Protection of Cap-dependent Protein Synthesis in Vivo and in Vitro with an eIF4G-1 Variant Highly Resistant to Cleavage by Coxsackievirus 2A Protease

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The shut off of host protein synthesis by certain picornaviruses is mediated, at least in part, by proteolytic cleavage of eIF4G-1. Previously, we developed a cleavage site variant of eIF4G-1, termed eIF4G-1DM, that was 100-fold more resistant to in vitro cleavage by Coxsackievirus 2A protease (2APro) than wild-type eIF4G-1 (eIF4G-1WT), but it was still digested at high protease concentrations. Here we identified a secondary cleavage site upstream of the primary site. We changed Gly at the P1-position of the secondary site to Ala to produce eIF4G-1aDM and eIF4G-1DM was 1,000–10,000-fold more resistant to cleavage in vitro than eIF4G-1WT. Full functional activity of eIF4G-1DM was demonstrated in vitro by its ability to restore cap-dependent translation to a 2APro-pretreated rabbit reticulocyte system. An isoform containing the binding site for poly(A)-binding protein, eIF4G-1eDM, was more active in this assay than an isoform lacking it, eIF4G-1aDM, but only with polyadenylated mRNA. Functional activity was also demonstrated in vivo with stably transfected HeLa cells expressing eIF4G-1 from a tetracycline-regulated promoter. Cap-dependent green fluorescent protein synthesis was drastically inhibited by 2APro expression, but synthesis was almost fully restored by induction of either eIF4G-1aDM or eIF4G-1eDM. By contrast, encephalomyocarditis virus internal ribosome entry site-dependent green fluorescent protein synthesis was stimulated by 2APro; stimulation was suppressed by eIF4G-1eDM but not eIF4G-1aDM.

Upon infection of mammalian cells with picornaviruses of the rhino-, aphtho-, and enterovirus genera, most host protein synthesis is shut off coincident with the appearance of viral proteins (1). The shut off is thought to be mediated by a switch from cap-dependent to cap-independent translation. Extracts from poliovirus-infected cells are unable to carry out cap-dependent translation, but this ability can be restored by addition of initiation factors from uninfected cells (2, 3), particularly preparations containing eIF4 polypeptides (4, 5). eIF4F, a complex of eIF4E, eIF4A, and eIF4G that is isolated from high salt extracts of ribosomes, was shown to restore cap-dependent translation in lysates of poliovirus-infected HeLa cells (6, 7). eIF4F from uninfected cells stimulates cap-dependent but not cap-independent translation in the messenger RNA-dependent lysate (MDL) system, whereas the opposite is true for eIF4F from poliovirus-infected cells (8).

eIF4G was discovered as a result of its proteolysis coincident with the loss of cap-dependent initiation during poliovirus infection (9). eIF4G is cleaved by the 2A protease (2APro) of Coxsackievirus B4 (CVB4) or human rhinovirus 2 (HRV2) into two functional domains, an N-terminal fragment (cpN) that binds eIF4E and poly(A)-binding protein (PABP) and a C-terminal fragment (cpC) that binds eIF4A and eIF3 (10–12) (see Fig. 1). The 5′-untranslated region of picornavirus mRNA contains an internal ribosome entry site (IRES) that allows translation to be initiated by the direct binding of the 40S ribosomal subunit (13, 14). Cleavage of eIF4G drastically inhibits translation of capped mRNAs in vitro, whereas initiation of IRES-containing and uncapped mRNAs is either unaffected or even stimulated (15–17).

cDNAs for eIF4G have been cloned from numerous sources, including human, rabbit, Drosophila melanogaster, wheat, Saccharomyces cerevisiae, and Schizosaccharomyces pombe (reviewed in Ref. 18). There are at least two different genes for eIF4G in yeast, wheat, and mammals (19–23). In humans, the two different genes encode eIF4G-1 (also known as p220, eIF4γ, eIF4I), and eIF4G-2 (also known as eIF4G-II and eIF4GII), which share 46% identity at the amino acid (aa) level (21, 23). The human cDNA initially isolated for eIF4G-1 contained an open reading frame for a protein of 154 kDa (21), referred to as eIF4G-1α (Ref. 24; see Fig. 1). Subsequently, two co-linear but longer cDNAs were isolated that diverged at the 5′-end (25, 26). These extended the open reading frame by an additional 156 codons to produce a theoretical polypeptide of 172 kDa, termed eIF4G-1α (24). Recently, an even longer form, eIF4G-1f, was detected by mass spectrometric analysis (24). eIF4G-1e and eIF4G-1f contain the binding
site for PABP, but eIF4G-1a does not (Fig. 1).

Cleavage of eIF4G-1 by 2APro was initially thought to be the major mechanism utilized by enteroviruses to shut down host protein synthesis and allow viral protein synthesis to continue by IRES-driven translation (1). Subsequently, evidence has been presented that eIF4G-1 cleavage is not responsible, or is only partially responsible, for the shut off of host protein synthesis. When cells are infected with poliovirus in the presence of a viral replication inhibitor, eIF4G-1 is completely degraded, but host protein synthesis is only partially shut off (27, 28). Similarly, ionophores block the host shut off but not eIF4G cleavage (29).

Expression of poliovirus 2APro in COS-1 cells has a much greater inhibitory effect on transcription by RNA polymerase II than on translation, suggesting that both processes may be involved in the host shut off (30). In vivo expression of 2APro alone activates viral mRNA translation, independent of its role in the host shut off (31, 32). Complete cleavage of eIF4G-1 in Xenopus oocytes (33) or HeLa cells (34) by introduction of exogenous 2APro results in only a modest reduction of protein synthesis. Cleavage of eIF4G-2 appears to correlate better with host shut off than that of eIF4G-1 (35).

Finally, PABP is also cleaved during Coxsackievirus B3 (CVB3; Ref. 36) and poliovirus (37) infection; partial cleavage coincides with the cessation of viral protein synthesis (36). Thus, it is possible that events accompanying picornavirus infection other than eIF4G-1 cleavage are responsible, wholly or partly, for the host shut off.

Creating an eIF4G-1 variant that is resistant to 2APro would provide a useful tool to study the mechanism of host protein synthesis shut off upon viral infection. Previously, we determined the cleavage site in eIF4G-1 for HRV2 and CVB4 2APro (10) and developed a variant form in which the Gly in the P1′-position was changed to Glu (38). Even though the variant (eIF4G-1aSM) was 100-fold more resistant to cleavage than eIF4G-1aWT, it was still digested, at sufficiently high protease concentrations and extended incubation times, into fragments of approximately the same size as those derived from eIF4G-1aWT. This raised the question of whether cleavage occurred at the same or a different site.

In the present study, we have mapped the CVB4 2APro cleavage site in eIF4G-1SM and found it to be different from the site in eIF4G-1WT. We used this information to create a variant in which both cleavage sites were altered, eIF4G-1DM. This variant is 1,000–10,000-fold more resistant to CVB4 2APro than eIF4G-1WT in vitro. eIF4G-1DM was capable of restoring cap-dependent protein synthesis after 2APro expression both in vitro and in vivo.

FIG. 1. Schematic diagram of the primary structure of human eIF4G-1. eIF4G-1a, eIF4G-1e, and eIF4G-1f are the 154- (21), 172- (25, 26), and 176-kDa (24) forms, respectively. N-terminal (cpN) and C-terminal (cpC) cleavage products are generated during infection of cells with picornaviruses of the entero-, rhino-, and aphthovirus genera or by in vitro cleavage with 2APro. The primary (1′) and secondary (2′) cleavage sites of CVB4 2APro are shown with arrows. Binding sites for other proteins are indicated by shaded boxes, bounded by aa residue numbers.

EXPERIMENTAL PROCEDURES

Materials—The C4 column for reverse phase purification of cpN was obtained from Vydac (Hesperia, CA). HPLC-grade acetonitrile and trifluoroacetic acid were obtained from Fisher and Pierce, respectively. MDL was prepared from New Zealand White rabbits (39). Globin mRNA was prepared from high salt-washed ribosomal pellets from rabbit reticulocytes (39). The vectors pIREs-EGFP, pEGFP-C1, and pTRE2p2ur were from Clontech (Palo Alto, CA). The vectors pET29a and pCITE2a as well as S-Protein-agarose were from Novagen (Madison, WI). The vector pFastBac HTb was purchased from Invitrogen. A plasmid expressing the VP-1-2APro domain of CVB3 from an encephalomyocarditis virus (EMCV) IRES was described previously (40) and is here referred to as pVP12A. Benzamidine, pepstatin, Pefabloc, and ExpandTM High Fidelity PCR systems were purchased from Roche Molecular Biochemicals. T7 RNA polymerase, T4 polynucleotide kinase, RNase H, S-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, all restriction enzymes unless otherwise specified, RQ DNase, 5-bromo-4-chloro-3-indolyl phosphate, and nitro blue tetrazolium chloride were obtained from Promega (Madison, WI). [35S]Met was obtained from ICN Biochemicals (Irvine, CA). Oligonucleotides used for site-directed mutagenesis and sequencing were acquired from Invitrogen. 15′-23PPlsCp was obtained from ICN Biochemicals (Irvine, CA). SgrAI and T4 RNA ligase were purchased from New England Biolabs (Beverly, MA). The T7-1 primer 5′-AAATGACGTCGCTCATTAGC-3′ was provided by the DNA sequencing facility at Iowa State University. The production of an antibody against peptide 7 of eIF4G-1 was described previously (21).

Construction of Recombinant Plasmids—The plasmids pS3N1 and pS3N1E were constructed by inserting the SmlI/XhoI fragments from pJP4 (41) and pAD4G G486E (38), respectively, into pET29A between the EcoRV and XhoI sites. This resulted in plasmids encoding a portion of eIF4G-1 (aa 590–1601) containing either the wild-type Gly or the variant Glu at aa position 683, each protein also containing an N-terminal tag consisting of the S-peptide of RNase A. These proteins are named S-eIF4G-1(590–1601)WT and S-eIF4G-1(590–1601)E.

Construction of the plasmid pCITE4G-1aSM reveals that codes eIF4G-1a with a second as substitution (G675A), was achieved by site-directed mutagenesis of pCITE4G-1aSM. A mutated PCR product from nucleotide (nt) 1221–2033 was synthesized from pCITE4G-1aSM using the sense primer 5′-GAGCCCCTGCGCACCACCG3′ (nt 1221–1237) and the antisense primer 5′-GTTCGTCCGGCAACAAGGT-TGGCAAGAGTAGGG3′ (nt 2003–2033). A second mutated PCR product from nt 2016–2952 was produced from pCITE4G-1aSM using the sense primer 5′-CAACCTTGGCCGCAACAACCTTACGACCCCG3′ (nt 2016–2045) and the antisense primer 5′-CACGTCTGGCAACAAGCTGACAGAATAAAGG3′ (nt 2931–2952). PCR was conducted with the Expand High Fidelity PCR system (Roche Molecular Biochemicals) for 20 cycles at 95°C (1 min), 61°C (30 s), and 72°C (30 s), with a final extension at 72°C (5 min). Two DNA fragments, one derived from the PCR product of nt 1221–2033 by cutting with BamHI and BseHI, and the other from the

3 The names of plasmids pCITE4G-1G486E and pAD4G G486E have not been changed from those used in the original publication (38). The site of the G486E substitution is aa 683 when numbered according to the longest known eIF4G-1 isoform, eIF4G-1f (24).
PCR product of nt 2016–3052 by cutting with BsmHI and NsiI, were ligated into pCTE4G-1DM that had been digested with BamHI and NsiI. The mutagenesis not only changed the encoded aa at position 675 from Gly to Ala but also created a restriction site for BsmHI at nt 2023–2028. Therefore, plasmids from colonies were initially screened by restriction digestion with BsmHI.

A plasmid expressing eIF4G-1eDM termed pCTE4G-1eDM, was constructed by inserting the DNA sequence that encodes the N-terminal extension of eIF4G-1e into pCTE4G-1aDM. This was accomplished by digesting a plasmid containing this sequence in pCR2.1 (Ref. 26; kindly donated by Peter Sarnow) with XmnI and EcoRV, treating the reaction mixture with the Klenow fragment of DNA polymerase I (Promega), and then ligating the gel-purified 501-bp fragment into pCTE4G-1aDM that had been digested with EcoRV.

pTRE2purG-1aDM and pTRE2purG-1eDM were constructed as follows. First, the plasmid pFastBac4G-1aDM was constructed by digesting pCTE4G-1aDM with EcoRV and XhoI. The gel-purified insert was ligated into pFastBac HTb after cutting with StuI and XhoI. pFastBac4G-1eDM was similarly constructed by digesting pCTE4G-1eDM with SgrAI and XhoI. pTRE2purG-1aDM and pTRE2purG-1eDM were constructed by digesting pFastBac4G-1aDM and pFastBac4G-1eDM, respectively, first with SpeI and then partially with NheI. The gel-purified inserts, consisting of the entire eIF4G-1aDM or eIF4G-1eDM coding regions, were ligated into pTRE2pur that had been previously cut with PcoI and NheI.

**Expression and Purification of the cpC Fragment—One-liner cultures of the Escherichia coli expression strain BL21(DE3)pLysS bearing the plasmids pΔN1 and pS3N1E were grown in LB medium (41 containing 25 μg/ml kanamycin and 34 μg/ml chloramphenicol to an A600nm of 0.3. Expression of S-eIF4G-1(590–1601)DM or S-eIF4G-1(590–1601)M was induced with 1 mM isopropyl-1-thio-β-D-galactoside for 3 h at 30 °C. Cells were cooled in an ice water bath, pelletted by centrifugation, and stored at −80 °C. Cells were thawed in the presence of 30 ml of Buffer A (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM dithiothreitol, 2 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mg/ml leupeptin, 1 mg/ml pepstatin, and 1 μg/ml Pefabloc. Lysis was completed by sonication (5–6 bursts of 10 s each), and insoluble debris was removed by centrifugation at 30,000 × g for 30 min. The supernatant was combined with 2 ml of S-Protein-agarose equilibrated in Buffer A and incubated with rotation at 4 °C for 2 h. The resin was washed with 80 volumes of Buffer A containing 0.1% Triton X-100. The protein, still bound to S-Protein-agarose, was subjected to digestion in 4 ml of Buffer A containing 50 μg/ml CVB4 2APro and 0.1% Triton X-100 at 30 °C for 2 h. The resin was allowed to settle, and 2 ml of the supernatant containing cpC, were removed and further fractionated by reverse phase HPLC on a 4.5 × 15 cm C4 column equilibrated in Buffer B (0.1% aqueous trifluoroacetic acid). The column was developed with 5 ml of Buffer B followed by a 45-ml linear gradient of Buffer B to 80% Buffer C (0.1% trifluoroacetic acid in 95% acetonitrile). Peak fractions were pooled and analyzed on an 8% gel before (lane 6) and after (lane 7) incubation with CVB4 2APro or S-eIF4G-1(590–1601)M. Lane 5, RNase H (5 units) was referred to as uncleaved S-eIF4G-1(590–1601)M. Lane 1, bacterial extract; lane 2, fraction unbound to S-Protein-agarose; lane 3, last wash fraction; lane 4, eluate after CVB4 2APro treatment; lane 5, CVB4 2APro alone. B, the eluate from A, lane 4, was fractionated by reverse phase HPLC on a C4 column. Proteins were monitored by absorbance at 214 nm. The peaks at 24 and 40 min were identified by SDS-PAGE as 2APro and cpC, respectively (data not shown).

**Establishment of Stable Cell Lines Expressing Tetracycline-regulated eIF4G-1DM and eIF4G-1eDM**—The Tet-On HeLa cell line (Clontech) was transfected by electroporation with pTRE2purG-1aDM or pTRE2purG-1eDM linearized with XhoI. After 24 h, the cells were selected in medium containing 2 μg/ml puromycin. The medium was changed every 3–4 days. Colonies were transferred to 6-well plates, and the culture was continued. Expression of eIF4G-1DM was detected by adding doxycycline to the culture medium (see figure legends). Cell lysates were treated with recombinant CVB4 2APro at 50 μg/ml for 2 h on ice. eIF4G-1 that was detected by immunoblotting with anti-eIF4G-1
antibodies but was resistant to 2APro digestion was scored as eIF4G-1SM. Colonies were selected that had the lowest expression of eIF4G-1SM in the absence of doxycycline but highest expression in its presence.

Expression and Detection of Green Fluorescent Protein (GFP) in Cell Limes Expressing eIF4G-1Sm—The stably transfected HeLa cell lines expressing eIF4G-1aSM and eIF4G-1eDM were cultured in 6-well plates. Cells were either induced by doxycycline to express exogenous eIF4G-1SM for 24 h or mock-induced. Cells were then co-transfected with pVP12A and either pEGFP-C1 or pIRE2-EGFP using Polyfectin (Qiagen, Valencia, CA). Culture was continued for 24 h, and then cells were observed with a Nikon Eclipse TE 300 fluorescence microscope (Melville, NY). In each well, five random sites were selected for quantitation of the number of GFP-expressing cells. In each site, cells were quantitated in 16 adjacent microscopic fields using the IPLab Scientific Imaging Software from Scanalytics, Inc. (Fairfax, VA).

Quantitation of GFP mRNA by Real Time PCR—RNA was isolated from the cells in each well and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). mRNA was transcribed into cDNA using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). Real time PCR was performed to detect GFP mRNA levels using an ABI PRISM 7700 Sequence Detection System. The antisense primer for GFP was 5'-GTACAGCTCGTCCATGCCGA-3', the sense primer, 5'-CAACGAGAAGCGCGATCACAT-3', and the probe 6-FAM-CTGCTGGAGTTCGTGACCGCCGC-TAMRA. GFP mRNA levels were normalized by taking the ratio to endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA using reagents from Applied Biosystems.

RESULTS

The Cleavage Site for CVB4 2APro in eIF4G-1aSM Is Different from the Site Previously Identified in eIF4G-1aWT—The fact that the eIF4G-1aSM variant is cleaved at sufficiently high CVB4 2APro concentrations (38) suggests one of two possibilities: either the single aa substitution is insufficient to completely prevent cleavage at the original location, or the protease cleaves eIF4G-1 at an alternate site close to the primary site. To distinguish between these possibilities, we determined the cleavage site in eIF4G-1SM, i.e., the site that is cleaved when the primary site in eIF4G-1 is altered. We expressed in E. coli truncated versions of eIF4G-1WT and eIF4G-1SM containing the primary site in eIF4G-1 is altered. We expressed in E. coli truncated versions of eIF4G-1WT and eIF4G-1SM containing the primary site in eIF4G-1 is altered. We expressed in E. coli truncated versions of eIF4G-1WT and eIF4G-1SM containing the primary site in eIF4G-1 is altered. We expressed in E. coli truncated versions of eIF4G-1WT and eIF4G-1SM containing the primary site in eIF4G-1 is altered. We expressed in E. coli truncated versions of eIF4G-1WT and eIF4G-1SM containing the primary site in eIF4G-1 is altered. We expressed in E. coli truncated versions of eIF4G-1WT and eIF4G-1SM containing the primary site in eIF4G-1 is altered.
A Variant of eIF4G-1 That Is Highly Resistant to 2A Protease

FIG. 4. Restoration of cap-dependent translation to CVB4 2A Pro-pretreated MDL by eIF4G-1 DM. Two-phase in vitro translation reactions were performed as described under “Experimental Procedures.” During phase I, either eIF4G-1a WT, eIF4G-1a DM, or eIF4G-1e DM was synthesized in the absence of radioactivity in 2A Pro-pretreated MDL using an EMCV virus IRES. Phase II was initiated by the addition of fresh 2A Pro-treated MDL, globin mRNA, and [35S]Met. Aliquots were removed after 30 min and subjected to SDS-PAGE on 6% and 15% gels. A, the 6% gel was immunoblotted with anti-eIF4G-1 antibodies. The positions of intact eIF4G-1 (4G) and the N- (cPn) and C-terminal (cPc) cleavage products are indicated. B, the 6% gel was subjected to autoradiography. C, the 15% gel was subjected to autoradiography. D, globin synthesis was quantitated from the autoradiograph in C with ImageQuant software (Amersham Biosciences).

directed mutagenesis to modify further eIF4G-1 SM (single mutant) and create a new variant, eIF4G-1 DM (double mutant), in which both of the P1 Gly residues are substituted (G675A and G683E). To test the effect of the two substitutions on susceptibility to CVB4 2A Pro, we synthesized various isoforms of eIF4G-1 in an MDL system programmed with mRNAs encoding eIF4G-1a WT, eIF4G-1a SM, eIF4G-1a DM, or eIF4G-1e DM. The predominant translation product was either full-length eIF4G-1a or eIF4G-1e (Fig. 3A, lower panel), whereas autodigestion with anti-eIF4G-1 antibodies detected both endogenous rabbit and exogenous (in vitro synthesized) human eIF4G-1 (Fig. 3A, upper panel), whereas autodigestion detected only the exogenous eIF4G-1 (Fig. 3A, lower panel). Newly synthesized eIF4G-1 WT was completely cleaved at 5 μg/ml CVB4 2A Pro, whereas endogenous eIF4G-1 WT was cleaved at 50 μg/ml. Most of the eIF4G-1 SM was cleaved at 500 μg/ml, as observed previously (38). However, newly synthesized eIF4G-1a DM and eIF4G-1e DM were only slightly cleaved at 500 μg/ml. The degree of cleavage of eIF4G-1 DM isoforms at 50 μg/ml was intermediate between that of eIF4G-1a SM at 5 and 50 μg/ml. Thus, eIF4G-1 DM was 10–100-fold more resistant than eIF4G-1 SM.

We also tested the resistance of eIF4G-1a WT, eIF4G-1a DM, eIF4G-1a DM, and eIF4G-1e DM to 100 μg/ml CVB4 2A Pro cleavage as a function of time (Fig. 3B). Newly synthesized eIF4G-1 WT (lower panel) was completely cleaved by 5 min, whereas the endogenous eIF4G-1 WT (upper panel) was cleaved somewhat more slowly. Most of the eIF4G-1a DM isoforms was digested by 60 min (Fig. 3B, lower panel), but the eIF4G-1a DM and eIF4G-1e DM variants were only slightly digested. The cleavage of eIF4G-1a DM and eIF4G-1e DM at 60 min was intermediate between that of eIF4G-1a SM at 0 and 5 min. By this criterion, eIF4G-1 DM was at least 12-fold more resistant than eIF4G-1 SM.

Restoration of eIF4G-1 Function by eIF4G-1e DM to 2A Pro-pretreated MDL—We sought to determine whether the two substitutions in eIF4G-1 DM interfered with its intrinsic activity as an initiation factor. We therefore conducted a two-phase in vitro translation assay to test if eIF4G-1 DM could restore activity to MDL that had been pretreated with CVB4 2A Pro. In phase I, exogenous eIF4G-1 WT isoforms were synthesized in 2A Pro-pretreated MDL by IRES-driven translation. In phase II, the newly synthesized eIF4G-1 WT was tested for its ability to restore translation of globin mRNA. Endogenous eIF4G-1 was cleaved in the 2A Pro-pretreated MDL (Fig. 4A, lanes 3–7) but not in mock-treated MDL (Fig. 4A, lanes 1 and 2). eIF4G-1a DM (lane 6) and eIF4G-1e DM (lane 7) were shown by immunoblotting (Fig. 4A) and autoradiography (Fig. 4B) to be resistant to CVB4 2A Pro. Globin was efficiently synthesized in mock-treated MDL (Fig. 4, C and D, lane 2) but not in 2A Pro-pretreated MDL in the absence of exogenous eIF4G-1 (lane 4) or in the presence of eIF4G-1a WT (lane 5). (The band for radioactive globin is broad because endogenous globin is ~100 mg/ml in MDL and runs as a broad band on SDS-PAGE.) Globin synthesis was partially restored by eIF4G-1a DM (lane 6) and completely restored by eIF4G-1e DM (lane 7). These results show that both eIF4G-1a DM and eIF4G-1e DM are functional but that eIF4G-1e DM has higher translational activity than eIF4G-1a DM. This comparison is even more striking because most eIF4G-1a DM was produced in the two-phase translation system than eIF4G-1e DM (Fig. 4, A and B). Apparently, synthesis of eIF4G-1e DM is less efficient than eIF4G-1a DM, because the phase I reactions contained the same amount of mRNA. In interpreting this and subsequent in vitro experiments, one must keep in mind that the eIF4G-1a and eIF4G-1e preparations contain full-length proteins but also smaller amounts of a variety of truncated forms. Thus, the assignment of activities to specific isoforms of eIF4G-1 cannot be made with complete confidence.

eIF4G-1a DM and eIF4G-1e DM Differ in Their Ability to Restore Translation of Polyadenylated mRNA in Vitro—eIF4G-1a DM and eIF4G-1e DM differ by 156 aa at the N terminus (Fig. 1). The extended N-terminal sequence contains a PABP-binding site (25). We reasoned that the PABP-binding site might account for the difference in translational activity between eIF4G-1a DM and eIF4G-1e DM. To test this, we deadenylated rabbit globin mRNA with oligo(dT) and RNase H. This treatment caused the loss of radioactivity from 3′-end-labeled globin mRNA (Fig. 5A). Concomitantly, ethidium bromide staining showed that untreated globin mRNA was heterogeneous but was resolved into distinct bands of faster mobility after RNase H treatment, representing α- and β-globin mRNA (Fig. 5B). These findings indicate that the poly(A) tract of globin mRNA was removed from the 3′-end.
The untreated and deadenylated globin mRNAs were then used to test the activity of eIF4G-1DM isoforms. Either eIF4G-1aDM or eIF4G-1eDM was synthesized in phase I (Fig. 6A). In phase II, the newly synthesized eIF4G-1aDM and eIF4G-1eDM were used to translate untreated or deadenylated globin mRNA (Fig. 6B). Deadenylated globin mRNA was translated at the same efficiency in the presence of either eIF4G-1aDM or eIF4G-1eDM (lane 9 versus 10, p = 0.36, n = 3). The polyadenylated globin mRNA, however, was translated at considerably higher efficiency in the presence of eIF4G-1eDM compared with eIF4G-1aDM (lane 8 versus 7, p < 0.008, n = 3). Furthermore, polyadenylated and deadenylated globin mRNAs were translated with the same efficiency with eIF4G-1aDM (lane 7 versus 9), presumably due to the absence of a PABP-binding site in eIF4G-1DM. It was possible that the deadenylated mRNA was less stable in the translation system, accounting for its lower activity with eIF4G-1aDM. To test this, we performed in vitro translation of natural or deadenylated globin mRNA, removed aliquots at 0 and 30 min, and tested both the concentration and integrity of globin mRNA by Northern blotting. The results indicated that untreated and deadenylated mRNA were intact and in the same concentrations at the beginning and end of the reaction (data not shown).

eIF4G-1DM Restores Cap-dependent Translation in Vivo—In order to test the translational activity of eIF4G-1DM variants in vivo, we established HeLa cell lines stably transfected with plasmids expressing either eIF4G-1aDM or eIF4G-1eDM under control of a tetracycline-regulated promoter. To distinguish endogenous eIF4G-1 from eIF4G-1DM, we treated cell extracts with CVB4 2AP before immunoblotting with anti-eIF4G-1 antibodies. The level of exogenous eIF4G-1aDM (Fig. 7A, + lanes) and eIF4G-1eDM (Fig. 7B, + lanes) increased with increasing doxycycline concentration. Unfortunately, the expression level of exogenous eIF4G-1DM was only a small fraction of endogenous eIF4G-1 (- lanes), despite the fact that we surveyed numerous clonal lines.

We tested the translational activity of eIF4G-1aDM and eIF4G-1eDM by co-transfecting the stable cell lines with vectors expressing GFP and CVB3 2AP. To distinguish between cap-dependent and cap-independent translation, we used vectors expressing GFP mRNA with either a normal 5'-untranslated region (pEGFP-C1) or an EMCV IRES (pIRES2-EGFP). The plasmid producing CVB3 2AP also contained an EMCV IRES, so the protease was produced regardless of the cleavage status of eIF4G. eIF4G-1DM isoforms were induced with doxycycline for 24 h. Cells were then transiently co-transfected with the GFP- and 2AP-expressing vectors.

It was necessary to control for differences in GFP mRNA levels under the various conditions tested, because eIF4G strongly enhances the rate of initiation and because the stability of an mRNA is affected by its state of translation. We therefore obtained quantitative estimations of GFP mRNA levels by real time PCR, normalizing for RNA yield and reverse transcription efficiency using GAPDH mRNA as endogenous control (Fig. 8). The threshold cycles (Cₚₗ) for all GAPDH cDNA samples fell between 18 and 20 (Fig. 8A), whereas those for GFP cDNA fell between 15 and 18 (Fig. 8B). Table II shows the average number of fluorescent cells in five randomly selected sites for each cell culture well. The levels of GFP mRNA for each cell type and condition was measured in duplicate. Rates of GFP synthesis were calculated by dividing the number of cells expressing GFP by the number of cells in each well.
fluorescent cells by the GFP mRNA level. The results are presented graphically in Fig. 9.

When mediated by cap-dependent translation, GFP synthesis was not significantly different in the presence or absence of doxycycline in eIF4G-1aDM cells not expressing 2APro (Fig. 9A, GFP; open versus filled bars; 0.5 < p < 0.8; eight degrees of freedom). This was also true for eIF4G-1eDM cells (Fig. 9B, GFP; 0.5 < p < 0.8). Expression of 2APro dramatically decreased GFP synthesis in eIF4G-1aDM cells uninduced with doxycycline (Fig. 9A, open bars, GFP + 2A versus GFP; p < 0.05). This was also true for eIF4G-1eDM cells (Fig. 9B, open bars, GFP + 2A versus GFP; p < 0.02). However, induction of eIF4G-1aDM significantly increased GFP expression (Fig. 9A, GFP + 2A, open versus filled bars, p < 0.01). Similarly, induction of eIF4G-1eDM significantly increased GFP expression (Fig. 9B, GFP + 2A, open versus filled bars; p < 0.001).

When GFP was expressed by IRES-dependent translation, the results with 2APro were quite different. In eIF4G-1aDM cells not expressing 2APro, GFP expression was not affected by doxycycline (Fig. 9C, IGFP, 0.2 < p < 0.5). This was also true in eIF4G-1eDM cells not expressing 2APro (Fig. 9D, IGFP, 0.1 < p < 0.2). Unlike cap-dependent GFP synthesis, IRES-dependent GFP synthesis increased dramatically with 2APro expression in uninduced eIF4G-1aDM cells (Fig. 9C, open bars, IGFP + 2A versus IGFP; p < 0.01). This was also true for uninduced eIF4G-1eDM cells (Fig. 9D, open bars, IGFP + 2A versus IGFP; p < 0.01). This increase in GFP expression was suppressed by the induction of eIF4G-1eDM by doxycycline (Fig. 9D, IGFP + 2A; p < 0.01) but not eIF4G-1aDM (Fig. 9C, IGFP + 2A; 0.5 < p < 0.8).

**DISCUSSION**

Comparison of the cleavage sites for 2APro in the viral polyproteins of 22 entero- and rhinoviruses yields the consensus sequence Leu-Thr-Thr-X; Gly-Pro... (P4-P3-P2-P1...; see Ref. 45). The most conserved positions are P1, which is Gly in all cases, and P2, which is Thr in 16 out of 22

![Image](image-url)
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Fig. 9. GFP synthesis in stable cell lines expressing eIF4G-1DM isoforms. GFP synthesis, as calculated in Table II, was measured in cells stably expressing either eIF4G-1aDM (A and C) or eIF4G-1eDM (B and D). Expression of eIF4G-1 was either uninduced (open bars) or induced with doxycycline for 24 h (filled bars). Cells were then co-transfected with vectors expressing 2APro (pVP12A) and GFP by either cap-dependent (pEGFP-C1) or IRES-dependent (pIRES2-EGFP) translation, and GFP synthesis was measured after an additional 24 h.

The 2APro of HRV2 and CVB4 cleave eIF4G-1 initially at Leu794-Ser-Thr-Arg↓Gly, a sequence that contains both of these consensus aa residues (10). Our previous observation that the G683E variant eIF4G-1aSM was still cleaved (38), albeit 100-fold more slowly than eIF4G-1aWT, meant either that CVB4 2APro had a preference but not an absolute requirement for Gly at P1 or that it cleaved at another site. Finding a secondary cleavage site between Leu794 and Gly877, 8-aa residues upstream from the primary site, supports the latter mechanism. Whereas the P1′ position in the secondary cleavage site is occupied by the highly conserved Gly, the P2 position is occupied by the less conserved Asn, which occurs in only 2 of the 22 viral polyprotein sequences (45). This may account for the slower rate of cleavage at the secondary site. It is possible that both sites are cleaved in eIF4G-1WT, but our method of analyzing cp by Edman sequencing detects only the downstream site (10). Even if this occurs, the results of Fig. 3 indicate that the secondary cleavage is 100-fold slower. Interestingly, the Lb protease of foot-and-mouth virus, which is a Papain-like protease, cleaves eIF4G-1 between Gly775 and Arg776 (46), only 1-aa residue from the secondary cleavage site of CVB4 2APro. Thus, this region of eIF4G-1 may be particularly accessible to proteases. Because of this proximity of sites, the eIF4G-1DM variant may have some resistance to Lb protease as well.

Careful examination of the immunoblots in Fig. 3 reveals that the newly synthesized eIF4G-1aWT is cleaved more readily than endogenous eIF4G-1 (Fig. 3, A and B, upper versus lower panels). There are several possible explanations for this as follows: human eIF4G-1 may be a better substrate for CVB4 2APro than rabbit eIF4G-1, because human matches the consensus CVB4 2APro-recognition sequence better than does rabbit (10); newly synthesized eIF4G-1 may associate more readily with eIF4E, because eIF4G-1 alone is a poorer substrate for HRV2 2APro than the eIF4G-1-eIF4E complex (47); or eIF4G-1a may be more susceptible to proteolysis than the larger forms.

We found that the ability of eIF4G-1eDM to restore translation to 2APro-pretreated MDL was significantly higher than that of eIF4G-1aDM when the system contained natural globin mRNA. By contrast, the activities of eIF4G-1eDM and eIF4G-1eDM were similar with deadenylated globin mRNA. This is mostly likely explained by the finding of Imataka et al. (25) that eIF4G-1e but not eIF4G-1a contains a PABP-binding site. It might be argued that 2APro-pretreated MDL lacks both functional eIF4G-1 and PABP, because the latter is also cleaved by 2APro (36, 37). However, we have previously shown that PABP cleavage occurs at only approximately one-tenth the rate of eIF4G-1 cleavage (36). Under the conditions actually used in the present study, we demonstrated, by immunoblotting with anti-PABP antibodies, that pretreatment of MDL with CVB4 2APro resulted in only one-third cleavage of PABP but complete cleavage of eIF4G-1 (data not shown). The fact that eIF4G-1eDM is able to fully restore translation of globin mRNA indicates that (i) any hypothetical changes to the translational system caused by 2APro treatment other than eIF4G cleavage do not affect the overall rate of translation, and (ii) the 2-aa substitution in eIF4G-1eDM does not affect its activity for in vitro translation of capped and polyadenylated mRNA.

Co-transfection of stable cell lines expressing eIF4G-1DM isoforms with plasmids that express GFP and 2APro indicated that eIF4G-1DM isoforms substantially protect cap-dependent protein synthesis from inhibition by 2APro in vivo. The response of GFP synthesis to 2APro was opposite depending on how translation of GFP was initiated. Cap-dependent GFP synthesis was dramatically inhibited by 2APro expression, whereas IRES-dependent GFP translation initiation was greatly increased. This latter result agrees with the observation that initiation of both IRES-containing and uncapped mRNAs is either unaffected or even stimulated upon eIF4G cleavage (15–17). This may be due to two factors as follows: (i) cp is more active than intact eIF4G-1 for initiation of IRES-containing mRNAs, and (ii) the inhibition of cap-dependent translation of exogenous mRNAs by 2APro makes limiting components of the translational machinery available for IRES-driven synthesis. Doxycycline-induced expression of eIF4G-1eDM substantially reversed the effects of 2APro in both cases; inhibition of cap-dependent translation by 2APro was relieved (Fig. 9B), and stimulation of IRES-dependent translation by 2APro was suppressed (Fig. 9D). For reasons we do not understand, eIF4G-1eDM was able to restore cap-dependent translation (Fig. 9A) but not suppress the IRES-dependent stimulation (Fig. 9C).

Initial attempts to protect cells against CVB3 infection were not successful. This may be because the amount of exogenous eIF4G-1 was low compared with the endogenous level (Fig. 7). With improved techniques for producing eIF4G-1DM at higher levels in intact cells, it should be possible to use the approach demonstrated here to gain further insight into the cytopathic effect of picornaviruses. Furthermore, because there are numerous isoforms of eIF4G-1 (24), this approach may provide a means of testing them for unique activities or preferential recruitment of mRNA populations.

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