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The effect of the vaccinia K1 protein on the PKR-eIF2α pathway in RK13 and HeLa cells

Kristen L. Willis[a], **Samir Patel**[a], **Yan Xiang**[b], and **Joanna L. Shisler**[a,*]

[a]Department of Microbiology, College of Medicine, University of Illinois at Urbana-Champaign, 601 S. Goodwin Avenue, Urbana IL 61801

[b]Department of Microbiology and Immunology, University of Texas Health Science Center and San Antonio, 7703 Floyd Curl Drive, San Antonio TX 78229

Abstract

Activated PKR protein regulates downstream anti-viral effects, including inhibition of translation. Thus, many viruses encode proteins to inhibit PKR. Here, we provide evidence that the vaccinia virus K1 protein, a host range protein, possesses this function. First, the expression of the wild-type K1 protein was necessary to inhibit virus-induced eIF2α phosphorylation, an indirect measure of PKR activation, in RK13 and HeLa cells. Second, virus-induced eIF2α phosphorylation no longer occurred in PKR-deficient HeLa cells, suggesting PKR was responsible for vaccinia virus-induced eIF2α modification. Third, in normal HeLa cells, K1 protein expression also prevented virus-mediated PKR phosphorylation (activation). Residues in the C-terminal portion of the ANK2 region of K1 were identified as necessary for this inhibitory phenotype. Interestingly, mutant viruses that failed to inhibit PKR activation, such as S2C#2, also did not replicate in HeLa cells, suggesting that K1's inhibition of PKR was required for a productive infection. In support of this theory, when PKR was absent from HeLa cells, there was a modest restoration of viral protein synthesis during S2C#2 infection. However, the increased protein synthesis was insufficient for a productive infection.

Keywords

vaccinia; PKR; eIF2α; K1L; host-range

Introduction

Vaccinia virus, a member of the poxvirus family, has historically been used as a vaccine to protect against smallpox, a disease caused by the closely related variola virus (Moss, 2007). Like all poxviruses, vaccinia virus is a complex virus that codes for approximately 200 proteins (Moss, 2007). Unique to this family of DNA viruses is a replication cycle that occurs in the cytoplasm of its host cell. As such, poxviruses produce many proteins to alter the cellular environment to allow for viral gene expression, DNA replication and morphogenesis (Moss,

*Corresponding author. B103 Chemical and Life Sciences Building, 601 S. Goodwin Avenue, Urbana, IL 61801. Phone: 217-265-6450; Fax: 217-244-6697; jshisler@illinois.edu.

2007). Identification of such mechanisms gives insight into the cellular anti-viral responses that must be inactivated for a productive infection.

The down-regulation of protein synthesis is a powerful cellular mechanism to prevent virus replication. One such protein to mediate this event is eukaryotic translation initiation factor 2 (eIF2α)(Proud, 2005). In normal cells, eIF2α remains unphosphorylated and therefore inactive. Cellular proteins identified to phosphorylate eIF2α include PKR, PERK, GCN2 and HRI (Proud, 2005). Once eIF2α is phosphorylated on serine residue 51, eIF2α prevents translation initiation, resulting in a dramatic decrease in protein synthesis (Proud, 2005).

In virus-infected cells, PKR is commonly the mediator of eIF2α phosphorylation (Garcia, Meurs, and Esteban, 2007). To inhibit this antiviral response, poxviruses encode proteins that inhibit the PKR-eIF2α phosphorylation pathway. For example, the vaccinia virus E3 protein binds to dsRNA or to PKR to prevent PKR activation (Rivas et al., 1998; Romano et al., 1998; Sharp et al., 1998; Shors et al., 1997). The cowpox virus CP77 (CHOhr) protein also inhibits PKR activation during poxvirus infection (Hsiao et al., 2004). Since CP77 lacks RNA binding domains, it likely utilizes an inhibitory mechanism distinct from the E3 mechanism. Yet another vaccinia protein, C7, prevents PKR phosphorylation (Meng, Chao, and Xiang, 2008), thereby inhibiting eIF2α phosphorylation (Najera et al., 2006).

Poxviruses possess host-range genes, a family of genes whose products are necessary for a productive virus infection(Werden, Rahman, and McFadden, 2008). The vaccinia virus K1 protein is one such host-range factor (Perkus et al., 1990). Infection of rabbit kidney epithelial RK-13 cells in the absence of the K1L ORF results in rapid inhibition of viral and cellular protein synthesis. The cowpox virus CP77 protein, another host-range product, is a functional homolog for K1L, and substitutes for the K1 protein to restore vaccinia virus replication in RK13 cells (Ramsey-Ewing and Moss, 1996). For human cells, such as the human cervical HeLa cell line, the K1L ORF is also required for virus replication (Perkus et al., 1990). In contrast to RK13 cells, either the non-homologous vaccinia C7L or the cowpox CP77 ORFs can substitute for the K1L ORF to allow for a productive infection in HeLa cells (Perkus et al., 1990).

How the K1, C7 and CP77 host-range molecules create an environment conducive for virus replication is unclear. An attractive hypothesis is that these proteins prevent PKR activation to allow for viral protein synthesis and viral production. However, the CP77 protein's function to inhibit PKR activation does not completely rescue virus replication (Hsiao et al., 2004), suggesting that additional mechanisms are required to ensure virus replication. It was unknown if the K1 protein, like other host-range proteins, affected PKR activation and if such a function was responsible for its host-range function. To test this, the effect of the K1 protein on eIF2α phosphorylation was assessed during virus infection, under conditions in which the K1 protein was required for replication in RK13 and HeLa cells. Using this strategy, we identified a novel eIF2α inhibitory function for the K1 protein. Further analysis showed that PKR activation was responsible for eIF2α phosphorylation during vaccinia infection, with the K1 protein inhibiting PKR activation as its mechanism for function. The absence of PKR during infection partially restored viral protein synthesis, but did not allow for plaque formation. Thus, PKR activation may be one of many cellular factors responsible for inhibiting viral protein synthesis.

Results

The K1 product is required and sufficient to inhibit virus-induced eIF2α phosphorylation in RK13 cells

The vaccinia K1 product is required for a productive vaccinia infection of RK13 cells (Perkus et al., 1990). The cowpox virus CP77 protein can compensate for K1 during infection of RK13

cells, restoring replication proficiency of vaccinia viruses lacking the K1L ORF (Ramsey-Ewing and Moss, 1996). Since the CP77 product was reported to possess eIF2α inhibitory function (Hsiao et al., 2004), we queried if the K1 product affected this event. To this end, RK13 cells were infected with viruses containing or lacking the K1L ORF, and cytoplasmic extracts were examined by using immunoblotting, incubating blots with antibodies that recognize eIF2α phosphorylated at serine 51 (p-eIF2α).

As shown in Figure 1A, infection of cells with the wild-type Western Reserve (WR) strain of vaccinia virus prevented eIF2α phosphorylation, as observed by low levels of p-eIF2α. In contrast, p-eIF2α levels were increased in cells infected with ΔK1L, a mutant WR virus that lacks the K1L ORF (Shisler and Jin, 2004), inferring that the presence of K1 proteins prevented virus-induced eIF2α phosphorylation. The highest amount of p-eIF2α present during infection, as detected by an increase in band intensity, occurred at 3 h post-inoculation (pi), and this form was detectable at all times tested. Treatment of mock-infected cells with thapsigargin, a known inducer of p-eIF2α (Prostko et al., 1995), resulted in the presence of a band with the same mobility. The relative quantities of unmodified eIF2α for each sample remained fairly constant, as measured by immunoblotting with antibodies recognizing only the native (unphosphorylated) form of eIF2α. Thus, the differences in p-eIF2α levels were not due to protein degradation. Similar results were observed if rabbit kidney epithelial LLC-RK1 cells or rabbit lung fibroblast R9AB cells were utilized instead (data not shown), indicating that the K1 protein's inhibitory effect occurred in several cell lines. However, p-eIF2α was not detected in ΔK1L–infected human kidney fibroblast 293 cells or mouse fibroblast NIH3T3 cells, inferring that the effect of the K1 protein was cell-type specific.

Modified vaccinia Ankara (MVA) is an attenuated vaccinia virus strain lacking a wild-type K1L ORF(Antoine et al., 1998). Thus, it was not surprising to observe eIF2α phosphorylation in lysates from MVA-infected RK13 cells, with p-eIF2α detected as early as 1 h pi (Figure 1B). eIF2α phosphorylation was long-lasting since this modified form of eIF2α was still detected in cells collected at 6 h pi. The re-insertion of a wild-type K1L ORF into MVA (MVA/K1L) resulted in a virus that no longer triggered eIF2α phosphorylation, suggesting that the K1L ORF was sufficient to prevent p-eIF2α in RK13 cells. As with Figure 1A, unmodified eIF2α levels in immunoblots were similar for all samples, inferring that the differences in p-eIF2α levels were not due to inadequate levels of proteins in each sample. Similar results were also observed during infection of either LLC-RK1 or R9AB cells (data not shown).

Mutations in the ankyrin repeat 2 affects the ability of the K1 product to inhibit eIF2α phosphorylation in RK13 cells

The K1 protein possesses 6 ankyrin (ANK) repeats, motifs important for protein-protein interactions (Meng and Xiang, 2006; Sedgwick and Smerdon, 1999). Substitution mutations of residues in the K1 ANK repeats 1, 2, 3 or 5 results in a nonproductive infection of RK13 cells (Meng and Xiang, 2006), inferring that these residues are critical for K1's molecular function. To assess if these same residues were important for K1's eIF2α inhibitory function, p-eIF2α was detected in lysates from cells infected with viruses expressing mutant K1 proteins. Studies were focused on mutations in the K1 ANK2 region, a region in which discrete substitution mutations had identified residues in the ANK2 region necessary for virus replication (Meng and Xiang, 2006).

Five recombinant viruses were utilized for this study. For this set of viruses, wild-type or mutant K1L ORFs were stably introduced into a WR-based virus lacking both the C7L and K1L ORFs (Meng and Xiang, 2006). Virus vC7L^{-}K1L^{+} contains a reinserted wild-type K1L ORF possessing a V5 epitope tag (Meng and Xiang, 2006). The S2N and S2C viruses, in contrast, express V5 epitope-tagged K1 proteins with mutations in the N or C terminus of ANK2, respectively (Meng and Xiang, 2006). S2C#2 and S2C#3 possessed mutations in the C-terminal

region of ANK2, with S2C#2 containing mutations at residues 81, 82 and 84, and S2C#3 containing mutations at residues 86–88 (Meng and Xiang, 2006).

As would be expected, vC7L^{-}K1L^{-} infection rendered the same phenotype as ΔK1L infection of RK13 cells: eIF2α was phosphorylated under these conditions (data not shown). In RK13 cells, the C7L ORF was not required to inhibit eIF2α phosphorylation: p-eIF2α levels were comparable in cells infected with a virus lacking only the C7L ORF (vC7L^{-}K1L^{+}) versus those infected with WR (Figure 2A and 2B), and were lower than those detected in ΔK1L-infected cells. Expression of a mutant S2C K1 protein, in which nine conserved and non-conserved amino acid substitution mutations were introduced into the C-terminal half of the K1L ANK2 region (Meng and Xiang, 2006), resulted in increased peIF2α levels, indicating that these residues were important for K1 function. P-eIF2α remained detectable if cells were instead infected with the S2C#2 virus, a virus in which only ANK2 residues 81, 82 and 84 were substituted (Meng and Xiang, 2006), indicating the importance of these three residues in preventing eIF2α phosphorylation. In contrast, p-eIF2α levels were greatly decreased in cells infected with S2C#3, in which residues 86–88 are mutated (Meng and Xiang, 2006), indicating that these C-terminal residues were not critical for the K1 inhibitory function. Interestingly, amino acids in the ANK2 N-terminus were also important for inhibitory function since p-eIF2α was detected in S2N–infected cells (Figure 2B). When comparing these results to residues important for K1's host-range function (Meng and Xiang, 2006), the same mutations that altered the K1 host-range function also altered the K1 function of preventing eIF2α phosphorylation in RK13 cells. Unmodified eIF2α was present in similar amounts for each immunoblotted sample, indicating that the variation observed in p-eIF2α levels was not due to uneven protein loading of gels.

Virus-induced eIF2α phosphorylation occurs despite vaccinia E3 protein expression

The K1L ORF is required for a productive vaccinia infection of RK13 cells (Perkus et al., 1990). If the K1L ORF is absent, there is a reduction in viral and cellular protein synthesis, with little nascent protein synthesis detected at 2 h pi (Ramsey-Ewing and Moss, 1996). Under this condition, there may be little to no expression of the vaccinia E3 product, a protein that binds to double-stranded RNA to prevent PKR activation and subsequent eIF2α phosphorylation (Chang, Watson, and Jacobs, 1992). Thus, one concern was that eIF2α phosphorylation in ΔK1L- or S2C#2-infected RK13 cells was due to the absence of E3 proteins, and not to an inherent inhibitory biochemical property of the K1 product.

To investigate this possibility, E3 protein levels were detected in virus-infected cells by using immunoblotting, probing membranes with antiserum recognizing the E3 protein (Weaver et al., 2007). E3 proteins were synthesized and stable during RK13 infection since E3 proteins were detected in all infected cells at all times measured pi, as was evidenced by the presence of a 25-kDa band (Figure 3). Thus, it was unlikely that virus-mediated eIF2α phosphorylation was due to the absence of E3 proteins. Other vaccinia early proteins (M2 and J3) were also detected in ΔK1L-infected cells (data not shown), suggesting that ΔK1L infection allows for the synthesis of other early gene products. In comparison to WR- or vC7L–K1L+-infected cells, it should be noted that E3 proteins were reduced in ΔK1L- and S2C#2-infected RK13 cells (Figure 3). Detection of similar levels of actin for each sample indicated that the differences observed for E3 protein expression was not due to uneven loading of samples.

Wild-type and mutant K1 proteins were expressed in similar levels in virus-infected cells (Figure 3), suggesting that the substitution mutations did not cause gross misfolding of K1 proteins. Whether these mutations resulted in more subtle changes in protein conformation cannot be excluded. However, it should be noted that the residues altered in the S2C#2 construct were predicted to be residues located on the surface of the K1 protein, where the mutations were likely to have the least impact on protein folding (Meng and Xiang, 2006). The K1

products from vC7L^{-}K1L^{+}-infected cells possessed a higher mobility than those from WR-infected cells, reflecting the presence of a V5 epitope tag (Meng and Xiang, 2006). Mutant S2C#2 proteins, which did not prevent eIF2α phosphorylation (Figure 2), were also detected at all times post-inoculation. Similar to a previous report, this V5 epitope-tagged mutant protein possessed a higher molecular mobility than its wild-type counterpart (Meng and Xiang, 2006).

As shown in Figure 3, E3 protein levels were decreased in cells infected with either ΔK1L or S2C#2, viruses that mediated eIF2α phosphorylation. To ensure that reduced E3 protein expression was independent of eIF2α phosphorylation, studies were repeated in RK13 cells stably expressing the vaccinia E3L product (RK13-E3L). As an indication that the E3 protein in RK13-E3L cells is functional, poly I:C-induced p-eIF2α levels were decreased as compared to those observed for similarly treated RK13 cells (data not shown). In contrast to RK13 cells, E3 protein expression in ΔK1L- or S2C#2-infected cells remained similar to that observed in infected cells expressing wild-type K1 proteins in RK13-E3L cells (Figure 4). Despite the equal E3 protein levels, p-eIF2α remained higher in ΔK1L- or S2C#2-infected RK13-E3L cells as compared to WR- or vC7L^{-}K1L^{+}-infected RK13-E3L cells, at all times pi measured, suggesting that E3 protein levels did not dictate eIF2α phosphorylation. As a control, regular RK13 cells were infected and processed in parallel with RK13-E3L cells (Figure 4). As would be expected, p-eIF2α levels were increased in regular cells infected with either ΔK1L or S2C#2 compared to WR or vC7L^{-}K1L^{+}, similar to results shown in Figure 2.

The K1 product inhibits virus-induced eIF2α phosphorylation in the human HeLa cell line

Continued studies of the K1 molecular function in RK13 cells were difficult due to the paucity of reagents that are biologically reactive with rabbit proteins. Thus, similar experiments were performed in human cells, in which more reagents are available. The HeLa cell line was chosen for further study since the K1L ORF acts as a host-range gene in this cell line (Perkus et al., 1990). In contrast to RK13 cells, in which removal of the K1L ORF is sufficient to inhibit vaccinia replication, in HeLa cells, the K1L host-range effect is only observed in the absence of the C7L ORF (Perkus et al., 1990). The C7 product was also reported to inhibit eIF2α phosphorylation (Najera et al., 2006). Thus, studies performed in these cells required viruses that lacked the C7L ORF (Meng and Xiang, 2006). Recombinant viruses expressing wild-type or mutant K1 proteins used for the above studies in RK13 and RK13-E3L cells indeed lacked the C7L ORF (Meng and Xiang, 2006), enabling us to evaluate the role of the K1 protein in the absence of C7L gene expression in HeLa cells. Studies comparing p-eIF2α in MVA versus MVA/K1L-infected cells were not performed using HeLa cells. MVA infection of HeLa cells does not result in eIF2α phosphorylation (Najera et al., 2006). Thus, MVA/K1L infection of HeLa cells would result in the same phenotype as MVA-infected cells.

eIF2α phosphorylation was examined in virus-infected HeLa cells, and p-eIF2α was detected by using immunoblotting. Results are shown in Figure 5. In HeLa cells, unmodified eIF2α was present in mock-infected cells or in cells infected with a virus containing the wild-type K1L ORF (vC7L^{-}K1L^{+}). The K1 ANK2 C-terminus was responsible for the inhibitory phenotype, since p-eIF2α was detected in HeLa cells infected with S2C, but not S2N. Of note, infection of HeLa cells with the S2N virus rendered a different eIF2α phenotype than observed with RK13 cells: the mutant S2N protein prevented eIF2α phosphorylation in HeLa cells at 4 h pi (Figure 5) or at 6 h pi (data not shown). In contrast, infection of RK13 cells with S2N no longer inhibited this action (Figure 2B). Upon further analysis of ANK2 C-terminal region, ANK2 residues 81, 82 and 84 were important for inhibiting eIF2α phosphorylation since S2C#2 infection resulted in eIF2α modifications. As with RK13 cells, the presence of p-eIF2α during infection correlated with a replication defective phenotype in HeLa cells (Meng and Xiang, 2006).

Inhibition of virus-induced eIF2α activation in HeLa cells is independent of E3 protein expression

As with RK13 cells, it was important to eliminate the possibility that eIF2α phosphorylation was due to a lack of E3 protein synthesis in virus-infected HeLa cells. Thus, E3 proteins were detected in vaccinia virus-infected HeLa cells by using immunoblotting, similar to experiments shown in Figure 3. Similar to results with RK13 cells, E3 proteins were detected at early and late times post-infection, in all virus-infected cells, suggesting that eIF2α phosphorylation was not due to an absence of E3 proteins (Figure 6). It was noted that E3 protein levels, while detectable, were lowest in cells infected with S2C#2, a virus that did not prevent eIF2α activation in HeLa cells (Figure 6). Although it cannot be ruled out that the decreased E3 protein levels are responsible for eIF2α phosphorylation, data from Figure 3 and Figure 4 would indicate that E3 protein levels are independent of the K1 phenotype in HeLa cells. Mutant and wild-type K1 proteins were detected in fairly similar amounts in infected cells (Figure 6). Thus, the reduction in E3 protein levels did not appear to be due to K1 protein synthesis or stability. As before, the V5 epitope-tagged K1 products expressed in vC7L^{-}K1L^{+}- or S2C#2-infected cells possessed a higher mobility than wild-type K1 products expressed during WR infection (Figure 6).

The PKR protein is responsible for activating eIF2α in vaccinia virus-infected cells

Several cellular proteins are known to activate eIF2α, including PKR, PERK, GCN2 and HRI (Proud, 2005). Commonly, PKR is responsible for eIF2α phosphorylation during virus infection, thus we assessed if the PKR protein was responsible for eIF2α activation in vaccinia virus-infected cells. To this end, we assessed virus-induced eIF2α phosphorylation in HeLa cells in which >95% of PKR protein expression is stably silenced (Zhang, Jacobs, and Samuel, 2008). This cell line is referred to as PKR^{kd}. Similar to previous studies (Zhang, Jacobs, and Samuel, 2008), PKR proteins were either undetectable or greatly reduced in lysates from PKR^{kd} cells versus normal HeLa cells, regardless of whether cells were mock-infected or infected (Figure 7). The decrease in PKR proteins did not grossly affect eIF2α protein stability since eIF2α was present in similar amounts in PKR^{kd} versus HeLa cells. P-eIF2α levels were greatly decreased when PKR^{kd} cells were infected with viruses that normally allowed for eIF2α activation in parental HeLa cells, such as S2C#2 and S2C, inferring that PKR proteins were required for virus-mediated eIF2α phosphorylation. Equally important, eIF2α remained unmodified in vC7L^{-}K1L^{+}-, S2C#3-, and S2N-infected PKR^{kd} cells, as would be expected.

The K1 function of preventing virus-mediated PKR activation is not related to its host range function in HeLa cells

It was noticed that only K1-expressing viruses that inhibited eIF2α phosphorylation allowed for a productive infection in RK13 and HeLa cells (Meng and Xiang, 2006). These data suggested that a block in eIF2α phosphorylation, which would allow for translation initiation, is the K1 mechanism for allowing a productive infection. If this is the appropriate mechanism, then PKR-deficient cells should lack a mechanism to phosphorylate eIF2α, and should be permissive for virus replication, regardless of the absence of a functional K1 product. To test this, we detected viral protein synthesis in HeLa cells lacking PKR proteins (PKR^{kd}), comparing the phenotypes to virus growth in normal HeLa cells possessing functional PKR proteins (Fig 8A). The infection of HeLa cells with S2C#2, a virus unable to replicate in HeLa cells (Meng and Xiang, 2006), resulted in the dramatic decrease of viral protein synthesis at 4 and 24 h pi. In PKR^{kd} cells infected with S2C#2, modest levels of protein synthesis were still observed at 4 and 24 h pi, suggesting that the elimination of PKR indeed allowed for some viral protein synthesis. For either cell line, viral protein protein expression was observed during infection with either vC7L-K1L+ or S2C#2. When detecting an individual early (E3) or late (L1) protein under these same conditions, E3 proteins were still detected (Fig. 8B). In contrast,

L1 proteins were no longer detected in S2C#2-infected PKR^{kd} cells, suggesting that the elimination of PKR, while allowing for partial restoration of viral protein synthesis, was not sufficient for allowing robust expression of viral late proteins. Due to the lack of late protein synthesis, no visible plaques were observed in PKR^{kd} monolayers infected with S2C#2 (or S2C) (Fig 8C). The recombinant viruses also express a GFP gene under the control of the p11 late promoter (Meng and Xiang, 2006), allowing for the visualization of individual plaques by fluorescent microscopy. In agreement with data from Figure 8C, no GFP-expressing plaques were observed for PKR^{kd} cells infected with S2C#2 (Fig 8D). The WR, vC7L–K1L+, S2C#3 and S2N viruses maintained their abilities to replicate in PKR^{kd} cells, as evidenced by the presence of microscopic (Fig 8C) and visual (Fig 8D) plaques. GFP-expressing plaques formed during vC7L–K1L+, S2C#3 or S2N infections were noticeably smaller in PKR^{kd} versus normal HeLa monolayers.

The K1 product prevents PKR phosphorylation, an event correlative with its ability to inhibit eIF2α phosphorylation

For PKR to become activated, it forms a dimer, resulting in its auto-phosphorylation (Garcia, Meurs, and Esteban, 2007). We observed that the K1 protein prevented eIF2α phosphorylation. There were two likely mechanisms responsible for this phenotype. First, the K1 protein may interact with eIF2α, thereby preventing activated PKR from phosphorylating eIF2α. Alternatively, the K1 protein may prevent PKR auto-phosphorylation as its mechanism for inhibition. To test the later possibility, we assessed if the K1 product prevented PKR activation. HeLa cells were infected with viruses expressing wild-type or mutant K1 proteins, and PKR activation was assessed by detecting the phosphorylated (activated) form of PKR by using immunoblotting. Wild-type K1L proteins prevented PKR activation, as judged by the absence of p-PKR (Figure 9). This function was also detected in cells infected with mutant viruses that inhibited eIF2α phosphorylation (S2C#3 and S2N), suggesting that the K1 protein affected the ability of PKR to auto-phosphorylate. Conversely, PKR was activated when cells were infected with either S2C#2 or S2C, viruses that no longer inhibited eIF2α phosphorylation. Anti-PKR antiserum that simultaneously recognizes the modified and unmodified PKRs was used to detect similar PKR levels in each well. Interestingly, although activated PKR was present in S2C#2- and S2C-infected cells, a population of inactive PKR proteins was also detected.

Discussion

Here, we report a novel function for the vaccinia K1 protein; it inhibits PKR activation. Since active PKR attenuates protein synthesis, one attractive hypothesis was that the K1 protein, in preventing PKR activation, would allow for virus replication in RK13 and HeLa cells. This model was bolstered by the observation that viruses that allowed PKR or eIF2α activation were not replication competent in HeLa or RK13 cells (Meng and Xiang, 2006), respectively. While PKR-deficient HeLa cells indeed allowed for a partial restoration of viral protein synthesis, these cells remained unable to support the synthesis of the late L1 protein or the replication of S2C#2. Thus, K1's PKR inhibitory phenotype only partially contributes to permissivity in HeLa cells. One conclusion from this study is that there are PKR-independent mechanisms for controlling viral protein synthesis during infection. For example, for translation to occur, capped mRNA must be complexed with eIF4F, a complex that is formed only upon the inactivation of eIF4E binding proteins. Vaccinia virus infection inactivates the 4E-BP1 binding protein to promote eIF4F complex formation. Whether this event is critical to the host-range phenotype is unknown (Walsh et al., 2008).

The E3 and K3 proteins are expressed early in infection, and each inhibit PKR activation (Rivas et al., 1998; Romano et al., 1998; Sharp et al., 1998; Shors et al., 1997). ΔK1L infection of RK13 cells results in a dramatic decrease in early gene translation (Ramsey-Ewing and

Moss, 1996), which would presumably include E3 and K3 protein synthesis. Thus, an initial concern was that the ΔK1L effect in these rabbit cells was indirect, due to a lack of E3 and K3 proteins. However, we found that E3 proteins were expressed in RK13 cells, regardless of the absence of the K1L ORF. Further examination revealed that other early proteins, such as the M2 and J3 products, were also detected in ΔK1L-infected RK13 cells (data not shown), suggesting that the K3 protein would also be synthesized during infection as well. Since early protein synthesis, as measured by the ^{35}S-methionine metabolic labeling of nascent proteins during virus infection, was detected for at least one hour after ΔK1L infection of RK13 cells (Ramsey-Ewing and Moss, 1996), our results were not surprising. Most important, these data indicate that the PKR activation phenotype in ΔK1L–infected RK13 cells is not indirect; i.e., PKR activation in ΔK1L–infected cells is not simply due to a lack of E3, or other known or undiscovered, PKR inhibitory proteins. For HeLa cells, infection with viruses lacking both the K1L and C7L ORFs results in a block in intermediate gene translation (Hsiao et al., 2004). Thus, E3 (and K3) proteins are expected to be synthesized, an event we detect in our system.

We identified that the ANK2 repeat of K1 is responsible for PKR inhibitory function, but cannot rule out that other K1 ANK repeats may also possess inhibitory function. Given that other ANK repeats of K1 are important for its host-range function (Meng and Xiang, 2006), one prediction is that mutations in other ANK repeats would be expected to affect K1's PKR inhibitory function. We purposefully limited our studies to the ANK2 region since the smallest number of amino acid substitutions was made in this repeat (Meng and Xiang, 2006). Further, two of these constructs (S2C#2 and S2C#3) yielded different host range phenotypes, characteristics that would enable us to easily assess if PKR activity and host range phenotype were related.

ANK repeats are 33 residues in length, and the motif is defined as a β-hairpin-helix-loop-helix structure (Sedgwick and Smerdon, 1999). These motifs are found in proteins in nearly all phyla, and have myriad functions (Sedgwick and Smerdon, 1999). Diversity in binding partners for ANK-containing proteins is attained by variation in the surface residues of ANK repeats, and by the number of ANK repeats stacked together to form a stable structure (Sedgwick and Smerdon, 1999). Substitution mutations were originally made in the ANK2 region that would only affect surface residues, affecting potential binding site for K1 interacting partners, but allowing for the ANK repeat to keep its structure, since structure is likely important for its ability to interact with its target protein (Meng and Xiang, 2006). The mutated residues in S2C#2 lie in the outer helix, a potential surface for protein-protein interactions, while the mutations in S2C#3 lie downstream of the outer helix (Meng and Xiang, 2006). Thus, the finding that the S2C#2 virus allowed for PKR activation likely reflects a loss of binding to a target protein. A previous study identified a rabbit homolog of the human cellular ACAP2 protein that interacts with K1 protein in vaccinia-infected RK13 cells (Bradley and Terajima, 2005). Whether K1-ACAP2 interactions are critical for its ability to inhibit PKR activity is an area of future research. It was noticed that the ANK2 regions responsible for PKR inhibition were different for RK13 versus HeLa cells. For RK13 cells, residues in both the N and C terminal portion of ANK2 were necessary for inhibitory function, while residues only in the C terminal portion of ANK2 were important for HeLa cells. These differences may indicate that the K1 protein interacts with a different subset of rabbit versus human proteins.

While activated PKR is best known for its anti-viral effect of inhibiting protein synthesis, it possesses other biological properties that neutralize virus infections (Garcia, Meurs, and Esteban, 2007). For example, PKR also induces apoptosis, a rapid form of cell death that eliminates virus-infected cells (Lee and Esteban, 1994). Whether the K1 protein would prevent PKR-induced apoptosis is unknown. PKR also activates the NF-κB transcription factor (Maran et al., 1994; Yang et al., 1995). Since this cellular transcription factor induced the expression of anti-viral immune molecules, this property of PKR could also inhibit virus replication. To

date, there are multiple vaccinia proteins that inhibit PKR activity, including K1, C7, E3 and K3 (Garcia, Meurs, and Esteban, 2007). For this model system, in which HeLa cells are used, it does not appear that the E3L or the K3L ORF, both of which are present and presumably expressed during virus infection, are involved in virus-mediated PKR activation. These data would argue that molecules in addition to dsRNA, including PACT and Mda7, activate PKR during poxvirus infection. Furthermore, the presence of myriad vaccinia proteins likely reflect the fact that different insults activate PKR.

The CP77, K1 and C7 proteins can functionally complement each other to allow for a productive vaccinia infection in HeLa cells (Perkus et al., 1990). These three proteins also prevent PKR activation (Meng, Chao, and Xiang, 2008) (Hsiao et al., 2004). The K1 and CP77 proteins both possess ankyrin repeats. The CP77 protein also has an F-box motif (Chang et al., 2009), a motif lacking in the K1 protein. In contrast, no obvious F-box or ankyrin motifs are present in C7 proteins. One prediction, then, is that the CP77 and K1 proteins will utilize similar molecular mechanisms to alter PKR activation, while the C7 product will likely utilize a mechanism distinct from CP77 or K1. As shown here, the K1 protein prevents PKR activation, as measured by an inhibition of PKR phosphorylation. The K1 protein lacks an obvious dsRNA binding motif, making it unlikely that K1 functions like the E3 protein, binding to dsRNA as its mechanism to inhibit PKR activation. A simple explanation for K1's molecular mechanism is that the K1 protein binds to PKR, preventing its dimerization and subsequent auto-phosphorylation. Ankyrin repeats, like the ones present in K1, are important for mediating protein-protein interactions (Bork, 1993), making it reasonable to suggest that the K1 protein could form a stable complex with PKR. A second possibility is that the K1 protein indirectly inhibits PKR activation by binding to cellular PKR activating proteins, such as PACT and Mda7 (Garcia, Meurs, and Esteban, 2007; Zhang et al., 2009). Thus, future directions include assessing K1 interactions with the above proteins and assessing if K1 binding function correlates with PKR inhibition.

Materials and methods

Cells and viruses

Human cervical carcinoma HeLa or rabbit kidney epithelial (RK13) cells were obtained from the American Type Culture Collection. RK13 cells stably expressing the vaccinia E3L product (RK13-E3L) were obtained from Dr. Bertram Jacobs (Department of Microbiology, Arizona State University). HeLa cells in which more than 95% of PKR is stably knocked down (PKR^{kd}) were obtained from Dr. Charles Samuel (Department of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara) (Zhang, Jacobs, and Samuel, 2008). These cells stably express short hairpin RNA specific for PKR mRNA. All cell lines were maintained in DMEM containing 10% fetal calf serum (FCS). PKR^{kd} cells were maintained in medium supplemented with 1 μg/ml puromycin (Sigma Aldrich).

The wild-type Western Reserve (WR) strain of vaccinia virus and the attenuated modified vaccinia Ankara (MVA) strain were used in these studies. Virus construct MVA/K1L contains the wild-type K1L ORF and its natural promoter stably inserted into the MVA genome (Shisler and Jin, 2004). Virus ΔK1L was created by replacing the K1L ORF of WR with the *Escherichia coli* gpt gene via homologous recombination (Shisler and Jin, 2004). WR and ΔK1L viruses were amplified in BSC-1 cells, while MVA-based viruses were amplified in chicken embryo fibroblasts (Charles Rivers Laboratories).

Five recombinant viruses containing stably re-introduced wild-type or mutated K1L ORFs were used in these studies: $vC7L^{-}K1L^{+}$, S2N, S2C, S2C #2 and S2C#3, and their construction was described previously (Meng and Xiang, 2006). The parent virus for each of these viruses was $vC7L^{-}K1L^{-}$, in which the C7L and K1L ORFs were deleted from the WR genome. For

the creation of vC7L^{-}K1L^{+}, a cassette containing the V5 epitope-tagged K1L ORF and its natural promoter and an adjacent green fluorescing protein (GFP) gene under control of the P11 late promoter were stably re-inserted into vC7L^{-}K1L^{-} (Meng and Xiang, 2006). Thus, cells infected with these viruses also express GFP. Virus S2N was created similarly, except that ankyrin repeat 2 of the K1L ORF was mutated at residues 63, 67 and 70–75, and then inserted into vC7L^{-}K1L^{-}(Meng and Xiang, 2006). S2C is a virus that codes for a mutated K1 product, in which the ankyrin repeat 2 C-terminal residues 78,79, 82, 83, 85 and 87–90 are mutated (Meng and Xiang, 2006). Viruses S2C#2 and S2C#3 express mutant K1L product in which residues 81,82 and 84 or 86–88 are mutated, respectively (Meng and Xiang, 2006). These recombinant viruses were amplified in Vero cells.

Immunoblot Analysis

Cytoplasmic extracts were collected according to a modified protocol described previously (Shisler and Jin, 2004). Confluent monolayers of cells in 6-well tissue culture dishes were infected with the indicated viruses at an MOI of 10, with an absorption incubation for 60 min in DMEM containing 2.5% FCS. At the times indicated post-infection, cellular monolayers were dislodged from their wells by scraping, and cells were collected by centrifugation (30 sec at 14,000 × *g*). As a positive control for eIF2α phosphorylation, a separate set of mock-infected RK13 cells was incubated in medium containing 10 μM thapsigargin (Sigma-Aldrich) for 4 h before cells were collected by scraping. Cellular pellets were resuspended and lysed in cytoplasmic extraction buffer (CE) that contained the HALT protease inhibitor cocktail (Pierce) for 5 minutes at 4°C. After a brief centrifugation (30 sec at 14,000 × *g*), the resultant supernatants were collected and transferred to new microfuge tubes. The protein concentration of each sample was determined using the bicinchoninic acid (BCA) assay (Pierce). An equal amount of protein (20 μg) from each sample was mixed with 5× non-reducing lane marker (Thermo Scientific) for a final 1× concentration that contained 5% 2-mercaptoethanol (Fisher Scientific), boiled for 5 min, and incubated on ice briefly. Next, proteins were electrophoretically separated by using SDS-12%PAGE. Following electrophoresis, proteins were transferred to a polyvinyl difluoride (PVDF) membrane (Millipore), and membranes were incubated for 1 h in TBS containing 0.05% Tween-20 (TTBS) and 5% non-fat milk. Next, membranes were incubated at 4°C overnight in primary antiserum in TTBS containing 5% non-fat milk. The following primary antibodies were used: mouse monoclonal anti-phospho-eIF2α S51 (1:500; Epitomics), mouse monoclonal anti-eIF2α (1:1,000; Santa Cruz), mouse anti-phospho PKR T446 (1:1,000; Epitomics), mouse monoclonal anti-PKR N-terminus (1:1,000; Epitomics), mouse monoclonal anti-E3L antibody (1:1,000; a gift from Dr. Stuart Isaacs, School of Medicine, University of Pennsylvania)(Weaver et al., 2007), mouse monoclonal anti-L1R antibody (1:1,000; a gift from Dr. Jay Hooper, Virology Division, United States Army Medical Research Institute for Infectious Disease, Fort Detrick, Maryland), rabbit polyclonal anti-K1L antiserum (1:500) or rabbit anti-actin antiserum (1:1,000; Sigma Aldrich). Immunoblots were washed in TTBS and incubated in a TTBS solution containing either goat-anti-rabbit immunoglobulin G (IgG; 1:10,000) or goat-anti-mouse IgG conjugated with horseradish peroxidase (1:10,000; Fischer Scientific). Immunoblots were developed using SuperSignal West chemilluminescence reagents per the manufacturers directions (Pierce).

Detection of GFP-expressing viruses and plaque phenotypes

Confluent monolayers of HeLa or PKR^{kd} cells in 6-well issue culture plates were infected at an MOI of 0.01 PFU/cell of either WR, vC7L^{-}K1L^{+}, S2N, S2C, S2C#2, or S2C#3. At 48 hours post-infection, GFP-expressing plaques were detected and visualized and photographed by using an inverted fluorescence microscope (Olympus, 4× objective). Following the microscopic examination of infected cellular monolayers, supernatants were removed from monolayers, and 2 ml of a 20% ethanol solution containing 1% crystal violet was added to each

well. After a 5 min incubation, the solution was removed and plates were air-dried to visualize virus plaques.

^{35}S-methionine labeling of virus infected cells

Confluent monolayers of HeLa or PKR^{kd} cells in 6-well tissue culture plates were mock-infected or infected at an MOI of 10 PFU/cell with either vC7L–K1L+ or S2C#2. Thirty minutes before harvesting, each cell was washed twice with DMEM lacking methionine and cysteine (DMEMMet-Cys-; Hyclone), and subsequently replenished with 0.5 ml of DMEMMet-Cys- containing 75 μCi EXPRE^{35}S^{35}S protein labeling mix (Perkin Elmer). Cells were harvested at 0.5, 2, 4 and 24 hours post-infection by scraping, and collected by centrifugation. Pelleted cells were lysed in CE buffer as described above. Equal volumes of clarified lysates were analyzed by using 10% SDS-PAGE. Gels were dried in a gel drier, exposed to phosphoimager screens, and images were developed by using phosphoimaging.

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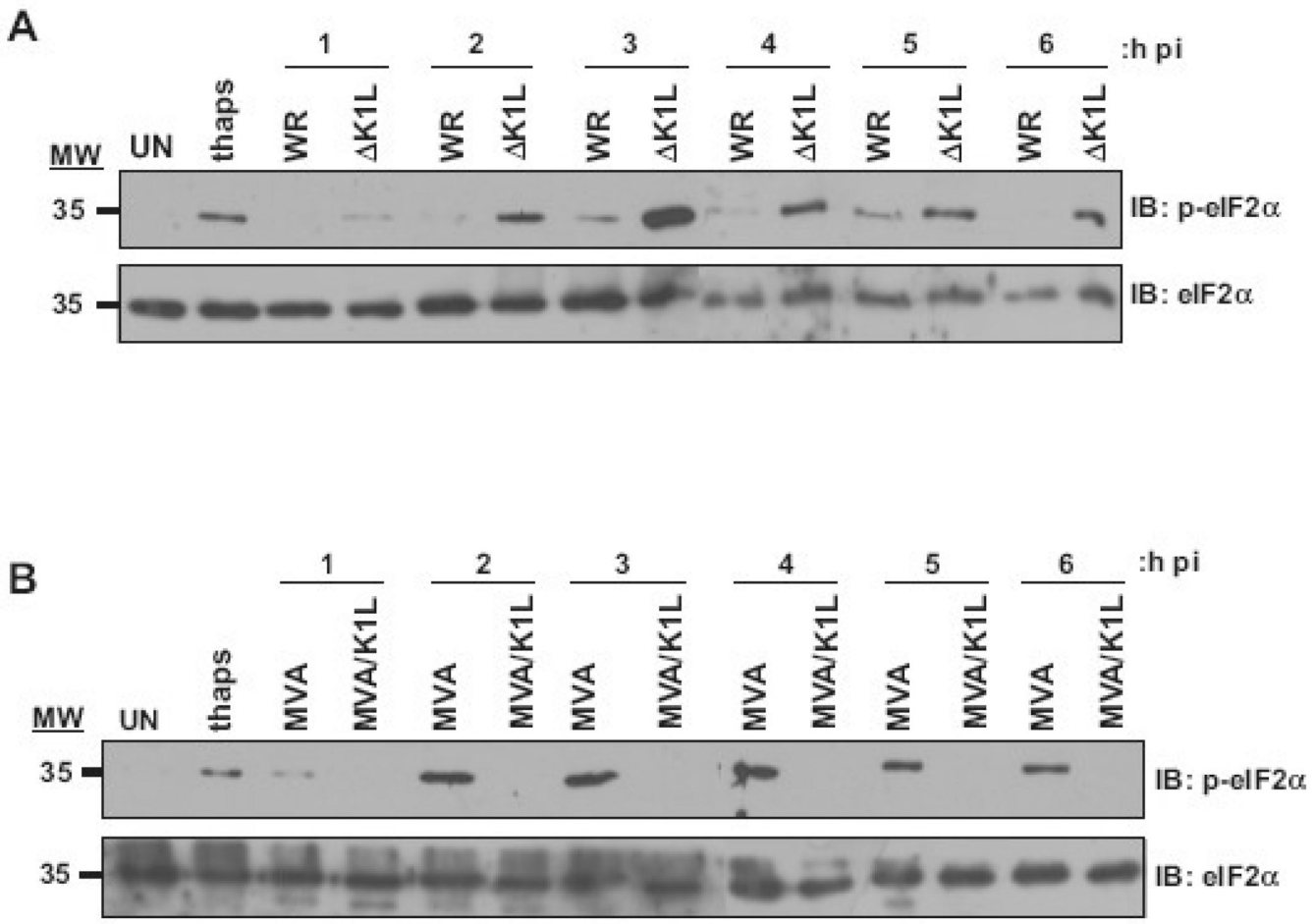


Figure 1. eIF2α phosphorylation in virus-infected RK13 cells

RK13 cellular monolayers were mock-infected (UN) or infected at an MOI of 10 with either (A) WR or ΔK1L, or (B) MVA or MVA/K1L (MOI = 10), and infections proceeded for the indicated times. For both experiments, a separate set of mock-infected RK13 cells were incubated in medium containing thapsigargin (thaps) for 4h as a positive control for eIF2α phosphorylation. Regardless of treatment, cells were collected and lysed in CE buffer. Equal amounts of prepared cytoplasmic extracts (20 μg) were electrophoretically separated by SDS-12% PAGE and transferred to PVDF membranes. Membranes were probed with antiserum recognizing either the phosphorylated (p-eIF2α) or unmodified forms of eIF2α. Molecular weight markers (MW) are indicated.

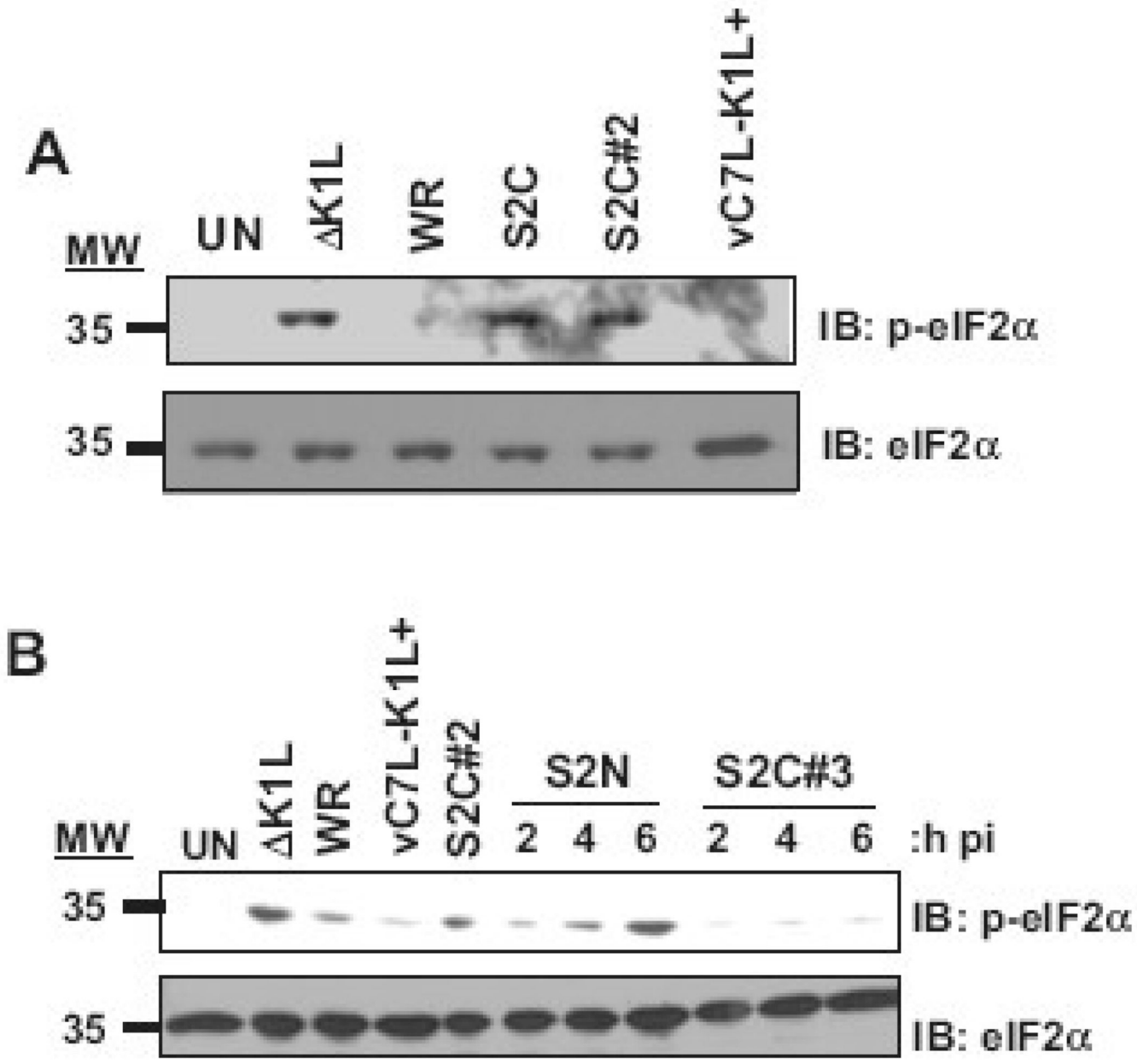


Figure 2. The effect of K1L mutations on eIF2α phosphorylation

(A) RK13 cellular monolayers were mock-infected (UN) or infected at an MOI of 10 with (A) ΔK1L, WR, S2C, S2C#2, or vC7L^{-}K1L^{+} or (B) ΔK1L, WR, vC7L^{-}K1L^{+}, S2C#2, S2N or S2C#3. Unless indicated otherwise, infections proceeded for 4 h pi. Cytoplasmically extracted proteins were separated by using SDS-12% PAGE and then transferred to PVDF membranes. Immunoblots were used to detect phosphorylated (top panel) or unmodified (bottom panel) forms of eIF2α. Molecular weight markers (MW) are indicated.

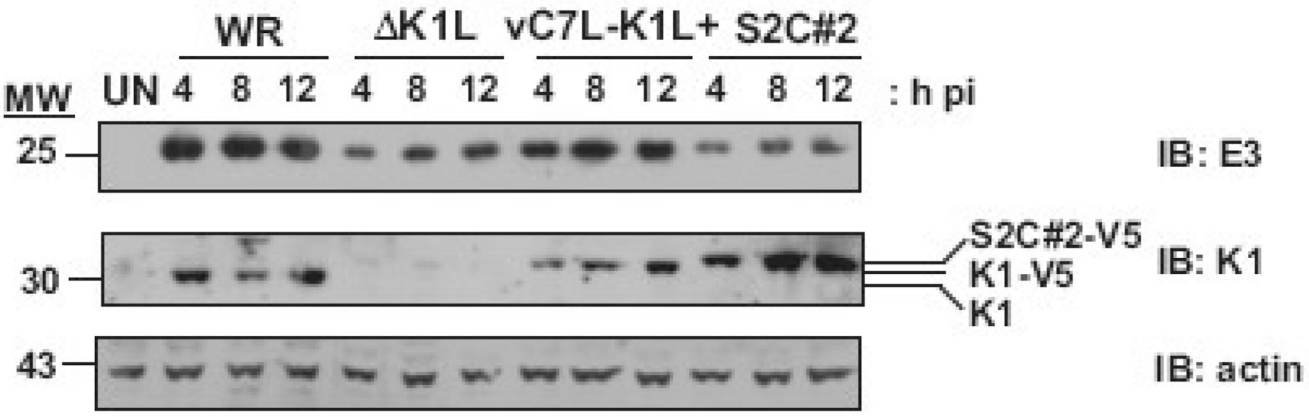


Figure 3. E3 and K1 product levels in virus-infected RK13 cells

Cellular monolayers were mock-infected (UN) or infected with WR, ΔK1L, S2C#2, or vC7L^{-}K1L^{+} (MOI = 10). At 4, 8 or 12 h pi, cells were harvested and immunoblotted lysates were probed with antiserum recognizing the vaccinia E3 product. The same membrane was re-probed for unmodified or V5 epitope tagged K1 products by incubating the immunoblot with anti-K1 antiserum. The mobilities of wild-type or mutant K1 proteins are indicated. Equal protein loading was confirmed by probing the same membrane with anti-actin antiserum. Molecular weight markers (MW) are indicated.

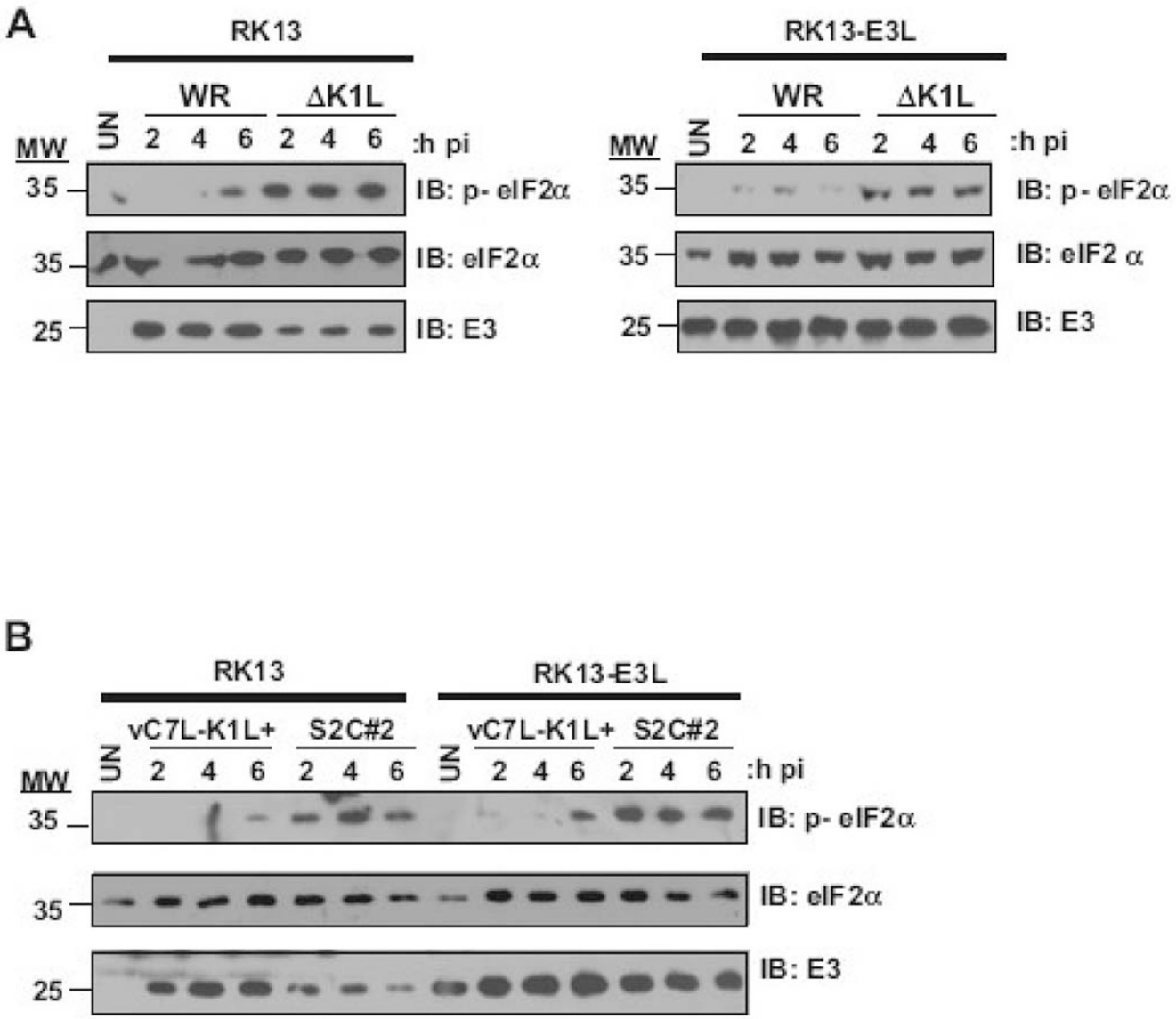


Figure 4. eIF2α phosphorylation in RK13 cells stably expressing the vaccinia E3 product

RK13 or RK13-E3L cellular monolayers were infected with either WR, ΔK1L, vC7L^{-}K1L^{+} or S2C#2 at an MOI of 10 PFU/cell. At 4 h post-infection, cells were harvested, and immunoblotted lysates were incubated with antiserum specific for the active (phosphorylated) form of eIF2α, and then re-probed with antiserum raised against the unmodified form of eIF2α and the vaccinia E3 protein.

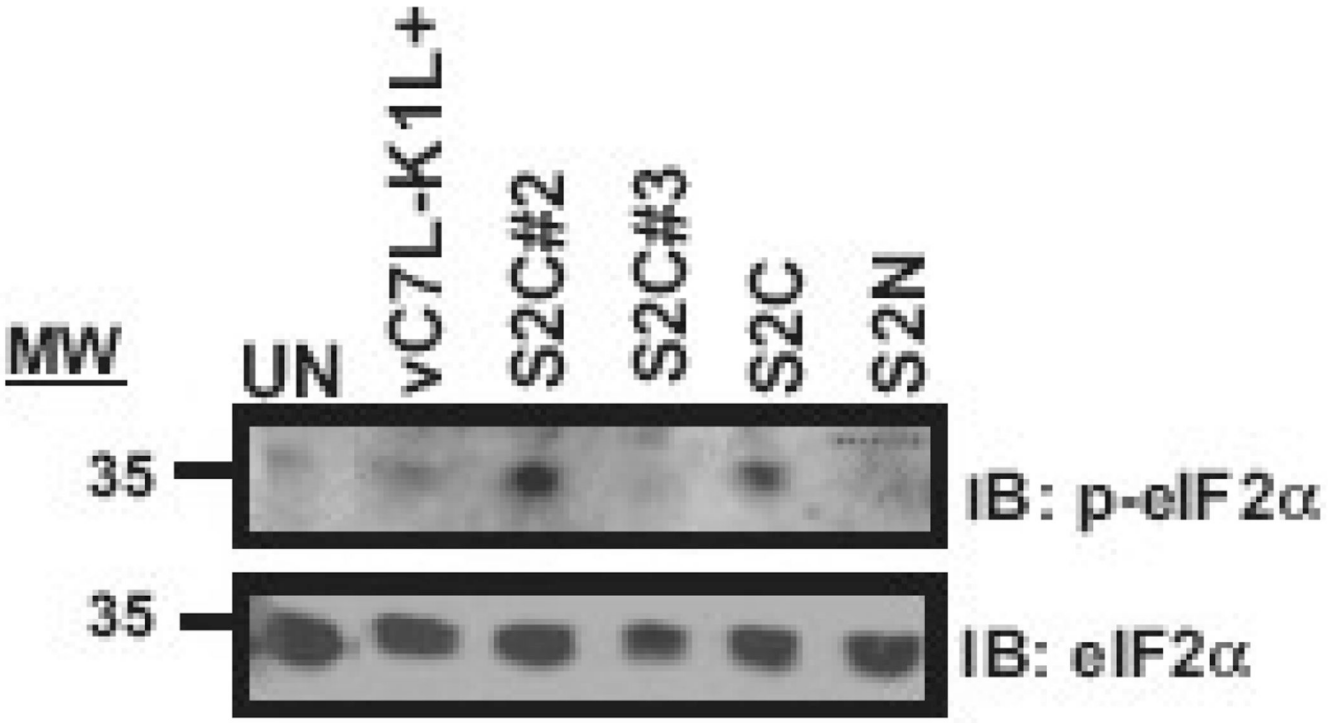


Figure 5. eIF2α phosphorylation in virus-infected HeLa cells

HeLa cellular monolayers were mock-infected (UN) or infected with vC7L^{-}K1L^{+}, S2C#2, S2C#3, S2C or S2N at an MOI = 10. Infections proceeded for 4 h pi. Immunoblots containing cytoplasmically extracted proteins were probed with antiserum recognizing either the phosphorylated (top panel) or unmodified (bottom panel) form of eIF2α. Molecular weight markers (MW) are indicated.

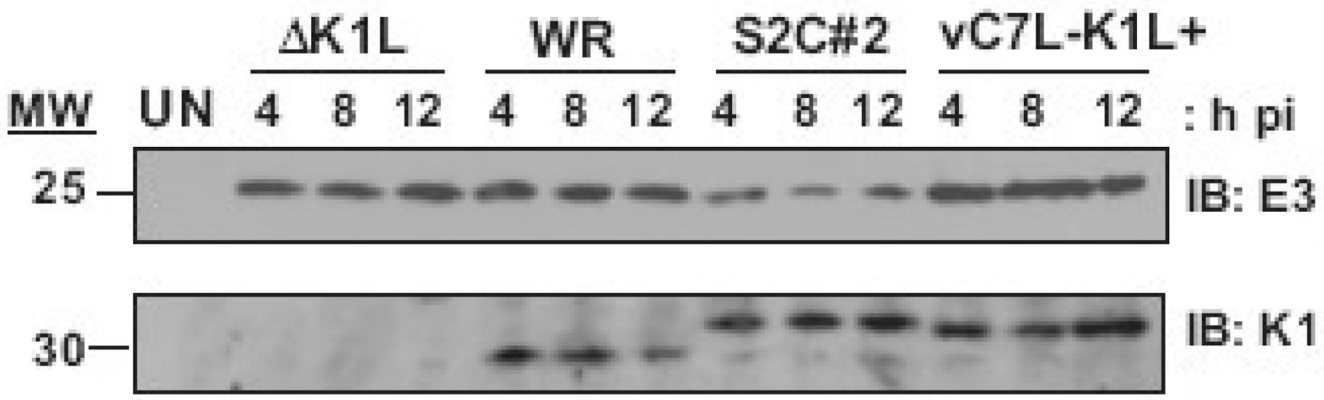
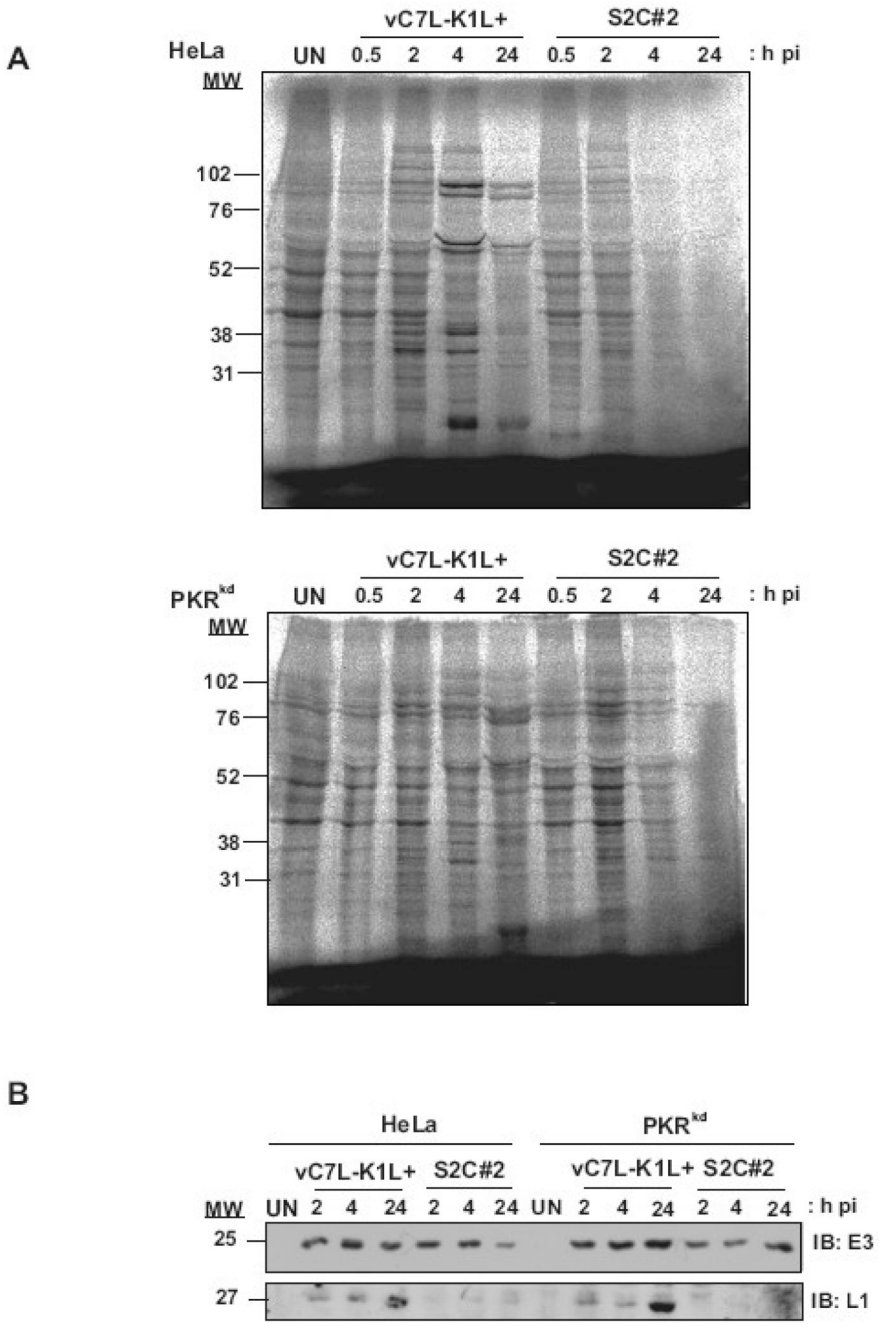


Figure 6. E3 protein levels in virus-infected HeLa cells

Cellular monolayers were mock-infected (UN) or infected with ΔK1L, WR, S2C#2, or $vC7L^{-}K1L^{+}$ (MOI = 10). At 4, 8 or 12 h post-infection, cells were processed, and immunoblots were probed with antiserum recognizing the vaccinia E3 product. The same membrane was re-probed for K1 products by incubating the immunoblot with anti-K1 antiserum. Molecular weight markers (MW) are indicated.

Figure 7. The effect of decreased PKR protein levels on eIF2α phosphorylation in virus-infected HeLa cells

HeLa cells expressing PKR (HeLa) or lacking PKR (kd) were either mock-infected (UN) or infected with either vC7L^{-}K1L^{+}, S2C#2, S2C#3, S2C or S2N (MOI = 10). At 4 h pi, cells were harvested and subjected to immunoblotting with antiserum recognizing either the phosphorylated or unmodified forms of eIF2α. The same immunoblot was probed with anti-serum specific for the PKR protein. Molecular weight markers (MW) are indicated.



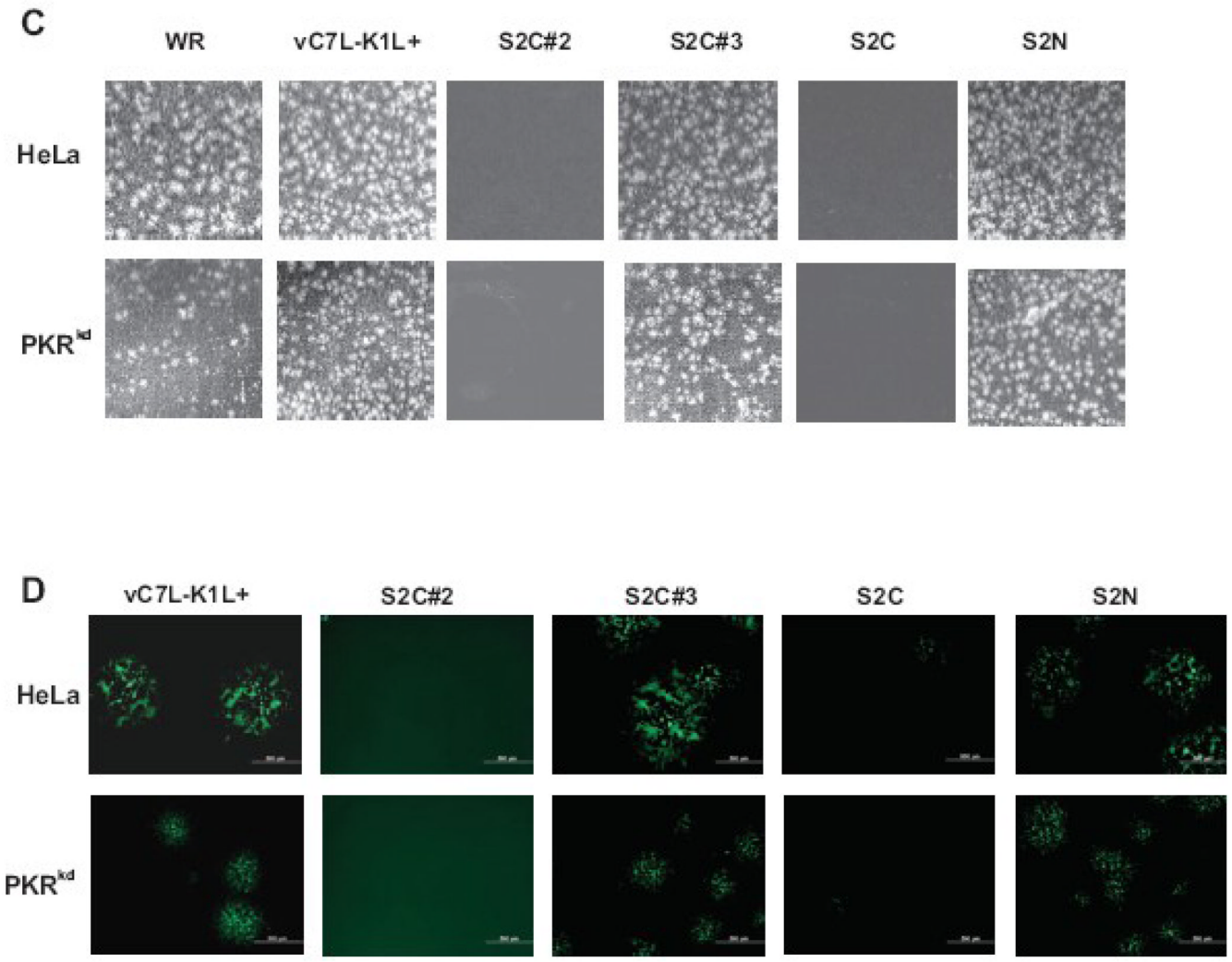


Figure 8. Virus plaque formation in HeLa cells expressing or lacking PKR proteins

HeLa cellular monolayers either expressing PKR proteins (HeLa) or deficient for PKR proteins (PKR^{kd}) were infected with the indicated viruses at an MOI of (A and B) 10 or (C and D) 0.01 PFU/cell. (A) At 30 min before harvesting, infected cellular monolayers were incubated in medium containing ^{35}[S]-methionine and ^{35}[S]-cysteine. Cells were collected and lysed, and an equal volume of each lysates was separated by SDS-10%PAGE. Dried gels were imaged by using phosphorimaging. (B) Infected HeLa or PKR^{kd} cells were collected at the times indicated, and lysed in CE buffer. Lysates were analyzed by SDS-12%PAGE, and proteins were transferred to PVDF membranes. Immunoblots were probed with antiserum recognizing either the L1 or E3 vaccinia proteins. Molecular weight markers (MW) are indicated. (C) At 48 h post-infection, media was replaced with a 20% ethanol solution that contained 1% crystal violet. After a 10 min incubation, supernatants were removed and plates were air-dried. (D) GPF-expressing infected cells were detected by using fluorescence microscopy at 48 h pi.

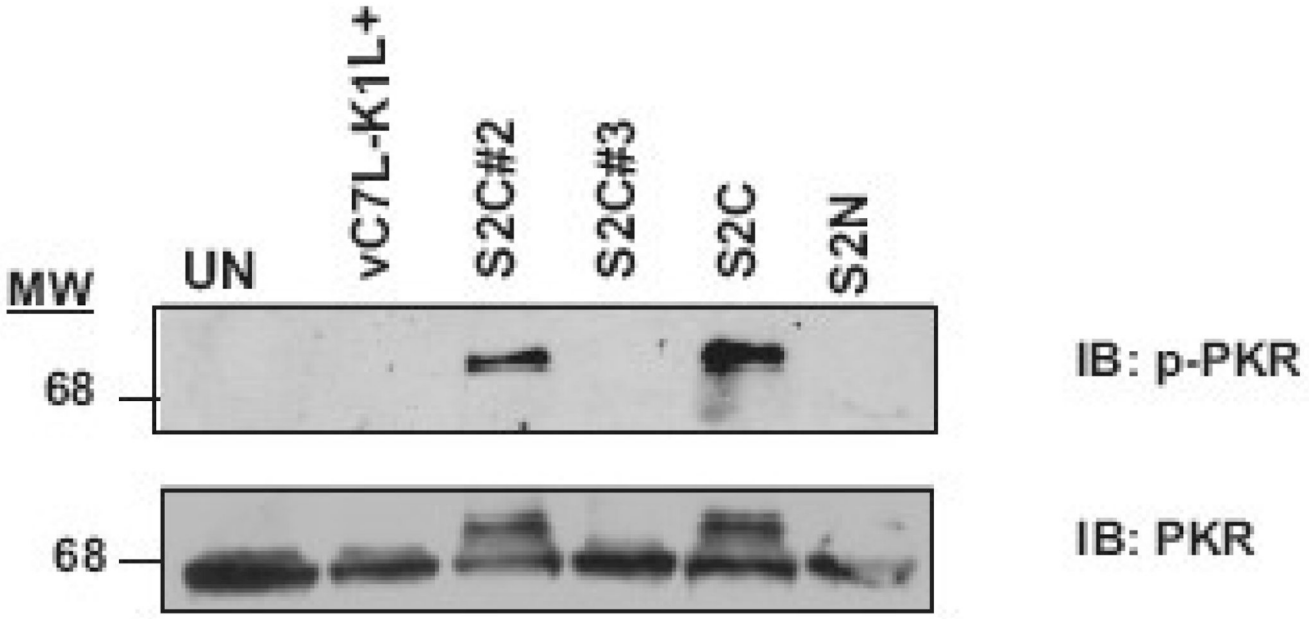


Figure 9. The effect of virus infection on PKR activation

HeLa cell monolayers were mock-infected (UN) or infected with either vC7L^{-} K1L^{+}, S2C#2, S2C#3, S2C or S2N at an MOI of 10. At 4 h post-infection, cells were harvested, prepared for immunoblotting and probed with antiserum recognizing either the phosphorylated form of PKR (p-PKR) or the unmodified form. Molecular weight markers (MW) are indicated.