Functional interaction of translation initiation factor eIF4G with the foot-and-mouth disease virus internal ribosome entry site

Lanja Saleh, René C. Rust,† Ralf Füllkrug, Ewald Beck, Gergis Bassili, Kerstin Ochs and Michael Niepmann

Institute of Biochemistry, Friedrichstr. 24, 35392 Giessen, Germany

In the life-cycle of picornaviruses, the synthesis of the viral polyprotein is initiated cap-independently at the internal ribosome entry site (IRES) far downstream from the 5′ end of the viral plus-strand RNA. The cis-acting IRES RNA elements serve as binding sites for translation initiation factors that guide the ribosomes to an internal site of the viral RNA. In this study, we show that the eukaryotic translation initiation factor eIF4G interacts directly with the IRES of foot-and-mouth disease virus (FMDV). eIF4G binds mainly to the large Y-shaped stem–loop 4 RNA structure in the 3′ region of the FMDV IRES element, whereas stem–loop 5 contributes only slightly to eIF4G binding. Two subdomains of stem–loop 4 are absolutely essential for eIF4G binding, whereas another subdomain contributes to a lesser extent to binding of eIF4G. At the functional level, the translational activity of stem–loop 4 subdomain mutants correlates with the efficiency of binding of eIF4G in the UV cross-link assay. This indicates that the interaction of eIF4G with the IRES is crucial for the initiation of FMDV translation. A model for the interaction of initiation factors with the IRES element is discussed.

Introduction

Foot-and-mouth disease virus (FMDV), a member of the family Picornaviridae, is the cause of a highly contagious disease of cattle and other cloven-hoofed animals (Brown, 1999). After infection of the cell and release of the viral plus-strand RNA, FMDV translation is mediated by an internal region of the viral RNA, the internal ribosome entry site (IRES). At the same time, the cap-dependent cellular translation is shut down by the viral leader (L) protease that cleaves the eukaryotic translation initiation factor (eIF) eIF4G (Liebig et al., 1993), which is the major component of the cap-binding protein complex eIF4F.

The IRES elements of picornaviruses are large cis-acting RNA regions that guide ribosomes to an internal site of the viral RNA (Jackson & Kaminski, 1995; Niepmann, 1999). They are classified in three groups, the type I elements of the entero- rhinovirus group (including poliovirus), the type II elements of the cardio-/aphthovirus group (including FMDV), and the type III element of hepatitis A virus. The IRES elements consist of highly conserved RNA structures with a core in their 3′ region (Le et al., 1996). At their 3′ borders, they have a characteristic tandem of an oligopyrimidine tract followed by a conserved AUG triplet. Most likely mediated by the help of standard initiation factors, the ribosome is guided to a starting window at the 3′ border of the IRES that contains the conserved AUG (Pilipenko et al., 1994). In cardio-/aphthoviruses, this AUG is usually used for translation initiation. FMDV also utilizes an additional start site 84 nucleotides further downstream after scanning (Belsham, 1992). In addition to the standard initiation factors, picornavirus IRES elements also recruit other cellular RNA-binding proteins, like the 57 kDa polypyrimidine tract-binding protein (PTB) that stimulates the IRES activities of encephalomyocarditis virus (EMCV) (Kaminski et al., 1995) and FMDV (Niepmann, 1996).

Almost the complete set of canonical initiation factors appears to be essential for internal initiation on picornaviral RNAs (Anthony & Merrick, 1991; Pause et al., 1994; Pestova et al., 1996a). eIF4B has been shown to bind to the large Y-shaped stem–loop 4 of the FMDV IRES (Meyer et al., 1995; Rust et al., 1999) and to interact with the FMDV IRES in both 48S and 80S ribosomal initiation complexes (Ochs et al., 1999). However, a key role in the process of internal initiation is attributed to eIF4G. It appears to take on the role of a...
multipurpose adapter that connects the RNA with the ribosome (Hentze, 1997). The N-terminal domain of eIF4G that interacts with the cap-binding protein eIF4E (Mader et al., 1995) is clipped off by the proteases of several picornaviruses (Lamphear et al., 1995; Liebig et al., 1993). The C-terminal domain interacts with the ribosome-bound eIF3 and the RNA helicase eIF4A (Imataka & Sonenberg, 1997) and is sufficient to confer internal translation initiation of several picornaviruses (Ohlmann et al., 1996). Cleavage of eIF4G even stimulates translation from the type I IRES elements of enteroviruses, whereas the FMDV and other type II IRES elements work well with the cleaved or the uncleaved form of eIF4G (Borman et al., 1995). eIF4G binds to the EMCV IRES (Pestova et al., 1996b) and contacts stem–loops J and K of the EMCV IRES, and the binding of eIF4G, eIF4B and eIF4A to the IRES of EMCV appears to be synergistic (Kolupaeva al., 1998).

In this study, we investigated the interaction of eIF4G with the FMDV IRES. We mapped the determinants for binding of eIF4G in the IRES by a series of deletion mutants in which each of the secondary structure elements of the FMDV IRES was removed, and also by smaller deletion mutants within the stem–loop that is the binding site for eIF4G. In addition, the efficiency of translation of these mutants was determined.

**Methods**

- **Plasmids.** pSP449 (Luz & Beck, 1991) contains the FMDV O, K IRES from position 363 to 804 plus 27 nucleotides of coding sequence. In pSP449-Δ2,3 and Δ4, sequences corresponding to the respective stem–loops of the FMDV IRES had been removed (Meyer et al., 1995) (see Fig. 2a). In pSP449-Δ4-1, -Δ4-2 and -Δ4-3 (Rust et al., 1999), sequences corresponding to the respective subdomains of IRES stem–loop 4 had been removed (boxed regions in Fig. 3a). pD12 (Ochs et al., 1999) is a dicistronic expression vector that contains the chloramphenicol acetyltransferase (CAT) gene and the FMDV IRES (positions 185 to 804) with the eleventh ATG of FMDV fused to the luciferase gene. In pD12- ΔA-, ΔA-1, -ΔA-2 and -ΔA-3 (Rust et al., 1999), the corresponding domains or subdomains of the IRES have been removed in a similar way to the pSP449 series of plasmids. pFMVDV14 was derived from the full-length FMDV clone pFMVDV-YEP-polyC (Zibert et al., 1990) by fusing FMDV nucleotides 1 to 114 with FMDV position 215, thereby removing part of the 5′-noncoding FMDV sequence, including the poly(C) tract.

- **Preparation of RNAs and translation.** For in vitro transcription, the plasmids of the pSP449 series were linearized with Smal in the linker downstream of the FMDV IRES sequence. The template for RNA Δ5 was generated by linearizing pSP449 with NcoI in the FMDV IRES stem–loop 5 sequence. Labelled RNAs were synthesized as described before (Ochs et al., 1999) using SP6 RNA polymerase in the presence of 2.5 mM [α-32P]UTP (400 Ci/mmol; Amersham) plus 10 µM unlabelled UTP. Dicistronic plasmids of the pD-series were linearized with BamHI downstream of the luciferase gene, and pFMVDV14 was linearized with BamHI in the FMDV 1D (VP1) coding region. Unlabelled RNAs were synthesized in the presence of 500 µM unlabelled nucleotides. Translation reactions contained 0.25 µg of RNA in a 20 µl reaction containing 10 µl rabbit reticulocyte lysate (RRL, Promega). Translations and measurement of reporter protein activities were performed as described (Niepmann et al., 1997).

**Results**

*eIF4G binds to the FMDV IRES*

For identification of the eIF4G protein binding to the FMDV IRES, the UV cross-link assay was used in combination with immunoblotting. RRL that is competent for FMDV translation (Kühn et al., 1990; Niepmann et al., 1997) was used as the source of IRES-binding proteins. Proteins from the RRL were allowed to bind to [α-32P]UTP-labelled IRES RNA for 10 min at 30 °C. Proteins directly contacting the IRES RNA were then covalently linked to the RNA by UV irradiation, and excess RNA was digested by RNase A. Half of the sample was analysed by gel electrophoresis and autoradiography (Fig. 1a). The other 50% of the sample was separated by gel electrophoresis, the proteins were blotted to a nitrocellulose membrane, and eIF4G was identified by immunostaining using an anti-eIF4G antiserum (Fig. 1b).

Several proteins binding to the complete FMDV IRES were detected in the UV cross-link assay (Fig. 1a, lane 1), including a 57 kDa protein identical to PTB (Niepmann et al., 1997) and a protein with an apparent molecular mass of 80 kDa identical to initiation factor eIF4B (Meyer et al., 1995; Ochs et al., 1999). The identities and binding specificities of several other proteins appearing in this cross-link assay are still unknown. In addition, a band in the range of about 200 kDa molecular mass, as expected for eIF4G (Lamphear et al., 1995), was detected (Fig. 1a, lane 1). In the immunoblot, a band appeared at exactly the same position of the gel (Fig. 1b, lane 1), providing evidence that the protein detected in the UV cross-link is identical to eIF4G. In addition, a second band appeared in the immunoblot below the eIF4G band, which may represent a degradation product of eIF4G.

In order to further confirm that the band appearing in the UV cross-link assay is identical to the eIF4G protein detected in the immunoblot, we used aliquots of RRL that had been preincubated for different times with an RNA encoding the FMDV L protease, which is known to cleave eIF4G (Lamphear et al., 1995). When L RNA was preincubated for only 2 min
Interaction of eIF4G with the FMDV IRES

**Fig. 1.** Identification of eIF4G binding to the FMDV IRES. (a) UV cross-link assay with normal RRL or RRL treated with FMDV L protease. For translation of L protease, unlabelled RNA transcribed in vitro from pFMDV14 was preincubated with the RRL at 30 °C for the times indicated on the top (min. L-prot.). In lane 1, no pFMDV14 RNA was added. This RRL was then used for UV cross-link reactions with [α-32P]UTP-labelled FMDV IRES RNA. Reactions were incubated at 30 °C for 10 min, irradiated with UV light for 20 min, and excess RNA was digested with RNase A. Half of each sample was applied to an SDS–8% polyacrylamide gel and analysed by autoradiography. 4G, eIF4G; 4GC, the C-terminal fragment of eIF4G. The molecular masses of marker proteins (M) are given in kDa. (b) Western blot. The other half of the same samples as loaded in (a) were separated on an SDS–8% polyacrylamide gel, proteins were transferred to nitrocellulose, and eIF4G was immunostained using an anti-eIF4G antiserum. (Fig. 1a, b, lanes 2), the band supposed to be identical to eIF4G remained unchanged in the UV cross-link assay (Fig. 1a) as well as in the immunoblot (Fig. 1b), except that an additional weak band migrating slightly faster than eIF4G appeared in the immunoblot. However, with aliquots of RRL that had been preincubated with L RNA for 5 min, 10 min or even for 30 min (lanes 3 to 5), the high molecular mass eIF4G disappeared, and a labelled band of about 110 kDa appeared both in the UV cross-link assay (Fig. 1a, lanes 3 to 5) and in the immunoblot (Fig. 1b, lanes 3 to 5). This 110 kDa band corresponds to the C-terminal cleavage product (‘4Gc’ in Fig. 1) of eIF4G (Lamphear et al., 1995). In the immunoblot, the intense labelling of the eIF4G cleavage product compared to the uncleaved eIF4G is most probably due to the fact that the smaller protein is transferred to the membrane much more easily than the large uncleaved eIF4G.

Taken together, these results demonstrate that the band of about 200 kDa which binds to the FMDV IRES is identical to eIF4G.

**IRES stem–loops 4 and 5 contribute to eIF4G binding**

For mapping the determinants in the FMDV IRES RNA required for binding of eIF4G, we used IRES deletion mutants in which the sequences corresponding to each of the large stem–loop structures of the IRES had been deleted (Fig. 2a). When the IRES stem–loop 2 or stem–loop 3 sequences were removed (Fig. 2b, lanes 2 and 3), eIF4G still bound well to the FMDV IRES. In contrast, when the stem–loop 4 sequence was removed, binding of eIF4G was completely abolished (lane 4), whereas removal of sequences downstream of the tip of the stem–loop 5 in RNA Δ5 affected binding of eIF4G only slightly (lane 5).

To confirm that the loss of detection of eIF4G in the UV cross-link assay was due to the loss of eIF4G binding and not only due to the loss of a cross-link site affecting radioactive label transfer, we performed binding competition assays (Fig. 2c). As to be expected, both unlabelled complete (wild-
Fig. 3. Involvement of stem–loop 4 subdomains in eIF4G binding and FMDV IRES activity. (a) Secondary structure of the FMDV IRES stem–loop 4 with its subdomains 4-1, 4-2 and the A-rich bulge 4-3. The subdomains which had been deleted separately in the respective IRES mutants are boxed. Deletion of single subdomains does not essentially change the predicted structures of the remaining parts of stem–loop 4 in the respective mutants. (b) UV cross-link assay with labelled FMDV IRES RNAs, either complete IRES (wt) or stem–loop 4 or subdomain deletion mutants as indicated on the top. 4G, eIF4G; 4B, eIF4B. (c) Translation assays. Unlabelled dicistronic RNAs with either the complete FMDV IRES (wt) or stem–loop 4 and subdomain deletion mutants directing luciferase translation were translated in RRL, and CAT and luciferase activities were determined (Niepmann et al., 1997). Readings were standardized, setting the reaction with complete IRES as 100%. The values shown represent % luciferase expression divided by % CAT expression in order to normalize luciferase expression with respect to the 5′-dependent translation. The arrow indicates the background level of translational activity of a control construct containing a linker sequence between the two genes. (d) Competition UV cross-link assays. The binding of complete labelled FMDV IRES RNA to RRL proteins was competed by unlabelled subdomain deletion mutant IRES RNAs in the molar excesses as indicated on the top. 4G, eIF4G.

Stem–loop 4 subdomains involved in eIF4G binding

Stem–loop 4 of the FMDV IRES is folded into a Y-shaped structure (Fig. 3a). The upstream subdomain, itself appearing as a stem–loop structure, is termed stem–loop 4-1, and the second is stem–loop 4-2. Downstream of stem–loop 4-2, a stretch of unpaired nucleotides forms the A-rich bulge 4-3. We used mutants in which these predicted subdomain structures had been exactly deleted from the IRES to obtain more information about the determinants of the eIF4G-binding site in the FMDV IRES. The subdomain deletions do not essentially affect the predicted secondary structure of the remaining parts of stem–loop 4 in the respective mutants.

These mutants were tested for eIF4G binding in the UV cross-link assay (Fig. 3b). The larger upstream subdomain, 4-1, of the Y-shaped stem–loop 4 structure is an essential determinant for binding of eIF4G since binding was completely lost when subdomain 4-1 was deleted (Fig. 3b, lane 3). Binding of eIF4G was also abolished when the unpaired A-rich bulge 4-3 was deleted from the IRES (Fig. 3b, lane 5). In contrast, the deletion of the small stem–loop subdomain, 4-2, resulted in markedly reduced eIF4G binding but not in a complete loss of the interaction (Fig. 3b, lane 4). Again, competition assays were used to confirm that the loss of detection of the eIF4G band with mutants Δ4-1 and Δ4-3 was actually due to the loss of eIF4G binding (Fig. 3d, lanes 1 to 4 and 9 to 12, respectively). Competition with the subdomain 4-2 mutant resulted only in a slight competition of eIF4G binding (lanes 5 to 8), confirming that subdomain 4-2 considerably contributes to binding of eIF4G.

Translation efficiencies correlate with the binding of eIF4G

Among the set of initiation factors involved in internal initiation of picornaviral RNAs (Anthony & Merrick, 1991; Pestova et al., 1996a), eIF4G is supposed to be the most important initiation factor that connects the RNA with the small ribosomal subunit (Hentze, 1997). According to this idea, variations in the efficiency of binding of eIF4G to the RNA to
be translated should result in equivalent variations in translation efficiency.

To investigate the relationship between the mere binding of initiation factor eIF4G and the translational activity of the viral IRES element, we generated the corresponding stem–loop 4 subdomain deletion mutants in a dicistronic expression vector, pD12. The translation efficiencies of the IRES variants were measured in RRL and compared with that of the wt IRES (Fig. 3c). All of the translation efficiencies of the mutants are below 20% (Fig. 3c, lanes 2 to 5) compared with the wt (Fig. 3c, lane 1). Remarkably, mutant Δ4-2 is the most efficient, approaching 20% translation efficiency (Fig. 3c, lane 4), whereas the deletion of the complete stem–loop 4 and both the deletion of each of subdomains 4-1 or 4-3 results in a reduction of translation efficiency to values near or below the background.

The clear correlation between the partial reduction of binding of eIF4G to IRES Δ4-2 in the UV cross-link and the partial reduction in translation efficiency with this mutant, compared with the complete reduction of both eIF4G binding and translation efficiency with the other mutants, suggests that eIF4G is the crucial factor for IRES activity.

The interaction of eIF4G with the IRES is independent of PTB

As a supporting non-canonical factor, the cellular RNA-binding protein PTB had been found to stimulate FMDV translation (Niepmann, 1996, 1997). PTB was found to contact not only stem–loop 2 of the IRES and the oligopyrimidine tract (Luz & Beck, 1991), but in addition the apical loop of subdomain 4-2 (Kolupaeva et al., 1996). Regarding the close vicinity of the binding sites for both eIF4G and PTB in the IRES stem–loop 4 region, we asked whether the interaction of eIF4G with the IRES would possibly be supported by PTB. To answer this question, we removed the endogenous PTB from RRL. This PTB-depleted lysate was then used to investigate the association of eIF4G with the FMDV IRES.

When a UV cross-link was performed with this depleted lysate (Fig. 4, lane 1), no endogenous PTB was detected binding to the IRES RNA. In contrast, eIF4G bound well to the IRES, confirming the idea that the binding of eIF4G is not dependent on the presence of PTB. When increasing amounts of purified recombinant PTB were added to the reaction (Fig. 4, lanes 2 to 4), no change in the intensity of the eIF4G band was observed. Thus, the interaction of eIF4G with the FMDV IRES is independent of the presence of PTB.

Discussion

In this study we show that the eukaryotic translation initiation factor eIF4G interacts directly with the IRES of FMDV. By using a series of deletion mutants and competition assays, we demonstrate that this binding of eIF4G is specific and that its affinity to the IRES RNA is in the same range as the affinity of other proteins. The determinants for the binding of eIF4G were delimited to stem–loops 4 and 5 of the FMDV IRES. No sequences in the upstream part of the IRES, including stem–loops 2 or 3 are required. At the functional level, the translational activity of stem–loop 4 subdomain mutants correlates with the efficiency of binding of eIF4G in the UV cross-link assay. This indicates that the interaction of eIF4G with the FMDV IRES is one of the crucial interactions of initiation factors with the IRES that lead to the initiation of translation of the viral RNA.

Within the Y-shaped stem–loop 4 structure, its subdomains 4-1 and the bulge 4-3 are essential components of the eIF4G-binding site. Deletion of subdomain 4-1 impairs binding of eIF4G and translation efficiency. Although only one contact site of eIF4G with this subdomain 4-1 has been mapped so far using footprinting assays, this contact site was assigned to the same unpaired dinucleotide sequence ‘GA’ in the FMDV IRES (Pilipenko et al., 2000) and in the related EMCV IRES (Kolupaeva et al., 1998), pointing to the importance of this element. Our findings that the binding of eIF4G as well as translation efficiency are severely affected by deletion of subdomain 4-1 support the idea that this subdomain must be an essential determinant of the eIF4G-binding site.

Our finding that the bulge 4-3 is an essential element of the eIF4G-binding site is consistent with the observation that almost all nucleotides in this bulge in the closely related EMCV IRES are protected by eIF4G binding (Kolupaeva et al., 1998). Moreover, the complete loss of translational activity of this
The binding site for eIF4G as defined in this study and in another study for EMCV (Kolupaeva et al., 1998) overlaps with the binding site for eIF4B (Meyer et al., 1995; Rust et al., 1999). Most likely, the binding of both factors, together with eIF4A, occurs synergistically. This idea is supported by three lines of evidence. First, eIF4B binds to the FMDV IRES only in the presence of ATP, although the eIF4B protein itself has no ATPase activity, pointing to the involvement of the ATP-dependent RNA helicase eIF4A (Meyer et al., 1995). Second, both eIF4A and eIF4B were found to bind to the EMCV IRES extremely weakly in the UV cross-link assay when added separately. However, their binding was greatly enhanced when both factors were added together in the presence of eIF4G (Pestova et al., 1996b). Third, in the absence of either eIF4F (which includes eIF4G), eIF4B or eIF4A, the efficiency of 48S complex formation with the EMCV IRES is markedly reduced but not completely abolished (Pestova et al., 1996a). Taken together, these observations point to a strong synergy in binding of eIF4G, eIF4B and eIF4A to the IRES, which could be in accord with overlapping binding determinants for eIF4G and eIF4B in stem–loop 4.

Fig. 5. Model for the binding of canonical initiation factors to the FMDV IRES. The features of the model are discussed in the text.

The first step is the ATP-dependent synergistic binding of eIF4G, eIF4B and eIF4A to the 3′ region of the IRES (Kolupaeva et al., 1998; Meyer et al., 1995; Pestova et al., 1996a). The binding site for these factors is the Y-shaped stem–loop 4, and binding is supported also by stem–loop 5 sequences. This first step is independent of both the presence of the small ribosomal subunit and the location of the initiator AUG (Ochs et al., 1999). The second step is the binding of the small ribosomal subunit to this complex of IRES RNA associated with initiation factors. This step is energy-independent (Ochs et al., 1999). Many interactions, including different initiation factors and the ribosome, may contribute to this step, like an interaction of the IRES-bound eIF4G with the ribosome-bound eIF3 (Imataka & Sonenberg, 1997) and an interaction of the IRES-bound eIF4B with eIF3 (Méthot et al., 1996). Moreover, an interaction of the oligopyrimidine tract in the 3′ region of the IRES with the ribosomal 18S RNA (Pilipenko et al., 1992; Schepet et al., 1994) may be facilitated by the RNA annealing activity of eIF4B (Allmann et al., 1995), perhaps together with the RNA helicase activity of eIF4A (Pause et al., 1994). The third step of ribosome assembly on the picornaviral IRES is the association of the large ribosomal 60S subunit. This step is again energy-dependent (Ochs et al., 1999) due to the hydrolysis of the eIF2-bound GTP by eIF5 (Pestova et al., 1979) and the GTPase activity of eIF5B (Pestova et al., 2000).

This work was supported by grants from the Deutsche Forschungsgemeinschaft (GK 370, SFB 535).

References


Received 17 August 2000; Accepted 20 December 2000