The mammalian host protein DAP5 facilitates the initial round of translation of Coxsackievirus B3 RNA

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Running Title: DAP5 substitutes eIF4GI function in type I IRES

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During enteroviral infections, the canonical translation factor, eukaryotic translation initiation factor 4 γ I (eIF4GI) is cleaved by the viral protease 2A. The resulting C-terminal fragment is recruited by the viral internal ribosome entry site (IRES) for efficient translation of the viral RNA. However, 2A protease is not present in the viral capsid and is synthesized only after the initial round of translation. This presents a conundrum of how the initial round of translation occurs in the absence of the C-terminal eIF4GI fragment. Interestingly, host protein DAP5 (also known as p97, eIF4GIII, or eIF4G2), an isoform of eIF4GI, closely resembles the C-terminal fragment produced after the 2A protease mediated cleavage. Using coxsackievirus B3 (CVB3) IRES as a model system, here we demonstrate that DAP5, but not the full length eIF4GI, is required for CVB3 IRES activity for the translation of input viral RNA. Additionally, we show that DAP5 is specifically required by type I IRES but not by type II or type III IRES, in which cleavage of eIF4GI has not been observed. We have observed that both DAP5 and C-terminal eIF4GI interact with CVB3 IRES in the same region, but DAP5 exhibits lower affinity for CVB3 IRES as compared to C-terminal eIF4GI fragment. It appears, that DAP5 is required for the initial round of viral RNA translation by sustaining a basal level of CVB3 IRES activity. This activity leads to expression of 2A protease and consequent robust CVB3 IRES mediated translation by the C-terminal eIF4GI fragment.

The enteroviruses such as EV71, Poliovirus and Coxsackievirus possess a type I IRES in their genomes, which is characterized by its conserved structure and the requirement of all canonical translation initiation factors except eIF4E (1). Other IRESs, such as the HCV IRES, which is a type III IRES, are completely independent of the eIF4F complex (consisting of eIF4G, eIF4A and eIF4E) (1). Interestingly, upon infection by enteroviruses, the viral protease 2A cleaves the scaffolding protein eIF4GI into two fragments (2-4). The C-terminal eIF4GI fragment directly interacts with the stem loop V in type I IRES via its MIF4GI domain and this interaction is critical for recruitment of the ribosome (5). The mutations in the 2A protease that inhibit the cleavage of eIF4GI lead to abrogation of the poliovirus life cycle (6). The aforementioned reports clearly
suggest that the 2A protease mediated cleavage of eIF4GI is essential for the successful life cycle of the virus and signify the importance of the interaction between the C-terminal eIF4GI and IRES in viral RNA translation.

Upon the entry of enteroviruses in cells, only the viral genomic RNA is released into the cells. This genomic RNA has to undergo its first round of translation in the absence of the 2A protease and hence, in the absence of the C-terminal eIF4GI. This suggests that the initial rounds of translation occur by an alternate mechanism in which the C-terminal eIF4GI is not required. It has been suggested that the full length eIF4GI may interact with type I IRES and mediate translation, however, no experimental evidence has been provided (7). In the current study, the Coxsackievirus B3 was used as a model system and the focus was on the functional relevance of the full length eIF4GI and its cellular isoforms on IRES-mediated translation of Coxsackievirus B3 genomic RNA. Interestingly, it was found that the host protein DAP5, an N-terminal truncated isoform of eIF4GI, resembles the C-terminal eIF4GI with respect to the domain organization. It contains a MIF4GI domain and eIF4A interacting domain, similar to the C-terminal eIF4GI that is produced post cleavage by 2A protease. DAP5 has previously been studied extensively with respect to its role in the IRES mediated translation of cellular messenger RNAs (mRNAs) (8-10). It has been shown that DAP5 undergoes caspase mediated cleavage during apoptosis, which leads to the production of a 86 KDa truncated protein, which acts as a stimulator for cellular IRESs like c-Myc, DAP5 and XIAP, etc. (11,12). Unlike its isoform eIF4GI, DAP5 does not have an eIF4E interacting domain and it has been shown to be dispensable for cap-dependent translation (8,13). However, it retains the regions involved in the interaction with eIF3 and eIF4A, which are essential for ribosome recruitment on the target RNA.

Unlike many nuclear resident host RNA binding proteins that act as IRES transacting factors (ITAFs) for viral IRES (14-21), DAP5 is a resident of cytoplasm, making it readily available for the incoming viral RNA. Considering the cytoplasmic localization and its domain organization similar to the C-terminal eIF4GI, we studied the role of DAP5, and full length eIF4GI, in the initial rounds of translation of viral IRES. Our study suggests that DAP5, but not the full length eIF4GI, is required for the initial round of translation during CVB3 infection. A comparative study was carried out with type I, type II and type III IRESs and it was found that only type I IRES requires DAP5. This is interesting because only after infection with type I IRES containing viruses, eIF4GI is cleaved by the 2A protease and not in closely related EMCV virus that contains type II IRES. The results suggest that DAP5 directly interacts with the CVB3 IRES, but not the HCV IRES. DAP5 also interacts with the stem loop V, similar to the C-terminal eIF4GI, but with a lower affinity. Based on these results, we propose a model in which the viral RNA undergoes two different stages of translation. The first stage is the basal level of translation during the initial stages (before the cleavage of eIF4GI) which is mediated by DAP5. Consequently, viral proteins are produced leading to the 2A protease mediated cleavage of eIF4GI. The C-terminal eIF4GI is then utilized by IRES to carry out robust protein synthesis. Our study provides new insights into the mechanism of translation initiation at the initial stages and provides a model to explain how input viral RNA could be translated in the milieu of limited resources (host factors) in the cytoplasm, with the better availability of ITAFs pending.

**Results**

DAP5 is required for the initial round of CVB3 IRES mediated translation

As discussed above, eIF4G and its isoforms (Figure 1A) are known to play a central role in both cap dependent and cap-independent internal initiation of translation. To understand the mechanism of the initial round of translation in CVB3 genomic RNA, the possible role of the isoforms of eIF4G was explored. DAP5 is an N-terminally truncated isoform of eIF4G that resembles the C-terminal part of eIF4GI and eIF4GII which is formed post cleavage by 2A protease. Interestingly, DAP5 contains the MIF4GI domain that is known to interact with the stem loop V of CVB3 5’UTR, and also retains the eIF3 and eIF4A interacting regions. The N-
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terminus region that interacts with eIF4E is absent in DAP5.

To study the role of DAP5 in the CVB3 life cycle, CVB3 replicon RNA was used, wherein a firefly luciferase gene substitutes the region encoding for structural proteins (figure 1B) (22). The *in vitro* transcribed RNA was directly transfected in cells and the efficiency of translation and replication were quantified by measuring the luciferase activity. Initially, siRNA-mediated knockdown of DAP5 was carried out in a dose dependent manner followed by the transfection of CVB3 replicon RNA. The luciferase activity from the replicon RNA transfected cells was measured at 8 hours post transfection. As compared to the non-specific siRNA transfected cells (si Nsp), the luciferase activity was found to be decreased in dose dependent manner in siDAP5-transfected cells, suggesting that DAP5 is important for the CVB3 life cycle (figure 1B). To study the role of full-length eIF4GI and DAP5 in the initial round of translation, the cells were harvested at 1.5 hours post CVB3 replicon RNA transfection. At this early stage, the luciferase activity is a measure of the translation alone as replication begins only after 2 hours post transfection of the CVB3 replicon RNA (figure 1C). The partial silencing of DAP5 resulted in a reduction of luciferase activity, suggesting that DAP5 regulates the CVB3 RNA translation during the early stages of the viral life cycle (figure 1C). Interestingly, the partial silencing of full length eIF4GI caused a marginal increase in translation of CVB3 RNA, suggesting that full length eIF4GI does not mediate CVB3 IRES mediated translation at the earlier stages (figure 1C). The partial knockdown of both DAP5 and eIF4GI rescued the translation that was inhibited by siDAP5 treatment (Figure 1C).

In order to eliminate the possibility of a low level of eIF4GI cleavage at the earlier stages of infection that could have been missed during detecting by western blots, an alternate strategy was adopted. A CVB3 replicon RNA harbouring mutation in the 2A protease region was generated; this is known to inhibit eIF4GI cleavage (Figure 1D) (23). Partial knockdown of DAP5 led to a reduction in CVB3 IRES mediated translation at 1.5 hours post transfection in both the wildtype as well as the mutant (Figure 1E), suggesting that DAP5 mediates CVB3 IRES mediated translation independently of eIF4GI cleavage. Of note, it was confirmed that the siRNAs targeting DAP5 and eIF4GI were specific to their targets (Supplementary figure S1). In order to rule out the non-specific effects due to the transfection of the CVB3 replicon RNA, the same assay was performed using CVB3 virus containing Renilla Luciferase gene in the genome (as described by Lanke et al., 2009)(24). The partial silencing of DAP5 led to a dramatic reduction in the CVB3 IRES mediated translation at 1.5 hours post infection. As expected, partial silencing of the full-length eIF4GI did not reduce the CVB3 IRES-mediated translation (figure 1F). In order to rule out the differences in the luciferase activity due to differential cell physiology due to siRNA treatment, MTT assay was performed. As observed in supplementary figure S2, no significant change in cell proliferation was observed due to siDAP5 or si4GI treatment (supplementary figure S2).

These results suggest that during the initial stages of the viral life cycle, DAP5, but not the full-length eIF4GI, is required for the CVB3 IRES-mediated translation.

**Role of DAP5 and eIF4GI in viral IRES mediated translation**

To study the role of the full-length eIF4GI and DAP5 proteins in CVB3 RNA translation, a bicistronic reporter was utilized. In the reporter construct, the renilla luciferase activity represents cap-dependent translation and the firefly luciferase activity represents CVB3 IRES mediated translation (Supplementary figure S3). Upon expression of the 2A protease, the CVB3 IRES activity was stimulated while the cap-dependent translation was reduced drastically (Supplementary figure S3). This suggests that the IRES activity measured from the bicistronic construct in the absence of 2A protease represents a basal level of IRES activity and is a good system to study the initial round of translation that happens in the absence of the viral proteins. The siRNA mediated knockdown of DAP5 and the full-length eIF4GI was performed in dose dependent manner followed by the transfection of capped CVB3 bicistronic RNAs. Cells were processed for measuring luciferase activity at 10
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hours post transfection (Figure 2A). The partial silencing of DAP5 reduced CVB3 IRES activity in a dose dependent manner but the cap-dependent translation was unaffected (figure 2A). Consistent with observation using CVB3 replicon, the partial silencing of eIF4GI led to a significant increase in the CVB3 IRES activity while reducing the cap-dependent translation (figure 2A). The HCV IRES, which belongs to the type III class of IRESs was used as a control as the type III IRESs are known to be independent of the eIF4F complex. As expected, the silencing of DAP5 had no effect on the HCV IRES activity (Figure 2B).

Both type I (CVB3 IRES) and type II IRES (e.g. EMCV IRES) both require all the canonical translation initiation factors except eIF4E. However, in the case of the EMCV virus infection, eIF4GI cleavage has not been observed. Unlike type I and type II IRES, type III IRESs (e.g. HCV IRES) does not require the eIF4F complex (eIF4E, eIF4G, eIF4A and eIF4B) for translation initiation. The study was extended to different IRESs to understand the role of DAP5 in IRES mediated translation. The partial silencing of DAP5 had no effect on the EMCV or HCV IRES mediated translation, suggesting that DAP5 is required only for type I IRES and not for type II and type III IRESs (figure 2B and 2C respectively). Interestingly, the silencing of eIF4GI increased the IRES activity of both the EMCV and HCV IRESs similar to CVB3 IRES (figure 2B and 2C respectively). It was surprising to observe the inhibitory role of full-length eIF4GI for all the IRESs. We reason that this could be due to the increased availability of other initiation factors (associated with eIF4GI) to viral IRES as a consequence of the silencing of eIF4GI.

**DAP5 interacts with CVB3 IRES**

From earlier studies, it is known that the C-terminal eIF4GI directly interacts with the stem loop V in CVB3 5’UTR via its MIF3GI domain (5). The crystal structures of the MIF4GI domain of eIF4GI and DAP5 have been solved earlier (PDB ID: 4IUL and 1HU3 respectively) (25,26). Overlaying these structures revealed a remarkable similarity in the overall folding of MIF4GI domains in DAP5 and eIF4GI (supplementary figure S4). The folding of the RNA interacting region in the MIF4GI domain of the C-terminal eIF4GI and the corresponding region in DAP5 was similar with minor differences in the composition of amino acids (figure S4). We tested the hypothesis whether DAP5 also interacts with the CVB3 5’UTR similar to C-terminal eIF4GI. The UV-crosslinking experiment was performed using radiolabelled CVB3 5’UTR RNA and the recombinant DAP5 protein. The unlabelled CVB3 5’UTR and HCV IRES RNAs were used as competitors in this experiment. As observed in figure 3A, DAP5 was able to specifically interact with CVB3 5’UTR (lanes 2 and 3) but not with HCV IRES (lanes 4 and 5). The bar graph represents the band intensities from three independent experiments (Figure 3A). This suggests that DAP5 specifically interacts with CVB3 IRES but not with the HCV IRES, which is independent of the eIF4F complex for its function. This result is also consistent with the experiments illustrated in figure 2B, which suggests that DAP5 is not required for HCV IRES.

A mutation in the stem loop V at the 541 nucleotide position has been reported to inhibit the C-terminal 4GI interaction with CVB3 IRES (figure 3B) (27). We investigated whether the same mutation could also inhibit the interaction of DAP5 with the CVB3 IRES. The UV-crosslinking experiment was carried out using radiolabelled CVB3 5’UTR and the recombinant DAP5 protein, in presence of wild type CVB3 5’UTR or the 541 CCG→AAA mutant 5’UTR competitor RNAs (Figure 3C). The bar represents the normalised band intensities in three independent experiments (Figure 3C). The mutant CVB3 RNA was also able to compete with the probe, but to a much lesser extent, suggesting that the mutation in stem loop V could partially inhibit the interaction of DAP5 with CVB3 IRES. The minor difference in the amino acid sequence in the MIF4GI domain of DAP5 and eIF4GI could account for this difference in the DAP5 and eIF4GI interactions. These results demonstrate that both DAP5 and C-terminal eIF4GI interacts with CVB3 IRES at the stem loop V region via their MIF4GI domains. Of note, when purifying the recombinant proteins from *E. coli* for carrying our UV-crosslinking assays, contaminant proteins were also co-purified (Supplementary figure S5). However, these proteins did not interact with the CVB3 IRES in the assay and hence we believe that the presence of the contaminants does affect the conclusions.
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In order to confirm whether DAP5 interacts with CVB3 IRES at the early stages of the virus life cycle, the Myc-tagged DAP5 was overexpressed and immunoprecipitation of DAP5 was performed at 1.5 hours post CVB3 replicon RNA transfection and checked its association with CVB3 genomic RNA. As observed in figure 3D, the CVB3 genomic RNA was found to be associated with DAP5 at the earlier stages in cells.

**Significance of DAP5 in CVB3 IRES-mediated translation**

The results suggest that DAP5 interacts with the CVB3 IRES and helps in the initial round of translation during the virus life cycle. However, previous studies have indicated that the inhibition of eIF4GI cleavage by a mutation in the 2A protease leads to an abrogative life cycle, suggesting that DAP5 alone is unable to sustain the entire life cycle and that cleavage of eIF4GI is required. We hypothesized that this could be because DAP5 is less efficient in translation as compared to the C-terminal eIF4GI. To address this question, surface plasmon resonance experiments were carried out with the recombinant DAP5 and C-terminal eIF4GI proteins to measure their affinity with CVB3 5’UTR. The affinity of DAP5 was found to be weaker as compared to the C-terminal eIF4GI with the CVB3 IRES (Figure 4A and 4B). It is known that the affinity of DAP5 with eIF4A is lower as compared to the affinity of eIF4GI with eIF4A, suggesting that DAP5 is weak in recruiting the downstream factors required for IRES mediated translation. Together these results highlight the relatively weak IRES trans activating potential of DAP5 as compared to the C-terminal eIF4GI because of its lower affinity with both the CVB3 IRES as well as eIF4A. We propose a model in which DAP5 interacts with the CVB3 IRES and mediates its basal level of translation during the early stages of the CVB3 life cycle. This leads to the synthesis of the 2A protease and hence, the cleavage of eIF4GI and the subsequent activated mode of translation that is mediated by the cleaved C-terminal part of eIF4GI (figure 5).

**Discussion**

The enterovirus life cycle takes up to 8 to 10 hours in the cell culture. In order to achieve this, viral RNA must undergo robust protein synthesis. The enteroviruses manage to accomplish this task by hijacking the cellular translation machinery for its own protein synthesis and inhibiting the host cell translation. A well-studied example is cleavage of eIF4GI and eIF4GII by the viral protease 2A, which benefits the virus in two ways: (i) It leads to the inhibition of host cell translation, making the ribosomes and other initiation factors available for viral IRES mediated translation (3,4). (ii) The cleaved C-terminal part of eIF4GI directly interacts with IRES, thereby bridging the ribosomes to viral RNA (5). Apart from the canonical translation initiation factors, several host RNA binding proteins are recruited to viral IRES to facilitate efficient translation (14-21). These RNA binding proteins are predominantly nuclear residents and upon viral infection, they relocatalize to the cytoplasm. However, the relocalization of these proteins is induced only at 3-4 hours post viral infection (14,28). Hence, the intracellular host factors that are required for viral IRES mediated translation are limiting during the initial stages of viral infection, due to the absence of viral protease and limited amounts of ITAFs in the cytoplasm. In light of these observations, DAP5 which is localized to the cytoplasm and resembles the cleaved eIF4GI is a vital host factor for viral IRES mediated-translation at the initial stages.

A recent report suggested that eIF4E is required for translation initiation by the picornavirus IRES implying that eIF4GI could also be required. However, this possibility has not been directly studied (29). One possible explanation could be that the loss of eIF4E could increase the ability of eIF4G to interact with the other members of eIF4F (eIF4A and eIF4B) ultimately leading to reduced availability of these factors that are required for the type I IRES-mediated translation. Another report mentioned that the overexpression of the eIF4G1 enabled the rescue of the translation of the mutant poliovirus (30). This is not surprising as the eIF4G1 would also increase the abundance of the C-terminal eIF4G1 and hence increase translation. However, due to partial silencing of eIF4G1 achieved, our study does not completely rule out that full-length eIF4G1 can also function in the input viral RNA translation in the first and
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DAP5 substitutes eIF4GI function in type I IRES. Further studies involving IRES-eIF4GI interaction are required to rule out the role of full length eIF4GI in type 1 IRES mediated translation. The results with regard to the role of DAP5 in the initial round of translation were consistent in all the systems used in the current study: CVB3 Replicon RNA, bicistronic reporter RNA as well as the CVB3-Rluc virus infection (24).

The type I (CVB3 IRES) and type II IRESs (EMCV IRES) require all the canonical initiation factors except eIF4E, while the type III IRES (HCV IRES) functions independently of the eIF4F complex. The results suggest that DAP5 does not interact with the HCV IRES and is not required for its activity. Interestingly, it was found that the full length eIF4GI is dispensable for EMCV IRES-mediated translation. The EMCV virus harbours a type II IRES for its translation and eIF4GI cleavage has not been observed during EMCV infection. Consequently, it was found in the current study that DAP5 is dispensable for EMCV IRES mediated translation. Interestingly, the partial silencing of intact eIF4GI led to an increase in the EMCV IRES activity. These results are in contrast with the earlier reports that suggest that eIF4GI interaction with the J-K loop in EMCV IRES is required for ribosome recruitment (31-33). However, more recently it has been shown that the EMCV IRES could directly recruit 40S ribosome subunit in the absence of eIF4G suggesting that alternative mechanisms of translation initiation may exist (34).

In another study, the depletion of the full-length eIF4GI by expression of the CVB3 2A protease during EMCV infection led to an increase in the EMCV IRES activity, pointing out the fact that intact eIF4GI may not be required for EMCV IRES activity (35). We propose that the downregulation of eIF4GI by siRNAs led to an increase in the available amount of eIF4A and eIF3 for viral IRESs and hence, facilitate the ribosome recruitment. This claim is supported by evidence that eIF4GI is predominantly present in the monosomal and polysomal fractions in the cell, rather than the ribosome-free fractions, suggesting tight associations with the initiation factors and ribosomes (36).

DAP5 has been previously studied with respect to CVB3 infection, however, its role in viral IRES mediated translation has not been studied. It was found that DAP5 is also cleaved by viral 2A protease into N and C-terminal parts, such that the N-terminal fragment retains the MIF4GI domain (37). Interestingly, it was shown that the N-terminal fragment of DAP5 containing the MIF4GI domain, translocated to the nucleus. The translocation of N-DAP5 to the nucleus post 2A protease mediated cleavage could favour the interaction of the C-terminal eIF4GI, as both the proteins share a common binding site on CVB3 IRES as observed in the current study. The sequence of the stem loop V seems to be conserved in type 1 containing viruses (figure S6), suggesting that the role of DAP5 could be conserved in all viruses containing the type 1 IRES.

The interaction of DAP5 with the CVB3 IRES is a key step in translation initiation at the early stages. Our study finds that DAP5 has a lower affinity towards CVB3 IRES as compared to C-terminal eIF4GI. However, the interaction with RNA alone is not sufficient for translation initiation. The recruitment of downstream initiation factors like eIF4A by DAP5 is also required for IRES function. It has been observed that DAP5 has a 10 times lower affinity to eIF4A as compared to eIF4GI (25). These observations suggest that DAP5 is not as potent as the C-terminal eIF4GI in ribosome recruitment on CVB3 IRES and explains why DAP5 alone is unable to sustain the entire life cycle. We propose a model in which a basal level of translation is carried out by DAP5 in the initial stages, followed by a robust mode of translation mediated by C-terminal eIF4GI in the later stages. In the subsequent stages, many ITAFs also relocalize from the nucleus to the cytoplasm and these proteins could also contribute to a robust mode of translation. This study provides novel insights into a previously-ignored but essential stage of the virus life cycle.

**Experimental Procedures**

**Cell line, transfections and luciferase assay**

For all cell culture experiments in this study, HeLa cells were used. CVB3 replicon RNA used for transfections was prepared from pRib-T7/luc plasmid (a kind gift from Prof. Frank van Kuppeveld, Utrecht University) as described...
DAP5 substitutes eIF4GI function in type I IRES earlier (14). CVB3 replicon RNA was transfected in HeLa cells and at indicated time points, cells were processed for luciferase assay using luciferase assay reagent (Promega) as per manufacturer’s protocol. siDAP5 and sieIF4GI were acquired from IDT (Integrated DNA Technologies) and were transfected in 50 nM or 100 nM concentration. All transfections were carried out using lipofectamine 2000 reagent using manufacturers protocol (Invitrogen).

pCDNA3-CVB3 bicis, pCDNA3-HCV bicis and pCDNA3-EMCV bicis plasmids were used to prepared various capped bicistronic RNAs. These plasmids were linearized with Pmel restriction enzyme and the linear plasmids were used as a template in in vitro transcription reactions. The luciferase assays for bicistronic RNAs was performed using dual luciferase reagent kit (Promega), as per manufacturer’s protocol.

Virus preparation and infection

pRLuc-CVB3 plasmid (a kind gift from Prof. Frank Van Kuppeveld) was used to prepare CVB3 virus containing Renilla luciferase gene. In vitro transcribed CVB3-Rluc RNA was transfected in HeLa cells and the virus was purified from the cell culture supernatant. Virus titre was calculated by performing plaque assays in Vero cells and PFU/ml was estimated. For experiments, HeLa cells were infected with M.O.I of 15.

In vitro transcriptions

CVB3 5'UTR RNA was prepared from pCDNA3-CVB3 5'UTR plasmid as described earlier (14). α-32P was included in in vitro transcription reactions carried out to prepared radiolabelled RNAs. In vitro transcription reactions were carried out using T7 RNA polymerase (Thermo Scientific) as per manufacturer’s protocol. To prepare biotin labelled CVB3 5'UTR RNA, biotin-11-UTP was included in in vitro transcription reaction carried out using Ribomax Kit (Promega).

Capped RNAs were prepared by adding cap-analog in in vitro transcription reactions carried out using the Ribomax kit (Promega) as per manufacturer's protocol. These RNAs were transfected in HeLa cells and at 10 hours post transfection cells were processed for luciferase assay. Dual luciferase assays were carried out using the DLR kit (Promega) as per manufacturer’s protocol.

Plasmids and protein purification

Recombinant DAP5 protein was purified using pET28a-DAP5 plasmid (a kind gift from Prof. Adi Kimchi, Weizmann Institute, Israel) as described earlier (13). C-terminal eIF4GI was cloned into pET28a vector from pCDNA3-eIF4GI-HA plasmid (a kind gift from Prof. Nahum Sonenberg, McGill University). The pET28a-C-terminal eIF4GI plasmid was transformed in E. Coli BL21 strain and protein expression was induced at 0.6 OD by 0.6 mM IPTG for 3 hours followed by purification of his-tagged protein using Ni-NTA beads. Myc tagged DAP5 was cloned in pCDNA3 vector for expression in HeLa cells.

Western blotting

Cells were lysed using radioimmunoprecipitation buffer (RIPA buffer) and the protein was quantified using Bradford reagent. Equal amounts of proteins were resolved on SDS-PAGE followed by transfer of proteins over PVDF membrane (Millipore). Primary antibodies used in this study are: anti-DAP5 antibody (Imgenex), anti-eIF4GI antibody (Cell signalling technologies) or HRP conjugated β-actin antibody (Sigma). Primary antibodies were incubated with the blots for 12 hours followed by washing with TBST buffer (20 mM Tris pH7.5, 137 mM NaCl and 0.1% tween-20) and subsequent incubation with secondary antibodies (anti-rabbit HRP conjugated (Sigma) or anti-mouse HRP conjugated (Sigma). Blots were developed by chemiluminescence using WesternBright (Advansta).

UV induced cross linking of RNA-Protein complexes

UV crosslinking assays were carried out essentially as described earlier (14). Briefly, recombinant DAP5 or C-terminal eIF4GI protein was incubated with radiolabelled CVB3 5’UTR RNA at 30°C for 30 minutes in presence of RNA binding buffer (25 mM HEPES (pH 7.6), 1.25 mM ATP and 2 mM MgCl2, 19% glycerol, 0.5 mM EDTA, 1.25 mM ATP, 2 mM GTP). The reaction also contained 10 μg of yeast tRNAs as non-specific RNAs. The RNA-proteins complexes were then cross-linked using UV light for 20
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minutes followed by RNase A treatment for 45 minutes at 37°C. Laemmlı’s buffer was added and the samples were boiled for 5 minutes and proteins were resolved on SDS-PAGE. The RNA bound proteins were visualized by autoradiography. For competition UV cross linking assays, indicated amount of unlabeled RNAs were additionally included in the reaction mixture. The radiolabelled RNAs were prepared by carrying out in vitro transcription in presence of α-32P UTP (BRIT, Hyderabad) and hence the RNAs were U labelled.

RNA Immunoprecipitation

RNA immunoprecipitation was essentially performed as previously described elsewhere (38). Briefly, cells were lysed in the IP buffer containing 100 mM KCl, 5 mM MgCl2, 10 mM HEPES (pH 7.0), 0.5% NP-40, 1 mM DTT and 100 U/ml RNase inhibitor. Protein G beads were saturated with antibodies (anti-Myc or IgG control) and incubated with an equal amount of lysate at 4°C overnight. The beads were washed with the wash buffer twice and resuspended in 100 µl of IP buffer. To analyse the proteins in the pull down, western blotting was performed using a 10% aliquot of the pull-down sample. To check the RNAs associated with the pulled down proteins, the sample was subjected to proteinase K treatment and subsequently, RNA was isolated using Trizol. The association of CVB3 genomic RNA was tested by semi-quantitative PCR using CVB3 specific primers. CVB3-F: 5′ GAATGCGGCTAATCCTAACTGC 3′; CVB3-R: 5′ GCTCTATTAGTCACCGGATGGC 3′.

Surface Plasmon resonance experiments

SPR spectroscopy was performed using BIAcore3000 optical biosensor (GE healthcare lifescience) to study the binding kinetics of DAP5 and C-terminal eIF4GI proteins with CVB3 5’UTR RNA. Biotin labelled CVB3 RNA was immobilized on streptavidin coated sensor chip (GE healthcare lifescience) to a final concentration of ≈1000 RU/flow cell. RNA-Protein interactions were carried out in a continuous flow of tris-buffer (25 mM Tris (pH-7.5), 100 mM KCl, 7 mM β-ME and 10% glycerol) at 25°C at a flow rate of 10 µl/min. Increasing concentrations of DAP5 or C-terminal eIF4GI protein was loaded on the biosensor chip for 100 seconds (characterized as association phase) followed by a dissociation phase of 300 seconds with the buffer alone. A blank surface, without any RNA, was used to determine the background non-specific interaction by simultaneous injections of the sample during the experiment. The on-rate $K_{on}$ (M⁻¹s⁻¹) and off rate $K_{off}$ (s⁻¹) was determined in BIA evaluation software (version 3.0) by using a 1:1 Langmuir binding model. The dissociation constant $K_d$ was determined using the following equation: $K_d = \frac{k_{off}}{K_{on}}$.

MTT Assay

To study the effect of siDAP5 and sieIF4GI on cell proliferation and growth, MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay was performed as described previously (39).

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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.
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Figures

Figure 1: DAP5 is required for CVB3 IRES mediated translation. A. Schematic representation of isoforms of eIF4GI in mammalian cells. Different domains in individual proteins, length of the proteins and 2A protease cleavage site are indicated. B. Effect of partial silencing of DAP5 on the CVB3 life cycle. The schematic of CVB3 replicon RNA is indicated. CVB3 replicon RNA was transfected in cells previously transfected with indicated siRNAs and cells were processed for luciferase activity at 8 hours post replicon RNA transfection. Luciferase activity is indicated in bar graphs. Western blot indicates the DAP5 and β-Actin protein levels. The band intensities were quantified and silencing efficiency is represented normalised to β-Actin. C. Effect of knockdown of DAP5 or eIF4GI or both on the early stage of CVB3 life cycle. Bar graph represents luciferase activity indicating the translation of CVB3 IRES and western blot shows the DAP5 and β-Actin protein levels upon treatment of si DAP5, si eIF4GI or both. D. Schematic of wildtype and 2A protease mutant CVB3 replicon RNAs. G122E mutation in the 2A protease region of the CVB3 replicon RNA is indicated. E. Effect of partial silencing of DAP5 on wild type and mutant CVB3 replica RNA translation at early stages. Bar graph represents luciferase activity indicating the translation of CVB3 IRES and western blot shows the DAP5 and β-Actin protein levels upon si DAP5 treatment. F. Effect of partial silencing of DAP5 and eIF4GI on CVB3 IRES activity at 1.5 hours post CVB3 infection. The CVB3-RLuc virus was used for this experiment and the bar graph represents percentage luciferase activity. All graphs represent the average of three or more independent experiments. Error bars represent standard deviation. * indicates $P \leq 0.05$ and ** indicates $P \leq 0.01$. Band intensity normalised to β-actin is indicated below the western blots. Similar set of blots were used to indicate the molecular weight position in the western blots.
Figure 2: Role of DAP5 in modulating functions of different IRES elements. A. Role of DAP5 and eIF4GI in CVB3 IRES mediated translation. The schematic of CVB3 bicistronic RNA is indicated above the graph. B. Role of DAP5 and eIF4GI in HCV IRES mediated translation. C. Role of DAP5 and eIF4GI in EMCV IRES mediated translation. F luc activity represents IRES mediated translation and R luc activity represents cap-dependent RNA translation throughout. * indicates $P<0.05$ and ** indicates $P<0.01$. D and E) Western blots to indicate the silencing of DAP5 (D) and eIF4GI (E). Band intensity normalised to β-actin is indicated below the western blots. Similar set of blots were used to indicate the molecular weight position in the western blots.
Figure 3: Interaction of DAP5 with CVB3 IRES. A. UV cross linking experiment carried out with radiolabelled CVB3 5’UTR and recombinant DAP5 protein in presence of unlabelled CVB3 5’UTR RNA (lanes 2 and 3) or HCV IRES RNA (lanes 4 and 5) as competitor RNAs. The graph indicates the average of normalized band intensities from three independent experiments. Error bars represent standard deviation. * indicates $P \leq 0.05$ and ** indicates $P \leq 0.01$. B. Schematic of stem loop V in the CVB3 IRES. The binding site of eIF4GI is indicated in the box and this site mutated from CCG to AAA. C. UV crosslinking experiment carried out using radiolabelled CVB3 RNA probe with recombinant DAP5 protein in presence of wild type unlabelled CVB3 5’UTR and CCG→AAA mutant CVB3 5’UTR RNA as competitor RNAs. Normalized band intensities are indicated under individual lanes. The graph indicates the average of normalized band intensities from three independent experiments. Error bars represent standard deviation. * indicates $P \leq 0.05$ and ** indicates $P \leq 0.01$. D. RNA immunoprecipitation experiment carried out at 1.5 hours post CVB3 replicon RNA transfection. The top panel indicates the CVB3 positive strand RNA after immunoprecipitation, and the bottom panel indicates the protein levels by western blot.
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Figure 4

Figure 4: Affinity of DAP5 and C-terminal eIF4GI proteins for binding CVB3 IRES. Sensorgrams representing the interaction of DAP5 (A) or C-terminal eIF4GI (B) with CVB3 5’UTR RNA at indicated amounts of protein concentrations. Y-axis represents the change in RUs during association and dissociation phases. The average affinity constant (K_d) is indicated in the inset.
Figure 5: Role of DAP5 in the initial round of translation. The proposed model for the initial round of translation in type I IRES. Upon infection, DAP5 interacts with CVB3 IRES and leads to the basal level of translation. This produces 2A proteases, which can now cleave eIF4GI. The C-terminal eIF4GI now interacts with CVB3 IRES and leads to a robust activation of translation that is essential for the rapid progression of the life cycle.
The mammalian host protein DAP5 facilitates the initial round of translation of Coxackievirus B3 RNA
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