Phosphorylation of eIF2α is responsible for the failure of the picornavirus internal ribosome entry site to direct translation from Sindbis virus replicons

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Translation directed by the poliovirus (PV) or encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) is very inefficient when expressed from Sindbis virus (SV) replicons. This inhibition can be rescued by co-expression of PV 2A protease (2Apro). Inhibition correlates with the extensive phosphorylation of eukaryotic initiation factor (eIF) 2α induced by SV replication. Confirmation that PV or EMCV IRES-driven translation can function when eIF2α is not phosphorylated was obtained in dsRNA-activated protein kinase knockout mouse embryonic fibroblasts (PKR−/− MEFs), where SV replication cannot induce eIF2α phosphorylation, and in variant S51A MEFs that express an unphosphorylatable eIF2α. In these cells, PV or EMCV IRES-dependent translation operated more efficiently than in wild-type MEFs. However, this translation was potently blocked when eIF2α was phosphorylated by the addition of thapsigargin to PKR−/− MEFs. In addition, when wild-type eIF2α was expressed in S51A MEFs or PKR was expressed in PKR−/− MEFs, PV IRES-dependent translation decreased. In both cases, the decrease in PV IRES-dependent translation correlated with the phosphorylation of eIF2α. Notably, PV 2Apro expression rescued PV IRES-driven translation in thapsigargin-treated PKR−/− MEFs. Taken together, these results demonstrated that PV IRES-driven translation can take place from SV replicons if eIF2α remains unphosphorylated. Remarkably, PV IRES-dependent translation was fully functional in this system when PV 2Apro was present, even if eIF2α was phosphorylated.

INTRODUCTION

Initiation of translation is a key step in the regulation of the production of viral and cellular proteins (Gale et al., 2000; Pestova et al., 2001; Sonenberg & Hinnebusch, 2009). Two initiation factors constitute the main target for regulation of translation in virus-infected animal cells: the eukaryotic initiation factor (eIF) 4F complex and eIF2 (Belsham, 2009; García et al., 2006; Lloyd, 2006). With regard to eIF2, the main regulatory mechanism is phosphorylation of the eIF2α subunit at serine 51 (García et al., 2006). The first amino acid to be incorporated in protein synthesis is methionine, which interacts with eIF2 and GTP in the form of Met-tRNA\textsubscript{Met}, leading to the formation of the ternary complex Met-tRNA\textsubscript{Met}–eIF2–GTP. Together with other eIFs, such as eIF1 and eIF5, this ternary complex recognizes the 40S ribosomal subunit and interacts with the AUG initiator codon present on the mRNA (Pestova et al., 2001; Sonenberg & Hinnebusch, 2009). This initiation complex joins the 60S ribosomal subunit and, together with eIF5B, catalyses GTP hydrolysis and the exit of eIFs, including eIF2–GDP, which in the presence of eIF2B recycles to eIF2–GTP. When eIF2α is phosphorylated, eIF2B remains bound to eIF2–GDP, blocking the recycling step. As the amount of eIF2B is about 10–20-fold less than eIF2, even a low percentage of eIF2α phosphorylation can arrest the initiation of translation (García et al., 2006; Proud, 2005; Schmitt et al., 2010). A number of stresses promote phosphorylation of eIF2α within minutes. This is also the case observed following infection of mammalian cells by some animal viruses (Kim et al., 2011; Montero et al., 2008; Qin et al., 2011; Raaben et al., 2007; Robert et al., 2006; Rojas et al., 2010; Schmitt et al., 2010; Welnowska et al., 2011; White et al., 2011). Thus, in alphavirus-infected cells, most of the eIF2α appears phosphorylated from about 2–3 h after infection (Gorchakov et al., 2004; McInerney et al., 2005; Sanz et al., 2009; Ventoso et al., 2006).

The presence of phosphorylated eIF2α in virus-infected cells poses an intriguing question about the mechanism by which the initiation of translation occurs under these conditions. The possibility that other factors replace eIF2 has been put forward. In the case of Sindbis virus (SV) and
RESULTS

Expression of eIF4G fragments in SV-replicating baby hamster kidney (BHK-21) cells

Viral mRNAs bearing IRES elements usually exhibit a low requirement for eIFs during the initiation of translation. Thus, it was surprising to find that SV sgmRNAs bearing picornavirus IRESs are translated inefficiently in SV-replicating cells (Sanz et al., 2010). This elimination of translation was rescued by co-expression of the picornavirus proteases 2APro of PV or 2PPro of FMDV. Both viral proteases share the ability to cleave eIF4G. We therefore started by testing whether co-expression of the PV IRES and the entire eIF4G, isoform I (eIF4GI) or the N- or C-terminal halves of eIF4GI was able to rescue IRES-driven translation in SV-replicating cells. For this purpose, the complete sequence of human eIF4GI, the N-terminal half of the molecule (aa 1–674) and the C-terminal half of the molecule (aa 675–1600) were cloned in SV replicons after the capsid (C) protein (Fig. 1a). (The nomenclature of the different plasmids, replicons and sgmRNAs used in this work is shown in Table 1, and details of their construction can be found in Supplementary methods, available at JGV Online.) Transcription of the corresponding plasmids produced RNA replicons that could be transfected into BHK-21 cells, leading to the generation of genomic and subgenomic mRNAs, as occurs during viral infection (Strauss & Strauss, 1994). Translation of the sgmRNA from replicons yielded C protein, plus the appropriate recombinant protein, which is released because the C protein has autoproteolytic activity at its C terminus. Therefore, cells were co-electroporated with the replicon rep LPol-luc and replicons expressing the entire eIF4GI or its N- or C-terminal halves. Replicons expressing PV 2APro (rep C+2A) or 2C protein (rep C+2C) were used as controls. Translation of sgmRNA from the replicons rep C+4G, rep C+4GNt or rep C+4Gct synthesized the SV C protein and the recombinant eIF4G, N-terminal half of eIF4G or C-terminal half of eIF4G, with six extra amino acids (SAHMSR) at the N terminus of each protein (see Supplementary Methods). At 5 h post-electroporation (p.e.), protein synthesis was analysed by radioactive labelling (Fig. 1b). Efficient synthesis of all recombinant proteins derived from replicons rep C+recombinant protein was shown in such a way that the synthesis of these proteins was higher than that observed for the majority of cellular proteins in control cells (Fig. 1b). Expression of the entire eIF4GI or its N- or C-terminal half was also determined by Western blotting (Fig. 1c). However, Luc synthesis could be detected by radioactive labelling only in the presence of PV 2APro (indicated by an asterisk in Fig. 1b). Overexpression of complete eIF4GI or its N- or C-terminal half did not enhance Luc expression, similar to what occurred when the PV 2C control protein was expressed (Fig. 1b). To assess further that Luc synthesis was stimulated by the co-expression of PV 2APro, the activity of Luc was measured (Fig. 1d). The measurements of Luc activity from cells co-electroporated with the different combinations of replicons were compared with the values obtained from cells electroporated with rep LPol-luc. Strikingly, there was a 27-fold stimulation of Luc activity in the presence of the viral protease, whereas eIF4GI or its fragments were unable to accomplish this stimulation. The differences in Luc activity estimated in Fig. 1(d) could have arisen from differences in the amount of SV sgmRNAs encoding Luc present in co-transfected cells. To test this possibility, in parallel, BHK-21 cells transfected with the different replicons were labelled with 3H]uridine in presence of actinomycin D, as detailed in Methods. Under these conditions, only viral mRNAs are synthesized and they were detected by separation in denaturing agarose gels. In BHK-21 cells transfected with rep LPol-luc, there were two types of viral mRNA, the largest corresponding to the genomic RNA (gmRNA) and the shortest corresponding to sgmRNA that directed the synthesis of Luc (Fig. 1e). In BHK-21 cells co-transfected with two different replicons, there were four RNAs, two corresponding to gmRNAs, and the other two to sgmRNAs. These four RNAs were distinguished and separated in agarose gels (Fig. 1e). When PV 2APro was expressed, the amount of sgmRNA encoding Luc was similar to the control and lower than the rest of samples examined (Fig. 1e). Thus, the increased Luc synthesis when PV 2APro was present was not due to an increased amount of LPol-luc sgmRNA. Therefore, the presence of eIF4GI or its N- or C-terminal half was not sufficient to increase PV IRES-driven translation to an extent similar to that observed with PV 2APro in SV-replicating cells.
To test whether the translation enhancer activity resulted from the combined participation of 2A\(^{pro}\) and one of the cleavage fragments of eIF4GI, co-expression of a proteolytically inactive mutant, 2A\((G60R)\), and each of the eIF4GI cleavage fragments was assayed. In addition, the expression of both eIF4GI fragments with or without the PV 2A\((G60R)\) variant was tested. To this end, BHK-21 cells were co-electroporated with rep LPol-luc and different combinations of other replicons, namely rep C + 2A, rep C +2A\((G60R)\), rep C + 4G\(Nt\) or rep C + 4G\(Ct\) as controls.
Table 1. Nomenclature of plasmids, replicons and sgmRNAs

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<th>Plasmid</th>
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<td>pT7 rep LPol-luc</td>
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<td>pT7 rep C+ 2A(G60R)</td>
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<td>pT7 rep LEMCV-luc</td>
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<td>pT7 rep C+ elf2a(S51A)</td>
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and a mixture of rep C+4Gnt plus rep C+4Gct, rep C+2A(G60R) plus rep C+4Gnt, rep C+2A(G60R) plus rep C+elf4Gct, or rep C+2A(G60R) plus a mixture of rep C+4Gnt and rep C+4Gct, and Luc activity was determined at 5 h p.e. (Fig. S1). Clearly, the co-expression of both cleavage fragments of elf4G or 2A(G60R) and the cleavage products of elf4GI did not enhance Luc expression compared with that obtained in 2Apro-expressing cells. Therefore, the co-expression of both cleavage fragments of elf4GI and the combination of a proteolytically inactive 2Apro and each of the cleavage fragments of elf4GI did not rescue PV IRES-driven protein synthesis. In conclusion, only when active PV 2Apro was synthesized was there a clear rescue of PV IRES-driven translation in SV-replicating cells.

Phosphorylation of eIF2α in SV-replicating BHK-21 cells

In alphavirus-infected cells, there is strong phosphorylation of eIF2α induced by the generation of viral dsRNA that activates PKR (Gorchakov et al., 2004; McInerney et al., 2005; Sanz et al., 2009; Ventoso et al., 2006). However, in the late phase of SV infection, the translation of sgRNA operates even in the presence of arsenite (Ars), an inducer of phosphorylation (Sanz et al., 2009). In addition, translation of mRNAs bearing a PV or EMCV IRES is blocked by eIF2α phosphorylation in the absence of picornavirus proteins (Welnowska et al., 2011). We therefore decided to analyse, by isoelectric focusing, the degree of phosphorylation of eIF2α in BHK-21 cells treated or not with Ars and infected or not with SV (Fig. 2a). In control BHK-21 cells, no phosphorylation of eIF2α was observed and practically all of the eIF2α remained unphosphorylated. However,Ars treatment led to 74 % phosphorylation of eIF2α (Fig. 2a, lane 2), which was concomitant with a strong inhibition of cellular protein synthesis (Sanz et al., 2009; Welnowska et al., 2011). When BHK-21 cells were infected with SV, almost all of the eIF2α was phosphorylated (Fig. 2a). Addition of Ars to SV-infected cells served to ensure that all of the eIF2α remained phosphorylated and that it was unlikely that a small percentage of this factor was dephosphorylated during virus replication. This experiment showed that eIF2α of each cell where there was replication of SV was phosphorylated. This is important because in transfected cells only some of

Fig. 2. Phosphorylation of eIF2α in SV-replicating BHK-21 cells.
(a) Mock-infected or SV-infected (5 h) BHK-21 cells were treated or not with 400 μM Ars for 45 min and then collected for isoelectrofocusing. Thus, the phosphorylated and unphosphorylated forms of eIF2α were separated, transferred to nitrocellulose membrane and detected by anti-eIF2α antibodies. The relative percentages of the phosphorylated and unphosphorylated forms of eIF2α are indicated. (b) BHK-21 cells were electroporated with transcription buffer as a control or co-electroporated with a mixture of in vitro-synthesized rep LPol-luc and rep C+2A or rep C+2A(G60R). At 5 h p.e., 400 μM Ars was added for 45 min and protein synthesis was analysed by incorporation of [35S]Met/Cys during the last 30 min of treatment, followed by SDS-PAGE, fluorography and autoradiography. (c–e) A duplicate of the samples in (b) was transferred to nitrocellulose and analysed by Western blotting with anti-Luc antibodies (c), anti-elf4G antibodies (d) or anti-phospho-eIF2α antibodies (e).
the cells incorporate the viral replicon, so the use of this technique produces less accurate results. In most studies on the phosphorylation of eIF2\(\alpha\), measurement of total eIF2\(\alpha\) and its phosphorylated form using specific antibodies is sufficient to determine the phosphorylation status of this factor, assuming that the phosphorylation of a small part of eIF2\(\alpha\) is sufficient to inactivate the eIF2B factor and prevent the recycling of eIF2–GDP to eIF2–GTP. Thus, it was of interest to analyse eIF2\(\alpha\) phosphorylation in BHK-21 cells transfected with rep LPol-luc, particularly in the presence of 2\(\text{A}^{\text{pro}}\). For this purpose, BHK-21 cells were co-electroporated with rep LPol-luc and rep C+2A or rep C+2A(G60R), a control comprising a proteolytically inactive 2\(\text{A}^{\text{pro}}\), and at 5 h p.e., cultures were treated with 400 \(\mu\)M Ars for 45 min or left untreated. Protein synthesis was analysed by radioactive labelling during the last 30 min of treatment. In agreement with the results shown in Fig. 1, synthesis of Luc could only be detected by radioactive labelling in cells expressing 2\(\text{A}^{\text{pro}}\) (Fig. 2b). Confirmation that 2\(\text{A}^{\text{pro}}\) but not 2\(\text{A}(\text{G60R})\) enhanced IRES-dependent

![Graph](image-url)

Fig. 3. Translation of SV sgmRNAs bearing a picornavirus IRES in wt MEFs, PKR\(^{-/-}\) MEFs and S51A MEFs. (a) Schematic representation of the replicons. (b, c) The different MEF cells were transfected with the indicated in vitro-transcribed replicons. Transfection was carried out with 5 \(\mu\)g mRNA of the different replicons and 2 \(\mu\)l Lipofectamine. At 4 h p.t., 1.5 \(\mu\)M Tg or 50 \(\mu\)M CHX was added. At 6 h p.t., untreated and treated cells were harvested and Luc activity was determined. Values obtained from CHX-treated cells were used to subtract the amount of Luc synthesized prior to Tg addition. The percentage values of Tg-treated cells relative to their respective untreated cells are shown in (b). The mean values of Luc activity obtained from untreated or treated cells are also indicated. The absolute values of Luc activity of untreated cells are showed in (c). Luc activity results are displayed as means \(\pm\) SD of three representative experiments performed in triplicate.
translation was obtained by Western blot analysis with anti-Luc antibodies (Fig. 2c). As expected, eIF4GI was cleaved when wild-type (wt) 2A was expressed but not after 2A(G60R) expression (Fig. 2d). The phosphorylation state of eIF2α was determined by Western blotting with anti-phospho-eIF2α antibodies (Fig. 2e). Ars treatment induced phosphorylation of eIF2α in mock cells, which correlated with the inhibition of protein synthesis. In cells electroporated with the replicons, eIF2α was always phosphorylated, presumably because virus replication activated PKR, and additionally as a consequence of the action of Ars. Notably, PV IRES-driven translation that produced Luc operated in 2Apro-expressing cells even in the presence of Ars but not in cells expressing 2A(G60R). Therefore, the expression of 2Apro promoted translation of mRNAs with a PV IRES, even though eIF2α was phosphorylated in BHK-21 cells.

Translation of SV sgRNAs bearing picornavirus IRES in wt murine embryonic fibroblasts (MEFs), MEFs deficient in PKR and S51A MEFs

Another way to analyse the involvement of eIF2α phosphorylation in PV IRES-driven translation is by using cell lines in which eIF2α cannot be phosphorylated. Particularly interesting is the PKR knockout (PKR−/−) MEF cell line, where SV infection does not induce phosphorylation of eIF2α (Gorchakov et al., 2004; Ventoso et al., 2006). However, phosphorylation of eIF2α can be induced in this cell line by thapsigargin (Tg), which activates PKR-like endoplasmic reticulum kinase (PERK), which activates PKR-like endoplasmic reticulum kinase (PERK) (Harding et al., 2000; Linero et al., 2011). Moreover we used MEFs with a S51A mutation in eIF2α (S51A MEFs), which express an unphosphorylatable form of the protein. In these cells, SV replication can activate PKR and treatment with Tg activates PERK, although phosphorylated eIF2α cannot be generated. Finally, eIF2α can be phosphorylated in wt MEFs both via PKR and PERK. Taking into account these considerations, we decided to compare the translational behaviour of the sgRNA C+ luc, whose translation is resistant to the phosphorylation of eIF2α (Sanz et al., 2009), with LPol-luc and LEMCV-luc sgRNAs (see Fig. 3a). To this end, the same number of cells from each line was transfected with the replicons rep C+ luc, rep LPol-luc or rep LEMCV-luc. Next, the cells were treated with 1.5 μM Tg from 4 h post-transfection (p.t.) and Luc activity was measured at 6 h p.t. As a control, 50 μM cycloheximide (CHX) was added in parallel at 4 h p.t., and values obtained from these cells were used to subtract the amount of Luc synthesized prior to Tg addition. In PKR−/− MEFs, where the phosphorylation of eIF2α is induced by Tg addition, Luc production from rep C+ luc dropped only 16% after Tg treatment, whilst it decreased by >95% from rep LPol-luc and rep LEMCV-luc (Fig. 3b). These results indicated that translation driven by the PV or EMCV IRES is eIF2α dependent in this system. The small decrease in Luc production after Tg treatment in S51A MEFs may reflect the secondary effects of Tg unrelated to eIF2α phosphorylation (Fig. 3b). Consistently, in this line, Luc production from all replicons was similarly affected by Tg treatment (9–24%). Finally in wt MEFs, Tg treatment had little effect on Luc production in all replicons (9–13% reduction). In this case, SV replication induced eIF2α phosphorylation via PKR. Thus, the addition of Tg only slightly increased the level of phosphorylation of eIF2α. Therefore, translation in this cell line was largely unaffected by the slight increase in phosphorylated eIF2α. In contrast, when comparing the absolute values of Luc activity obtained in the different cell lines, it was clear that PKR exerted an inhibitory effect on Luc production that could not be assigned exclusively to the induction of eIF2α phosphorylation (Fig. 3c). In such cases, Luc activity values obtained in untreated PKR−/− MEFs and S51A MEFs should be similar. However, Luc production was approximately 6.9-, 3.9- and 5.8-fold higher in PKR−/− MEFs than in S51A MEFs for rep C+ luc, rep LPol-luc and rep LEMCV-luc, respectively. Notably, Luc production was approximately 6.5- and 5.7-fold higher in S51A MEFs than in wt MEFs for rep LPol-luc and rep LEMCV-luc, respectively, whilst it was about 1.9-fold higher for rep C+ luc. The increased translatability of the sgRNAs with IRESs in S51A MEFs agrees with the idea that IRES-driven translation is eIF2α dependent. As an additional control, we examined the effects of expression of eIF2α and its S51A variant on Luc synthesis from rep LPol-luc in S51A MEFs (Fig. 4). We measured both Luc activity and the phosphorylation of eIF2α. To this end, wt eIF2α or its S51A variant were cloned after the sequence of the C protein in SV replicons (Fig. 4a). When cells were co-transfected with rep LPol-luc and rep C+ eIF2α/wt), there was clear phosphorylation of the newly produced eIF2α from the SV replicon, indicating that PKR can be activated in S51A MEFs (Fig. 4b). In contrast, overexpression of both forms of eIF2α, wt and S51A, in the co-transfected cells was evident after analysis of total eIF2α (Fig. 4b). Notably, in cells co-transfected with rep C+ eIF2α/wt), there was a decrease in Luc synthesis concomitant with the increase in phosphorylated eIF2α, providing further evidence that phosphorylation of eIF2α is the cause of the inhibition of LPol-luc sgRNA translation (Fig. 4c). This inhibition was not observed when S51A MEFs were co-transfected with rep LPol-luc and the replicon encoding the S51A eIF2α variant. In this case, no phosphorylation of this factor was observed and there was also no apparent decrease in Luc synthesis.

We also carried out another complementation experiment by expression of PKR in PKR−/− MEFs. To this end, human PKR was cloned after the sequence of the C protein in a SV replicon (Fig. 5a). To analyse the translation of sgRNA LPol-luc in PKR−/− MEFs that synthesize PKR, cells were co-transfected with rep LPol-luc and rep C+ PKR. As a control, cells were co-transfected with rep LPol-luc and rep C, which only expresses C protein. As additional controls, wt MEFs and S51A MEFs were also co-transfected. Next, Luc production was evaluated at 3 and 6 h p.t. (Fig. 5b). In parallel, human PKR production and the phosphorylation of eIF2α were analysed by Western
cells, even though eIF2α is phosphorylated. To analyse the translation of sgRNA LPol-luc in MEFs that synthesize PV 2Apro, the three different cell lines of MEFs were co-transfected with the replicons rep LPol-luc and rep C+2A. As a control, cells were co-transfected with rep LPol-luc and rep C+2A(G60R). The presence of PV 2Apro enhanced translation of sgRNA LPol-luc in all MEFs lines compared with expression of the 2A(G60R) variant (Fig. 6a). A higher enhancer activity of PV 2Apro was obtained in wt MEFs (19.2-fold), whilst in S51A MEFs and PKR−/− MEFs, the increase was 4.5- and 3.2-fold, respectively. It is probable that the better translation ability of LPol-luc sgRNA in these cell lines, where eIF2α is not phosphorylated, explains the lower transactivation by 2Apro. It is also noteworthy that the enhancement of PV IRES-dependent translation exerted by 2Apro in wt MEFs occurred in an environment where eIF2α was phosphorylated by virus replication independently of Tg treatment (see P-eIF2α in wt MEFs in Fig. 6c). The fact that 2Apro allowed IRES-dependent translation even when eIF2α was phosphorylated could be observed more clearly in PKR−/− MEFs treated with Tg (Fig. 6b). In these cells, phosphorylation of eIF2α occurred only after Tg treatment (Fig. 6c). Notably, addition of 1.5 μM Tg drastically blocked Luc synthesis when the 2A(G60R) variant was expressed in PKR−/− MEFs (4% of control), whilst Tg addition had little effect on Luc production in PV 2Apro-expressing cells (80–90% of control) (Fig. 6b). Addition of Tg had little effect on the phosphorylation of eIF2α in wt MEFs and S51A MEFs, as already indicated above. Altogether, these results indicated that, in the presence of 2Apro, sgRNA LPol-luc was translated efficiently even when eIF2α had been phosphorylated. In conclusion, PV 2Apro not only stimulates translation directed by sgRNA LPol-luc but also provides eIF2 independence during the initiation of translation of this mRNA.

**DISCUSSION**

Translation of the majority of picornavirus mRNAs can take place with intact eIF4G, or with the C terminus of this factor after PV 2Apro cleavage (Belsham, 2009). In addition, these mRNAs can be translated without some eIFs, such as eIF1, eIF1A and eIF4E (Pestova et al., 2001). Therefore, there is a widespread idea that picornavirus mRNAs are translated efficiently under adverse conditions. In this sense, it was surprising to find that alphavirus sgRNAs bearing picornavirus IRESs are very poorly translated from alphavirus replicons (Rausalu et al., 2009; Sanz et al., 2010). We found that the presence of picornavirus proteases such as PV 2Apro or FMDV Lpro increased this translation (Sanz et al., 2010). As both proteases cleave eIF4G, one possibility to explain these results was that the generation of the C terminus of eIF4GI could rescue IRES-driven translation. However, this is not the case, as illustrated in the present work, as individual or joint expression of the N- and C-terminal moieties of eIF4GI in SV-replicating cells did not

**Effect of 2Apro expression on translation of SV sgRNAs bearing a picornavirus IRES in MEFs**

Our above observations demonstrated that sgRNA LPol-luc can be translated when PV 2Apro is present in BHK-21...
increase protein synthesis directed by PV IRES. This finding is in good agreement with previous results indicating that the cleavage fragments of eIF4GI do not enhance picornavirus IRES-dependent translation (Redondo et al., 2011; Roberts et al., 1998), and nor did the expression of complete eIF4GI rescue translation directed by PV IRES. Moreover, the expression of a proteolytically inactive variant of 2Apro, 2A(G60R), plus the cleavage fragments of eIF4GI also could not rescue this translation. Therefore, we concluded that PV 2Apro exerts a different activity, in addition to the cleavage of eIF4GI. One possibility is that PV 2Apro participates in the initiation of PV mRNA translation by interacting with the C terminus of eIF4G, or even by direct binding to PV IRES. In this regard, it is of interest to note that PV variants in the IRES region can be compensated by mutations in the 2Apro gene (Macadam et al., 1994). As well as these considerations, it is important to note that eIF4GI as well as its moieties contained six extra amino acids at the N terminus that may have affected their functioning.

Several laboratories have demonstrated that eIF2α becomes phosphorylated during alphavirus infection (Gorchakov et al., 2004; McNerney et al., 2005; Sanz et al., 2009; Ventoso et al., 2006). In contrast, initiation of translation on the PV or EMCV IRES requires eIF2 when assayed out of the infection context or at the beginning of infection (Welnowska et al., 2011). Therefore, the inability of PV or EMCV IRESs to direct translation may be due to the phosphorylation of eIF2α in SV-replicating cells. As demonstrated in this work, this seems to be the case, as the replication of rep LPol-luc or rep LEMCV-luc gave rise to the synthesis of significant amounts of Luc in PKR−/− MEFs and S51A MEFs. This translation utilized the replication of rep LEMCV-luc and rep C+PKR, and at 3 or 6 h p.t. were collected and the Luc activity determined. As controls, wt MEFs and S51A MEFs were also co-transfected. The percentage values of C+PKR-expressing cells relative to their respective C-expressing cells are indicated in the figure. (c) PKR expression was analysed in parallel by Western blotting with a mixture of rabbit antibodies against mouse PKR (MusPKR) and rabbit antibodies against human PKR (HuPKR). The amount of phospho-eIF2α and eIF2α was also determined.
eIF2α is strongly phosphorylated, extensive Luc synthesis directed by PV IRES takes place.

**METHODS**

**Cell lines and viruses.** MEFs derived from normal and PKR−/− mice have been described previously (Yang et al., 1995). S51A MEFs containing an unphosphorylatable form of eIF2α were kindly provided by D. Ron (MRC Laboratory of Molecular Biology, Cambridge, UK) and R. J. Kaufman (University of Michigan School of Medicine, Ann Arbor, MI, USA) and have been described previously (Scheuner et al., 2001). BHK-21 cells were obtained from ATCC. SV stock was prepared from a pT7 SV wt infective cDNA clone (Sanz et al., 2009).

**Antibodies.** A mixture of rabbit polyclonal antibodies raised against peptides derived from the N- and C-terminal regions of human eIF4GI were used to detect eIF4GI, eIF4GNt and eIF4GCt (Feduchi...
et al., 1995). Specific rabbit polyclonal antibodies raised against phospho-eIF2α (serine 51) (Cell Signaling Technology) or total eIF2α (Santa Cruz Biotechnology) were used in Western blot analysis. The rabbit polyclonal antibodies raised against Luc, mouse PKR and human PKR were purchased from Santa Cruz Biotechnology.

**In vitro transcription and electroporation.** Plasmids digested with XhoI enzyme were used as templates for *in vitro* RNA transcription with T7 RNA polymerase (Promega). The transcription mixture always contained an m'G(5')ppp(5')G cap analogue. For electroporation, subconfluent BHK-21 cells were harvested, washed with ice-cold PBS and resuspended at a density of ~2.5 × 10^6 cells ml^-1 in the same buffer. Subsequently, 20 μg in *in vitro*-transcribed RNA was added to 0.4 ml cell suspension and the mixture was transferred to a 2 mm cuvette. Electroporation was carried out at room temperature by generating two consecutive 1.5 kV, 25 μF pulses with a Gene Pulser apparatus (Bio-Rad), as described previously (Liljestrom et al., 1991).

**Transfection of MEFs.** The mRNAs used for transfection were *in vitro* transcribed, treated with DNase I and purified using an RNA cleanup kit (Qiagen). Transfection was carried out in 200 μl Opti-MEM I Reduced Serum Medium (Invitrogen) for each L-24 plate well using 2 μl Lipofectamine 2000 (Invitrogen) and 5 μg mRNA, as described previously (Welnowska et al., 2011).

**Analysis of protein synthesis by radioactive labelling.** Protein synthesis was analysed as described elsewhere (Sanz et al., 2009). The cells were then collected in the appropriate gel loading buffer and analysed by SDS-PAGE and autoradiography.

**Measurement of Luc activity.** Cells were lysed in buffer containing 0.5 % Triton X-100, 25 mM glycylglycine (pH 7.8) and 1 mM DTT. Luc activity was determined using a Monolith 2010 luminometer (Promega).

**Isoelectric focusing and analysis by radioactive labelling of SV RNAs synthesized in SV-replicating cells.** Isoelectric focusing was carried out as described previously (Ventoso et al., 2006). Uridine-[3-H] incorporation in cells treated with actinomycin D (2.5 μg ml^-1) was used to detect viral RNA synthesis by agarose gel electrophoresis of labelled RNA, as described previously (Sanz et al., 2010).

**ACKNOWLEDGEMENTS**

This study was supported by a DGCIT grant no. BFI2009-07352. N.R. and M.G.M. are holders of FPI Fellowships. The Institutional grant awarded to the Centro de Biología Molecular ‘Severo Ochoa’ by the Fundación Ramón Areces is acknowledged.

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