DIFFERENTIAL CLEAVAGE OF eIF4GI AND eIF4GII IN MAMMALIAN CELLS. EFFECTS ON TRANSLATION.

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SUMMARY

Two isoforms of the translation initiation factor eIF4G, eIF4GI and eIF4GII, have been described in eukaryotic cells. The exact function of each isoform during the initiation of protein synthesis is still under investigation. We have developed an efficient and reliable method of expressing poliovirus 2A pro, which differentially proteolyzes eIF4GI and eIF4GII in a time- and dose-dependent manner. This system is based on the electroporation of an in vitro transcribed mRNA that contains the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) followed by the sequence of poliovirus 2A pro. In contrast to HeLa cells, expression of this protease in BHK-21 cells induces delayed hydrolysis kinetics of eIF4GI with respect to eIF4GII. Moreover, under these conditions the polyadenylate binding protein is not cleaved. Interestingly, de novo synthesized Luciferase mRNA strongly requires eIF4GI integrity, whereas ongoing translation is inhibited at the same time as eIF4GII cleavage. Moreover, reinitiation of translation on luciferase mRNA after polysome run-off is dependent on the integrity of eIF4GII. Notably, de novo translation of heat shock protein 70 mRNA exhibits a low requirement for eIF4GI integrity, being more susceptible to eIF4GII hydrolysis. Finally, EMCV IRES driven
translation when the two isoforms of eIF4G are differentially hydrolyzed has been examined.

INTRODUCTION

The initiation of translation is a major target for the regulation of gene expression in eukaryotic cells. A number of initiation factors participate in this process leading to the interaction of the small ribosomal subunit with the mRNA. The initiation factor 4F (eIF4F) plays a central role in the early steps of protein synthesis. eIF4F is composed of three polypeptides: eIF4E, eIF4A and eIF4G (1,2). eIF4E is the cap-binding subunit. eIF4A is an RNA helicase which, together with eIF4B, unwinds the secondary structure present at the 5′ end of mRNAs. eIF4G is a scaffolding protein that physically links the cap structure and the poly(A) tail of mRNAs with the small ribosomal subunit by means of its interaction with eIF4E, poly(A) binding protein (PABP), and eIF3. In addition, eIF4G interacts with other cellular and viral proteins involved in the regulation of translation (2). There are two isoforms of eIF4G in mammalian cells, known as eIF4GI and eIF4GII, which are 46% identical in their primary amino acid sequence. It has been proposed that these two isoforms possess similar biochemical activities and are functionally interchangeable (2). eIF4G contains three domains of a similar size: (i) the N-terminal region contains the eIF4E and PABP binding sites, which are needed for cap and poly(A) recognition (3,4); (ii) the middle portion participates in the recruitment of the 43S preinitiation complex upon interaction with eIF3 (5); and (iii) the C-terminal domain binds Mnk1, a mitogen-activated protein kinase that enhances cap-dependent translation, by phosphorylation of eIF4E (6,7). Two eIF4A interaction sites have been found in eIF4G, located in the middle and C-terminal domain, respectively (8). Thus, eIF4G is essential for coordinating a number of components of the translation machinery to build up the initiation complex.

Infection of mammalian cells with most cytopathic animal viruses induces a marked inhibition of host transcription and translation. Many viruses have evolved mechanisms that employ viral proteases to manipulate the host translational machinery to maximize the selective translation of viral mRNAs compared to endogenous host transcripts (9). Although it was found some time ago that picornavirus
infection induces a marked shutoff of host protein synthesis, the mechanisms involved are still being investigated. Previous reports revealed that hydrolysis of eIF4GI, which comprises most of the total eIF4G (10,2), is not sufficient to fully inhibit host translation (11-13). Proteolysis kinetics of eIF4GII were delayed with respect to cleavage of eIF4GI in poliovirus (PV)- and human rhinovirus-infected cells. In this regard, disappearance of intact eIF4GII correlated with the abrogation of host translation in PV- and rhinovirus-infected and apoptotic cells (14-16). Cleavage of both eIF4GI and eIF4GII strongly blocked the initiation of newly made mRNAs (17). In addition, PV and coxsackievirus 2A pro and 3C pro are able to cleave PABP during infection (10). More recently, it has been reported that hydrolysis of PABP by 3C pro in the absence of eIF4G degradation, blocks the translation of endogenous mRNAs in HeLa cell extracts (18). 3C pro preferentially hydrolyzed the PABP associated with the translational machinery, although the hydrolysis of PABP did not correlate with the shutoff of cellular translation in PV-infected cells (10). Therefore, the individual contribution of eIF4GI, eIF4GII and PABP proteolysis to the shutoff of cellular protein synthesis needs further investigation.

We describe an effective system to differentially hydrolyze eIF4GI and eIF4GII on PV 2A pro expression without affecting PABP. Hydrolysis of eIF4GI by 2A pro occurs before proteolysis of eIF4GII in HeLa cells, whereas the opposite is true for BHK cells. The effect of differential degradation of each eIF4G isoform in the translation of cellular mRNAs in culture cells has been examined.

**MATERIALS AND METHODS**

*Cel l cultures.* HeLa and BHK-21 (baby hamster kidney) cells were grown at 37°C in Dulbecco’s Modified Eagle medium (DMEM) supplemented with 5% fetal calf serum (FCS) and non-essential amino acids. HeLa clone X1/5 cells express luciferase (Luc) in a tetracycline-dependent manner (19,17). Repression of Luc gene transcription was obtained with 20 ng/ml of tetracycline in the culture medium (17).

*Plasmids.* The plasmids pKs-Luc, pTM1-Luc, pTM1-2C, pTM1-2A and the plasmids pTM1-2AG60R, pTM1-2AG121E, pTM1-2AD135N and pTM1-2AV119M, which encodes a PV
(PV) 2A<sup>pro</sup> mutant, have been described previously (20,21).

RNA transcription. The in vitro transcription was carried out with T7 polymerase (Promega) according to the indications of the manufacturer, and the corresponding plasmid as a template. In vitro polyadenylation was performed with polyA polymerase (Invitrogen). The mRNA was purified using the chroma spin columns kit (BD Biosciences). The amount of mRNA was analyzed with the NanoDrop ND-1000 spectrophotometer.

Transfection of HeLa and BHK-21 cells. HeLa cells were electroporated with in vitro synthesized mRNAs. Subconfluent cells were harvested, washed with ice-cold phosphate-buffered saline (PBS), and resuspended in PBS at a density of about 2.5x10<sup>6</sup> cells/ml. Fifty µl of transcription mixture (Promega) with the amounts of RNA indicated in each figure legend were added to 0.8 ml of cells suspension and the mixtures were transferred to 4 mm electroporation cuvettes (Bio-Rad). Electroporation was performed at room temperature by one 350 V, 975 µF pulse using a Gene Pulser apparatus (Bio-Rad). BHK-21 cells were electroporated as previously described (22). Coupled infection/transfection by vaccinia virus T7 (VVT7)/pTM1 system has been described previously (23). Protein synthesis was analyzed by metabolic labeling with 50 µCi of [<sup>35</sup>S]Met-[<sup>35</sup>S]Cys/ml (Promix; Amersham Pharmacia) for 1 h, followed by polyacrylamide gel electrophoresis (SDS-PAGE), fluorography and autoradiography. The integrity of translation initiation factors was analyzed by Western blotting with anti-eIF4GI antisera raised against peptides derived from the N- and C-terminal regions of human eIF4GI at a 1:1000 dilution, rabbit antisera raised against the N-terminal and C-terminal region of eIF4GII (a gift from N. Sonenberg, McGill University, Montreal, Canada) at a 1:500 dilution, mouse monoclonal anti-PABP antibody (Abcam) at a 1:250 dilution or anti-eIF4A at a 1:50 dilution (a gift from Dr. H. Trachsel, Institute for Biochemistry and Molecular Biology, University of Berne, Switzerland). Heat shock protein 70 (Hsp70) was detected using rabbit anti-Hsp70 antisera at a 1:200 dilution (Santa Cruz). Anti-rabbit and anti-mouse immunoglobulin G antibodies coupled to peroxidase (Pierce) were used at a 1:10000 dilution. Percentage of protein synthesis and % of intact eIF4G were determined by densitometric scanning of the corresponding protein band.
Analysis of mRNA by real-time RT-PCR. β-Actin, Luc and Hsp70 mRNA levels in transfected HeLa cells were determined by real-time quantitative reverse transcription RT-PCR. Total RNA was extracted from 2x10^5 cells at the times indicated in each figure using the RNeasy commercial kit (Qiagen) according to the manufacturer’s recommendations (22). Analysis of the actin mRNA level was performed using the Hs 99999903-m1 assay, whereas the Hsp70 mRNA level was estimated with the Hs00359163-s1 assay. As a control, 18S rRNA was measured using the Hs 99999901-m1 assay (Applied Biosystems). In the case of Luc mRNA, primers and probe were designed and provided by Applied Biosystems. The amount of the different mRNAs was obtained taking into consideration the 18S rRNA levels. RT-PCR was carried out in 20 µl of reaction mixture containing 0.9 µM of each primer and 0.25 µM of TaqMan probe. Reverse transcription was performed at 25°C for 10 min and 37°C for 2 h. Afterwards, PCR amplification was started by incubation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min using the ABI PRISM 7000 (Applied Biosystems). Data analysis was carried out using the SDS-7000 software (version 1.1). 95% of confidence interval obtained from three independent experiments was indicated as error bars in the corresponding figure.

Measurement of Luc activity. HeLa X1/5 and HeLa cells electroporated with the in vitro synthesized Luc mRNAs were lysed in a buffer containing 0.5% Triton X-100, 25 mM glycylglycine (pH 7.8) and 1 mM dithiothreitol at different post-electroporation times. Luc activity was determined using a Monolight 2010 apparatus (Analytical Luminiscence Laboratory) as described previously (23). Standard deviations determined from three independent experiments are indicated as error bars in each figure.

RESULTS

Trasfection of HeLa cells with mRNAs containing PV 2A sequence. We initially constructed a plasmid that encode for the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) followed by the PV 2APro sequence (pTM1-2A) (21). In vitro transcription from this plasmid leads to the synthesis of EMC-2A mRNA. Electroporation of 9 µg of this mRNA was sufficient to drastically inhibit translation in HeLa cells, accompanied by hydrolysis of eIF4G (Fig. 1A and B).
The cleavage products were similar to those found after transfection of cells with pTM1-2A plasmid and infection with recombinant vaccinia virus T7 (VVT7) but differed from the eIF4G peptides produced by caspase-3 activity in apoptotic cells (data not shown). Notably, PABP remained intact (Fig. 1C), while a potent hydrolysis was found using this antibody in cells transfected with pTM1-3C, pTM1-2A or pTM1-HIV-1PR (24). In this regard, PV 2A exhibit low proteolitic activity against PABP (25,10). This method yields low levels of 2Apro, since this protease was not detected by autoradiography or Western blotting with specific antibodies (data not shown). The IRES region from EMCV confers a high translatability on the mRNAs and so transfection of EMC-2A could compete with cellular mRNAs, leading to the inhibition of host translation. To test this possibility, HeLa cells were transfected with mRNAs that encodes for inactive point mutants of PV 2Apro (M2: G60R and M15: G121E) (21). These mRNAs did not affect the level of host protein synthesis as compared to control cells and both isoforms of eIF4G remained intact at 8 hpe (Fig. 1A and B). These data suggest that the inhibition of host mRNA translation and the modifications of eIF4G are, indeed, due to the synthesis of PV 2Apro and not to competition by the transfected mRNAs.

**Transfection of HeLa X1/5 cells with EMC-2A: effect on translation of preexisting and de novo synthesized mRNAs.** Previous observations have shown that eIF4GI is preferentially hydrolyzed as compared to eIF4GII in PV-infected HeLa cells (14). Our aim was to establish conditions under which these two isoforms were differentially cleaved in the absence of PABP hydrolysis in culture cells. We wanted to analyze the effect of differential eIF4GI and eIF4GII hydrolysis on the initiation of translation of de novo synthesized mRNAs as compared to the translation of mRNAs already engaged in the protein synthesis machinery. For this, we used Luc inducible HeLa X1/5 cells (19). These cells were electroporated with different amounts of in vitro transcribed EMC-2A mRNA: 1, 2, 4 or 9 µg. As controls, cells were electroporated with transcription buffer or with 9 µg of EMC-2C mRNA. During electroporation, tetracycline was removed from the culture medium to trigger Luc mRNA synthesis. Host protein synthesis, Luc expression, integrity of initiation factors and mRNA levels were analyzed at 4, 6 and 8 hpe.
(Fig. 2 and Fig. 9 of supplementary information). The 2A<sup>pro</sup> expression induced a time- and dose-dependent inhibition of host protein synthesis (Fig. 2A). To assure that similar amounts of proteins were loaded in each lane of the gel, the level of eIF4A was also estimated (Fig. 2B). eIF4GI and eIF4GII were proteolyzed in a time- and dose-dependent manner. As expected, eIF4GII was cleaved by 2A<sup>pro</sup> with delayed kinetics as compared to eIF4GI hydrolysis (Fig. 2B and Fig. 6C) (14). Interestingly, PABP remained intact during this assay (Fig. 2C). Significant hydrolysis of eIF4GI accompanied by partial cleavage of eIF4GII had only a limited effect on host translation of preexisting mRNAs (Fig. 2A and B: Lane 8). However, extensive inhibition of ongoing protein synthesis was observed when both eIF4G isoforms were hydrolyzed by 2A<sup>pro</sup> (Fig. 2A and B). The shutoff of protein synthesis was not produced by a reduction in the total amount of preexisting mRNAs since significant differences in the level of actin mRNA were not observed in these cells (Fig. 9 of supplementary information). These findings indicate that a strong abrogation of translation of preexisting mRNAs takes place when 2A<sup>pro</sup> substantially cleaves both eIF4GI and eIF4GII. Moreover, PABP degradation is not essential for this inhibition to occur.

Luc mRNA translation was measured to analyze the effect on translation of de novo synthesized mRNAs when eIF4GI and eIF4GII are differentially hydrolyzed. Synthesis of Luc mRNAs was not significantly affected by the expression of PV 2A<sup>pro</sup> (Fig. 9 of supplementary information) (17). Notably, 1 µg of EMC-2A mRNA sufficed to abrogate Luc mRNA translation (Fig. 2D). This amount of EMC-2A mRNA induced efficient cleavage of eIF4GI but only partially hydrolyzed eIF4GII (Fig. 2B: Lane 2, 7 and 14). These results suggest that translation of de novo synthesized mRNAs is more affected by eIF4GI proteolysis than mRNAs engaged in the translation machinery. As the amounts of actin and Luc mRNAs were similar in transfected cells, regardless of the EMC-2A dose employed (Fig. 9 of supplementary information), the inhibition on actin or Luc synthesis could not be ascribed to a decrease in the level of these mRNAs.

Translation of de novo synthesized Luc mRNA exhibited a dependence on eIF4GI integrity higher than preexisting actin mRNAs. To determine the eIF4G requirement to translate preexisting Luc mRNA, tetracycline was removed from
the culture medium 4 hours before electroporation. Thus, Luc mRNA started to be synthesized previously to transfection of EMC-2A mRNA. Next, cells were electroporated with 0.1, 0.5, 1, 3 and 9 µg of EMC-2A and actinomycin D (ActD), an inhibitor of transcription (26), was immediately added at a final concentration of 0.5 µg/ml. Luciferase activity and eIF4G integrity were measured at 0 hpe and 4 hpe in ActD treated as well as untreated cells (Fig. 3A). eIF4GI was significantly hydrolyzed at 0.1 µg of EMC-2A mRNA in a dose-dependent manner. However, eIF4GII was only proteolyzed in cells electroporated with 9 µg of EMC-2A (Fig. 3B). Transfection of 0.1 µg of EMC-2A was sufficient to fully block Luc synthesis in non treated cells, whereas 9 µg of this mRNA was required to inhibit Luc synthesis in ActD treated cells (Fig. 3A). Therefore, hydrolysis of eIF4GI is sufficient to inhibit Luc synthesis in cells that synthesize Luc mRNA continuously. However, hydrolysis of both isoforms of eIF4G is required to fully block Luc mRNA translation when Luc mRNAs were engaged in the translation machinery.

Involvement of eIF4GI and eIF4GII in the translation of transfected Luc mRNAs. The next step was to determine the extent of translation of different mRNAs transfected in cells where both isoforms of eIF4G had been differentially cleaved. This was done by co-electroporating HeLa cells with different amounts of EMC-2A and 9 µg of different Luc mRNAs: capped (cap-Luc), capped and polyadenylated (cap-Luc-polyA) (Fig. 4A), uncapped (Luc) or uncapped and polyadenylated (Luc-polyA) (Fig. 10 of supplementary information). In this assay, PV 2A pro was synthesized at the same time as Luc mRNA was engaged in translation. Moreover, using this Luc mRNA transfection method, we could analyze the Luc activity derived from the translation of this mRNA over time. Interestingly, this mRNA transfection method proved very reliable, given that a similar amount of Luc mRNA was detected in cap-Luc transfected cells at 2 hpe regardless of the EMC-2A dose used (Fig. 4B). Luc activity and the integrity of initiation factors were analyzed at 4, 6 and 8 hpe. Total hydrolysis of eIF4GI with partial cleavage of eIF4GII was induced by electroporation of HeLa cells with 1 µg of EMC-2A, whereas total proteolysis of both isoforms of eIF4G was achieved.
in cells electroporated with 9 µg of this mRNA. Cleavage of PABP did not occur in either case (data not shown).

Luc activity increased up to 6 hpe in cells electroporated with cap-Luc in the absence of EMC-2A mRNA. Partial inhibition of Luc activity was detected in cells co-electroporated with 1 µg of EMC-2A, whereas in cells co-electroporated with a higher amount of this mRNA (9 µg), Luc activity was strongly inhibited (Fig. 4C). Expression of cap-luc-polyA was six-fold higher than that obtained with cap-Luc and thirty-fold higher than with Luc-PolyA at 6 hpe (Fig. 4C and D and Fig. 9 of supplementary information), revealing the concerted action between the cap structure and the poly(A) tail. In the case of cap-luc-polyA, Luc activity increased with time, indicating longer-lasting stability than cap-luc. Cap-luc-polyA mRNA translation was partially affected (about 50-62% inhibition) in cells co-electroporated with 1 µg of EMC-2A. Transfection of 9 µg of this mRNA completely repressed Luc activity (Fig. 4D). As expected, uncapped Luc mRNA was not translated in HeLa cells (Fig. 10 of supplementary information). Uncapped and polyadenylated Luc mRNA exhibited a low translation capability in control cells. The expression of 2Apro from EMC-2A mRNA provoked partial inhibition of Luc activity in a dose-independent manner (Fig. 10 of supplementary information). These findings lend support to the idea that cleavage of eIF4GI partially affects translation of mRNAs present in polysomes and are in agreement with the data on actin synthesis presented in Figure 2. In contrast, the cleavage of both isoforms of eIF4G strongly blocked the translation of these mRNAs (Fig. 4C and D). Moreover, proteolysis of eIF4GI and eIF4GII in cells containing intact PABP is sufficient to abrogate translation of capped and polyadenylated mRNAs (Fig. 4D).

Translation of an IRES-containing mRNA under conditions of eIF4G cleavage. The mRNAs of picornavirus are uncapped and polyadenylated. The 5' untranslated region (UTR) of these mRNAs allows recruitment of the 40S ribosomal subunit under conditions in which eIF4F complex is disrupted by viral protein activity (2). Translation of picornavirus IRES-containing mRNAs is enhanced by the presence of a poly(A) tail at its 3' end (27,28). EMCV IRES has been classified as type II on the basis of primary sequence and secondary structure conservation and its requirements for optimal internal
initiation *in vitro* (29). We therefore used the IRES of EMCV to address the effect of differential cleavage of eIF4GI and eIF4GII in IRES-driven translation in transfected cells.

Thus, two mRNAs were obtained by *in vitro* transcription from the pTM1-Luc plasmid. One mRNA contained the 5'-UTR of EMCV followed by the Luc gene (EMC-luc), whereas in the other mRNA, the poly(A) tail was added by *in vitro* polyadenylation (EMC-luc-polyA) (Fig. 5A). HeLa cells were co-electroporated with 9 µg of each EMCV IRES-containing mRNA together with 1 µg or 9 µg of EMC-2A or transcription buffer as a control. The Luc activity and the integrity of initiation factors were analyzed at 4, 6 and 8 hpe. The PV 2A<sup>pro</sup> expression system described above is highly reproducible. Thus, 1 µg of EMC-2A mRNA brought about significant cleavage of eIF4GI, whereas eIF4GII remained largely intact. However, electroporation of 9 µg of this mRNA led to total cleavage of both eIF4G isoforms (data not shown). Most of the Luc synthesized from EMC-Luc was produced before 4 hpe, after which time, only a slight increase in Luc activity was achieved (Fig. 5B). The same effect was found with this mRNA when it was co-transfected with low dose of EMC-2A. However, powerful stimulation of the Luc activity was observed when a high dose of EMC-2A was employed (Fig. 5B). Translation of this mRNA was enhanced at all times compared to control cells. These data suggest that significant hydrolysis of eIF4GI and eIF4GII is required to induce the total transactivation of the EMCV IRES-driven translation in culture cells. To our knowledge these findings provide the first evidence in culture cells illustrating that the massive cleavage of both isoforms of eIF4G is necessary for full stimulation of EMCV IRES-driven translation. Synthesis of luc from EMC-luc-polyA was significantly stimulated compared with its counterpart lacking the poly(A) tail (approximately seven-fold at 8 hpe) (Fig. 5B). Only slight enhancement was observed when this mRNA was co-expressed with 2A<sup>pro</sup>, regardless of the EMC-2A dose employed (Fig. 5B). Therefore, EMC-Luc is more extensively transactivated by 2A<sup>pro</sup> activity than EMC-luc-polyA. Notably, the differences in the Luc activity obtained from EMC-Luc and EMC-luc-polyA disappeared when a high dose of EMC-2A was used (Fig. 5B).

*Synthesis of heat-shock proteins.*

*Effect of the differential cleavage of eIF4GI and eIF4GII.* Several host
mRNAs can be translated by a cap-independent mechanism. This is the case of Hsp70 mRNA (17), which may contain an IRES element at its 5'-UTR (30). Alternatively, it may be translated by a “shunting mechanism” under heat shock conditions (31). We therefore considered it of interest to assay the action of the differential cleavage of both eIF4G isoforms in the initiation of translation of Hsp70 mRNA. HeLa cells were electroporated with 1, 2, 4 or 9 µg of EMC-2A mRNA. As controls, cells were transfected with 9 µg of EMC-2C mRNA or with transcription buffer. At 5 hpe, cells were incubated at 42°C for 3 h to trigger the heat shock response. Host protein synthesis and the integrity of the different initiation factors were analyzed at 4 hpe and at 8 hpe (3 h post heat-shock). As shown in Figure 2, the repression of host protein synthesis and proteolysis of both eIF4G isoforms took place in a dose- and time-dependent manner (Fig. 6A and B). Once again, significant hydrolysis of eIF4GI without eIF4GII cleavage had only a slight effect on cellular protein synthesis. Notably, a strong shutoff of host translation was observed, which correlated with eIF4GII hydrolysis (Fig. 6A and B). At 3 h post heat-shock, Hsp70 synthesis was detected (Fig. 6A). Under conditions in which eIF4GI was totally cleaved and eIF4GII remained largely intact, de novo synthesized Hsp70 mRNA can be translated at a level similar to that observed in control cells (Fig. 6A and B: Lane 7). However, a decrease in Hsp70 synthesis was detected coinciding with an increase in the dose of EMC-2A (Fig. 6A and C). When eIF4GI and eIF4GII were significantly cleaved by PV 2Apro, Hsp70 was synthesized by about 35-25%, whereas endogenous mRNAs were strongly inhibited (about 10% of actin synthesis) (Fig. 6A and B: Lane 11). The amount of Hsp70 mRNA was analyzed by RT-PCR. At 3 h post heat-shock, the amount of this mRNA decreased in a dose-dependent manner in transfected cells (Fig. 11 of supplementary information). A decrease in the amount of Hsp70 mRNA had been observed previously when hybrid proteins that contain PV 2Apro were introduced in HeLa cells (17). Nevertheless, the electroporation of 9 µg of EMC-2A induced a 30-40% decrease in the amount of Hsp70 mRNA, whereas the synthesis of the heat-shock protein was greatly inhibited (65-75%). Therefore, blockade of the initiation of Hsp70 mRNA translation was not entirely due to decreased mRNA concentrations. These findings reveal that the first initiation event in
some host mRNAs might be more susceptible to eIF4GII proteolysis than to eIF4GI degradation. These results, taken together with the findings reported earlier, suggest that eIF4GI and eIF4GII may have differential roles in the translation of different cellular mRNAs.

Two PV 2Apro variants, D135N (M6) and V119M (M7), that failed to repress host transcription but maintained their capacity to proteolyze eIF4GI, have been described (21). Since Hsp70 mRNA transcription was partially inhibited by 2Apro expression, mRNAs that encode these two 2Apro mutants were transfected to analyze their effect on Hsp70 synthesis. Transfection of a high dose of these mRNAs largely hydrolyzes both eIF4G isoforms leading to the blockade of actin as well as Hsp70 synthesis. Analysis of the Hsp70 mRNA by real-time RT-PCR revealed that 2Apro M6 and M7 failed to repress host transcription (Fig. 12 of supplementary information). Thus, the inhibition of Hsp70 synthesis in 2Apro M6 and M7 expressing cells is not due to the blockade of transcription but also to their direct effect on translation.

Ongoing mRNA translation and Hsp70 synthesis in BHK cells transfected with EMC-2A. To analyze the differential cleavage of the two isoforms of eIF4G by 2Apro in another cell line, BHK cells were electroporated with 1, 2, 4, 6, 9 or 18 µg of EMC-2A. At 5 hpe, cells were incubated at 42°C for 3 h to trigger the heat-shock response. Host protein synthesis and integrity of translation initiation factors were analyzed at 8 hpe in cells incubated at 37°C and in cells subjected to heat-shock for 3 h (7 hpe and 3 h post heat shock). In both cases a gradual dose-dependent inhibition of protein synthesis was achieved (Fig 6A). eIF4GI and eIF4GII were also proteolyzed in a dose-dependent manner, whereas PABP remained intact in each case (Fig. 7B and C). Previous analyses of eIF4GI using specific antibodies have revealed the existence of two proteins of ~220 and ~150 kDa, respectively, in BHK cells (22). As described earlier, eIF4G exhibits different mobility patterns in SDS-PAGE in mammalian cells, possibly due to post-translational modifications (32). Alternatively, it has been proposed that it could be a breakdown product of eIF4G. Both 220- and 150-kDa polypeptides disappeared in 2Apro expressing cells. Only the C-terminal proteolytic fragment could be detected with anti-eIF4GI antibodies (22). Surprisingly, proteolysis kinetics of eIF4GI was delayed with respect to
eIF4GII in BHK cells (Fig. 7B). eIF4GII was extensively proteolyzed at 8 hpe using 18 μg of EMC-2A, whereas under these conditions 40-50% of the eIF4GI remained intact (Fig. 7B). Notably, the inhibition of ongoing protein synthesis correlated well with the proteolysis of eIF4GII (Fig. 7A, B and D). Under conditions where 75-85% of eIF4GII was cleaved by 2Apro and about 100-60% of eIF4GI remained intact, host translation dropped to nearly 35% (Fig. 7A and B: Lanes 6 and 13). These results provide further evidence for the essential role that eIF4GII plays in the translation of mRNAs already engaged in translation.

Translation of Hsp70 mRNA was also analyzed in BHK cells. The heat-shock treatment was carried out at 5 hpe. Thus, Hsp70 mRNA synthesis started when eIF4GI and eIF4GII were already hydrolyzed (data not shown). Synthesis of Hsp70 was detected at 3 h post heat-shock treatment. Hsp70 synthesis was also inhibited in a dose-dependent manner (Fig. 7A). Interestingly, significant inhibition of the initiation of translation of Hsp70 mRNA (about 60-65%) was observed when 6 μg of EMC-2A was used. In these cells, eIF4GII was fully cleaved, although all eIF4GI remained intact (Fig. 7A and B: Lane 12). These results were reproduced by western blotting with an antibody against Hsp70 (Data not shown). Notably, inhibition of Hsp70 and actin synthesis correlated well with the proteolysis of eIF4GII in this cell line (Fig. 7D).

**Cytoplasmic initiation of translation in EMC-2A transfected HeLa and BHK Cells.** To determine the involvement of eIF4GI and eIF4GII in the first event of translation on preexisting mRNAs, run-off of polysomes was carried out both in HeLa and BHK cells (Fig. 8). To this aim, cells were incubated with hypertonic medium leading to the inhibition of the initiation of translation, while elongation still takes place. This treatment provokes the run-off of polysomes, whereas a return to normal medium leads to initiation of translation on ribosome-stripped mRNA (22,17). HeLa or BHK cells were electroporated with the EMC-2A at the doses indicated (Fig. 8). At 8 hpe the culture medium was supplemented with 150 mM NaCl for 2 hours, giving rise to polysome run-off. At 10 hpe normal ionic conditions were restored to test the first translation initiation event on preexisting cytoplasmic mRNAs. Protein synthesis, eIF4GI and eIF4GII integrity (Fig. 8) and the amount of eIF4A (data not
shown) were determined at 8, 10 and 12 hpe.

A potent proteolysis of eIF4GI was achieved in HeLa cells transfected with 1 and 3 µg EMC-2A. Under these conditions the level of protein synthesis was similar to control cells (Fig. 8A). Remarkably, a high dose of EMC-2A induced a deep inhibition of ongoing translation concomitantly with eIF4GI and eIF4GII hydrolysis. When these cells were incubated with hypertonic medium, translation was abrogated indicating that the run-off of polysomes took place (Fig. 8A). Notably, protein synthesis was recovered in control cells and cells electroporated with low doses of EMC-2A after restoration to normal conditions, even though in the last cases eIF4GI was cleaved. Only cells transfected with high doses of EMC-2A did not recover translation (Fig. 8A). These data suggest that integrity of eIF4GI is not essential for cytoplasmic initiation of translation to occur.

The inhibition of endogenous translation in BHK-cells correlated with eIF4GII inactivation at 8 hpe (Fig 8B). Treatment of BHK-cells with hypertonic medium blocked the translation in all cases irrespective of eIF4GII integrity. When normal conditions were restored, preexisting mRNAs were engaged in translation both in control cells and BHK cells transfected with a low dose of EMC-2A in which eIF4G remained intact. However, a significant inhibition of initiation of translation was observed in cells electroporated with 9 or 18 µg EMC-2A. In cells transfected with 9 µg mRNA eIF4GII was hydrolyzed, remaining about 60% of eIF4GI intact. These results indicate that eIF4GII is essential for the first events of initiation on cytoplasmic mRNAs.

DISCUSSION

A wide variety of animal viruses such as several picornaviruses, retroviruses and caliciviruses bring about the cleavage of initiation factors in infected cells to modulate host and viral translation. In this respect, eIF4GI, eIF4GII and PABP are some of the most common cellular targets for viral proteases (2,9). Nevertheless, the particular contribution of hydrolysis of each of these initiation factors to the inhibition of host protein synthesis is not yet well established. The method of PV 2Apro expression described here differentially cleaves eIF4GI and eIF4GII, leaving PABP intact. Such a system can help elucidate the exact role played by cleavage of these two
isoforms of eIF4G during the initiation of translation. This assay is easy to perform and reproducible, and led to efficient cleavage of eIF4G very soon after transfection of the majority of culture cells. Moreover, the proportion of the two eIF4G isoforms cleaved varied according to dose of EMC-2A mRNA transfected. Interestingly, the kinetics of eIF4GI cleavage in BHK cells is delayed from that in HeLa cells. Thus, hydrolysis of eIF4GI is produced after eIF4GII cleavage. This method is much more efficient, rapid and reliable than other methods previously described, such as HeLa cell lines that can be induced to express PV 2A pro, direct penetration of hybrid proteins that contain this protease or coupled infection with recombinant VVT7 and transfection with pTM1-2A (20,17,21).

The different kinetics of cleavage of the two isoforms of eIF4G observed in HeLa and BHK cells can be accounted for by the differences in the primary structure of the corresponding initiation factors. Comparison of the amino acid sequence of human and mouse eIF4GI (since the sequence of hamster eIF4G is not available) reveals several changes in the amino acid residues around the cleavage site of 2A pro (Thr by Ser at P2, Thr by Ala at P5 and Thr by Pro at P6) (Berger and Schechter notation). Variations at the P2 position of the cleavage site recognized by 2A pro are very restrictive for trans substrate proteolysis (33). On the other hand, the proteolysis site of human eIF4GII recognized by 2A pro has not yet been identified. In this regard, the rhinovirus 2A pro cleavage site (another enterovirus) is well-conserved in mouse eIF4GII (34). These data may account for the different kinetics of eIF4GI hydrolysis observed in the two cell lines and the similar susceptibility of eIF4GII to PV 2A pro expression.

Translation of Luc mRNA on induction of the HeLa cell line X1/5 is strongly inhibited by transfection of EMC-2A mRNA. This blockade correlated well with eIF4GI inactivation, despite the fact that eIF4GII remained largely intact. The Luc mRNA synthesized in HeLa X1/5 cells is capped and polyadenylated by the host enzymes and contains a leader sequence typical of most cellular mRNAs (17). These data reveal that eIF4GI could participate in the recognition of newly synthesized cellular mRNAs. eIF4GI not only interacts with the cytoplasmic translation initiation complex (known as the steady-state complex) but it is also present in the pioneer translation initiation complex in the nucleus, bound
to the nuclear cap binding proteins CBP80 and CBP20, and it is associated with pre-mRNAs (35-38). Hydrolysis of eIF4GI by HIV-2 PR or 2A<sup>pro</sup> blocks the steady state of translation as well as the pioneer round of protein synthesis on virgin mRNAs (37). However, in the present work we describe that hydrolysis of eIF4GI did not inhibit the first initiation event after polysome run-off. These data suggest that hydrolysis of eIF4GI could inhibit the pioneer translation initiation complex from mRNAs transported from the nucleus, whereas this isoform is not required for the steady-state complex.

Previous findings indicated that translation of mRNAs already engaged in the protein-synthesis machinery is resistant to eIF4GI cleavage (11-13,39). Earlier reports showed a good correlation between eIF4GII hydrolysis and the shutoff of host protein synthesis (14-16). As shown in this work, individual expression of PV 2A<sup>pro</sup> hydrolyzes eIF4GII with delayed kinetics as compared to eIF4GI in HeLa cells. In this cell line, the decrease in ongoing protein synthesis coincides with eIF4GII cleavage. Moreover, translation of a capped and polyadenylated Luc mRNA previously engaged in polysomes was fully blocked when both initiation factor isoforms were proteolyzed, whereas the effect was partial when only eIF4GI was cleaved in Luc mRNA transfected as well as ActD treated HeLa cells. These observations are further reinforced in BHK cells, where eIF4GII was hydrolyzed by 2A<sup>pro</sup> more rapidly than eIF4GI. Therefore, in both HeLa and BHK cells, the inhibition of translation on preexisting mRNAs correlates well with the inactivation of eIF4GII. In this respect, a recent report suggests that both forms of eIF4G can be differentially recruited to the mRNA cap structure at the same time as the onset of cell differentiation (40). Therefore, eIF4GII integrity could be essential for ongoing translation given the fact that when eIF4GII was hydrolyzed and a large proportion of eIF4GI remained intact, the translation of endogenous mRNAs was blocked by about 60-80% in BHK cells. Besides, cleavage of eIF4GI by 2A<sup>pro</sup> in HeLa cells while maintaining eIF4GII intact only decreases the endogenous translation by about 5%-30%. These findings do not support the possibility that variations in the amount of total eIF4G affects ongoing translation since eIF4GII is minority compared with eIF4GI (10-15%) (25,10,2) (10,2).

PV, coxsackievirus and calicivirus infection induce cleavage of PABP (10).
It has been suggested that PABP may play an important role in the PV induced shutoff of protein synthesis (18). Nevertheless, significant cleavage of PABP is only detected at 4-5 hpi, whereas block of cellular protein synthesis by PV occurs earlier, at the same time as hydrolysis of eIF4G. The possibility that 3C<sup>pro</sup> may exhibit a high affinity for ribosome-associated PABP has been put forward to support this hypothesis (10). Notably, the addition of a recombinant PABP with the four RRM motifs but lacking the CTD domain in a PABP depleted Krebs-2 extract partially restored translation (41). This truncated protein is similar to the N-terminal product obtained by the proteolysis of PABP by 2A<sup>pro</sup> or 3C<sup>pro</sup> (10), although the C-terminal product of PABP was not present. In the system described here, 2A<sup>pro</sup> strongly inhibited ongoing host translation without affecting PABP integrity. Moreover, under these conditions, there was a strong inhibition of translation of a capped and polyadenylated Luc mRNA. In this regard, the interaction between eIF4G and PABP has been described as a requirement for the efficient assembly of the translation machinery and for the poly(A) tail-dependent stimulation of translation (41). As proteolysis of eIF4G by 2A<sup>pro</sup> separates the PABP interaction domain, the absence of eIF4E and PABP binding sites in this initiation factor may abrogate the translation of a capped and polyadenylated mRNA. Therefore, the hydrolysis of either eIF4G or PABP could, in principle, contribute independently to the shutoff of host protein synthesis.

PV 2A<sup>pro</sup> has two opposite effects in gene expression. On one hand, this protease abrogates host protein synthesis, but it can also stimulate PV translation (20, 2, 9). The findings described in the present work certainly support these opposing effects, since there is a correlation between eIF4G hydrolysis and the inhibition of cellular protein synthesis, whereas stimulation of translation of IRES-driven mRNAs is observed under these conditions. Interestingly, although eIF4G is not hydrolyzed in cells infected with EMCV, translation driven by its IRES element is stimulated (high EMC-2A dose) or, at least, is not affected (low EMC-2A dose) when this factor is cleaved by 2A<sup>pro</sup> in transfected HeLa cells. In agreement with previous data obtained using Krebs-2 extracts, stimulation of translation when eIF4G is hydrolyzed by 2A<sup>pro</sup> of an mRNA containing EMCV IRES is higher than for its counterpart carrying the poly(A) tail (28). As previously described, Paip-
2 and rotavirus NSP3, two inhibitors of poly(A) tail-dependent stimulation (42,43), strongly blocked the translation of polyadenylated EMCV IRES-containing mRNA in vitro, whereas it was not affected in the presence of rhinovirus 2A pro (27,28). These data suggest that the transactivation of EMCV IRES, under conditions of eIF4G cleavage, replaces the stimulation provided by poly(A) tail. Cleavage of eIF4G by 2A pro separates its PABP binding domain breaking the circular conformation of the mRNA and abolishing the conformational changes induced by the eIF4G-PABP interaction. Under these conditions, poly(A) tail-dependent stimulation is abrogated (41,27). However, 2A pro activity enhances EMCV IRES-driven translation, counteracting the stimulation provided by poly(A) tail. Nevertheless, the molecular mechanism by which this protease enhances IRES-driven translation is still under investigation. Probably, the proteolytic activity of this enzyme produces a transactivator or inactivates an inhibitor of IRES translation. As observed in this work, significant expression of 2A pro is required to stimulate IRES-driven translation. This effect could be produced by significant hydrolysis of eIF4G achieved in cells electroporated with high dose of EMC-2A mRNA. Nevertheless, we cannot rule out that 2A pro itself or the proteolysis of an unknown substrate of this protease enhances EMCV IRES driven-translation.

Another mRNA classified as IRES-containing is Hsp70 mRNA (30). The dependence of translation of this mRNA on some initiation factors is low, and translation was significant when eIF4GI was cleaved by 2A pro (17). In good agreement with these data, our present findings suggest that the initiation of translation of de novo synthesized Hsp70 mRNA is not dependent on eIF4GI integrity. However, an increase in the dose of EMC-2A induced a partial reduction in the Hsp70 mRNA level, although this decrease did not fully account for inhibition of the Hsp70 synthesis. Strikingly, a gradual block of the translation of Hsp70 mRNA occurs at the same time as eIF4GII cleavage. Moreover, high doses of EMC-2AM6 and EMC-2AM7 also induced the cleavage of the two isoforms of eIF4G and strongly inhibited Hsp70 and actin synthesis, although host transcription was only slightly affected. These findings could support the view that eIF4GI and eIF4GII have a differential
participation in the initiation of translation of different cellular mRNAs.

Therefore, the effect of eIF4GI and/or eIF4GII hydrolysis in translation varies according to the mRNA analyzed. The use of different viral proteases that cleave the two isoforms of eIF4G and PABP in a differential manner may help to elucidate the exact role that each of these factors plays in the translation of cellular and viral mRNAs.

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REFERENCES


**FOOTNOTES**
The abbreviations used are: mRNA, messenger RNA; rRNA, ribosomal RNA; eIF, eukaryotic initiation factor; PV, poliovirus; EMCV, encephalomyocarditis virus; IRES, internal ribosome entry site; Hsp 70, heat shock protein 70; PABP, poly(A)-binding protein; Luc, luciferase; VVT7, vaccinia virus T7.

**FIGURE LEGENDS**
Figure 1. Expression of PV 2A\textsuperscript{pro} in HeLa cells on transfection of EMC-2A mRNA. HeLa cells were electroporated with 9 µg of EMC-2A, EMC-2C, EMC-2AM2, EMC-2AM15 mRNAs or transcription buffer. Proteins were labelled from 7 to 8 hpe and processed as described in materials and methods. A) Analysis of protein synthesis. B) Western blotting against eIF4GI (upper panel) and eIF4GII (lower panel). C) Western blotting against eIF4A (upper panel) and PABP (lower panel). Ac, actin. N-t, N-terminal fragments of eIF4GI or eIF4GII. C-t, C-terminal fragments of eIF4GI or eIF4GII. Mr (KDa) molecular weight markers.

Figure 2. Differential hydrolysis of eIF4GI and eIF4GII by PV 2A\textsuperscript{pro}. Effects on translation of preexisting and de novo synthesyzed mRNAs. HeLa X1/5 cells were electroporated with 1, 2, 4 or 9 µg of EMC-2A, 9 µg of EMC-2C or transcription buffer. One third of the cells were used to analyze protein synthesis and the integrity of initiation factors. A) Analysis of protein synthesis. B) Western blotting against eIF4GI (upper panel), eIF4GII (middle panel) and eIF4A (lower panel). C) Western blotting against PABP. The rest of the cells were used to analyze the Luc activity (D) and mRNA levels (Fig. 9 of supplementary information). RLU, relative light units.

Figure 3. Effect of eIF4GI hydrolysis on translation of preexisting Luc mRNA. X1/5 HeLa cells were washed twice with PBS and then culture medium was added. 4 hours after Luc induction, cells were electroporated with 0.1, 0.5, 1, 3 and 9 µg of EMC-2A. Before electroporation cells were treated or not with 0.5 µg/ml ActD. Luciferase activity and integrity of initiation factor were analyzed at 0 and 4 hpe. A) Relative representation of luciferase activity from ActD treated or untreated cells at 4 hpe compared to control cells. B) Comparative representation of the level of intact eIF4GI and eIF4GII.

Figure 4. Effect of eIF4GI and eIF4GII cleavage by PV 2A\textsuperscript{pro} in the translation of different Luc mRNAs. A) Schematic representation of the different transfected Luc mRNAs. HeLa cells were co-electroporated with 9 µg of a Luc mRNA and with 1, 9 µg of EMC-2A or transcription buffer. Luc activity was measured at 4, 6 and 8 hpe. B) Total RNA was isolated at 2 hpe from cells co-electroporated with cap-Luc mRNA and the different doses of EMC-2A. The amount of Luc mRNA was quantified as described
in materials and method. The relative level of RNA was represented with respect to control cells. C) Luc activity obtained at times indicate from cells co-transfected with different doses of EMC-2A and cap-Luc mRNA; D) cap-Luc-polyA mRNA.

**Figure 5. IRES-driven translation upon differential cleavage of eIF4GI and eIF4GII.** HeLa cells were co-electroporated with 9 μg of EMC-Luc or EMC-Luc-polyA together with 1 or 9 μg of EMC-2A or transcription buffer. The Luc activity in each case was measured at 4, 6, 8 hpe. A) Schematic representation of the IRES-containing mRNAs. (B) Luc activity obtained from EMC-Luc or EMC-Luc-polyA in co-transfected cells.

**Figure 6. Translation of Hsp70 mRNA in HeLa cells containing the two isoforms of eIF4G differentially cleaved.** HeLa cells were electroporated with 1, 2, 4 or 9 μg of EMC-2A, 9 μg of EMC-2C or transcription buffer. At 5 hpe, cells were incubated at 42°c for 3h. Proteins were labeled from 3 to 4 or from 7 to 8 hpe. A) Analysis of protein synthesis by SDS PAGE, fluorography and autoradiography. B) Western blotting against eIF4GI (upper panel), eIF4GII (middle panel) and eIF4A (lower panel). C) Comparative representation of the level of intact eIF4GI and eIF4GII with the translation of de novo synthesized Luc or Hsp70 mRNAs and actin synthesis. These data were obtained from five independent experiments including the results shown in Figs. 2A, 2B, 2D, 5A and 5B. hpHS, hours post heat shock.

**Figure 7. Effect of PV 2A pro in BHK-21 cells.** BHK cells were electroporated with 1, 2, 4, 6, 9 or 18 μg of EMC-2A or with transcription buffer alone. One half of the cells were subjected to heat-shock treatment (42°c) at 5 hpe for 3 h. The remaining cells were incubated at 37°c throughout the time course. In both cases the proteins were labeled at 7 hpe for 1h. A) Analysis of protein synthesis by SDS PAGE, followed by fluorography and autoradiography. B) Western blotting against eIF4GI (upper panel) and eIF4GII (lower panel). C) Western blotting against eIF4A (upper panel) and PABP (lower panel). D) Comparative representation of the level of intact eIF4GI and eIF4GII; translation of de novo synthesized Hsp70 mRNAs and preexisting actin mRNA in cells transfected with different amounts of EMC-2A. These data were obtained from three independent experiments and represented as percentage with respect to control cells.
Figure 8. Effect of eIF4GI and eIF4GII cleavage on the initiation of translation after exposure to hypertonic medium. HeLa and BHK cells were electroporated with 1, 2, 4 and 9 or 1, 4, 9 and 18 µg of EMC-2A, respectively. In both cases transcription buffer and high dose of EMC-2C were used as controls. At 8 hpe 150 mM of NaCl was added giving rise to a final concentration of 300 mM in the culture medium. These conditions were maintained for 2 hours. At 10 hpe hypertonic medium was removed and the cells were washed twice with PBS. Then, normal medium was restored. Protein synthesis and the integrity of initiation factors were analyzed at 8, 10 and 12 hpe. A) Hypertonic treatment in HeLa cells. Analysis of protein synthesis by SDS PAGE, followed by fluorography and autoradiography (upper panel). Western blotting against eIF4GI (middle panel) and eIF4GII (lower panel). B) Hypertonic treatment in BHK cells. Analysis of protein synthesis by SDS PAGE, followed by fluorography and autoradiography (upper panel). Western blotting against eIF4GI (middle panel) and eIF4GII (lower panel).

Figure 9. Schematic representation of the differential mechanism of action of both isoforms of eIF4G. The mRNAs synthesized in the nucleus are transported and presented to the protein synthesizing machinery by eIF4GI, while eIF4GII is necessary to initiate translation of cytoplasmic mRNAs.