Translational resistance of late alphavirus mRNA to eIF2α phosphorylation: a strategy to overcome the antiviral effect of protein kinase PKR

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The double-stranded RNA-dependent protein kinase (PKR) is one of the four mammalian kinases that phosphorylates the translation initiation factor 2α (eIF2α) in response to virus infection. This kinase is induced by interferon and activated by double-stranded RNA (dsRNA). Phosphorylation of eukaryotic initiation factor 2α (eIF2α) blocks translation initiation of both cellular and viral mRNA, inhibiting virus replication. To counteract this effect, most viruses express inhibitors that prevent PKR activation in infected cells. Here we report that PKR is highly activated following infection with alphaviruses Sindbis (SV) and Semliki Forest virus (SFV), leading to the almost complete phosphorylation of eIF2α. Notably, subgenomic SV 26S mRNA is translated efficiently in the presence of phosphorylated eIF2α. This modification of eIF2 does not restrict viral replication; SV 26S mRNA initiates translation with canonical methionine in the presence of high levels of phosphorylated eIF2α. Genetic and biochemical data showed a highly stable RNA hairpin loop located downstream of the AUG initiator codon that is necessary to provide translational resistance to eIF2α phosphorylation. This structure can stall the ribosomes on the correct site to initiate translation of SV 26S mRNA, thus bypassing the requirement for a functional eIF2. Our findings show the existence of an alternative way to locate the ribosomes on the initiation codon of mRNA that is exploited by a family of viruses to counteract the antiviral effect of PKR.

Keywords: Translation, eIF2, eIF2A, PKR, alphaviruses, antiviral response

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The activity of eukaryotic initiation factor 2 (eIF2) is a key target in the overall control of protein synthesis in mammalian cells [Dever 2002]. eIF2 is an oligomer composed of three subunits (α, β, γ) that interact with GTP and initiator methionyl-tRNA (Met-tRNAi). This ternary complex associates with the small 40S ribosomal subunits to form the 43S initiation complex [Hershey 1991; Pestova et al. 2001]. According to the scanning model, the 43S complex binds to the 5′ end of mRNA through the interaction of the cap-binding complex (eIF-4F). The resulting 48S complex moves downstream to reach the first AUG in an appropriate context [Kozak 1980; Gingras et al. 1999]. Once positioned on the initiation codon (AUGi), the 60S subunit joins to the small ribosomal subunit to form the 80S ribosome; concomitantly, eIF2 is released following GTP hydrolysis. The eIF2-GDP complex is continuously recycled by GDP–GTP exchange in a process catalyzed by eIF-2B [Yang and Hinnebusch 1996; Kimball et al. 1998; Kimball 1999]. The function of eIF2 in protein synthesis is thus the delivery of Met-tRNAi to the P ribosomal site to initiate protein synthesis starting at AUGi. Recent data also indicate that initiation factors 1 and 1A are required for correct ribosome location on the initiation codons [Pestova et al. 1998].

Several stress signals induce transient inactivation of eIF2α by phosphorylation, leading to a general downregulation of protein synthesis, accompanied by the activation of genes implicated in stress response [Harding et al. 2000; Dever 2002]. Four different kinases regulate eIF2 activity in response to specific environmental stresses in mammalian cells: HRI, RNA-dependent protein kinase [PKR], GCN2, and PERK [de Haro et al. 1996; Dever 2002]. These kinases catalyze the phosphorylation of eIF2α at Ser 51; phosphorylated eIF2-GDP binds eIF2B.
in an irreversible manner, thus preventing the regeneration of active eIF2-GDP, which results in a general inhibition of protein synthesis (Sudhakar et al. 2000). Translation directed by certain cellular and viral mRNAs is nonetheless induced by eIF2α phosphorylation. The best-illustrated examples are the expression of three genes involved in the response to nutrient deprivation: yeast GCN4, and ATF4 and Cat-1 in mammalian cells (Mueller and Hinnebusch 1986; Harding et al. 2000; Yaman et al. 2003; Vattem and Wek 2004). In these three cases, eIF2α phosphorylation may promote leaky scanning of ribosomes through the small open reading frames (uORF) at the 5′ leader sequence of these mRNAs to initiate translation at the downstream bona fide AUG codon (Dever 2002). One of the most striking cases of eIF2 independence for initiation of protein synthesis is the IRES-driven translation of the second cistron of the cricket paralysis virus (CrPV) genomic RNA. This cistron directs incorporation of the first amino acid [Ala], rather than the canonical methionine, into the A ribosomal site (Wilson et al. 2000).

The double-stranded RNA (dsRNA)-activated PKR has been implicated in antiviral defense due to its ability to respond to viral infection. PKR binds to and is activated by double-stranded RNA, a molecule usually generated responding to viral infection. PKR binds to and is activated by double-stranded RNA, a molecule usually generated in an irreversible manner, thus preventing the regeneration of active eIF2-GDP, which results in a general inhibition of protein synthesis (Sudhakar et al. 2000). Translation directed by certain cellular and viral mRNAs is nonetheless induced by eIF2α phosphorylation. The best-illustrated examples are the expression of three genes involved in the response to nutrient deprivation: yeast GCN4, and ATF4 and Cat-1 in mammalian cells (Mueller and Hinnebusch 1986; Harding et al. 2000; Yaman et al. 2003; Vattem and Wek 2004). In these three cases, eIF2α phosphorylation may promote leaky scanning of ribosomes through the small open reading frames (uORF) at the 5′ leader sequence of these mRNAs to initiate translation at the downstream bona fide AUG codon (Dever 2002). One of the most striking cases of eIF2 independence for initiation of protein synthesis is the IRES-driven translation of the second cistron of the cricket paralysis virus (CrPV) genomic RNA. This cistron directs incorporation of the first amino acid [Ala], rather than the canonical methionine, into the A ribosomal site (Wilson et al. 2000).

The double-stranded RNA (dsRNA)-activated PKR has been implicated in antiviral defense due to its ability to respond to viral infection. PKR binds to and is activated by double-stranded RNA, a molecule usually generated during replication and transcription of viral genomes. eIF2α phosphorylation by PKR leads to inhibition of translation, blocking viral replication [Meurs et al. 1990; Manche et al. 1992; Gunncery and Mathews 1998; Williams 1999]. A large body of evidence supports the idea that PKR activity is intimately linked to the antiviral effect of interferons (IFN) [Stark et al. 1998]. PKR activation is induced by type I IFN, and PKR-deficient mice are not protected against several animal viruses, lacking the antiviral response after IFNγ priming [Yang et al. 1995; Balachandran et al. 2000; Stojdl et al. 2000]. The importance of PKR in antiviral defense is further supported by the majority of animal viruses, which have evolved diverse strategies to prevent PKR activation in infected cells [Kaul and 1999]. PKR is thus rapidly degraded in picornavirus-infected cells [Black et al. 1993], whereas other animal viruses encode proteins that directly or indirectly block PKR activation. Some of these viral proteins, such as influenza [FLU] NS1, vaccinia E3L, or reovirus σ3, are able to sequester the dsRNA generated in the infected cells [Carroll et al. 1993; Davies et al. 1993; Lu et al. 1995; Yue and Shatkin 1997; Bergmann et al. 2000]. Other viral products, such as adenovirus VA1 RNA or HCV NS5A and E2 proteins, appear to prevent PKR activation by direct binding to the kinase [Kitajewski et al. 1986; Gale et al. 1998; Taylor et al. 1999]. On the contrary, HSV-1 expresses a gene that promotes dephosphorylation of eIF2α by activating cell phosphatase P101 [He et al. 1997]. In contrast to these strategies followed by most animal viruses, we describe that alphavirus [Sindbis and Semliki forest virus] infection induces strong PKR activation, which results in almost complete phosphorylation of eIF2α. Notably, translation of alphavirus 26S mRNA takes place efficiently in the presence of phosphorylated eIF2α. Our findings support a novel model for the initiation of translation, in which eIF2 activity appears dispensable. This represents a new strategy, used by this group of viruses to overcome the antiviral effect of PKR.

**Results**

PKR activation and eIF2α phosphorylation in sindbis (SV)-infected cells

Alphaviruses are a group of positive single-stranded RNA (ssRNA) viruses that infect several invertebrate and mammalian hosts. After uncoating, genomic 49S RNA is translated to produce the nonstructural proteins, involved in the synthesis of viral genomes and subgenomic 26S mRNA. Beginning at ~3–5 h post-infection (hpi), subgenomic mRNAs are efficiently synthesized and translated, generating the precursors of structural proteins (p130) that are proteolytically processed to the mature virion structural proteins [Strauss and Strauss 1994]. During the course of our experiments, we observed that translation of subgenomic 26S mRNA proceeded at very high rates in the presence of phosphorylated eIF2α in SV-infected cells. To examine this in detail, we analyzed the time course of eIF2α phosphorylation and protein synthesis in SV-infected 3T3 cells. Extensive phosphorylation of eIF2α was already apparent at 4 hpi, and did not increase further with time [Fig. 1A]. Using a phosphospecific antibody, we estimated that the level of phosphorylated eIF2α increased 10- to 15-fold in SV-infected cells at 4–6 hpi compared with mock-infected cells. Despite this, viral structural proteins were able to accumulate in infected cells at a very high rate [Fig. 1A]. Western blotting of total or Thr 451 phosphospecific PKR forms showed strong activation of the kinase following infection. The level of phosphorylated PKR at residue Thr 451 increased early in time (3 h), diminishing to basal levels at 5–6 hpi. This transient increase precedes the change in electrophoretic mobility observed for PKR at 3–4 hpi, indicative of extensive autophosphorylation and activation [Gorchakov et al. 2004].

To better quantify the percentage of eIF2α that became phosphorylated upon SV infection, IEF analysis of protein extracts was performed. We found that virtually all eIF2α (>95%) was phosphorylated in SV-infected cells at 4 hpi [Fig. 1B]. Similar results were obtained using MEF [data not shown].

Translation of subgenomic 26S mRNA is resistant to eIF2α phosphorylation as compared with genomic RNA and reporter (EGFP) mRNA

Two possibilities were considered to explain how 26S mRNA translation proceeds in the presence of phosphorylated eIF2α. SV could express a protein that replaces eIF2 function in trans, or SV 26S mRNA does not require eIF2 to initiate translation. To distinguish between these options, we engineered a recombinant SV expressing the EGFP gene under the control of a second
subgenomic promoter (Fig. 2A, Levis et al. 1990). The recombinant SV simultaneously expresses RNA from the genuine subgenomic promoter, 26S mRNA, and from a duplicate subgenomic promoter, EGFP mRNA (Fig. 2A). Moreover, EGFP mRNA contains the 5’ and 3’ UTRs present in SV 26S mRNA. Notably, EGFP-encoding mRNA was efficiently translated in PKR\(^{+/+}\), but not in PKR\(^{-/-}\) cells. In addition to the 33-kDa band corresponding to whole EGFP, we also detected a smaller 26-kDa band that reacted with anti-EGFP antibodies (Fig. 2B,D); this band may correspond to a proteolyzed form of EGFP. Moreover, EGFP mRNA contains the 5’ duplicate subgenomic promoter, EGFP mRNA (Fig. 2A; Levis et al. 1990). The bulk of eIF2\(^{\alpha}\) phosphorylation in infected cells (3 h), it appears that genomic RNA stops translation at 6–7 hpi. Notably, the late glycoprotein E1 began to accumulate when translation of genomic RNA had ceased. These data support the concept that translation of genomic and subgenomic RNA is subject to a strict temporal regulation. Luciferase activity increased more rapidly in SV-luc-infected PKR\(^{0/0}\) cells than in PKR\(^{-/-}\) cells, reaching maximal activity at 4–5 hpi. SV structural proteins consequently appeared earlier in the infection in PKR\(^{0/0}\) cells. These findings agree well with the results described above (Fig. 2) and suggest that translation of genomic SV RNA is improved in PKR\(^{0/0}\) cells.

It was of interest to analyze whether SV 26S mRNA was able to initiate translation at the initiator AUG codon in the virtual absence of a functional ternary complex. We developed a protocol to measure the synthesis of methionyl-puromycin [Met-Pur] catalyzed by the 80S initiation complex directed by SV and Semliki Forest virus (SFV) 26S mRNA. Infected cells were first treated with hypertonic medium to induce polysome run-off, followed by a recovery period in normal medium to allow reassembly of the 80S initiation complex in the presence of puromycin and \(^{[35]S}\)-Met [Fig. 4A]. Since infected cells exclusively translate 26S mRNA from 3 to 4 hpi, \(^{[35]S}\)-Met-Pur synthesis after polysome run-off reflects translation initiation of viral mRNAs. During hypertonic shock, trans-
lation was completely inhibited in both mock and infected cells, recovering to control levels after 1 h of incubation in normal medium (Fig. 4B). Strikingly, hypertonic medium induced strong but reversible eIF2\(\alpha\)/H9251 phosphorylation, as described for yeast (Goossens et al. 2001). \(\text{[35S]}\)-Met-Pur synthesis increased progressively during the recovery period in control cells (Fig. 4C). As a control of \(\text{[35S]}\)-Met-Pur synthesis, we used cycloheximide (CHX), another inhibitor of the elongation step of protein synthesis, which does not form a peptidyl bond with Met. No radioactivity was recovered in the organic phase of CHX-treated cells, validating the experimental protocol. Notably, \(\text{[35S]}\)-Met-Pur synthesis was blocked in cells treated with DTT during the recovery period, indicating that functional eIF2 is required to reinitiate translation of cellular mRNA. In SV- or SFV-infected cells, progressive \(\text{[35S]}\)-Met-Pur accumulation was found during the recovery period at levels comparable to those in uninfected cells, despite massive eIF2\(\alpha\) phosphorylation. Moreover, only small differences in \(\text{[35S]}\)-Met-Pur synthesis were observed between SV- or SFV-infected PKR\(^{+/+}\) and PKR\(^{0/0}\) cells (Fig. 4D,E). These data suggest that SV and SFV 26S mRNAs initiate translation with methionine in the presence of high levels of phosphory-

Figure 2. SV 26S mRNA is specifically translated in PKR\(^{+/+}\) cells. (A) Scheme of recombinant SV expressing the EGFP gene under a second subgenomic promoter. Note that EGFP mRNA also contains the natural 5’ and 3’ UTRs present in 26S mRNA. Arrows indicate transcription initiation sites. (B) PKR\(^{+/+}\) and PKR\(^{0/0}\) cells were infected with SV-EGFP virus and metabolically labeled at indicated times. Bands corresponding to EGFP and a truncated form of the protein (\(\Delta\text{EGFP}\)) are marked (see text for explanation). (C) Comparative analysis of EGFP versus SV C protein levels synthesized in PKR\(^{+/+}\) and PKR\(^{0/0}\) cells. Protein bands from the film shown in B were quantified by densitometry and plotted in arbitrary units. (D) Western blot analysis of EGFP accumulated in PKR\(^{+/+}\) and PKR\(^{0/0}\) cells. The blot was probed with a monoclonal anti-EGFP antibody (Clontech).

Figure 3. Premature phosphorylation of eIF2\(\alpha\) blocks translation of genomic SV RNA. (A) Scheme of recombinant SV expressing luciferase from the genomic RNA. (B) Timing of eIF2\(\alpha\) phosphorylation and translation of genomic and subgenomic 26S mRNA in PKR\(^{+/+}\) and PKR\(^{0/0}\) cells. Cells were infected with TotoLuc1101 virus (MOI: 10 PFU/cell), and extracts were prepared at the times indicated. Luciferase activity was measured and used to quantitate translation from genomic SV RNA. Arrows indicate the time at which genomic RNA stopped translating. Translation from subgenomic mRNA was measured by Western blot of viral glycoprotein E1. (C) Effect of premature eIF2\(\alpha\) phosphorylation on SV genomic RNA translation. PKR\(^{0/0}\) cells were infected with TotoLuc1101 and treated with 0.1 mM DTT from 0 hpi. At the times indicated, luciferase activity and the phosphorylation status of eIF2\(\alpha\) were measured in extracts.
lated eIF2α. To confirm this, we carried out sequence determination of the N-terminal tryptic peptide of capsid proteins from SV and SFV virions synthesized in PKR+/+ and PKR0/0 cells. The N-terminal sequence, acetyl-MNYIPTQTFYGR, was identical for SFV capsid protein synthesized in PKR+/+ and PKR0/0 cells [see Supplementary Material]. We were unable to determine the N-terminal peptide sequence for the SV capsid protein due to the presence of Arg at position 3, which yields a tryptic peptide too small for MALDI-TOF detection. N-terminal blockade by acetylation in SV and SFV capsids did not permit direct sequencing of the N terminus of capsid protein by Edman degradation. Finally, TLC chromatographic analysis was carried out of [35S]Met-labeled tryptic peptides of capsid protein synthesized in PKR+/+ and PKR0/0 cells (see Supplemental Material). We were unable to determine the N-terminal tryptic peptide of capsid proteins from SV and SFV virions synthesized in PKR+/+ and PKR0/0 cells. The N-terminal sequence, acetyl-MNYIPTQTFYGR, was identical for SFV capsid protein synthesized in PKR+/+ and PKR0/0 cells [see Supplementary Material]. We were unable to determine the N-terminal peptide sequence for the SV capsid protein due to the presence of Arg at position 3, which yields a tryptic peptide too small for MALDI-TOF detection. N-terminal blockade by acetylation in SV and SFV capsids did not permit direct sequencing of the N terminus of capsid protein by Edman degradation. Finally, TLC chromatographic analysis was carried out of [35S]Met-labeled tryptic peptides of capsid protein synthesized in PKR+/+ and PKR0/0 cells (see Supplemental Material). Altogether, the data show that in the presence of phosphorylated eIF2α, SV and SFV are able to initiate translation of subgenomic mRNA with Met.

A hairpin loop RNA structure (DLP) downstream of AUGi promotes translational resistance of SV 26S mRNA to eIF2α phosphorylation

We attempted to determine how 26S mRNA initiates protein synthesis in the presence of phosphorylated eIF2α. Since our data [Fig. 2] ruled out the involvement of 5′ or 3′ UTR regions in translational resistance to eIF2α phosphorylation, we considered that sequences within the SV 26S mRNA-coding region might promote eIF2-independent translation. Previous works by Frolov and Schlesinger [1994b, 1996] showed that the first 180 nucleotides [nt] of 26S mRNA act as a translational enhancer of subgenomic mRNA in SV-infected cells. This region includes the 50-nt 5′ UTR, followed by 130 nt corresponding to the capsid protein-coding sequence. Site-directed mutagenesis revealed a DLP involved in the translation, located downstream of the initiation codon [AUGi + 50] [Frolov and Schlesinger 1996]. This loop encompasses nucleotides 77–139, containing an extensive G-C pairing stretch that could form a very stable structure ($\Delta G^\text{m} = -45$ kcal/mol). The existence of this loop was confirmed by enzymatic probing using RNAses followed by primer extension analysis of the fragments generated [Fig. 5A]. The RNA sequences encompassing nucleotides 77–102 and 109–139, predicted to form the dsRNA stretch of DLP, were resistant to single-strand-specific RNase A and T1, whereas the loop itself was sensitive to these enzymes. In addition, primer extension detected a premature elongation halt of RT at 26S mRNA nucleotide 139, corresponding to the 3′ base of the hairpin loop [Fig. 5A]. We also analyzed ribosomal initiation complex formation by primer extension using RRL programmed with SV-CA mRNA in the presence of CHX. This showed two major toeprints at positions U99, C70, U72, and G73 [Fig. 7B, below]. The data indicate that 80S initiation complexes immobilized on SV-CA mRNA in the presence of CHX protected 18–19 nt 3′ from the AUGi [where A is +1], concurrently with results reported for other mRNAs [Pestova and Hellen 2003]. Furthermore, given the significant protection observed at G73, our data suggest that the leading edge of the 80S complex could be extended a few nucleotides downstream of the AUGi.
To test the role of DLP in translation initiation of 26S mRNA, we engineered an SV mutant lacking the DLP. This was achieved by changing C (or G) residues to A to destroy the G-C pairings of the loop, with no effect on the coding sequence except a conservative Leu-to-Phe change at position 14 of the capsid protein. RNA folding programs predicted no stable structures for viral RNA lacking the DLP. RNA from wild-type or /H9004 DLP cDNA was electroporated in BHK-21 cells, and the resulting viruses were amplified in these cells to obtain high-titer stocks. SV /H9004 DLP virus was viable, although the viral yield was 10-fold less than wild-type SV in BHK-21 cells.

We compared protein synthesis of these viruses in PKR+/+ and PKR0/0 cells; notably, translation of /H9004 DLP 26S mRNA was impaired in PKR+/+, but not in PKR0/0 cells (Fig. 5C). Consequently, replication of DLP virus was greatly diminished in PKR+/+ cells, giving ~2 log less progeny than wild type. On the contrary, DLP virus replicated at similar levels to SV wild type in PKR0/0 cells (Fig. 5D). We found that host translation shut-off occurred in SV /H9004 DLP-infected cells, suggesting that the initial steps of viral replication took place in these cells.

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accurate translation initiation in the absence of functional eIF2α.

To analyze whether the 5′ end of SV 26S mRNA confers translational resistance to eIF2α phosphorylation, we examined the translation of a hybrid mRNA containing the first 140 nt of SV 26S mRNA followed by the EGFP sequence. The resulting construct [p5’26S-EGFP] expresses a protein with the first 31 amino acids of the SV capsid protein fused to EGFP. As predicted, the hybrid protein shows delayed electrophoretic mobility compared with EGFP alone [Fig. 6]. To test the effect of eIF2α phosphorylation on translation of mRNA derived from pEGFP and p5’26S-EGFP constructs, BHK-21 cells were transfected with the plasmids, then infected with SV. EGFP synthesis was estimated by immunoprecipitation [Fig. 6]. The presence of 140 nt of SV 26S mRNA had little effect on EGFP translation in mock-infected cells. Nonetheless, in SV-infected cells with phosphorylated eIF2α, translation of p5’26S-EGFP mRNA resisted inhibition, whereas translation of EGFP alone was greatly reduced. These data show that the first 140 nt of SV 26S mRNA were sufficient to confer translational resistance to eIF2α phosphorylation.

Evidence that initiation factor 2A is involved in translation initiation of SV 26S mRNA in the absence of functional eIF2

The data presented above raised the question as to how ribosomes incorporate the first methionine on initiation complex of SV subgenomic mRNA. We tested the possibility that eukaryotic initiation factor 2A (eIF2A) could act by delivering the Met-tRNAi on initiation complex containing SV 26S mRNA in the absence of functional eIF2. Initiation factor 2A has been shown to directly bind the Met-tRNAi to 40S ribosomal subunits in a AUG codon-dependent manner [Merrick and Anderson 1975]. In contrast to eIF2, mammalian eIF2A consists of a single polypeptide of 68 kDa that does not require GTP to bind the Met-tRNAi [Adams et al. 1975]. To test the involvement of eIF2A in translation of SV 26S mRNA, we silenced the expression of murine eIF2A by means of small interfering RNA (siRNA) interference. A murine cDNA clone [GenBank: NM_001005509] is predicted to encode a 65-kDa polypeptide that shows 90% identity in amino acid sequence to human eIF2A [Zoll et al. 2002; see Supplementary Figure S3]. Giving this high degree of sequence homology, we considered this gene as the murine ortholog of human eIF2A. Cells were transfected with a siRNA targeted to eIF2A mRNA as described in Materials and Methods, and the effect on SV translation was assayed 50 h post-transfection. As a control, we transfected in parallel an unrelated siRNA labeled with FITC fluorochrome. Silencing of eIF2A expression was confirmed by Northern blot [Fig. 7A]. Hybridization of blots with a specific probe revealed a single mRNA transcript with the expected size (∼2 kb). Transfection with specific siRNA gave a consistent 70%–80% reduction in the amount of eIF2A mRNA presented at 50 h post-transfection in both PKR+/+ and PKR−/− cells. This agrees well with the percentage of transfection estimated by using FITC-labeled control siRNA [data not shown]. Silencing of eIF2A neither induced any apparent phenotype in uninfected cells, nor affected steady-state general protein synthesis. This agrees with previous data showing that deletion of yeast eIF2A did not affect translation [Komar et al. 2005]. Interestingly, interference of eIF2A expression led to a considerable reduction in the synthesis of SV structural proteins in PKR+/+ cells, but not in PKR−/− cells. Densitometric quantification revealed a 80% reduction in the synthesis of SV capsid protein, which agrees well with the percentage of transfection achieved. As expected, eIF2α phosphorylation was only observed in PKR−/− cells infected with SV irrespective of siRNA treatment. The effect of eIF2A silencing on SV was restricted to translation of 26S mRNA and did not affect translation of genomic mRNAs as demonstrated by using the recombinant SV expressing the luciferase

Figure 6. Translation resistance to eIF2α phosphorylation promoted by the 5′ extreme of SV 26S mRNA. Diagram of EGFP constructs. p5’26S-EGFP contains the first 140 nt of SV before the EGFP-coding sequence. Arrows indicate translation initiation sites. BHK-21 cells were transfected with 2 µg of the indicated plasmids using JetPEI (Poly-Plus Transfection) and infected with SV [MOI: 25 PFU/cell]. [Upper panel] At 5 hpi, cells were labeled with [35S]Met/Cys [1 h] and immunoprecipitated with anti-EGFP antibodies. The autoradiogram of labeled products is shown. The protein band that cross-precipitated with anti-EGFP antibodies probably corresponds to actin and serves as an internal control. Western blot analysis of eIF2α phosphorylation and Northern blot analysis of EGFP mRNA levels are also shown [middle panel], as well as ethidium bromide staining of total RNA loaded in each sample [bottom panel]. For Northern blot analysis, the membrane was probed with a 32P-labeled DNA fragment corresponding to the first 600 nt of the EGFP gene.
PKR0/0 cells was arbitrarily assigned as 1

hours later, poly(A)+ mRNAs were isolated

A described in Materials and Methods. (eIF2A-specific or control siRNAs as de-
cated cell type was transfected with

of eIF2A does not affect translation of genomic SV mRNA. Transfected cells were infected with recombinant SV-Luc, and the luciferase

activity of cell extracts was assayed 6 hpi.

Discussion

Considering the role of PKR kinase in antiviral defense, it is not surprising that viruses have evolved mecha-
nisms to prevent activation of this kinase in infected
cells. Inhibition of PKR activity could favor viral repli-
cation at two levels. The deleterious effect of early eIF2α phosphorylation by dsRNA on viral protein synthesis would be avoided, and production of IFN and proinflam-
matory cytokines through activation of IRF and NFκB, respectively, would be also limited (Yang et al. 1995;
Stark et al. 1998). Here we report that infection with alphaviruses (SV and SFV) induces PKR activation, re-
sulting in phosphorylation of virtually all eIF2α. Consistent with this, RNA folding pro-
duction of dsRNA replicative forms in SV-infected
cells; it is difficult to envisage how a very small percent-
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sis of viral structural proteins in SV-infected cells. Sec-
ond, the recycling of eIF2 necessary to support viral
translation would be limited due to the inhibitory effect
of phosphorylated eIF2α on eIF2B activity. Third, trans-
lation of EGFP and other viruses (Bischoff and Samuel 1989). The synthesis of large amounts of 26S mRNA from –3–4 hpi may also contribute to PKR activation and subsequent eIF2α phos-
phorylation. Furthermore, our data indicate that unlike
other animal viruses, alphaviruses express no specific
PKR inhibitor. Expression of these PKR-blocking agents in other viruses frequently enhances viral replication by preventing the deleterious effect of dsRNA accumula-
tion. In some cases, these inhibitors can even confer re-
sistance to IFN (Gale et al. 1998; Xiang et al. 2002).

Phosphorylation of eIF2α impairs translation initia-
tion in mammalian cells (Kimball et al. 1998; Kimball
1999). Since the amount of eIF2B in the cell is limited
with respect to eIF2, small increases in phosphorylated
eIF2α levels could cause severe inhibition of protein syn-
thesis due to eIF2B sequestration (Yang and Hinnebusch
1996; Sudhakar et al. 2000; Krishnamoorthy et al. 2001;
Balachandran and Barber 2004). Here we show that vir-
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infection of 3T3 cells. Phosphorylation of this factor se-
verely impairs translation of cellular or reporter (EGFP)
mRNA, but not translation directed by viral SV subge-
momic mRNA. That the remaining ~5% of intact eIF2α
might support 26S mRNA translation seems unlikely for
three reasons. First, translation of SV structural proteins
represents 30%–40% of protein synthesis in uninfected
cells; it is difficult to envisage how a very small percent-
age of functional eIF2α could support the massive synthe-
sis of viral structural proteins in SV-infected cells. Se-
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of phosphorylated eIF2α on eIF2B activity. Third, trans-
lation of EGFP and ΔDLP 26S mRNAs was abrogated in
PKR+/+ cells infected with recombinant SV-EGFP and SV
ΔDLP, respectively, indicating that the small fraction of
unphosphorylated eIF2α that remaining in these cells
cannot support canonical translation.

In contrast to 26S mRNA, we found that translation of
SV genomic mRNA was very sensitive to eIF2α phos-
phorylation. Consistent with this, RNA folding pro-
gams did not predict a similar structure as DLP at the 5’
end of SV genomic mRNA. However, given the temporal
regulation of translation found for the different types of

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phorylation. Furthermore, our data indicate that unlike
other animal viruses, alphaviruses express no specific
PKR inhibitor. Expression of these PKR-blocking agents in other viruses frequently enhances viral replication by preventing the deleterious effect of dsRNA accumula-
tion. In some cases, these inhibitors can even confer re-
sistance to IFN (Gale et al. 1998; Xiang et al. 2002).

Phosphorylation of eIF2α impairs translation initia-
tion in mammalian cells (Kimball et al. 1998; Kimball
1999). Since the amount of eIF2B in the cell is limited
with respect to eIF2, small increases in phosphorylated
eIF2α levels could cause severe inhibition of protein syn-
thesis due to eIF2B sequestration (Yang and Hinnebusch
1996; Sudhakar et al. 2000; Krishnamoorthy et al. 2001;
Balachandran and Barber 2004). Here we show that vir-
ually all eIF2α is phosphorylated following alphavirus
infection of 3T3 cells. Phosphorylation of this factor se-
verely impairs translation of cellular or reporter (EGFP)
mRNA, but not translation directed by viral SV subge-
momic mRNA. That the remaining ~5% of intact eIF2α
might support 26S mRNA translation seems unlikely for
three reasons. First, translation of SV structural proteins
represents 30%–40% of protein synthesis in uninfected
cells; it is difficult to envisage how a very small percent-
age of functional eIF2α could support the massive synthe-
sis of viral structural proteins in SV-infected cells. Se-
cond, the recycling of eIF2 necessary to support viral
translation would be limited due to the inhibitory effect
of phosphorylated eIF2α on eIF2B activity. Third, trans-
lation of EGFP and ΔDLP 26S mRNAs was abrogated in
PKR+/+ cells infected with recombinant SV-EGFP and SV
ΔDLP, respectively, indicating that the small fraction of
unphosphorylated eIF2α that remaining in these cells
cannot support canonical translation.

In contrast to 26S mRNA, we found that translation of
SV genomic mRNA was very sensitive to eIF2α phos-
phorylation. Consistent with this, RNA folding pro-
gams did not predict a similar structure as DLP at the 5’
end of SV genomic mRNA. However, given the temporal
regulation of translation found for the different types of
SV mRNAs in infected cells, translation of genomic mRNA can efficiently proceed before the extensive phosphorylation of eIF2α. Although SV genomic and subgenomic mRNAs appear to have different requirements for eIF2 to initiate translation, phosphorylation of eIF2α does not seem to be the event that switches translation from genomic to subgenomic mRNAs, since temporal regulation of SV mRNAs translation was also observed in the absence of eIF2α phosphorylation [PKR0/0 cells]. It is interesting to note that the translational switch from genomic to subgenomic mRNA of SV is temporally correlated with the onset of host translation shut-off. Moreover, SV replicons lacking the structural region of the genome can induce an inhibition of host translation similar to wild-type virus [Frolov and Schlesinger 1994a]. These observations suggest that the activity of one or more nonstructural proteins of SV is involved in this process, which is largely independent of eIF2α phosphorylation.

Mechanism of translation resistance to eIF2α phosphorylation

To counteract the effect of PKR activation, our data support a translational mechanism that promotes the synthesis of SV structural proteins irrespective of the functional status of eIF2. mRNA that is translated after eIF2α phosphorylation was reported for yeast GCN4 and mammalian Cat-1 and ATF-4 genes, and for the second cistron of CrPV genomic RNA [Mueller and Hinnebusch 1986; Dever 2002; Yaman et al. 2003; Vattem and Wek 2004]. Translation of mRNA for the first three genes is very low under normal [unstressed] conditions, but is induced by eIF2α phosphorylation. The mechanism involved is still poorly understood, but appears to involve reinitiation of ribosomes from upstream short ORFs under limited concentrations of active eIF2 [Dever 2002]. For CrPV, however, translation is initiated by Ala on the leading edge of 80S ribosomes could be extended several nucleotides, which is reminiscent of reinitiation at downstream initiation codons on GCN4 or ATF-4 mRNAs under limiting concentrations of active eIF2 [Mueller and Hinnebusch 1986; Vattem and Wek 2004]. Toeprin analysis of immobilized 80S/SV-CA mRNA complexes showed that ribosomes located on AUG, protected mainly +18–19 nt downstream of the initiation codon. Nonetheless, we also detected weak arrests of primer extension at positions +20, +23, and +24 with respect to AUG. Assuming that RT can penetrate a few nucleotides into the 80S complex [Kozak 1998], the data indicate that the leading edge of 80S ribosomes could be extended several nucleotides forward, to locate just behind DLP, or contacting the base of this structure [Fig. 7]. In this model, AUG, and DLP in 26S mRNA would be sufficiently separated to allow precise accommodation of ribosomes on the AUG. In fact, the AUG–DLP distance (5–28 nt) is conserved among members of the alphavirus group, despite their lack of sequence homology.

Here we present evidence that, in the absence of functional eIF2, eukaryotic initiation factor 2A (eIF2A) can support initiation of SV 26S mRNA. This is based on the fact that silencing of eIF2A expression inhibited translation of SV 26S mRNA in PKR+/+ cells, but not in PKR0/0 cells. Initial characterization of eIF2A from rabbit reticulocytes showed that this factor can bind and transfer the Met-tRNAi to 40S subunits only in the presence of the AUG codon [Komar et al. 2005]. We propose that eIF2A could deliver the Met-tRNAi to the 40S ribosome stalled on the 26S mRNA by the effect of DLP structure.
According to this model, both DLP and eIF2A factor would be necessary to confer eIF2-independent initiation of SV 26S mRNA, as supported by the data presented here.

Taken together, these results reveal the existence of an alternative mechanism to locate ribosomes on the initiation codon. We identified a novel mechanism by which the alphaviruses escape the antiviral action of PKR. This mechanism has implications for a better understanding of the function of eIF2 and eIF2A in mammalian cells, as well as for unraveling the intricate strategy developed by viruses to counteract the cellular antiviral response. Our findings open the way to analyze if a similar translation initiation mechanism operates in cellular mRNAs, in particular those involved in the stress response.

Materials and methods

Cells and virus infection
Murine embryonic fibroblasts (MEFs) derived from normal and PKR knockout mice were described previously (Yang et al. 1995); these mice have a mixed C57BL/6 and 129 SV genetic background. SV, SFV, encephalomyocarditis virus (EMC), VSV, and FLU were grown in 3T3 cells and purified through sucrose cushions. Virus was titrated by the standard plaque assay method. For infections, ~5 × 10⁵ cells were infected with viruses (multiplicity of infection [MOI]: 25–50 plaque-forming units [PFU]/cell) in serum-free DMEM. After 30 min of adsorption, viral inoculum was removed and fresh medium containing 10% fetal serum was added to the plates. At the times indicated, cells were washed briefly with cold PBS and lysed in 100 µL of sample buffer (0.15 M Tris-HCl at pH 6.8, 2% SDS, 10% glycerol, 0.1 M DTT, 0.02% bromophenol blue).

Plasmids and recombinant DNA procedures
The SV-EGFP virus expressing the green fluorescence protein from a second 26S subgenomic promoter was constructed as follows: A plasmid encoding the EGFP gene (pEGFP-N1; Clontech) was digested with BglII and BamHI and religated to eliminate the XhoI site of the polylinker. The EGFP gene was then cloned into the SV-2p26S infectious clone of SV using the XbaI site (Levis et al. 1990). The Sindbis virus expressing the luciferase gene as part of NSP3 protein (Toto1101/Luc) was generously provided by Charles Rice (Rockefeller University, New York).

The p5'-EGFP plasmid was constructed by placing the first 140 nt of the SV 26S mRNA before the EGFP-coding region. Virus was titrated by the standard plaque assay method. For infections, ~5 × 10⁵ cells were infected with viruses (multiplicity of infection [MOI]: 25–50 plaque-forming units [PFU]/cell) in serum-free DMEM. After 30 min of adsorption, viral inoculum was removed and fresh medium containing 10% fetal serum was added to the plates. At the times indicated, cells were washed briefly with cold PBS and lysed in 100 µL of sample buffer (0.15 M Tris-HCl at pH 6.8, 2% SDS, 10% glycerol, 0.1 M DTT, 0.02% bromophenol blue).
tion mixtures (2 µg of RNA) were used directly to electroporate in the presence of cap analog (New England Biolabs). Transcribed in vitro with T7 or SP6 RNA polymerases, respectively, Toto1101/luc plasmids were linearized with XhoI and transcribed protein fused to EGFP.

26S mRNA and includes the first 31 amino acids of the capsid protein fused to EGFP, respectively. For pRed-26S-EGFP, respectively. The distance between the stop codon of the EGFP-coding regions were placed as the first and second cistron, respectively. The distance between the stop codon of the EGFP-coding regions were placed as the first and second cistron, respectively.

Electroporation of cells with viral RNA

To generate viruses from infectious clones, SV-EGFP and Toto1101/luc plasmids were linearized with XhoI and transcribed in vitro with T7 or SP6 RNA polymerases, respectively, in the presence of cap analog (New England Biolabs). Transcription mixtures (2 µg of RNA) were used directly to electroporate ~10⁶ cells in a Bio-Rad electroporator [1500 V, 25 µF]. Virus was collected from medium after 48 h and further amplified to obtain high-titer stocks. Toto1101/luc has a slow-growth phenotype compared with SV or SV-EGFP viruses.

Metabolic labeling

Cells were labeled with [³⁵S]-Met/Cys (25 µCi/mL, Promix, Amersham) in medium lacking Met (30 min), washed with complete medium, and lysed in sample buffer. Proteins were analyzed by SDS-PAGE followed by fluorography and autoradiography.

IEF and Western blot

To analyze eIF2α by isoelectric focusing (IEF), infected cells were lysed in buffer (20 mM HEPES-KOH at pH 7.5, 150 mM NaCl, 10% [v/v] glycerol, 1% [v/v] Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 100 mM sodium fluoride, 17.5 mM β-glycerophosphate, 10 mM tetrasodium diphosphate, and a protease inhibitor cocktail [Complete; Boehringer Mannheim]). Cell debris was removed by centrifugation (15,000 × g for 5 min at 4°C). Supernatant was snap-frozen on dry ice and stored at −70°C. Equivalent amounts of protein extract [50 µg] were precipitated with trichloroacetic acid (10%). Protein pellets were resuspended in 60-µL vertical slab isoelectric focusing (VSIEF) gel sample buffer and resolved as described (Savinova and Jagus 1974). To control eIF2α phosphorylation status, we used rabbit reticulocyte lysate (RRL) incubated with 500 µM hemin and 2 mM EDTA (5 min at 30°C, nonphosphorylated) or minus hemin (10 min), followed by 5 min in the presence of 17.5 mM β-glycerophosphate (phosphorylated). RRL samples (2 µL) were added to 100 µL of VSIEF sample buffer, 20 µL of which was resolved. After VSIEF, proteins were transferred to Immobilon-P membranes and probed with eIF2α antibody (Santa Cruz). The rest of the antibodies used were anti-PKR (polyclonal no. sc-1702; Santa Cruz), anti-phospho eIF2α and anti-phospho PKR [Bio-source], and anti-EGFP [Clontech]. The rabbit antibody to the SV E1 glycoprotein has been described (Sanz et al. 2003). Plasmid colonization to the SV capsid protein was generously provided by Dr. M.J. Schlesinger [Karolinska Institute, Stockholm, Sweden]. Blots were developed using the ECL system [Amersham Biosciences].

Proteomic analysis

[³⁵S]Met/Cys-labeled peptide maps of alphavirus capsid proteins were analyzed by TLC as described [Maroto et al. 2000]. Briefly, PKR+/+ and PKR0/0 cells were infected with SV or FSV [MOI: 50 PFU/cell]. At 4.5 hpi, cells were labeled with 50 µCi/mL of [³⁵S]Met/Cys [1 h], extracted, and analyzed by SDS-PAGE followed by transfer to nitrocellulose membrane. The membrane was exposed to X-ray films [2 h] and the band corresponding to capsid proteins was excised, blocked with polyvinylpyrrolidone, and trypsin-digested [18 h, Promega]. Peptides were then oxidized with performic acid, rinsed in distilled water, and analyzed by one-dimensional TLC electrophoresis, and the TLC plates were exposed to X-ray films.

For N-terminal sequence determination of SFV capsid proteins, PKR+/+ and PKR0/0 cells were infected with SFV [MOI: 10 PFU/cell]; virus released to the culture medium at 16 hpi was purified by sedimentation through a 20/50% sucrose cushion [160,000 × g for 3 h]. Bands corresponding to capsid protein were excised from SDS-PAGE gels, then subjected to tryptic digestion and fingerprint analysis [MALDI-TOF], followed by sequence verification using fragmentation coupled to ESI [electrospray ionization] analysis.

Met-Pur synthesis assay

Synthesis of Met-Pur dipeptide in vivo was carried out as follows: Cells were incubated in hypertonic medium containing 0.31 M NaCl [40 min] to dissociate ribosomes from mRNA. 80S initiation complexes were then allowed to reassemble in normal medium [0.12 M NaCl containing 25 µCi/mL [³⁵S]Met and 50 µg/mL puromycin for 40–140 min. Cells were washed extensively in cold PBS and lysed in TNE buffer containing 1% Triton X-100. Post-nuclear supernatant was treated with 1 µg/mL RNase A [15 min, 37°C] and diluted fivefold in 0.1 M phosphate buffer [pH 8.0]. Samples were extracted twice with ethyl acetate, and the radioactive material in the organic phase was counted in a liquid scintillation counter as described previously [Suzuki and Goldberg 1974].

Toeprinting analysis

We used a nonradiative modification of primer extension analysis [Anthony and Merrick 1992, Kozak 1998]. In brief, 0.1 µg of in vitro synthesized SV CA mRNA was preannealed to 5'-fluorochrome-labeled [VIC] primer: GCCTGTCCAAATGGAC TAGGGCAGTGCAGCG by heating (1 min, 65°C), then cooling slowly to 37°C. For enzymatic probing, the RNA-primer mixture was treated with 0.01 U of RNase A or T1 [10 min, room temperature], extracted twice with phenol/chloroform, precipi-
tated with ethanol, and resuspended in RT buffer [50 mM Tris-HCl at pH 7.5, 40 mM KCl, 6 mM MgCl₂, 5 mM DTT, 0.5 mM dNTPs]. Following addition of 2 U of SuperScript II reverse transcriptase ( Gibco-BRL), primer extension reactions were incubated [20 min at 25°C], phenol-extracted, precipitated with ethanol, resuspended in 40% formaldehyde/8 mM EDTA, and heated (5 min at 95°C). Samples were analyzed in an ABI Prism 3700 DNA Analyzer using GeneScan software [Applied Biosystems]. To analyze formation of ribosomal initiation complex, nuclease-treated RRL (Promega) was programmed with RNA/primer mixture in the presence of 100 µg/mL CHX and incubated (25°C, 10 min) in a final volume of 25 µL. Samples were then diluted 20-fold in RT buffer supplemented with 100 µg/mL CHX, and primer extension analysis was carried out as described above.

**Gene silencing by siRNA**

To knock down the expression of murine eIF2α gene, an siRNA was designed (GUAAAGAUGGGACAUUGUUU) corresponding to nucleotides 177–195 from the 5′ extreme of mRNA sequence of murine eIF2α cDNA (GenBank: NM_001005509). PKR⁺/⁺ and PKR⁻/⁻ cells were transfected with 40 pmol of siRNA eIF2α or with an unrelated siRNA labeled with FITC using oligofectamine [Gibco-BRL] according to the manufacturer's recommendation. At 50 h post-transfection, cells were infected with SV or VSV at an MOI of 25 PFU/cell. Six hours later, cells were metabolically labeled and analyzed as described above. Silencing of eIF2α expression was confirmed by Northern blot analysis using an [α-thiol]dCTP-labeled probe encompassing nucleotides 287–886 of murine eIF2α cDNA derived from clone ID 30606936 provided by the I.M.A.G.E consortium (MRC Geneservice). Poly(A)⁺ mRNAs were isolated by the QuickPrep micro mRNA purification kit [Pharmacia Biotech]. Hybridization was performed in ExpressHyb solution (BD Biosciences) according to the manufacturer’s recommendation.

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Translational resistance of late alphavirus mRNA to eIF2α phosphorylation: a strategy to overcome the antiviral effect of protein kinase PKR

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