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Functional impairment of eIF4A and eIF4G factors correlates with inhibition of influenza virus mRNA translation

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ABSTRACT

Influenza virus mRNAs contain a 5′-cap structure followed by short cell-derived heterogeneous oligonucleotides and they are polyadenylated. However, selective translation of viral mRNAs occurs upon infection. Thus, we have studied whether differential requirements for the eIF4F components on viral and cellular translation could mediate this selectivity. We have previously reported that influenza virus infection proceeds efficiently upon functional impairment of the cap-binding factor eIF4E. Now, the requirements for the eIF4A helicase and the eIF4G scaffolding factor have been examined. The two proteins are essential for viral translation both in *in vivo* and *in vitro* analysis. Consequently, viral mRNAs do not contain *cis*-acting signals that could mediate eIF4A and eIF4G independence and *trans*-acting viral proteins do not replace their function. Thus, eIF4A and eIF4G proteins are not responsible for the selective translation of viral mRNAs and the translational shut-off of cellular protein synthesis observed in influenza virus infected cells.

Introduction

Due to their limited coding capacity, viruses do not possess the required components to initiate mRNA translation. Thus, they are obliged to utilize host cell factors and, therefore, to compete for and manipulate the translation apparatus to their own benefit. Initiation of translation is a major target for the regulation of gene expression (Gray and Wickens, 1998; Pestova et al., 2001) and viruses have evolved numerous unconventional mechanisms to recruit the cellular translational machinery to the viral messengers. Translation initiation of most cellular mRNAs requires an active eIF4F complex and this requirement has provided viruses with a readily accessible target and opportunity to block host-cell protein synthesis. Cellular mRNAs possess a 7-methyl guanine structure (cap) at their 5′ ends, which plays a critical role in the recruitment of the ribosome to the mRNA. The cap structure is firstly recognized by eIF4E, the cytosolic cap-binding protein that allows the subsequent binding of eIF4G and eIF4A, the other two components of the eIF4F complex. This complex efficiently recruits the 40S ribosomal subunit by direct interaction of eIF3 with the eIF4G subunit (for review, see (Gingras et al., 1999)) and, consequently, eIF4F constitutes one of the key factors on the control of protein synthesis. Accordingly, many viruses have developed alternative initiation mechanisms that circumvent or decrease the requirement for this complex. The strategies focused on eIF4F complex used by the different viruses depend on the nature of the corresponding viral mRNAs, such as whether or not they are capped or uncapped and whether or not their 3′ end are polyadenylated. For instance, a wide range of picornaviruses encode a protease that cleaves the eIF4G protein in two, impairing the cap-dependent translation initiation of cellular mRNAs, whereas cap-independent initiation of viral mRNAs is preserved via the utilization of internal ribosome entry sites (IRESs) (see reviews (Bushell and Sarnow, 2002; Schneider and Mohr, 2003)). Among the strategies used by viruses whose mRNAs are capped, disruption of the eIF4G–PABP1 interaction by rotavirus (Piron et al., 1998), displacement of eIF4G–Mnk1 interaction by adenovirus (Schneider and Mohr, 2003; Xi et al., 2004) or dephosphorylation of eIF4E and 4E-BP1 proteins, which results in reduced eIF4E–eIF4G association, by vesicular stomatitis virus (Connor and Lyles, 2002), have been broadly reported and widely studied.

Influenza virus mRNAs possess a 5′ cap structure and a 3′ polyA tail. Synthesis of capped and polyadenylated viral mRNAs is performed by the viral polymerase complex and is primed by short-capped oligonucleotides of around 10 to 12 nucleotides, which are scavenged from host cell nuclear mRNAs by viral polymerase endonuclease activity (Plotch et al., 1981). Following this cell-derived heterogenous sequence, viral mRNAs contain a common sequence that many contribute to translational regulation in the infected cell

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(Garfinkel and Katze, 1993). The viral polymerase complex, which is responsible for both the transcription and replication of the viral genome, is composed of three subunits named PA, PB1 and PB2. The PB2 subunit is responsible for the binding to the cap structures (Blaas et al., 1982), while the endonucleolytic activity required for the cap-snatching process resides in the PA subunit (Dias et al., 2009).

Although influenza virus mRNAs possess a 5′-cap structure, some experimental evidences strongly suggest that viral mRNAs are not translated by the classic cellular cap-dependent mechanism. For instance, influenza virus infection induces a significant dephosphorylation of eIF4E and a moderate phosphorylation of eIF4G (Feigenblum and Schneider, 1993), which have been associated with an inhibition of the cellular protein synthesis in different situations (Ling et al., 2005; Ross et al., 2006; Scheper and Proud, 2002). These alterations of the components of the eIF4F complex may contribute to the host cell translational shut-off induced by influenza virus in the infected cell. Moreover, we have previously shown that influenza infection proceeds efficiently upon functional impairment of the eIF4E factor (Burgui et al., 2007). In addition, influenza virus polymerase binds to translation preinitiation complexes and viral infection increases the binding of eIF4GI factor to cap-structures upon induced eIF4E–eIF4GI disassociation (Burgui et al., 2007). All together, the observed eIF4E-independence for viral mRNAs translation, the cap-binding nature of the polymerase complex and the ability of the viral polymerase to recruit eIF4G to cap structures, suggest that the viral polymerase could replace the functionally of eIF4E during infection. However, the contribution of the two other components of the eIF4F complex to viral translation remained unknown. In the present study, we have characterized whether translation of influenza messengers depends on the two other components of the eIF4F translation initiation factor, eIF4A and eIF4G proteins, to entirely characterize the dependence for the eIF4F complex during translation of influenza virus mRNAs.

Results

Influenza virus translation requires functional eIF4A

The eIF4A protein is a member of the DEAD box family of putative ATPase/helicases and is the RNA helicase component of the eIF4F complex. eIF4A is recruited to the 5′-end of mRNA molecules by an interaction with two independent binding sites located in the middle and C-terminal regions of eIF4G, which increases the RNA-stimulated helicase activity of eIF4A (Grifo et al., 1984; Korneeva et al., 2000; Pause et al., 1994). The relative requirement for eIF4A of a particular mRNA has been proposed to be at least partially determined by the level of RNA structure present in the 5′-end of the mRNA (Svitkin et al., 2001). Since the 5′-UTR sequences of influenza virus mRNAs contain between 20 and 50 nucleotides and according to secondary structure predictions, are poorly structured, a low requirement for the activity of the eIF4A helicase on viral transcription might be expected. To analyze the contribution of eIF4A on viral protein synthesis, different experimental approaches were used both in *in vitro* and *in vivo* assays.

Effect of hippuristanol on in vitro viral mRNA translation

To start characterizing the requirement for eIF4A on influenza mRNA translation, *in vitro* translation experiments were performed using hippuristanol. Hippuristanol is a sterol isolated from the coral *Isis hippuris* and identified via a high throughput screening for general translation inhibitors. It has been shown to block eIF4A-dependent translation by inhibiting RNA binding, ATPase, and helicase activities by the interaction with the C-terminal domain of eIF4A (Bordeleau et al., 2006). Moreover, hippuristanol appears to be highly selective because it has no effect on other RNA and DNA helicases tested and does not affect *in vitro* RNA splicing reactions (Bordeleau et al., 2006).The translation efficiency of viral mRNAs isolated from influenza virus infected cells was evaluated in the presence or absence of hippuristanol. A dicistronic cap-CAT:PTV-IRES-Luciferase RNA, containing the eIF4A-independent porcine teschovirus internal ribosome entry site (IRES),and rabbit β-globin mRNA were used as controls. Rabbit reticulocytes extracts were incubated or not with increasing concentrations of the inhibitor prior to the addition of *in vitro* transcribed cap-CAT-PTV:IRES-Luc RNA, rabbit β-globin mRNA or purified total RNA from influenza infected cells. Viral mRNAs extracted from infected cells have been synthesized by the viral polymerase and therefore, they would contain the described heterogeneity of the 5′UTR ends. After 90 min of incubation in the presence of ^{35}S-Met, the synthesized proteins were analyzed. As can be seen in Fig. 1, hippuristanol treatment resulted in a significant inhibition of cap-dependent CAT and influenza virus protein translation, while the synthesis of the IRES-dependent luciferase protein was unaffected. In addition, inhibition of β-globin mRNA translation was observed with hippuristanol treatment (Supp. Fig. 1A).

Effect of eIF4A dominant negative mutants in vitro

Next, to further characterize the requirement for eIF4A and confirm the previous results, *in vitro* experiments were carried out using different dominant negative mutants of eIF4A. Three different mutants were used: DQAD, R362Q and PRRVAA. DQAD has a mutation in the ATPase B motif of eIF4A, which results in inactivation of the ATPase and helicase activities (Pause et al., 1994). Mutant R362Q in the conserved 362 arginine of the C-terminal region of eIF4A, exhibits drastically reduced RNA binding and no RNA helicase activity (Pause et al., 1993). The dominant negative PRRVAA contains a mutation in the conserved Ia region (PTRELA to PRRVAA) and is inactive in ATP hydrolysis and RNA unwinding activities (Svitkin et al., 2001). The wild type or different mutants forms of eIF4A were purified from bacteria as described (Chard et al., 2006) and added to the reticulocyte lysates at different concentrations. *In vitro* transcribed cap-CAT:PTV-IRES-Luc RNA, rabbit β-globin mRNA or purified total RNA from infected cells were added to these extracts and the *in vitro* labeled proteins were analyzed. Addition of wild type eIF4A did not inhibit protein translation, while the addition of any of the eIF4A mutants resulted in a dramatic reduction in cap-dependent CAT and influenza virus proteins synthesis (Fig. 2) or β-globin mRNA translation (Supp. Fig. 1B) without affecting the IRES-mediated translation of the luciferase protein (Fig. 2).

Collectively, these data indicate that, although the 5′UTR of influenza messengers are short and should not be highly structured, the helicase activity of eIF4A is required for *in vitro* viral mRNAs translation.

Effect of hippuristanol in in vivo experiments

Although isolated viral mRNAs behaved as cellular capped transcripts in the *in vitro* translation assays, the activity of eIF4A could be replaced by a viral protein in the infected cell. In order to evaluate this possibility, we examined the effect of hippuristanol treatment on influenza virus protein synthesis *in vivo*. Due to its ability to inhibit cap-dependent translation, hippuristanol can be cytotoxic when cells are treated with high concentrations for long periods of time. In order to control for any indirect effects caused by a decrease in cell viability, the cytotoxicity of hippuristanol was evaluated. The viability of HEK293T cells was examined after 10 h exposure to increasing concentrations (0.03–1 μM) of the inhibitor using Cell Titer-Blue (Fig. 3A). The viability of the cell cultures was not significantly reduced at the different concentrations of hippuristanol assayed.

Next we assayed the hippuristanol effect on cellular translation and eIF4A-independent PTV-IRES activity as control. Thus, HEK293T cells were treated with hippuristanol for 1 h (0.5 and 1 μM concentration) and then were left untransfected or transfected with an *in vitro* transcribed PTV-IRES-luciferase RNA. 8 h post-transfection, luciferase activity was measured and compared with the general cellular translation quantified by ^{35}S-Met incorporation (Fig. 3B). As expected,

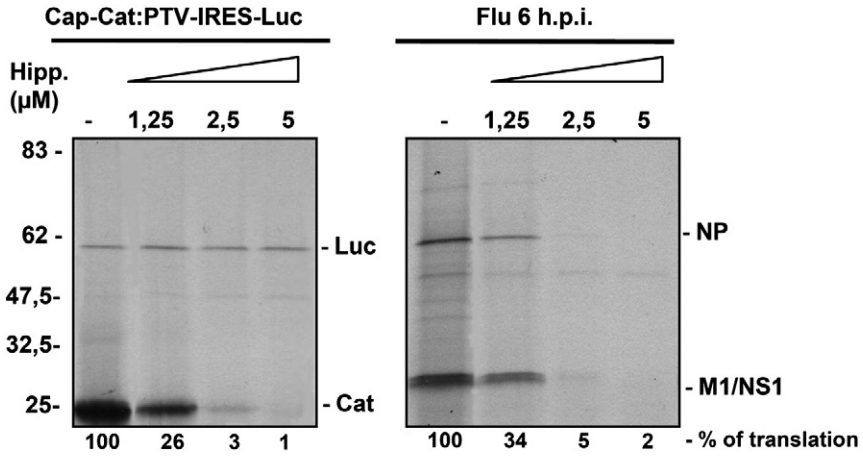

Fig. 1. *In vitro* translation of influenza virus mRNA is inhibited by the eIF4A small molecule inhibitor hippuristanol. Rabbit reticulocyte lysates were pretreated with either DMSO (−) or the eIF4A inhibitor hippuristanol at final concentrations of 1.25, 2.5, and 5 μM. The proteins synthesized from bicistronic cap-CAT:PTV-IRES-Luc or total RNA extracted from influenza infected cell 6 hpi were metabolically labeled and examined by SDS gels. The effect of the inhibitor was quantified by ^{35}S-Met incorporation and translation efficiency was expressed as a percentage of the translation in the untreated extracts.

cellular translation was significantly reduced upon hippuristanol addition, while PTV-IRES-mediated luciferase expression was slightly increased in these conditions, indicating that hippuristanol inhibits eIF4A-dependent translation initiation without affecting the general translation capacity of the treated cells. These results are in agreement with previously published data (Bordeleau et al., 2006), and the enhancement in luciferase expression upon eIF4A inhibition could reflect a reduction in the competition and an increase in the accessibility of the PTV-IRES-Luc RNA to the protein synthesis machinery.

To evaluate the effect of hippuristanol on influenza infection, HEK293T cells were treated with the inhibitor at 1 μM concentration for 1 h and either mock-infected or infected with influenza virus at 3 pfu/cell. The hippuristanol concentration in the culture medium was maintained over the duration of experiment. At various times post infection, the cells were metabolically labeled with ^{35}S-Met for 1 h and the synthesized proteins were analyzed by SDS-polyacrylamide gels (Fig. 3C). Both, cellular and viral protein synthesis were severely reduced by the hippuristanol treatment indicating that, besides the observed *in vitro* requirement, eIF4A is also essential for a correct influenza protein synthesis. Therefore, influenza virus mRNAs translation occurs following the behavior of a conventional cap-dependent pathway in respect to the eIF4A helicase activity.

Influenza virus translation requires functional eIF4G

The eIF4G protein has a pivotal role within the eIF4F complex, since it is a scaffold protein that possesses binding sites for eIF4E, eIF4A, polyA binding protein1 and Mnk1 kinase (Fig. 5A). In addition, it directly binds eIF3, the factor that mediates ribosome recruitment. Thus, eIF4G plays an essential role by acting as a molecular focal point upon which the translation initiation complex is assembled, bringing together the mRNA and ribosome. Co-infection experiments revealed that influenza virus do not progress in poliovirus infected cells (Garfinkel and Katze, 1992), in which eIF4G is cleaved by the poliovirus 2A protease. However, as poliovirus infection results in many intracellular changes, the previous experiments do not exclude that some of the observations may be the result of alterations non-directly related with translation initiation. Thus, to directly analyze the requirements for the different eIF4F components on influenza translation, the contribution of eIF4G was evaluated both in *in vitro* and *in vivo*.

In vitro requirements for eIF4G factor on influenza virus mRNA translation

The eIF4G factor is structurally divided in an N-terminal domain, which contains the eIF4E binding site required for cap-dependent translation, and a C-terminal domain, which mediates the association with the eIF3 complex and the ribosome recruitment. Several viral proteases, including the L-protease from foot and mouse disease virus, cleave eIF4G in two, separating the eIF4E and the eIF3 binding sites, inhibiting cap-dependent translation and permitting a cap-independent translation. To analyze whether translation of viral mRNAs could take place upon eIF4G cleavage, *in vitro* experiments were performed. Rabbit reticulocyte lysates were incubated with increasing amounts of purified L-protease from foot and mouth disease virus. *In vitro* transcribed cap-CAT:EMCV-IRES-Luc RNA, rabbit β-globin mRNA or RNAs isolated from influenza virus infected HEK293T cells were added and after metabolic labeling, the synthesized proteins were examined. As can be seen in Fig. 4A, L-protease efficiently cleaved eIF4GI, resulting in the separation of the N and C-terminal domains. As expected, whereas IRES-mediated luciferase synthesis was unaffected by eIF4G cleavage (Fig. 4B, left panel), cap-dependent translation of the CAT gene (Fig. 4B, left panel) and β-globin (Supp. Fig. 1C) decreased as the concentration of L-protease was increased. Similarly, the translation of influenza virus proteins decreased concomitantly with the increase in the protease concentration (Fig. 4B, right panel). Therefore, the translation of influenza virus mRNAs depends on an intact eIF4G protein, at least *in vitro*, confirming that *cis*-acting sequences resembling an IRES element that would confer independence of eIF4Gare not present in its mRNAs.

Specific requirement for eIF4G isoforms on in vivo influenza mRNA translation

The eIF4G protein is expressed in mammalian cells as two main isoforms, namely eIF4GI and eIF4GII, which share 46% identity at the amino acid level. A schematic representation of both proteins is depicted in Fig. 5A. To date, no clear evidence has been found for differential activities between eIF4GI and eIF4GII, since the two proteins can functionally complement each other in various translation assays (Coldwell and Morley, 2006; Prevot et al., 2003). However, some data support the notion that, although eIF4GI and eIF4GII can be at least in part functionally interchangeable, there are differences in their response to several signals that regulate translational, such as the selective recruitment of eIF4GII to eIF4F complex during megakaryocytic differentiation (Caron et al., 2004).

It is worth noting that a differential role for eIF4GI and eIF4GII in influenza virus mRNA translation cannot be inferred from previous work using co-infection or *in vitro* experiments, as viral proteases (including the poliovirus 2A protease) efficiently cleaves both isoforms. In order to evaluate the role of eIF4G and the specific contribution of

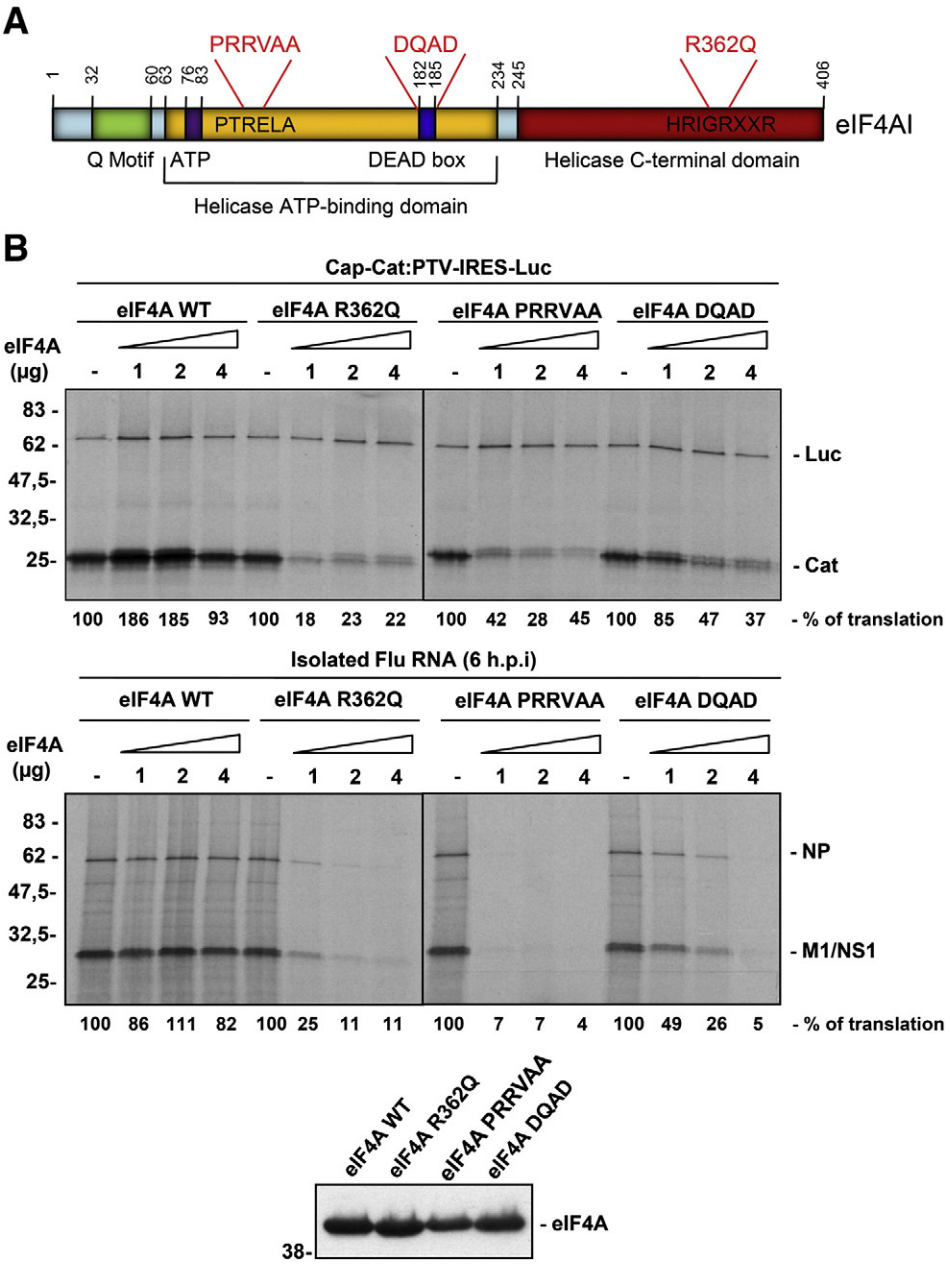

Fig. 2. *In vitro* translation of influenza virus mRNA is inhibited by eIF4A dominant negative mutants.(A)The sequence of eIF4A is schematically represented indicating the more relevant conserved regions. The position of the different mutations in the three negative dominants (DQAD, R362Q and PRRVAA) is shown in red. (B)Rabbit reticulocyte lysates were preincubated with either control buffer or recombinant eIF4A dominant negative mutants (1, 2, 4 µg of protein per reaction) and the effect on control dicistronic cap-CAT:PTV-IRES-Luc or total RNA extracted from influenza infected cell 6 hpi was assayed. The levels of translation observed were quantified by phosphorimaging and expressed as a percentage of the untreated control lysate.

eIF4GI and eIF4GII to viral translation *in vivo*, we performed RNA silencing experiments using siRNAs designed to discriminate between eIF4GI and eIF4GII mRNAs. Cultured A549 cells were transfected with specific siRNAs for eIF4GI or eIF4GII or with a random siRNA sequence and the effectiveness of the silencing was evaluated by measuring the accumulation levels of these proteins in the corresponding silenced cells (Fig. 5B). Twenty-four hours after the siRNAs transfection, the cells were re-transfected to improve the silencing efficiency and 48 h after from the last transfection, mock-infection, or infection with influenza virus was carried out. To discard possible indirect effects on influenza virus translation derived from variations in the amount/activity of other factors that could significantly reduce translation initiation efficiency, infection with vesicular stomatitis virus (VSV) was used as control, as it is able to initiate protein synthesis in the absence of eIF4G (Connor and Lyles, 2002). The cells were metabolically labeled at different times post-infection with ^{35}S-Met for 1 h and the synthesized proteins were analyzed in SDS-polyacrylamide gels. Quantitative analyses of the translation efficiency of cellular mRNAs after silencing of eIF4G showed that the siRNA directed against the eIF4GI isoform produced an inhibition of 40–60%, whereas the silencing of the eIF4GII isoform inhibited the cellular mRNAs translation by only 15–20% (Fig. 5C, Mock). When translation of influenza virus mRNAs was analyzed, we observed an inhibition on viral protein translation that mainly correlated with the observed decrease on cellular translation by each specific siRNA (Fig. 5C, Flu).It should be mentioned that the particular contribution of eIF4G isoforms on cellular protein synthesis is not yet well established and varies according to the mRNA analyzed (Castello et al., 2006). In agreement with our previous publications (Welnowska et al., 2009),

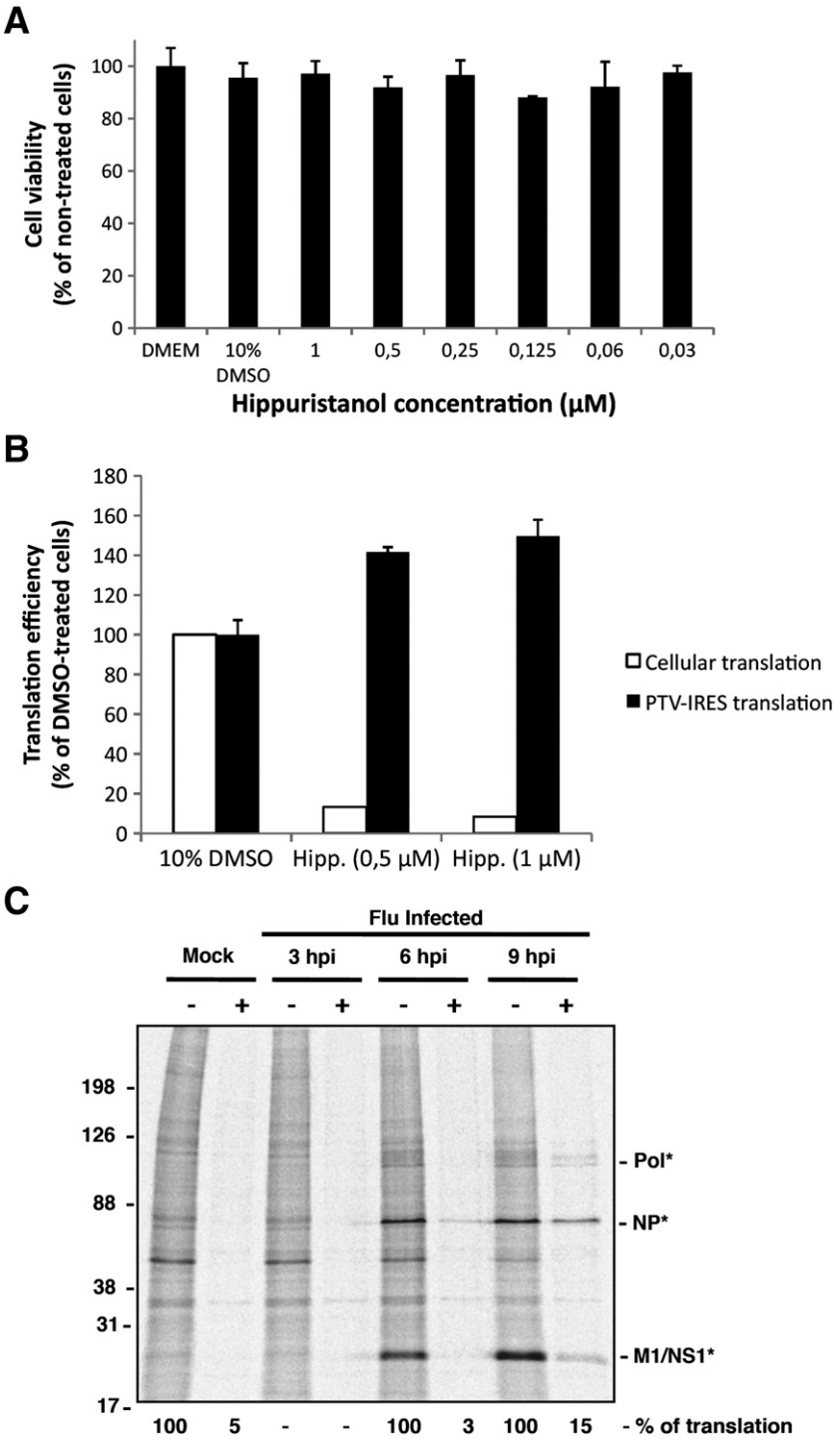

Fig. 3. The eIF4A inhibitor hippuristanol inhibits influenza virus translation. (A) The viability of HEK293T cells was examined after 10 h exposure to increasing concentrations (0.03–1 μM) of hippuristanol using Cell Titer-Blue (Promega). Cell viability is expressed as percentage of the untreated cells. (B) HEK293T cells were treated with hippursitanol for 1 h (0.5 and 1 μM concentration) and then transfected with an *in vitro* transcribed PTV-IRES-luciferase mRNA. 8 h post-transfection, luciferase activity was measured and compared with the general cellular translation determined by ^{35}S-Met incorporation. Translation efficiency is expressed as a percentage of the untreated control cells. (C) HEK293Tcells were treated with DMSO (−) or hippuristanol (1 μM) (+) for 1 h and either mock-infected or infected with influenza virus at 3 pfu/cell. The hippuristanol concentration in the culture medium was maintained all over the experiment. At different hpi, the cells were metabolically labeled with ^{35}S-Met for 1 h and the synthesized proteins were analyzed by SDS-polyacrylamide gels. The cellular and viral proteins (*) synthesized were quantified by phosphorimaging and translation efficiency was expressed as a percentage of the untreated control lysate.

VSV protein synthesis was not affected in these conditions (Fig. 5D), suggesting that silencing specifically inhibited eIF4G-dependent initiation without affecting general translation capacity of the silenced cells.

Since a significant reduction on viral mRNAs translation was observed upon eIF4GI silencing, we asked whether accumulation of viral proteins, which represents the summative effects on viral protein synthesis of each translation event, would be more substantially reduced in these conditions. To examine the accumulation of influenza virus proteins, the amount of PB2 polymerase subunit was determined at 6 hpi by Western-blot in control or eIF4G silenced cells (Fig. 6A). In addition, immunofluorescence analyses were performed in parallel to evaluate the presence and distribution of PB2 protein (Fig.6B). The inability of the eIF4GII antibody to detect the native protein did not allow performing similar analysis with this isoform. The results showed

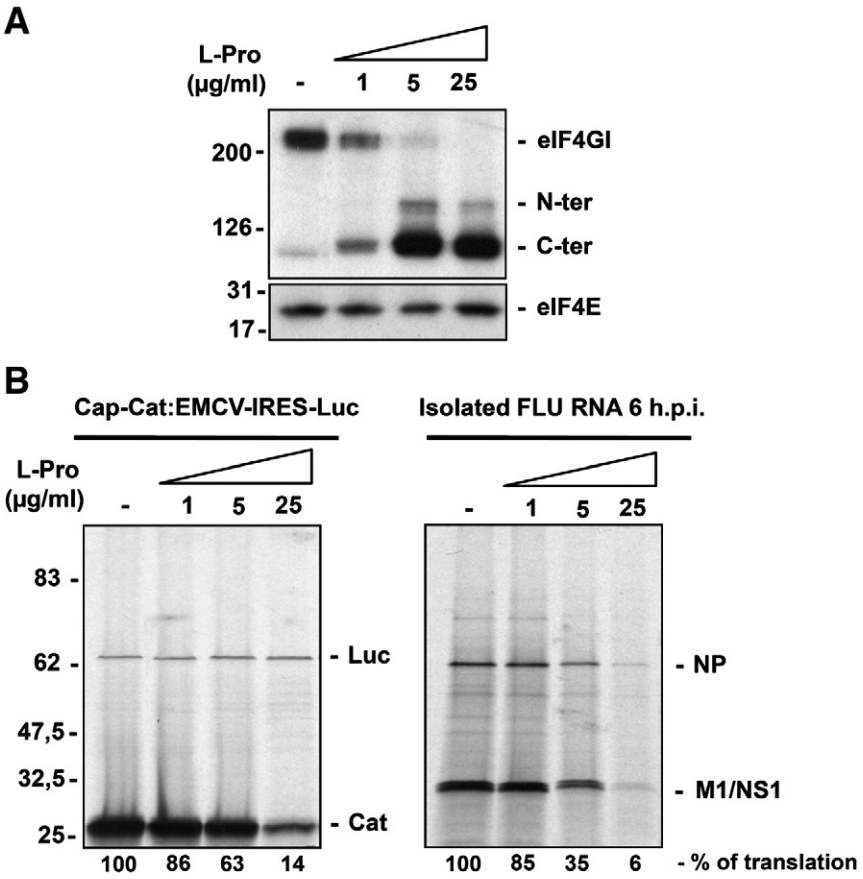

Fig. 4. *In vitro* translation of influenza virus mRNA requires intact eIF4G.Rabbit reticulocyte lysates were preincubated with or without recombinant FMDV Lb protease at a final concentration of 1, 5 and 25 μg/ml. Samples of treated lysates were either Western blotted with anti-eIF4GI (A) or used to translate control dicistronic cap-CAT:EMCV-IRES-Luc or total RNA extracted from influenza infected cell 6 hpi. (B) The effects on translation were quantified by phosphorimaging and expressed as a percentage of the translation levels observed in non-treated control.

a decrease of around 10 fold on PB2 accumulation upon eIF4GI silencing and, accordingly, PB2 was barely detectable by immunofluorescence in eIF4GI-silenced cells. These data suggest that eIF4GI is an absolute requirement for efficient influenza virus infection.

Discussion

Undoubtedly, the control of viral proteins synthesis in the host-cell is a key mechanism that viruses utilize to the benefit of their replication cycle and, at the same time, to regulate host cell gene expression. As mentioned, the control of the translation initiation step and, specifically, of the eIF4F factor is a mechanism widely used by numerous viruses. Accordingly, we have previously proposed an alternative cap-dependent mechanism used to initiate influenza virus mRNAs translation based on the observed normal progression of influenza virus infection during functional impairment of cap-binding eIF4E factor (Burgui et al., 2007). In that model, the influenza polymerase, bound to the viral 5′UTR common sequence, would remain associated to the capped 5′end of the viral mRNAs, functionally replacing eIF4E and recruiting translation initiation complexes. The proposed mechanism could also contribute to the host-cell shut-off observed in the infected cell. However, in addition to the reported eIF4E independence, other differential requirements between cellular and viral mRNAs for the other components of the eIF4F complex could occur, cooperatively resulting in the reported selective translation of influenza mRNAs in the infected cells.

Dependence of eIF4A helicase

Here, we provide evidence that translation of influenza virus mRNAs is fully dependent on a functional eIF4A factor both, in *in vivo* and *in vitro* studies. Since potential secondary structures in the 5′ UTR need to be resolved for landing and scanning of the small ribosomal subunit, the relative requirements for eIF4A are thought to correlate with the degree of RNA structure contained at the 5′ end of the corresponding mRNA (Svitkin et al., 2001). Interestingly, dependence of eIF4A occurs even though the 5′ end of influenza virus mRNAs is rather short and probably not highly structured. These results are in agreement with previous reports suggesting additional roles for eIF4A, such as its activity enhancing ribosome binding to unstructured mRNAs (Pestova and Kolupaeva, 2002). In addition, since the mRNAs in the cytoplasm are associated with proteins, it has been proposed that the eIF4A helicase activity could work removing proteins from the 5′ UTR of the RNA–protein complexes (Jankowsky et al., 2001). Some of the activities proposed for eIF4A are clearly required for efficient influenza virus mRNA translation, but the precise role of eIF4A in the process remains elusive. Moreover, the reported high abundance of eIF4A in mammalian cells (Hershey, 1994) makes unlikely that competition for this factor between cellular and viral mRNAs would contribute to the shut-off and the selective translation of influenza virus mRNAs in the infected cell.

Dependence of eIF4G

A wide variety of animal viruses such as picornaviruses, retroviruses and calicivirus express proteases that induce the cleavage of eIF4GI or/and eIF4GII proteins among other translation initiation factors (Schneider and Mohr, 2003). These viruses have provided very valuable tools to examine the specific roles of these eIF4G isoforms on the viral induced cellular shut-off and, in addition, to establishing their specific contribution to viral infection. Several reports revealed that hydrolysis of eIF4GI is not sufficient to fully inhibit host cell translation (Bonneau and

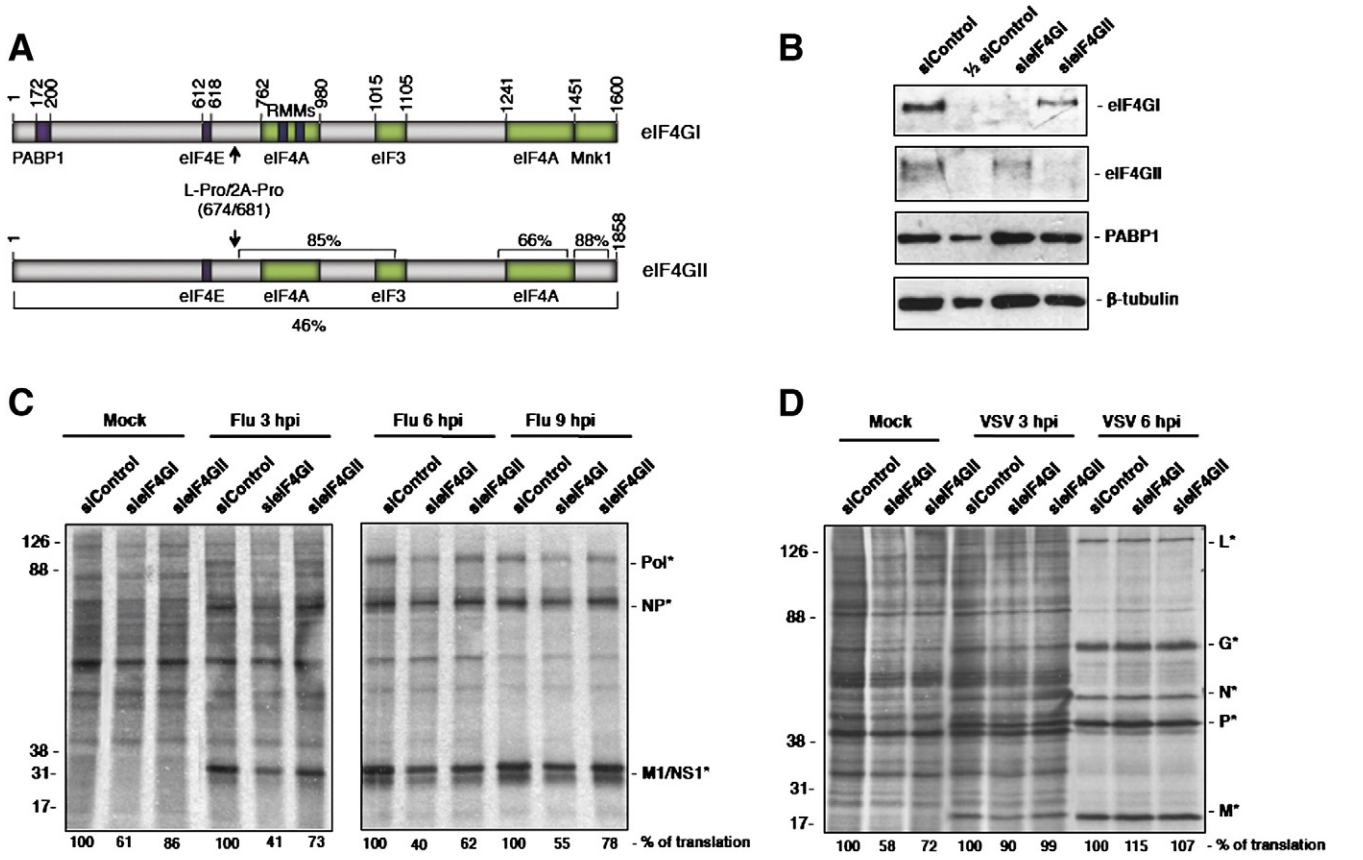

Fig. 5. *In vivo* translation of influenza virus mRNA requires eIF4G. (A) Schematic representation of eIF4GI and eIF4GII. N-terminal domains that mediate interaction with other proteins are represented in purple, while C-terminal interaction domains are represented in green. (B) A549 cells were transfected with specific silencers for eIF4GI or eIF4GII or with a random siRNA sequence and western-blot analysis was performed to control the efficiency of eIF4GI and eIF4GII silencing. Silenced cells where then either mock-infected or infected with influenza virus (C) or vesicular stomatitis virus (D) at 3 pfu/cell. At different hpi, the cells were metabolically labeled with ^{35}S-Met for 1 h and the synthesized proteins were analyzed by SDS-polyacrylamide gels. The cellular and viral proteins (*) synthesized were quantified by phosphorimaging and translation efficiency was expressed as a percentage of the untreated control lysate.

Sonenberg, 1987; Castello et al., 2006; Keiper and Rhoads, 1997). In addition, degradation of eIF4GII correlates with the abrogation of host cell protein translation in poliovirus and rhinovirus-infected cells, as well as in apoptotic cells (Gradi et al., 1998; Marissen et al., 2000; Svitkin et al., 1999). Therefore, it seems that cleavage of both eIF4G isoforms contribute to the viral-induced cellular shut-off indicating that translation of cellular mRNAs rely on both eIF4G factors with different degrees of contribution. Accordingly, it has been reported that translation of *de novo* synthesized mRNAs is highly dependent on eIF4GI integrity, whereas ongoing translation mainly dependent on eIF4GII integrity (Castello et al., 2006).

Influenza virus mRNA translation shows a similar degree of eIF4G dependence to that observed for host-cell mRNAs in infected A549cells (Fig. 5). Previous reports have quantified the relative amount of the two eIF4G isoforms in HeLa cells, and eIF4GI is the most abundant constituting around the 80% of the total eIF4G in the cell (Svitkin et al., 1999). With this information, if A549 cells behave similar, it seems plausible that an important inhibition on viral mRNAs translation takes place upon eIF4GI knock-down since it would represent a drastic reduction in the total amount of eIF4G factor. However, both eIF4G isoforms clearly contribute to efficient translation of influenza virus mRNAs that seems undistinguishable of the behavior of host-cell mRNAs.

Concluding remarks

The requirements of influenza virus mRNAs translation for the eIF4F components have shown that a functional cellular cap-binding factor eIF4E is dispensable (Burgui et al., 2007), whereas the eIF4A helicase and the eIF4G protein are absolutely needed (this report). Influenza virus infection induces an important cellular shut-off where the inhibition of translation initiation of host-cell mRNAs plays an important role (Katze et al., 1986). Thus, those alterations related with the impairment of eIF4E probably represent the most important viral induced activity in the process, as our data indicate that impairment of eIF4A or eIF4G would not allow efficient influenza infection.

Materials and methods

Biological materials

The HEK293T and A549 cell lines and the vesicular stomatitis virus and the influenza virus A/Victoria/3/75 (VIC) strain were used throughout these studies. L-protease was kindly provided by T. Skern. Hippuristanol was kindly provided by J. Pelletier. Rabbit β-globin mRNA was acquired from Sigma. Complete protease inhibitors and RNase (human placenta RNAse inhibitor) inhibitor were acquired from Roche.

Transfection and virus infection

All infections were carried out at a multiplicity of infection of 3 pfu/cell. Transfection of *in vitro* synthesized PTV-IRES-Luc mRNA in HEK293T cells was performed using Lipofectamine 2000 (Invitrogen). For eIF4G silencing A549cells were transfected twice (0 and 24 h) with 25 nM of control siRNAs (siControl), specific siRNA for eIF4GI (si4GI-31) [CCCAUACUGGAAGUAGAAGTT] (Coldwell and Morley, 2006) or eIF4GII

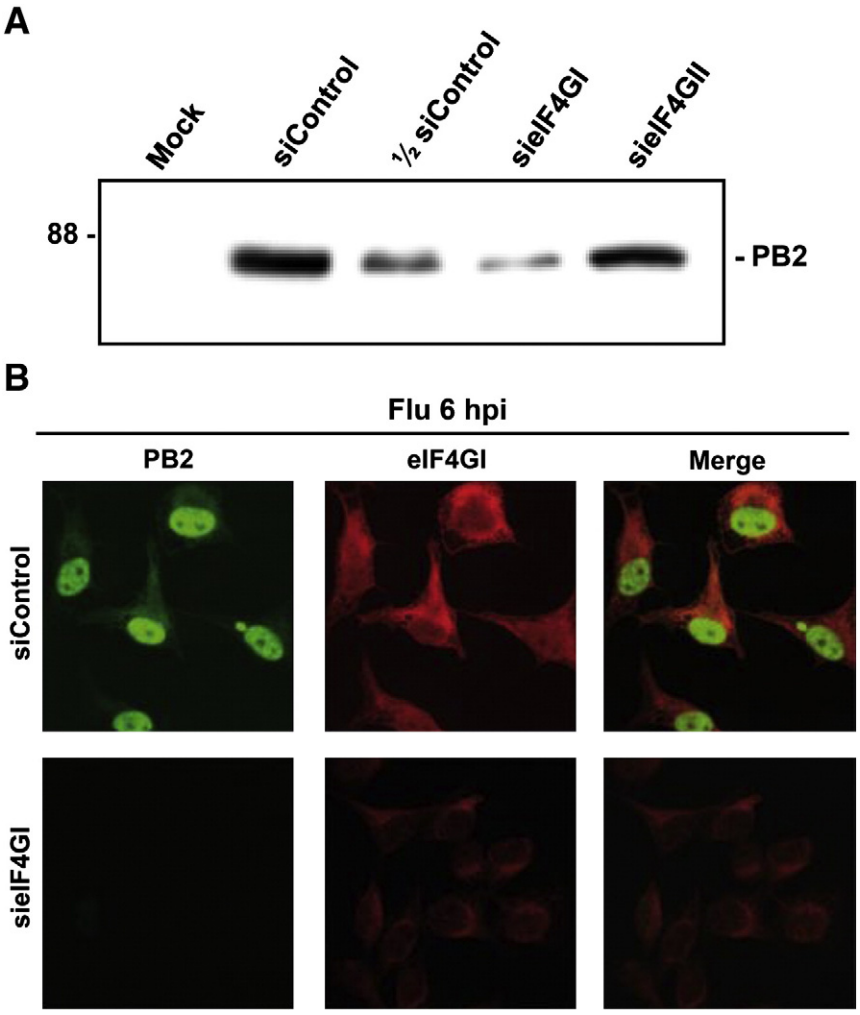

Fig. 6. Effect of eIF4G silencing on viral protein accumulation. A549 cells were transfected with specific silencers for eIF4GI or eIF4GII or with a random siRNA sequence and either mock-infected or infected with influenza virus at 3 pfu/cell. At 6 hpi, cells were used to determine the amount of PB2 polymerase subunit by Western-blot (A) or immunofluorescence (only for eIF4GI silenced cells) (B).

(si4GII-2) [CAAAGACCTGGACTTTGAA] (Welnowska et al., 2009) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations.

Western blotting

Western blotting was performed as described previously (Aragón et al., 2000). The following primary antibodies were used: for translation initiation factor eIF4GI, a mixture of four rabbit polyclonal antibodies (1:8000 each) (Aragón et al., 2000); for eIF4GII, a mixture of two rabbit polyclonal antibodies against the N-terminal and C-terminal regions (a gift from N. Sonenberg) (1:750 each);for PABP1 protein, a rabbit antiserum raised against GST–PABP1 fusion protein (1:1000) (Burgui et al., 2003); for eIF4A, a goat polyclonal antibody (1:200) from Santa Cruz; for eIF4E, a monoclonal antibody from Transduction Laboratories(1:2.000); and for ß-tubulin, a mouse monoclonal antibody (1:50,000) from Sigma.

In vitro translation

For *in vitro* translation reactions, transcription of control capped dicistronic mRNA was prepared from XhoI linearized pGEM-Cap-CAT: EMCV-IRES-Luc (which expresses the IRES element of the encephalomyocarditis virus) (Pisarev et al., 2004) or pGEM-Cap-CAT:PTV-IRES-Luc (which expresses the IRES element of the porcine teschovirus) in the eIF4A studies since this last IRES is insensitive to eIF4A (Chard et al., 2006), using the Megascript transcription system (Ambion). 7-mGTP cap 0 structure addition was performed using ScriptCap^{TM} m^{7}G Capping System (Epicentre Biotechnologies) and mRNA was poly-adenylated using poly-A polymerase (PAP) following the supplier recommendations. RNA was purified by lithium chloride precipitation, resuspended in RNA Storage Buffer (Ambion) and quantified by spectrophotometry. To obtain influenza virus RNAs for *in vitro* translation reactions, cytosolic extracts of infected cells were obtained 6 hpi. Total RNA was isolated from the extracts using Ultraspec reagent (Biotecx Laboratories), resuspended in RNA Storage Buffer and quantified by spectrophotometry.

In vitro translation reactions were performed using the Flexi rabbit reticulocyte lysate (RRL) system (Promega), using 200 μg/ml of cytosolic RNA from influenza infected cells, 80 μg/ml of rabbit β-globin mRNA or 10 μg/ml of control dicistronic RNAs. These concentrations of RNA were previously determined to give a linear yield of translated product over the time course of the translation (90 min). In reactions that required the addition of L-protease, the wild type or the dominant negative mutant forms of eIF4A or hippuristanol, the reactions were preincubated with the recombinant proteins or with the eIF4A inhibitor at 30 °C for 15 min prior to the addition of RNA. After 90 min, the reactions were terminated by the addition of SDS-PAGE sample buffer and subsequently resolved on 12.5% SDS-gels.

In vivo metabolic labeling

For continuous labeling of synthesizing proteins, after 1 h depletion in methionine free medium, HEK293T and A549 cells were incubated with medium containing 50 μCi/ml of ^{35}S-Met during 1 h at the indicated times.

Luciferase determination

Luciferase activity was determined using Luciferase Assay System (Promega), according to the manufacturer's recommendations.

In vivo hippuristanol assays

First, HEK293T cultured cells, were treated with 30 nM to 1 μM hippuristanol supplemented medium and after 10 h of treatment, cell viability was determined using CellTiter-Blue Cell Viability Assay (Promega). To evaluate hippuristanol effects on PTV-IRES activity, HEK293T cells were treated with hippursitanol for 1 h (0.5 and 1 μM concentration) and then transfected with an *in vitro* transcribed PTV-IRES-luciferase RNA. 8 h post-transfection, luciferase activity and general ^{35}S-Met incorporation were determined. To assess hippuristanol effect on influenza virus protein synthesis, HEK293T cells were pretreated with hippuristanol (30 nm to 1 μM) or Me_2SO as a control for 1 h prior to infection. Infections were carried out in the presence of hippuristanol or Me_2SO for 6 h at 37 °C, after which metabolic labeling was performed.

Immunofluorescence

A549 cells were fixed in 4% paraformaldehyde, permeabilized, and incubated with the following primary antibodies: rabbit polyclonal antibodies raised against eIF4GI (Aragón et al., 2000) at 1:500 dilution and mouse monoclonal antibodies 8 and 28 against PB2 (Bárcena et al., 1994) at 1:5 dilution. Microscopy was performed with a Zeiss LSM510 inverted confocal laser-scanning microscope (Bio-Rad/Zeiss) with 63×/1.4 oil Plan-Apochromat.

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