of PKR activation. The most likely interpretation of our results is that the NS1A protein, via a site that includes amino acids 123 and 124, functionally interacts with the viral polymerase to mediate temporal regulation of viral RNA synthesis during virus infection. This conclusion is consistent with previous studies that suggested a role for the NS1A protein in viral RNA synthesis (Falcon et al., 2004; Marion et al., 1997; Scholtissek and Spring, 1982; Shimizu et al., 1994). Although our genetic experiments show that there is a functional interaction between the NS1A protein and the viral polymerase, other experimental approaches are needed to elucidate the mode of this interaction. For example, does the NS1A-polymerase functional interaction involve direct binding of the NS1A protein to the viral polymerase? Does the NS1A protein suppress vRNA and/or viral mRNA synthesis at early times or enhance synthesis at late times? Is this functional interaction responsible for the selective replication and transcription of the NS and NP vRNAs at early times after infection?

As shown here, amino acids 123 and 124 of the NS1A protein mediate two different functions in influenza A virus-infected cells: binding of PKR that results in inhibition of its activation; and functional interaction with the viral polymerase that results in deregulation of the time course of viral RNA synthesis. It is therefore not surprising that the amino acids at these two positions (I123 and M124) in the NS1A protein, which are at the surface of the protein in a loop between two β-sheets (Bornholdt and Prasad, 2006), are highly conserved (approximately 95%) in human influenza A viruses (Macken et al., 2001). PKR binding and functional interaction with the polymerase would not be expected to compete for this site on the NS1A protein because these two interactions most likely occur at two different locations in the cell. PKR is located in the cytoplasm (Vattem et al., 2001; Wu et al., 1998; Zhu et al., 1997), where it would be expected to bind to NS1A proteins prior to their import into the nucleus. In contrast, the viral RNA polymerase that carries out viral RNA synthesis is located in the nucleus (Krug et al., 1989), where it would be expected to interact with NS1A proteins, which are also predominately localized in the nucleus (Greenspan et al., 1988; Min and Krug, 2006). Our identification of two new functional interactions of

the NS1A protein highlights the participation of the highly expressed NS1A protein in multiple functions in influenza A virus-infected cells.

Materials and methods

In vitro PKR assays

To generate C-terminal truncated NS1A proteins, PCR mutagenesis was used to introduce stop codons at the indicated positions in DNA encoding the NS1A protein. PCR mutagenesis was also used to substitute alanines (or in one case, a threonine) for the indicated wild-type amino acids in the NS1A protein. Mutated and wild-type DNA were inserted into the unique *Bam*H1 and *Eco*RI sites of the pGEX-3X plasmid, and GST-NS1A proteins were produced in BL21 *E. coli* and purified by glutathione Sepharose chromatography (Nemeroff et al., 1995). To assay binding of NS1A proteins to PKR *in vitro*, equimolar amounts of the indicated GST-NS1A protein was mixed with a cell extract containing FLAG-tagged PKR, followed by glutathione Sepharose chromatography and immunoblots using FLAG monoclonal antibody (Li et al., 2006a). For the *in vitro* PKR activation assays, V5-tagged PKR expressed in 293T cells was immunoprecipitated with anti-V5 monoclonal antibody (Li et al., 2006a). The immobilized PKR (~15 nM) was first incubated with the indicated GST-NS1A protein (270 nM) for 5 min at 4 °C, followed by the addition of the PKR activator (purified PACT domain 3, 10 nM) and (γ-^{32}P) ATP. After incubation for 30 min at 30 °C, the amount of ^{32}P-labeled PKR was determined by SDS-polyacrylamide gel electrophoresis and autoradiography.

Cells and viruses

The procedures for plaque assays in MDCK cells and for growth of influenza A/Udorn/72 virus in MDCK and A549 cells have been described (Min and Krug, 2006). Recombinant Udorn influenza viruses expressing mutant NS1A proteins were generated using the 12-plasmid transfection procedure (Min and Krug, 2006; Noah et al., 2003; Takeda et al., 2002). The pHH21 plasmid encoding NS genomic RNA contained the indicated mutations in the NS1A reading frame generated by PCR mutagenesis. All eight genome RNA segments of recombinant viruses were sequenced to verify that the only mutation in the recombinant viruses was the introduced NS mutation. Mouse PKR+/+ and PKR−/− cells were kindly provided by Bryan Williams, and were infected with influenza A/Udorn/72 virus under the same conditions used for A549 cells.

Measurement of influenza viral mRNAs and vRNAs by real-time quantitative RT-PCR

RNA was isolated from infected cells by using the Trizol reagent at the indicated times after infection of A549 cells. For each sample, 1 μg of total RNA, which corresponds to equal cell equivalents, was reverse transcribed using either an oligo(dT) primer (for viral mRNAs) or a primer specific for the common 3′ ends of influenza vRNAs. The amounts of particular viral mRNAs and vRNAs were determined using the TaqMan Gene Expression Assay (Applied Biosystems) with 5′ and 3′ primers specific for the particular viral mRNA or vRNA and a 6-carboxyfluorescein dye-labeled TaqMan MGB (minor groove binder) internal probe (Twu et al., 2006). Real-time PCR analysis was carried out using the Perkin-Elmer/Applied Biosystems 7900HT sequence detector.

Acknowledgments

This investigation was supported by the National Institutes of Health grants AI-11772 (RMK) and CA-68782 (GCS). We thank Gregory Peters for advice and help with the PKR activation assay.

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