Translation Directed by Hepatitis A Virus IRES in the Absence of Active eIF4F Complex and eIF2

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Abstract
Translation directed by several picornavirus IRES elements can usually take place after cleavage of eIF4G by picornavirus proteases 2Apro or Lpro. The hepatitis A virus (HAV) IRES is thought to be an exception to this rule because it requires intact eIF4F complex for translation. In line with previous results we report that poliovirus (PV) 2Apro strongly blocks protein synthesis directed by HAV IRES. However, in contrast to previous findings we now demonstrate that eIF4G cleavage by foot-and-mouth disease virus (FMDV) Lpro strongly stimulates HAV IRES-driven translation. Thus, this is the first observation that 2Apro and Lpro exhibit opposite effects to what was previously thought to be the case in HAV IRES. This effect has been observed both in hamster BHK and human hepatoma Huh7 cells. In addition, this stimulation of translation is also observed in cell free systems after addition of purified Lpro. Notably, in presence of this FMDV protease, translation directed by HAV IRES takes place when eIF2α has been inactivated by phosphorylation. Our present findings clearly demonstrate that protein synthesis directed by HAV IRES can occur when eIF4G has been cleaved and after inactivation of eIF2. Therefore, translation directed by HAV IRES without intact eIF4G and active eIF2 is similar to that observed with other picornavirus IRESs.

Introduction
A variety of animal viruses with positive-stranded RNA genomes contain internal ribosome entry sites (IRESs) in their 5’ untranslated region (5’-UTR) [1,2]. These IRES elements are highly structured and are involved in ribosome recruitment to promote viral mRNA translation. IRESs have been classified according to their phylogenetic origin, secondary structure and functionality. Thus, four major classes of IRESs from picornaviruses, flaviviruses, dicistroviruses and retroviruses have been defined. In addition, picornavirus IRESs have been divided into at least four types or classes. Poliovirus (PV) and human rhinovirus (HRV) IRESs are representative members of class I, while encephalomyocarditis virus (EMC) and foot-and-mouth disease virus (FMDV) IRESs belong to class II. Hepatitis A virus (HAV) IRES has been grouped in class III and, finally, porcine teschovirus-1 IRES with similarities to hepatitis C virus (HCV) is a representative member of class IV. In addition to differences in the length and structure of these elements, they exhibit different requirements for initiation factors during translation. Protein synthesis directed by all picornavirus mRNAs, with the exception of HAV mRNA, takes place efficiently when eIF4G is cleaved by picornavirus proteases. Thus, translation driven by EMCV and PV IRESs do not require eIF4E or intact eIF4F complex to initiate protein synthesis [3], whereas HAV IRES depends on eIF4F including eIF4E [4,5,6]. In fact, the requirement for eIF4E and intact eIF4F complex of HAV IRES constituted one major characteristic to justify placing it in a different group to the other picornaviruses.

The initial report by Whetter et al. (1994) examined translation of monocistronic and dicistronic mRNAs bearing the HAV IRES in monkey kidney cells permissive for HAV, which expressed the T7 RNA polymerase. Protein synthesis directed by these mRNAs was very inefficient and severely inhibited by co-expression of PV 2Apro. Subsequent in vitro experiments using RRL revealed that cleavage of eIF4G by HRV 2Apro or FMDV Lpro strongly reduced HAV IRES-directed translation [4,7]. This inhibition was rescued by addition of eIF4F, supporting the idea that HAV IRES required intact eIF4G to direct translation. Similar conclusions were reported by Wyeth et al. (1994) describing that inhibition of eIF4E by cap analogs or the presence of 4E-BP blocked HAV IRES-driven translation in RRLs [8]. Apart from these differences in the requirement of eIF4G between HAV and other picornavirus IRESs [9], translation directed by HAV exhibits other features. Thus, cleavage of poly-(A)-binding protein (PABP) and polypyrrolidine tract-binding protein (PTB) by HAV 3Cpro blocks translation of its cognate mRNA [10,11]. In addition, La autoantigen blocks HAV IRES [12] in contrast to the evidence that this RNA binding protein is a trans-acting factor on PV translation [13].

Recently, we found that translation of different picornavirus mRNAs can take place when eIF2α becomes phosphorylated at late times of infection [14]. In this sense, a dual mechanism is responsible for picornavirus mRNA translation. At early times of
infection picornavirus mRNA is translated following a canonical mechanism that employs intact eIF4G and active eIF2; whereas at late times inactivation of eIF2 does not abrogate viral protein synthesis [14]. Moreover, synthesis of PV 2Apro at high levels in culture cells makes translation of mRNAs containing EMCV or PV IRESs independent of eIF2 [15]. Therefore, the presence of PV 2Apro and the cleavage of eIF4G change the mode of initiation of protein synthesis to an eIF2-independent mechanism. The suggestion that cleavage of eIF5B by PV 3Cpro renders eIF2-less translation of PV mRNA [16], was not supported by the demonstration that, apart from PV 2Apro, none of the PV non-structural proteins provided eIF2-independence for picornavirus IRES-directed protein synthesis [15]. In view of these findings, we decided to analyze the mechanism of translation directed by HAV IRES in the presence of high levels of picornavirus proteases. Surprisingly, PV 2Apro and FMDV Lpro exhibit opposing effects on HAV translation. In accord with previous findings, PV 2Apro, none of the PV non-structural proteins provided eIF2-independence for picornavirus IRES-driven translation [6], while FMDV Lpro strongly inhibited HAV IRES-driven translation [6], while FMDV Lpro enhanced this translation by several fold. These findings strongly suggest that cleavage of eIF4GI by PV 3Cpro renders eIF2-less translation of PV mRNA. Moreover, synthesis of PV 2Apro at high levels in culture cells makes translation of mRNAs containing EMCV or PV IRESs independent of eIF2 [15]. Therefore, the presence of PV 2Apro and the cleavage of eIF4G change the mode of initiation of protein synthesis to an eIF2-independent mechanism. The suggestion that cleavage of eIF5B by PV 3Cpro renders eIF2-less translation of PV mRNA [16], was not supported by the demonstration that, apart from PV 2Apro, none of the PV non-structural proteins provided eIF2-independence for picornavirus IRES-directed protein synthesis [15]. In view of these findings, we decided to analyze the mechanism of translation directed by HAV IRES in the presence of high levels of picornavirus proteases. Surprisingly, PV 2Apro and FMDV Lpro exhibit opposing effects on HAV translation. In accord with previous findings, PV 2Apro strongly inhibited HAV IRES-driven translation [6], while FMDV Lpro enhanced this translation by several fold. These findings illustrate that contrary to previous ideas, HAV IRES can efficiently direct translation when eIF4G is phosphorylated.

Materials and Methods

Cell Cultures

Huh7-T7 cells (Human Hepatoma) [17] and Baby Hamster Kidney (clone BSR-T7/5, designated as BHK-T7) [18] were used in this work. Both cell types constitutively express the T7 RNA polymerase. Huh7-T7 cells were kindly provided by R. Bartenschlager (University of Heidelberg, Germany). Cells were grown at 37°C in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% or 5% fetal calf serum (FCS) and non-essential amino acids. BHK-T7 cells were additionally incubated with Geneticin G418 (Sigma) on every third passage at a final concentration of 2 mg/ml. For Huh7-T7 cells the medium was supplemented with 5 μM Zeocin.

Plasmids and Transfections

The plasmid encoding HAV(IRES)-luc has been described previously [11,19]. The construct pTM1-luc has also been already described [20]. pTM1 bears the EMCV IRES element before the corresponding gene. Plasmid T7 Rhc AEMC IGR-Fluc (pIGR CrPV-luc) is kindly provided by P. Sarnow (Stanford University, USA). Plasmid pFMDV-L was kindly provided by G. Belsham (Technical University of Denmark, Denmark). The different plasmids and mRNAs employed in this work are listed in Table 1. Huh7-T7 and BHK-T7 cells were transfected using Lipofectamine 2000 (Invitrogen). Cells were transfected or co-transfected with the plasmids as indicated in each experiment or with in vitro transcribed mRNA. These plasmids or RNAs were added along with 2 μl lipofectamine per well in Opti-mem medium (Invitrogen) and incubated at 37°C for 3 h in the case of the Huh7-T7 cells and 2 h for BHK-T7 cells. The lipofectamine was then removed and the cells were supplemented with fresh medium containing 10% or 5% FCS, respectively.

In vitro Transcription and Translation

pHAV-luc, pTM1-2C and pTM1-L were linearized prior to in vitro transcription with T7 RNA polymerase (BioLabs) according to the manufacturer’s instructions. In vitro transcription was carried out in RRL (Promega). To ensure the cleavage of eIF4G, the lysates were pre-incubated at 30°C for 1 h in the case of EMCV(IRES)-L mRNA or for 20 min with the purified protein FMDV Lpro. Extracts were then treated with 0.5 μg/ml poly(I:C) (PharmaciaBiotech) for 30 min to induce phosphorylation of eIF2α. Subsequently, 100 ng of different mRNAs were added and incubated for 1 h at 30°C. Protein synthesis was estimated by measuring luc activity and by Western blot to analyze the eIF4GI cleavage.

Inhibitor Treatments and Analysis of Protein Synthesis by Radioactive Labelling

BHK-T7 cells were transfected or co-transfected with the plasmids indicated in each experiment. At 2 hpt, cells were pre-treated with 200 μM sodium arsenite (Ars) (Riedel-de Haen) for 15 min at 37°C, or left untreated. Next, proteins were radiolabelled for 45 min with [35S]Met/Cys (Promix; Amersham Pharmacia) in methionine/cysteine-free DMEM in the presence or absence of 200 μM Ars. Finally, cells were collected in sample buffer, boiled for 4 min and analysed by SDS-PAGE (17.5%) and fluorography. Protein synthesis was quantified by densitometry using a GS-710 calibrated Imaging Densitometer (Bio-Rad). In the case of hippuristanol, Huh7-T7 cells were transfected with the indicated plasmids. Hippuristanol was a generous gift of J. Pelletier (McGill University, Canada). The cells were subsequently preincubated with different concentrations of hippuristanol for 20 min then radiolabelled for 60 min with [35S]Met/Cys in methionine/cysteine-free DMEM with the same concentrations of the inhibitor. Finally, the cells were processed as described above.

Purification of FMDV Lpro

Active Lpro (FMDV amino acids 29 to 201) was expressed as described previously [21]. Briefly, E.coli BL21 Lys E cells containing the plasmid pet11d/Lb were grown to an OD590 of 0.5. Expression was induced with 0.1 mM IPTG and cells were incubated at 30°C for a further 5 h. Cells were lysed by sonication, cleared by low-speed centrifugation and an ammonium sulphate

| Table 1. Plasmids used in this study. |
|--------------------------------------|
| Plasmid     | Description                      | mRNA               |
| pTM1-2A     | Plasmid containing PV 2A gene after EMCV IRES | EMCV(IRES)-2A      |
| pTM1-L      | Plasmid containing FMDV Lb gene after EMCV IRES | EMCV(IRES)-L       |
| pFMDV-L     | Plasmid containing FMDV L gene after FMDV IRES | FMDV(IRES)-L       |
| pTM1-2C     | Plasmid containing PV 2C gene after EMCV IRES | EMCV(IRES)-2C      |
| pHAV-luc    | Plasmid containing luc gene after HAV IRES | HAV(IRES)-luc      |
| pIGR CrPV-luc | Plasmid containing luc gene after IGR IRES | CrPV(IRES)-luc     |

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cut of 40–80% made. The pellet was resuspended in buffer A (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM EDTA, 5% glycerol), dialysed and loaded onto a 10/10 MonoQ column. Lbpro fractions eluted at around 300 mM NaCl. These were pooled and further fractionated on a superdex 75 HiLoad 26/60 column. Lbpro containing fractions were identified, pooled and stored in buffer A containing 50% glycerol at –80°C. Typical yields were between 5 and 7 mg of Lbpro per liter of culture.

**Figure 1. HAV IRES translation in BHK cells after cleavage of eIF4G.** A) BHK-T7 cells were transfected or co-transfected for 2 h with 1 µg plasmid encoding HAV(IRES)-luc alone or in presence of 1 µg pTM1-2A or pFMDV-L, respectively. After 2 hpt, cells were treated with 200 µM Ars for 15 min and then metabolically labeled with 0.2 µCi per well [35S]Met/Cys in presence (+) or absence (–) of Ars for 45 min. Finally, cells were processed by SDS-PAGE, fluorography and autoradiography. B) The same samples were used to analyze eIF4GI, eIF2α phosphorylation and total eIF2α by western blot using specific antibodies as detailed in Materials and Methods. C) BHK-T7 cells were transfected under the conditions described above. Cells were then collected and processed to assay for luc activity as described in Materials and Methods. The bars represent the luc activity in presence (+) or absence (–) of Ars. The RLUs values obtained were as follows: pHAV-luc in absence (–) or presence (+) of Ars were 3.9 × 10^5 and 1.8 × 10^5, respectively. pHAV-luc co-transfected with pTM1-2A (–) or (+) Ars were 0.2 × 10^5 and 0.1 × 10^5, respectively, and finally pHAV-luc co-transfected with pFMDV-L (–) or (+) Ars were 2.5 × 10^5 and 1.7 × 10^5, respectively. Error bars indicate standard deviation (SD). D) BHK-T7 cells were transfected with cap-luc, HAV(IRES)-luc or PV(IRES)-luc mRNAs. At 2 hpt cells were collected and luc activity was measured. The RLUs values obtained were as follows: cap-luc: 1.13 × 10^6; HAV(IRES)-luc: 1.46 × 10^6 and PV(IRES)-luc: 0.44 × 10^6.

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Western Blotting

Transfected cells were collected in sample buffer, boiled and analyzed by SDS-PAGE. After electrophoresis, proteins were transferred to a nitrocellulose membrane as described previously [22]. To detect eIF4GI, rabbit antibodies against the N-terminal and C-terminal portion of this protein [23] were used at a 1:1000 dilution. Rabbit antisera were raised against firefly luciferase (Promega). Incubation with primary antibodies was performed for 2 h at room temperature, except for phosphorylated eIF2α, which was incubated overnight at 4°C. Next, the membrane was washed three times with PBS containing 0.2% Tween-20 and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG antibodies (Amersham) at a 1:1000 dilution.
cells were transfected or co-transfected with 1 μg plasmid encoding pFMDV-L (upper panel). eIF4GI cleavage was analyzed by western blot (lower panel). B) pFMDV-L was linearized and transcribed 3 h with 1 μg plasmid pHAV-luc alone or with different concentrations of FMDV(IRES)-L mRNA. After 3 h in presence of transfection mixture and 3 h in fresh medium, cells were recovered and luc activity was measured and represented in the graph (upper panel). Error bars represent SD. The same samples were employed to analyze eIF4GI cleavage (lower panel).

**Results**

Opposite Effects of PV 2Apro or FMDV Lpro on HAV IRES-driven Translation

Several reports have established that HAV IRES-driven translation is abrogated by PV 2Apro or FMDV Lpro, both in culture cells and in cell free systems [4,5,6]. This abrogation is due to the bisection of eIF4G by these proteases, since addition of intact eIF4F complex restores this inhibition. Therefore, HAV IRES seems to be an exception among the other picornavirus IRES analyzed, as regards its requirement for intact eIF4G. This finding together with other differences in IRES structure provided the rationale for classification of the HAV IRES in the type III group [1]. Recently, using the system described in our previous work we found that PV 2Apro had the ability to modify the mechanism of initiation of PV- or EMCV IRES-directed translation, as regards its requirement for active eIF2 [15]. This system used a BHK cell line that stably expresses T7 RNA polymerase (BHK-T7). After co-transfection of BHK-T7 cells with plasmids encoding luciferase (luc) and PV 2Apro or FMDV Lpro, respectively, the luciferase reporter gene was transcribed from a T7 promoter. This system is used to analyze both total luc and PV or EMCV IRESs-directed translation, as regards their requirement for intact eIF2a [15]. This system and luciferase activity assay were used to study the effect of PV 2Apro and FMDV Lpro on HAV IRES-driven translation.

Measurement of Luciferase Activity

Cells were transfected in a buffer containing 25 mM-glycyglycine [pH 7.8], 0.5% Triton X-100 and 1 mM dithiothreitol. Luc activity was determined using luciferase assay system (Promega) and Monolight 2010 apparatus (Analytical Luminescence Laboratory) as described previously [24,25].

**Figure 3. Stimulation of HAV(IRES)-luc mRNA translation is dependent on FMDV Lpro concentration.** A) Huh7-T7 were transfected for 3 h with 1 μg plasmid encoding HAV(IRES)-luc alone and co-transfected with different concentrations of plasmid encoding FMDV Lpro. After 3 hpt, cells were harvested, washed in PBS and resuspended in luc buffer. The graph represents luc synthesis in presence of increasing concentrations of pFMDV-L (upper panel). eIF4GI cleavage was analyzed by western blot (lower panel). B) pFMDV-L was linearized and transcribed in vitro. Huh7-T7 cells were transfected or co-transfected with 1 μg plasmid pHAV-luc alone or with different amounts of FMDV(IRES)-L mRNA. After 3 h in presence of transfection mixture and 3 h in fresh medium, cells were recovered and luc activity was measured and represented in the graph (upper panel). Error bars represent SD. The same samples were employed to analyze eIF4GI cleavage (lower panel).

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1:5000 dilution. After washing three times, protein bands were visualized with the ECL detection system (Amersham).

**Discussion**

We have previously shown that treatment of BHK-T7 cells with 250 mM Ars inhibits luc synthesis by about 60% in BHK-T7 cells transfected with pTM1-2A or pFMDV-L. In addition, to analyze the participation of eIF2 in HAV-driven translation, cells were treated with Ars. This compound induces the activation of the protein kinase HRI that phosphorylates eIF2α [8,26,27,28]. Therefore, at 2 hpt, cells were pre-treated with Ars for 15 min and then radiolabeled by incubating with [35S]Met/Cys from 2–3 hpt in presence (+) or absence (−) of 200 μM Ars (Fig. 1A). These same samples were also analyzed by Western blotting to detect eIF4G or eIF2 (Fig. 1B). Synthesis of luc in this system can be detected by estimating luc activity. The amount of luc synthesized after transfection with pHAV-luc is much lower than that obtained by transfection of pTM1-luc, which is about 20–50 fold higher (results not shown). Therefore, under these conditions, luc detection by radiolabeling is not observed (Fig. 1A). In agreement with previous results, co-transfection of pHAV-luc with pTM1-2A strongly blocks the synthesis of luc. In this case, the synthesis of PV 2Apro is detected by SDS-PAGE of the radiolabeled proteins (Fig. 1A), as well as by cleavage of eIF4G (Fig. 1B, upper panel). To our surprise, co-transfection of pHAV-luc and pFMDV-L leads to a clear stimulation of luc synthesis. Indeed, high levels of luc synthesis can be detected by radiolabeling (Fig. 1A), even though eIF4G has been substantially cleaved (Fig. 1B, upper panel). This finding provided initial evidence that luc synthesis directed by HAV IRES at low detectable level is actually increased after eIF4G cleavage by FMDV Lpro. As regards eIF2α, we analyzed both total eIF2 and phosphorylated eIF2α (Fig. 1B). Addition of Ars clearly induces the phosphorylation of eIF2α in control cells and in cells that express the picornavirus protease. Interestingly, the expression of FMDV Lpro partially increased the phosphorylation of eIF2α, even in the absence of Ars (Fig. 1B). In previous work, we have found that treatment of BHK-T7 cells with 200 μM Ars induces the phosphorylation of virtually all eIF2α present in cells [14,15]. In parallel, cells were transfected under the same conditions and were collected at 3 hpt to measure luc activity. Treatment with 200 μM Ars inhibited luc synthesis by about 60% in BHK-T7 cells transfected with pTM1-2A or pFMDV-L. In addition, to analyze the participation of eIF2 in HAV-driven translation, cells were treated with Ars. This compound induces the activation of the protein kinase HRI that phosphorylates eIF2α [8,26,27,28]. Therefore, at 2 hpt, cells were pre-treated with Ars for 15 min and then radiolabeled by incubating with [35S]Met/Cys from 2–3 hpt in presence (+) or absence (−) of 200 μM Ars (Fig. 1A). These same samples were also analyzed by Western blotting to detect eIF4G or eIF2 (Fig. 1B). Synthesis of luc in this system can be detected by estimating luc activity. The amount of luc synthesized after transfection with pHAV-luc is much lower than that obtained by transfection of pTM1-luc, which is about 20–50 fold higher (results not shown). Therefore, under these conditions, luc detection by radiolabeling is not observed (Fig. 1A). In agreement with previous results, co-transfection of pHAV-luc with pTM1-2A strongly blocks the synthesis of luc. In this case, the synthesis of PV 2Apro is detected by SDS-PAGE of the radiolabeled proteins (Fig. 1A), as well as by cleavage of eIF4G (Fig. 1B, upper panel). To our surprise, co-transfection of pHAV-luc and pFMDV-L leads to a clear stimulation of luc synthesis. Indeed, high levels of luc synthesis can be detected by radiolabeling (Fig. 1A), even though eIF4G has been substantially cleaved (Fig. 1B, upper panel). This finding provided initial evidence that luc synthesis directed by HAV IRES at low detectable level is actually increased after eIF4G cleavage by FMDV Lpro. As regards eIF2α, we analyzed both total eIF2 and phosphorylated eIF2α (Fig. 1B). Addition of Ars clearly induces the phosphorylation of eIF2α in control cells and in cells that express the picornavirus protease. Interestingly, the expression of FMDV Lpro partially increased the phosphorylation of eIF2α, even in the absence of Ars (Fig. 1B). In previous work, we have found that treatment of BHK-T7 cells with 200 μM Ars induces the phosphorylation of virtually all eIF2α present in cells [14,15]. In parallel, cells were transfected under the same conditions and were collected at 3 hpt to measure luc activity. Treatment with 200 μM Ars inhibited luc synthesis by about 60% in BHK-T7 cells.
transfected with pHAV-luc (Fig. 1C). On the other hand, the presence of PV 2Apro inhibited luc synthesis by 95% in presence or absence of Ars. Remarkably, Ars treatment had no effect on luc synthesis in the presence of FMDV Lpro (Fig. 1C). In this case, FMDV Lpro stimulated luc synthesis by 6.4fold in absence of Ars and the stimulation was 9.4fold in its presence. This finding supports the idea that translation directed by HAV IRES can occur not only when eIF4G has been cleaved, but also in the absence of active eIF2. For comparative purposes cells were transfected with different mRNAs, in order to analyze their translatability. Thus, cells were transfected with cap-luc, HA-V(IRES)-luc and PV(IRES)-luc mRNAs and luc synthesis was estimated after 2 h. As observed in Figure 1D, the level of translation of HAV(IRES)-luc mRNA is similar to that found with cap-luc and even higher than that obtained with PV(IRES)-luc mRNA.

Figure 4. Co-transfection of HAV(IRES)-luc mRNA and FMDV(IRES)-luc mRNA induces a strong stimulation of luc synthesis. A) pTM1-L was linearized and transcribed in vitro. EMC(IRES)-L mRNA was obtained. Then, 1 μg pHAV-luc was transfected alone or co-transfected with increasing concentrations of EMC(IRES)-L mRNA for 3 h. At 3 hpt cells were processed as described in Materials and Methods to measure luc activity. Values are represented in the graph (upper panel). Error bars represent SD. The same samples were analyzed by western blot with specific antibodies against eIF4GI (lower panel). B) pHAV-luc was linearized and transcribed in vitro to obtain HAV(IRES)-luc mRNA. Then, 1 μg HAV(IRES)-luc mRNA was transfected alone (C) or co-transfected with 1 μg EMC(IRES)-2A mRNA, 1 μg EMC(IRES)-L or 1 μg FMDV(IRES)-L. At 3 hpt cells were processed to measure luc activity. The values of luc activity are indicated on the graph (upper panel). Error bars represent SD. eIF4GI cleavage was analyzed by western blot (lower panel). C) 1 μg HAV(IRES)-luc mRNA was transfected alone (C) or co-transfected with 1 μg EMC(IRES)-2A mRNA or 1 μg FMDV(IRES)-L mRNA for 3 h. Moreover, an mRNA mixture containing 1 μg HAV(IRES)-luc mRNA, 1 μg EMC(IRES)-2A mRNA and increasing concentrations of FMDV(IRES)-L mRNA were transfected for the same time. At 3 hpt cells were collected and luc activity was measured and plotted. Error bars indicate SD. D) Huh7-T7 cells were transfected with 1 μg HAV(IRES)-luc mRNA alone (C) or co-transfected sequentially with both mRNAs, i.e. first 1 μg EMC(IRES)-2A mRNA or 1 μg FMDV(IRES)-L mRNA was added and incubated for 2 h and then cells were transfected with 1 μg FMDV(IRES)-L mRNA or 1 μg EMC(IRES)-2A mRNA, respectively, together with 1 μg HAV(IRES)-luc. After 2 h of incubation cells were collected and luc activity was measured. The values obtained are represented in the graph.
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transfected with pHAV-luc (Fig. 1C). On the other hand, the presence of PV 2Apro inhibited luc synthesis by 95% in presence or absence of Ars. Remarkably, Ars treatment had no effect on luc synthesis in the presence of FMDV Lpro (Fig. 1C). In this case, FMDV Lpro estimated luc synthesis by 6.4fold in absence of Ars and the estimation was 9.4fold in its presence. This finding supports the idea that translation directed by HAV IRES can occur not only when eIF4G has been cleaved, but also in the absence of active eIF2. For comparative purposes cells were transfected with different mRNAs, in order to analyze their translatability. Thus, cells were transfected with cap-luc, HAV(IRES)-luc and PV(IRES)-luc mRNAs and luc synthesis was estimated after 2 h. As observed in Figure 1D, the level of translation of HAV(IRES)-luc mRNA is similar to that found with cap-luc and even higher than that obtained with PV(IRES)-luc mRNA.
Since the natural hosts for HAV replication are liver cells, we tested HAV(IRES)-luc mRNA translation in the human hepatoma cell line that stably expresses T7 RNA polymerase (Huh7-T7 cells). Analysis of protein synthesis by SDS-PAGE, fluorography and autoradiography showed that synthesis of luc is only apparent when FMDV Lpro is present (Fig. 2A). When Huh7-T7 cells were transfected with pTM1-2A, the synthesis of this protease was clearly apparent, but no luc synthesis was detected. Cleavage of eIF4G was found when PV 2Apro or FMDV Lpro were present, as analyzed by western blotting (Fig. 2B). Treatment of these cells with 200 μM Ars leads to a substantial inhibition of cellular protein synthesis, however, synthesis of PV 2Apro was more resistant to this inhibition, as well as the synthesis of luc when FMDV Lpro was present (Fig. 2A). Indeed, phosphorylation of eIF2α took place when cells were treated with Ars (Fig. 2B, middle panel). The synthesis of luc in this system was also tested by measuring luc activity after transfection with pHAV-luc (Fig. 2C). The results obtained were similar to those found with BHK-T7 cells (Fig. 1). In agreement with the above results, co-expression of PV 2Apro blocks HAV(IRES)-luc mRNA translation, but luc synthesis was clearly stimulated by the co-expression of FMDV Lpro. To further assess that PV 2Apro was inhibitory for HAV IRES-driven translation and also to analyze if there is a correlation between the protease activity and this inhibition, a concentration curve of pTM1-2A on luc synthesis was carried out. Figure 2D shows that increasing concentrations of pTM1-2A are inhibitory for luc synthesis after co-transfection with pHAV-luc. Strikingly, even the transfection of very low concentrations of pTM1-2A were inhibitory for luc synthesis in this system, suggesting that the entry of a few copies of this plasmid into cells leads to its efficient transcription, giving rise to PV 2Apro that is able to partially cleave eIF4G (Fig. 2D, lower panel). Taken together these findings indicate that PV 2Apro and FMDV Lpro exhibit opposite effects on translation directed by HAV IRES. Moreover, this translation may occur when eIF2α has been phosphorylated.

Translation of HAV(IRES)-luc mRNA after eIF4G Cleavage

To further assess whether HAV luc mRNA can be translated when eIF4G is cleaved, we have analyzed different expression systems in Huh7-T7 cells. First we assayed luc synthesis in cells

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Figure 5. HAV(IRES)-luc mRNA translation is inhibited by hippuristanol. A) Effects of hippuristanol in Huh7-T7 cells. Cells were treated for 30 min with increasing concentrations of hippuristanol and then metabolically labelled with 0.2 μCi per well [35S]Met/Cys for 1 h in presence of the inhibitor. Whole-cell extracts were analyzed by SDS-PAGE, fluorography and autoradiography. Dried gels were exposed to X-ray film. B) Huh7-T7 cells were transfected for 3 h with 1 μg plasmid bearing HAV(IRES)-luc in absence (upper panel) or presence (lower panel) of 1 μg pFMDV-L. Then, at 3 hpt, luc activity was measured in presence of increasing concentrations of hippuristanol. C) As control, Huh7-T7 cells were transfected during 3 h with 1 μg pCrPV IGR-luc. After, at 3 hpt, cells were treated with increasing concentrations of hippuristanol for 90 min. Finally, luc activity was measured and the values represented in the graph. Error bars indicate SD. doi:10.1371/journal.pone.0052065.g005
transfected with pHAV-luc and co-transfected with increasing concentrations of pFMDV-L. Fig. 3A shows that when the amount of pFMDV-L is increased, there is a partial cleavage of eIF4G and this cleavage is higher when 1 µg pFMDV-L is transfected. Notably, there is an increase in the production of luc by as much as 4 fold. Another system employed to synthesize FMDV L\textsuperscript{poso} was by transfection of the in vitro synthesized mRNA. Two different mRNAs were used, FMDV(IRES)-L and EMC(IRES)-L mRNAs. We have observed that the latter of these mRNAs, which contains HAV(IRES)-luc mRNA (Fig. 3B and 4A). These results reinforce the idea that cleavage of eIF4G by FMDV L\textsuperscript{poso} stimulates the translation of HAV(IRES)-luc mRNA.

To rule out the possibility that FMDV L\textsuperscript{poso} specially affected transcription directed by T7 RNA polymerase, cells were co-transfected with both types of mRNAs. To this end, pHAV-luc were linearized and transcribed in vitro to obtain HAV(IRES)-luc mRNA. Huh7-T7 cells were then transfected with HAV(IRES)-luc mRNA alone or with EMC(IRES)-2A, EMC(IRES)-L, or FMDV(IRES)-L mRNAs for 3 h. After transfection, the normal medium is restored and further incubated for 2 h. At this time, cell extracts are collected to measure luc activity. As occurs with DNA transfection, HAV(IRES)-luc mRNA translation is strongly inhibited by EMC(IRES)-2A mRNA but stimulated by both EMC(IRES)-L and FMDV(IRES)-L mRNAs (Fig. 4B). Substantial cleavage of eIF4G was observed in presence of proteases (Fig. 4B, lower panel).

Our next goal was to analyze the possibility that expression of FMDV L\textsuperscript{poso} might rescue HAV(IRES)-luc mRNA translation in presence of PV 2A\textsuperscript{poso}. To assay this, 1 µg HAV(IRES)-luc mRNA was transfected alone or co-transfected with 1 µg EMC(IRES)-2A mRNA or 1 µg FMDV(IRES)-L mRNA. Moreover, HAV(IRES)-luc mRNA was co-transfected with a mixture of 1 µg EMC(IRES)-2A mRNA and different concentrations of FMDV(IRES)-L mRNA (Fig. 4C). At 2 hpt, cells were harvested and lysed to measure luc activity. As expected, the presence of EMC(IRES)-2A diminishes luc synthesis around 8 fold whereas expression of FMDV(IRES)-L mRNA stimulates HAV(IRES)-luc mRNA translation by more than 3 fold. However, when both proteases are present, expression of FMDV(IRES)-L mRNA cannot rescue HAV(IRES)-luc mRNA translation in presence of EMC(IRES)-2A (Fig. 4C). A similar inhibition of HAV(IRES)-luc mRNA by PV 2A\textsuperscript{poso} is observed when both proteases are expressed sequentially, i.e. when PV 2A\textsuperscript{poso} is expressed prior to FMDV L\textsuperscript{poso} or viceversa (Fig. 4D). This result could indicate the possibility that PV 2A\textsuperscript{poso} hydrolyzes some cellular protein necessary for HAV IRES-driven translation.

**Requirement of eIF4A for HAV IRES-driven Translation**

In recent years, the compound hippuristanol has been used as a selective inhibitor of eIF4A [29,30]. It is known that both intact eIF4G as well as the carboxy fragment of this factor can interact with HAV IRES [3]. It is also known that eIF4A interacts with this carboxy fragment of eIF4G [31]. For this reason, it was of interest to analyze the participation of eIF4A in the translation of HAV(IRES)-luc mRNA when eIF4G has been cleaved by L\textsuperscript{poso}. Addition of different concentrations of hippocristain to Huh7-T7 cells blocks cellular translation (Fig. 5A), as expected for a selective inhibitor of eIF4A. Luc production in cells transfected with pHAV-luc were also strongly blocked by hippuristanol irrespective of the presence of FMDV L\textsuperscript{poso} (Fig. 5B). As control, pIGR CrPV-GFP.
Translation of HAV(IRES)-luc mRNA in Cell Free Systems

Some of the results on the inhibition of translation directed by HAV IRES with picornavirus proteases were obtained in RRL [7,9]. Therefore, we now decided to use RRL programmed with HAV(IRES)-luc mRNA. The effect of FMDV Lpro on this translation was tested using two approaches, one of them provides fresh Lpro by the translation of EMC(IRES)-L mRNA whereas the other employs the direct addition of purified Lpro to the cell free system. Moreover, we also analyzed the eIF2 requirement for translation of HAV(IRES)-luc mRNA under these conditions using poly(I:C), a compound that induces activation of PKR and eIF2α phosphorylation. Initially, a titration curve of poly(I:C) was carried out in order to obtain the optimal concentration of this compound that blocks translation of a cap-luc mRNA in our system (Fig. 6A). 50 ng/ml poly(I:C) was found to be the optimal concentration that blocked translation in RRL. To analyze eIF2α phosphorylation, RRL treated with this optimal poly(I:C) concentration at different times was tested. Clearly, incubation with this inhibitor leads to phosphorylation of eIF2α, even when FMDV Lpro was present (Fig. 6B). The first approach produces newly made Lpro by translation of the mRNA encoding this protease under the EMCV IRES sequence. After translation of this mRNA for 60 min, 50 ng poly(I:C) for 30 min was added. Then, HAV(IRES)-luc mRNA was incubated with 1h. As shown in Fig. 6C, left panel, eIF4G becomes cleaved under these conditions. Surprisingly, inhibition of luc synthesis was observed using this approach (Fig. 6C). Most probably, this inhibition was due to competition of HAV(IRES)-luc mRNA translation by EMCV(IRES)-L mRNA. To assay this possibility, a control EMCV(IRES)-2C mRNA was tested. This mRNA encodes for PV 2C protein, which is devoid of protease activity under the EMCV IRES. In this case, luc synthesis was also inhibited when the concentration of EMCV(IRES)-2C mRNA was increased, suggesting the existence of competition between both mRNAs (Fig. 6C, right panel). Notably, the effect of poly(I:C) was significantly different when EMCV(IRES)-L or EMCV(IRES)-2C mRNAs were assayed. Indeed, when EMCV(IRES)-L mRNA was present no inhibition by poly(I:C) was observed whereas in the case of EMCV(IRES)-2C the presence of poly(I:C) led to over 70% inhibition of luc synthesis (Fig. 6D). This result indicates that FMDV Lpro can confer translatability to HAV IRES when eIF2α is phosphorylated.

The other approach consisted of direct addition of purified Lpro to RRL. After pre-incubation for 20 min with the purified protease, 50 ng poly(I:C) was added and further incubated for 30 min. Then, HAV(IRES)-luc mRNA was added to RRL for 1 h. Clearly, a stimulation of about 3 fold of luc synthesis was found when eIF4G cleavage took place (Fig. 7A and 7B). In conclusion, these findings are in contrast to those previously reported indicating that FMDV Lpro blocks translation directed by HAV IRES in RRL [4]. Phosphorylation of eIF2α inhibits HAV(IRES)-luc mRNA translation by around 60%, but no inhibition was found when 40 μM Lpro was present (Fig. 7A). We conclude that in vitro translation of HAV(IRES)-luc mRNA can take place after eIF4G cleavage by Lpro and in presence of phosphorylated eIF2α.

Discussion

Picornavirus mRNAs contain rather long 5′-UTRs that are highly structured and bear an IRES element. These IREs drive translation by an initiation mechanism in which ribosomes directly interact with an internal region at or upstream to the initiator AUG, [1,2]. This mechanism of initiation does not require intact eIF4G, thus, cleavage of this factor by picornavirus proteases does not impair and, in some instances, even stimulates IRES-directed translation [32]. For many years, it has been thought that HAV IRES was an exception to this rule, since cleavage of eIF4G by PV 2Apro or FMDV Lpro abrogated translation of mRNAs containing HAV IRES [5,6,7]. In addition, the inhibition of eIF4E by 4E-BP1 impairs translation directed by HAV IRES [8], but surprisingly, these authors reported that HAV IRES can be translated in presence of the carboxy fragment of eIF4G in RRL depleted of this factor. One possible explanation for this result is that high concentrations of the carboxy fragment of eIF4G can restore translation of capped mRNAs in eIF4G-depleted RRL [33]. In the present work we provide evidence that HAV IRES translation can occur when eIF4G is cleaved by FMDV Lpro and thus HAV IRES does not represent an exception to the rest of picornavirus IRES functioning in this regard. We can now conclude that translation directed by all picornavirus IRESs tested can occur when eIF4G has been cleaved. The divergence in the functioning of the different picornavirus IREs analyzed may be lower than previously suspected [1]. Perhaps, the classification of HAV IRES in a different group (type III) can now be reconsidered. Although we do not know the reason why our results are so different from those previously reported, we believe that our findings with FMDV Lpro are very clear. Thus, this protease not only does not block HAV IRES-luc mRNA, but it stimulates its translation by several fold when eIF4G has been virtually totally cleaved. Previous works testing the requirement for intact eIF4F complex to translate HAV IRES mRNAs mostly used dicistronic mRNAs, bearing a capped structure in the first cistron and followed by the HAV IRES. In these works FMDV Lpro strongly inhibited (over 80%) translation driven by HAV IRES [4,16]. Perhaps, the use of dicistronic mRNAs have provided misleading results. However, in some of these studies, monocistronic mRNAs bearing the HAV IRES were also analyzed. In our present work we have used monocistronic mRNAs, as this approach is, in our opinion, more physiological than the use of dicistronic mRNAs. Although these mRNAs have been very useful for providing evidence of internal initiation, monocistronic mRNAs should be a better option for understanding the mechanism of IRES functioning [2,34]. Another possibility to account for the discrepancies between previous reports and our present observations is that the amount of FMDV Lpro employed was too high or even it contained an inhibitor unrelated to the protease itself.

It is puzzling to observe that PV 2Apro and FMDV Lpro exhibit opposite effects as regards to the translation of HAV(IRES)-luc mRNA. One obvious possibility is that apart from eIF4G, PV 2Apro cleaves a factor that is necessary for HAV IRES-driven translation. However, addition of purified eIF4F complex restores the inhibition of PV 2Apro on HAV translation [4]. Another possibility is that the carboxy fragments of eIF4G generated by its protease are not exactly similar. Thus, PV 2Apro cleaves eIF4G at position 681–682, which is located seven residues upstream from
the position used by FMDV I^pro, 674–675 [32]. Thus, the eIF4G carboxy terminal fragment generated by FMDV I^pro is seven residues longer than the one originated by PV 2A^pro. However, we believe that this possibility is very unlikely and most probably PV 2A^pro cleaves a factor that is necessary to translate HAV(IRES)-luc mRNA. Our findings support with this the transient simultaneous translation of mRNAs encoding PV 2A^pro and FMDV I^pro strongly blocks HAV IRES. Our findings also indicate that the C-terminal fragment of eIF4G generated by FMDV I^pro is employed to translate HAV IRES, since this fragment bound to eIF4A is necessary to translate mRNAs bearing picornavirus IRESs [31,35]. In this regard, hippuristanol, a selective inhibitor of eIF4A, blocks translation directed by picornavirus mRNAs [29,36]. As demonstrated in this work, eIF4A participates in protein synthesis directed by HAV IRES when eIF4G is intact or even after its cleavage.

Efforts to understand the mechanism by which picornavirus mRNAs are translated have been made over the past four decades. It is surprising that there are still novel and unsuspected findings about the mechanism of initiation of protein synthesis on mRNAs bearing picornavirus IRESs. In this respect, we recently found that PV 2A^pro made translation of mRNAs containing PV or EMCV IRESs independent of eIF2 [14,15]. Since the early days of picornavirus translation, it was thought that this mRNA required eIF2 to initiate translation [3,37,38]. Now, we provide evidence that another picornavirus protease, FMDV I^pro, modifies the requirement for eIF2 to translate HAV(IRES)-luc mRNA. Protein synthesis directed by this mRNA is inhibited by Ars in culture cells or by poly(LC) treatment of RRL, but this inhibition is not observed if I^pro is present. Therefore, translation directed by HAV IRES can occur not only when eIF4G has been cleaved by FMDV I^pro, but also when eIF2a has been inactivated by phosphorylation. To achieve eIF2 independence for this translation, high levels of FMDV I^pro are necessary. Thus, low concentrations of this protease that lead to cleavage of eIF4G do not render translation independent of eIF2. This finding indicates that the simple cleavage of eIF4G by FMDV I^pro does not suffice to confer eIF2-independent translatability of HAV(IRES)-luc. This result is in good agreement with our previous observations, demonstrating that high levels of PV 2A^pro are necessary for eIF2-independent translation directed by EMC or PV IRESs [15]. In this regard, translation of HAV IRES without intact eIF4G and eIF2 is similar to picornavirus IRES type I (PV) or type II (EMC) [15]. Our present results can serve to promote further research on the mechanism of picornavirus mRNA translation. Future studies could aim to understand the exact mechanism by which the initiation of picornavirus mRNA translation occurs when PV 2A^pro or FMDV I^pro is present.

Author Contributions
Conceived and designed the experiments: NR MAS LC. Performed the experiments: NR MAS. Analyzed the data: NR MAS LC. Contributed reagents/materials/analysis tools: NR MAS JS TS YK. Wrote the paper: NR LC.

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