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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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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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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